Helicobacter – species classification and identification

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The genus *Helicobacter* was created in 1989 with *H. pylori* as the type species. Since then the genus has expanded to include about 18 species. Some species were reclassified from *Campylobacter*, but most were newly discovered micro-organisms from gastric or intestinal sites in mammalian host animals. The essential property of almost all helicobacters is the presence of sheathed flagella. Most species possess strong ureolytic ability, particularly those associated with gastric mucosa, and exhibit considerable diversity in cell morphology with respect to cell length, number and location of flagella, and presence of periplasmic fibrils. *H. pylori* has a global distribution and infects human gastric mucosa exclusively but there is some evidence for infection in cats. Genomes of isolates from different individuals are unusual in their diversity in gene order and sequences within individual genes. '*H. heilmannii*' is another gastric spiral shaped organism less frequently infecting humans but commonly found in cat and dog gastric tissue. *H. felis* is important in the mouse model of infection. A range of conventional phenotypic tests as well as some new PCR based assays are available for identifying isolates of *Helicobacter* from clinical specimens.

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Over the past century, curved, spiral micro-organisms or 'spirochaetes' have been observed from time to time in gastric specimens of dogs, other carnivorous animals, and humans. As these gastric organisms were generally unculturable, no identification was possible until 1982 when Barry Marshall and colleagues in Perth (Western Australia), successfully cultured a small curved s-shaped bacillus observed microscopically in the antral biopsy material from patients with gastritis and gastric ulcers[1,2].

This discovery provided the impetus for a rapidly expanding area of microbiology with the recognition of a variety of new species with distinctive microbiological properties and disease associations.

From *Campylobacter* to *Helicobacter*

Classification in Campylobacter

When Marshall and Warren first isolated and described their novel gastric bacterium in 1983, it was logical that it should be classified as a new species in the genus *Campylobacter*, despite its unusual flagellar morphology[1,2]. The original idea for naming the new gastric bacteria was possibly first documented by Skirrow[3] who commented'.... their specific location and association makes the provisional name of 'pyloric campylobacter' particularly apt; *pylorus* is Greek for gatekeeper – one who looks both ways. Should these bacteria prove to be campylobacters then *Campylobacter pyloridis* would be an appropriate name'. Marshall and Warren[4] cautiously concluded that their bacilli appeared to be a new species closely resembling the campylobacters, but suggested that it was premature to talk of '*C. pyloridis*' and better to use the term '*pyloric Campylobacter*'.

Nevertheless, the name *Campylobacter pyloridis* was proposed and the culture Royal Perth Hospital 13487 (= NCTC 11637) was designated as the type strain[5]. The 'type strain' is an important concept because it defines the strain to which the name is attached but it does not necessarily follow that particular strain is typical of the species as a whole. Surprisingly, the new species was described as being unable to hydrolyse urea, although an active urease was later found to be a distinctive and unique diagnostic feature of *C. pyloridis*[6].

The name *C. pyloridis* was subsequently validated although two years later it was pointed out that the specific epithet *pyloridis* was grammatically incorrect and should be *pylori* (the genitive of the noun *pylorus*). The species name was subsequently revised in 1987 to *C. pylori* to conform to the rules of nomenclature[7].

Phylogenetic analysis

The taxonomy of *Campylobacter* and allied organisms was in a state of considerable flux after the genus was formed in 1963 with most species having uncertain associations. Their classification underwent a dramatic transformation with the introduction of novel chemotaxonomic methods, the most important being ribosomal (r)RNA analysis. Such molecules are universal and have a highly conserved structure and were identified as potential molecular chronometers, undergoing constant but random change with time, that could provide a record of evolution (phylogeny). From 1987, a phylogeny of prokaryotes began to emerge based on the study of 16S rRNA and its gene sequences[8]. Partial

sequencing of rRNA (oligonucleotide catalogues) was used initially but, as biochemical techniques improved, full sequencing of the 16S rRNA genes became possible. The detailed relationships between individual species of *Campylobacter* and allied taxa including *C. pylori* were initially unravelled by the determination of partial 16S RNA sequence homologies, which were in close agreement with DNA-23S rRNA hybridization analyses. The latter approach provided novel evidence that species of *Campylobacter* including *C. pylori*, as well as *Wolinella* and *Flexispira*, belonged to rRNA superfamily VI, a new grouping within the Gram-negative bacteria.

Classification in Helicobacter

The most important stage in the development of the taxonomy of gastric microorganisms was the proposal in 1989 to establish a new genus called *Helicobacter* – to mean a spiral rod – and that *C. pylori* should be transferred to that genus as *H. pylori*. *H. mustelae* was also included in the genus in a revision that provided the foundations for the development of a new field of microbiology[9]. Key features ascribed to the genus *Helicobacter* were: (i) cell motility by means of sheathed flagella; (ii) an external glycocalyx produced *in vitro* in liquid media; (iii) menaquinone-6 (MK-6) present as the major isoprenoid quinone; and (iv) G+C content of chromosomal DNA of 35–44 mol%.

In 1991, Vandamme *et al*[10] proposed an amended description of *Helicobacter* with the inclusion of two further species – *H. cinaedi* and *H. fennelliae*, which were previously classified as *Campylobacter*. Despite the distinctions evident from phylogenetic analysis, only two phenotypic taxonomic markers were found that clearly differentiated *Helicobacter* from other genera in rRNA superfamily VI – these were the presence of sheathed flagella, and the absence of hexadecanoic acids in the major fatty acid profiles. *Campylobacter* and *Arcobacter* were later classified in the new family *Campylobacteraceae* based on the results of these rRNA gene analyses but the family did not encompass *Helicobacter*, which currently does not have any formal position in the taxonomic hierarchy[11]. In the comprehensive phylogenetic tree derived by maximum likelihood analysis of small (16S) subunit rRNA sequences of 253 representative species of bacteria, *H. pylori* was positioned in the Delta and Epsilon subdivision of the Purple Bacteria (Proteobacteria)[12]. The most closely associated genera were *Wolinella* (represented by *W. succinogenes*) and *Campylobacter*.

Since the creation of *Helicobacter*, the genus has undergone significant expansion to include about 18 named and associated species such as *'Flexispira rappini'* (Tables 1 & 2). Several names are in quotations to indicate that they have not yet been formally validated.

Features of *H. pylori*

Cellular morphology

H. pylori is a Gram-negative, s-shaped or curved rod (0.5–0.9 μm wide by 2–4 μm long) with 1 to 3 turns when observed *in vivo*. No spores are formed in blood agar cultures (*in vitro*), and spiral forms are less obvious with cells appearing more frequently as singly curved rods. Cells of *H. pylori* typically have up to six polar flagella filaments. Cells are mostly actively motile although some cultures may appear to be non-motile in hanging drop preparations. Other forms of *H. pylori* reported in culture and occasionally *in vivo* include spherical, V-shaped, U-shaped (ox-bow) and straightened forms.

Colonial morphology

Colonies of *H. pylori* from primary culture on supplemented blood agar at 37°C usually take 3–5 days to appear and are circular (1–2 mm),

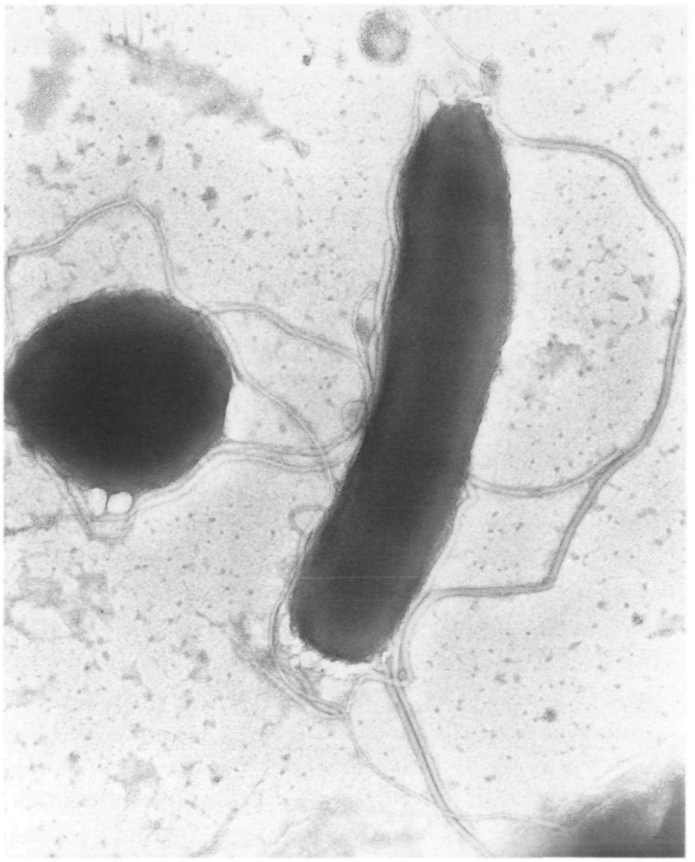

Fig. 1 Electron micrograph (EM) of *Helicobacter pylori* showing the typical rod and coccoid forms with associated multiple polar sheathed flagella. Magnif. x 55 000 [With acknowledgements to Dr H. Chart]

convex and translucent in appearance. There is slight haemolysis in blood agar around colonies, which are greyish in colour.

Ultrastructure features

Flagella of *H. pylori* are sheathed with a covering that is continuous with the outer membrane components of the body wall. Freeze-fracture ultrastructure studies suggest that the normal configuration of flagella is seven. Flagella are each about 30 nm in diameter with a filament of 12–15 nm. Some flagella have distinctive terminal bulbs but no function has been assigned to such structures. Electron microscopy also reveals the presence of a 40 nm thick glycocalyx or capsule-like polysaccharide rich layer external to the cell wall unit membrane, which is thicker *in vivo* than in cultured bacteria.

Coccoid bodies

In older cultures, *H. pylori* undergoes a morphological change from bacillary to coccoid form (Fig. 1) with an associated loss in culturability. Older cultures can consist wholly of such forms which may be viable but more resistant and dormant forms of *H. pylori* and could reflect a temporary adaptation to a hostile environment – stress caused by nutrient deprivation, exposure to antibiotics or extended incubation. It is speculated, but not yet clearly established, that coccoid forms can revert to an infectious bacillary form under appropriate conditions. However, the coccoid form alternatively may be degenerative and pose no infection risk.

General physiological properties

H. pylori is a microaerophile, growing best in an atmosphere of 5% oxygen with 5–10% CO_2 on blood containing media such as Oxoid brain heart infusion agar (BHI) and 5% horse blood agar enriched with 1% IsoVitaleX, which is a chemically defined supplement containing vitamin B_{12}, L-glutamine, L-cysteine, and various other growth promoting compounds. It has a respiratory type of metabolism. The cultures grow optimally at 37°C after 3–5 days. All strains grow over a relatively narrow temperature range of 33–40°C, whereas some grow poorly at30°C and 42°C, none grow at 25°C. *H. pylori* will grow on a suitable culture medium over a wide pH range (5.5–8.5) with good growth between pH 6.9 and 8.0. *H. pylori* does not tolerate low pH *in vitro*.

Table 1 Hosts and key morphological features of *Helicobacter* species associated with gastric mucosa

Species	Main host	Cell size (μm)	Periplasmic fibrils	No. of flagella	Distribution	Sheath
Simple cell morphology						
H. pylori	Human	2.0–4.0	–	4–6	Polar	+
H. acinonyx	Cheetah	2.0–5.0	–	2–5	Polar	+
H. mustelae	Ferret	2.0–5.0	–	4–8	Peritrichous	+
H. nemestrinae	Macaque monkey	2.0–5.0	–	4–8	Polar	+
'H. suis'	Pig	1.5–5.2	–	up to 6	Biopolar	ND
Complex cell morphology						
'H. heilmannii'	Cat, dog, (human)	3.5–7.5	–	12	Biopolar	+
H. felis	Cat, dog, (human)	5.0–7.5	+	14–20	Biopolar	+
H. bizzozeronii'	Dog	5.0–10.0	–	10–20	Biopolar	+

ND = not determined

Biochemical characteristics

H. pylori is inactive in most of the conventional biochemical tests. Carbohydrates are neither oxidized nor fermented. *H. pylori* produces catalase and cytochrome oxidase but is most notable for its high level of urease and alkaline phosphatase activity. *H. pylori* is a homogeneous species in its enzymic profile, with the exception of some minor strain differences in aminopeptidase and other preformed enzyme activities. Typical strains are positive for alkaline phosphatase, acid phosphatase, leucine arylamidase, naphthol-AS-B1-phosphohydrolase, esterases C4 (butyrate) and C8 (caprylate), and gamma glutamyl transpeptidase. Strains are usually negative in hippurate hydrolysis, nitrate reduction,

Table 2 Hosts and key morphological features of *Helicobacter* species associated with intestinal mucosa

Species	Main host	Cell size (μm)	Periplasmic fibrils	No. of flagella	Distribution	Sheath
Simple cell morphology						
H. cinaedi	Human, hamster	1.5–5.0	–	2	Biopolar	+
H. fennelliae	Human	1.5–5.0	–	2	Biopolar	+
H. canis	Dog, (human)	4.0	–	2	Biopolar	+
H. pullorum	Poultry, (human)	3.0–4.0	–	1	Polar	–
H. pametensis	Wild birds, pig	1.5	–	2[a]	Biopolar	+
'H. cholecystus'	Hamster[b]	3.0—4.0	–	1	Polar	+
H. hepaticus	Mice[b]	1.5–5.0	–	2	Biopolar	+
Complex cell morphology						
H. muridarum	Rat, mice	3.5–5.0	+	10–14	Biopolar	+
H. trogontum	Rat	4.0–6.0	+	5–7	Biopolar	+
'H. bilis'	Mice[b]	4.0–5.0	–	3–14	Biopolar	+
'Flexispira rappini'	Dog, pig, sheep	6.5	+	10–20	Biopolar	+

[a]Some strains have a third flagellum – all were located subterminally.
[b]Also isolated from livers.

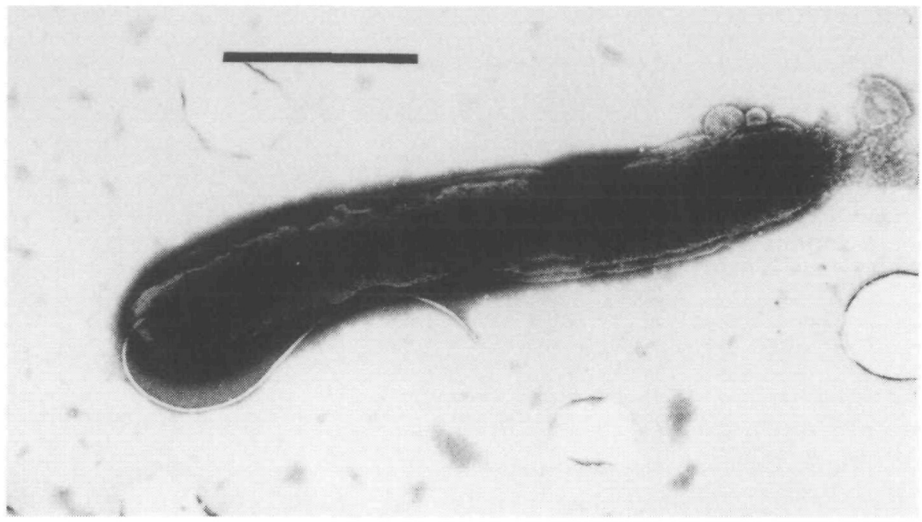

Fig. 2 Electron micrograph of a freeze-dried preparation of *H. pullorum* NCTC 12827 isolated from an HIV-positive patient. The cell has a unipolar flagellum lacking a sheath. Bar 1μm [From Stanley *et al*[28]; with permission from the Society for General Microbiology]

indole formation, arylsulphatase activity, growth in the presence of 1% and 3.5% NaCl, and indoxylacetate hydrolysis. Some *H. pylori* have been reported to be negative for catalase and urease production but, in general, the isolation of such strains directly from clinical material is rare. Another important difference between strains is their ability to produce a vacuolating cytoxtoxin in human and animal cell lines.

Antibiotic activity

Antibiotic susceptibilities (*in vitro*) are unreliable taxonomic features because resistance may develop during treatment, for example different isolates of the same strain can differ in their susceptibilities to metronidazole and clarithromycin. Polymyxin B activity is possibly of use as a taxonomic marker because most (95%) *H. pylori* are resistant (300 IU disk) and it has been suggested as an additional test to discriminate between *Helicobacter* and *Campylobacter*[13]. Nalidixic acid and cephalothin are important in *Campylobacter* identification and most (about 86%) of *H. pylori* are resistant to nalidixic acid (30 mg disk) and susceptible to cephalothin (30 mg disk).

Macromolecular characteristics

H. pylori is an homogeneous species with respect to a number of important molecular chemotaxonomic markers:

Genomic DNA The genomic DNA is a single circular molecule with a mean size of 1.71 Mb ranging from 1.40–1.73 Mb, and with a base

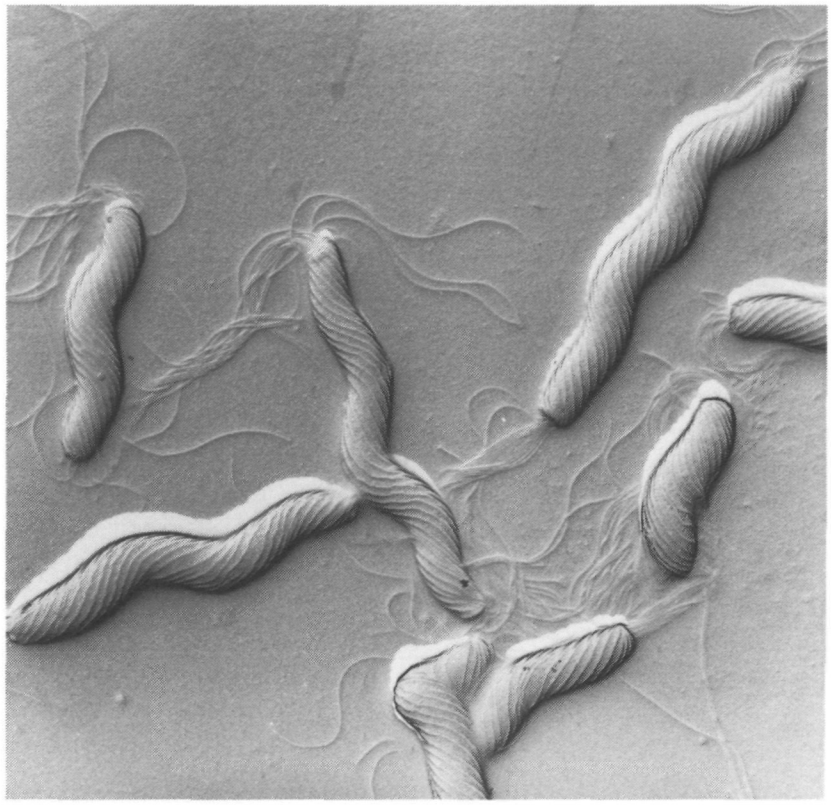

Fig. 3 Electron micrograph of a freeze-dried preparation of *Helicobacter muridarum* showing characteristic S shape, tufts of polar flagella and periplasmic fibers [From Lee *et al*[34] with permission from the American Society for Microbiology]

composition in the range 35–37 mol% G+C. DNA–DNA hybridizations show a high level (≥65%) of sequence homology between strains despite evidence for extensive re-arrangements in gene order and sequence variation within genes[14]. The complete sequences of the genomic DNA from several strains have been determined.

Fatty acid composition The major cellular fatty acids are tetradecanoic acid (14:0) and *cis*-11,12-methylene octadecanoic acid (19:0 cyc), with smaller amounts of hexadecanoic acid (16:0) and 3-hydroxydecanoic acid (3-OH-18:0), but 3-OH-14:0 and 16:1 are lacking. The main respiratory quinone is menaquinone-6 (MK-6) but thermoplasmoquinone-6 (TPQ-6) is lacking.

Extrachromosomal DNA Plasmid DNA is present in about 45% of strains although the type strain (NCTC 11637) is plasmid free. The number and size of plasmids can vary considerably from strain to strain but many strains have a single plasmid with sizes from 1.8–63 kbp.

Lipopolysaccharides (LPS) The LPS of about 80% of strains is unusual in that it expresses Lewis x and y blood group antigens which are generally

not found in the LPS of other Gram-negative bacteria. Structural analysis of the O-specific polysaccharide chains show mimicry of fucosylated Lewis x and y antigens. For example, the O-chain LPS of the type strain (NCTC 11637) exhibits mimicry of Lewis x. Expression of these determinants and the number of repeat oligosaccharide units in the O-chain may vary in LPS of different strains.

Host range and ecology

Man is the principal host of *H. pylori* and its distribution is world-wide. Occasional strains identified as *H. pylori* have been isolated from domestic cats and other animal hosts that include pig, baboon and rhesus monkeys. The site of isolation in man is almost exclusively the gastroduodenal mucosa with rare isolates from dental plaque, faeces and blood. *H. pylori* has not been cultured from food, drinking water or the natural environment, although there is evidence of its presence there from PCR assays.

H. pylori *may be a complex of related species*

Genetic variation in *H. pylori* is well documented[14] and it has been suggested that the level of diversity is sufficient to classify isolates into distinct species within a complex of associated species[15]. However, evidence to support this idea from multilocus enzyme electrophoresis and DNA sequencing is contradictory[16].

Viability and preservation

H. pylori is difficult to maintain by repeated sub-culture and viability is usually lost after about 4 sub-cultures on conventional media. Low temperature is the most practical method of long term storage. Cells are suspended in 10% (v/v) glycerol in Nutrient Broth No.2 (Oxoid CM 67) on glass beads at –70°C or in liquid nitrogen (–196°C).

Characteristics of other gastric Helicobacters

Seven other species of *Helicobacter* show *in vitro* urease activity and are associated with gastric mucosa. Table 1 lists the main cell morphological differences and Table 3 the key biochemical tests between each species. Features of those species infecting man are considered below.

Table 3 Key biochemical tests for identification of *Helicobacter* species associated with gastric mucosa

Species	Characteristic[a]						Reference
	Active urease	42°C growth	CEP	IA	NO_3	Mol %GC	
Simple cell morphology							
H. pylori	+	–	S	–	–	35–37	5
H. acinonyx	+	–	S	–	–	30	17
H. nemestrinae	+	+	S	–	–	24	18
H. mustellae	+	+	R	+	+	36	19
'H. suis'	+	NC					20
Complex cell morphology							
H. felis	+	+	S	–	+	43	21
H. bizzozeronii	+	+	S	+	+	ND	22
'H. heilmannii'	+	NC					23

[a]ND, no details available; NC, not culturable; CEP, cephalothin susceptibility (30 μg disk); R, resistant; S, susceptible; IA, indoxyl acetate hydrolysis; NO_3, nitrate reduction.

'H. heilmannii'

This species is associated with chronic gastritis in humans and was first named '*Gastrospirillum hominis*'[17]. It was not cultured but microscopy revealed it to be helical (tightly spiralled), 3.5–7.5 μm long and 0.9 μm in diameter. Up to 12 sheathed flagella (28 nm in diameter) were present at

Table 4 Key biochemical tests for identification of *Helicobacter* species associated with intestinal mucosa

Species	Characteristic[a]								Reference
	Active urease	42°C growth	Cat	CEP	IA	NO_3	AP	Mol %GC	
Simple cell morphology									
H. cinaedi	–	–	+	I	–	+	–	37	26
H. fennelliae	–	–	+	S	+	–	+	35	26
H. canis	–	+	–	I	+	–	+	48	27
H. pullorum (Fig. 2)	–	+	+	R	–	+	–	35	28
H. pametensis	–	+	+	S	–	+	+	38	29
'H. cholecystus'	–	+	+	R	–	+	ND	ND	30
H. hepaticus	+	–	+	R	+	+	ND	ND	31
Complex cell morphology									
H. trogontum	+	–	+	R	ND	+	–[b]	ND	32
'H. bilis'	+	+	+	R	–	+	ND	ND	33
H. muridarum (Fig. 3)	+	–	+	R	+	–	+	34	34
'Flexispira rappini'	+	+	+	R	ND	–	–	34	35

[a]See Table 3 for abbreviations. AP, alkaline phosphatase; Cat, catalase.
[b]One isolate positive.

each pole but it had no axial filament. The organisms are present in the gastric mucosa of approximately 1% of patients with gastritis and, recently, the first successful culture on artificial medium of an organism resembling '*H. heilmannii*' from a human stomach was reported[24]. Complete remission of gastric MALT lymphoma in patients with chronic gastritis associated with the species was achieved during treatment with omeprazole and amoxycillin for 14 days[25].

H. felis

This helical shaped bacterium colonizing the cat stomach has a complex tightly coiled (5–7 coils) cellular morphology with tufts of 10–17 sheathed flagella positioned slightly off centre at each end of the cell[20]. The body of the cell is entwined with unique periplasmic fibrils that usually occurred in pairs. Strains also have been isolated from the gastric mucosa of dogs and from infections in humans associated with acute gastritis.

H. felis is most important because of its use in a successful animal model – *H. felis* in the specific pathogen-free mouse. *H. felis* readily colonises mice, inducing an active/chronic gastritis. The model has been used as an *in vivo* antimicrobial screening system for potential anti-*H. pylori* agents, to investigate the pathology caused by gastric *Helicobacter* species, and in the study of potential human anti-*H. pylori* vaccine strategies. The type strain is CS1 (NCTC 12436 = ATCC 49179).

Characterisation of intestinal Helicobacters

Ten species of *Helicobacter* and '*F. rappini*' fall in this category. The key morphological and biochemical test differences between species are shown in Tables 2 and 4, respectively. The main species infecting man are considered below.

H. cinaedi

This organism was first described in association with enteric disease in homosexual men, when it was referred to as a *Campylobacter*-like organism (CLO-1A)[26]. Strains have since been recovered from blood, faeces and rectal swabs of patients, including young children with gastrointestinal symptoms and other serious underlying conditions. It is also a normal inhabitant of the intestinal tract of hamsters. Phylogenetic analyses showed that the species was a *Helicobacter* as did DNA-23S rRNA hybridization, immunotyping and numerical analysis of electrophoretic protein patterns. Type strain is NCTC 12423 (= ATCC 35683).

H. fennelliae

This organism was first described from rectal swabs of homosexual men with enteric disease and initially referred to as a *Campylobacter* like organism (CLO-2)[26]. It was classified in *Campylobacter* but later reclassified as a member of *Helicobacter* on the basis of phylogenetic analyses. The species is similar to *H. cinaedi* – both are nonureolytic – but is less frequently encountered in human clinical specimens. Type strain is NCTC 11612 (= ATCC 35684).

H. canis

This species was described from a polyphasic taxonomy of strains provisionally termed the HC (*Helicobacter*, canine) group[27]. The four domestic dog isolates (healthy and diarrhoeic animals), and one human isolate were classified as *H. canis*. Their pathogenic potential was not known but the organism has since been cultured from liver tissue of a dog with active multifocal necrotizing hepatitis, and from human blood. Cells have a simple cellular morphology with single bipolar sheathed flagella. Urease is not produced. A distinctive feature of the species is a G+C content of 48 mol%. Type strain is NCTC 12739.

H. pullorum

This species comprises isolates from liver, duodenum and caecum of poultry, and from faeces of patients with gastroenteritis[28]. It forms a distinct phylogenetic lineage within *Helicobacter* and its phenotype most closely resembles *H. cinaedi*. Distinctive features of *H. pullorum* are lack of urease activity and one polar flagellum (Fig. 2), which is unusual in not being sheathed, despite similarities to *Helicobacter* in other taxonomic markers. The type strain is NCTC 12824.

Key points for clinical practice

- The genus *Helicobacter* has expanded rapidly over the past decade and, as more animal hosts are investigated, other new species will undoubtedly be discovered and the concept of *Helicobacter* will continue to expand.

- Although identification of *H. pylori* should not pose a problem in clinical practice, the differentiation of other species is more difficult, given

the limited number of available test characteristics. This applies in particular to the intestinal species associated with human infections. More information is needed on such organisms to assess their pathogenic importance to man and the role of animals, particularly domestic pets and livestock, as reservoirs of human infection.

• Progress is being made with the development of molecular (PCR-based) methods of species identification. For example, species-specific PCR assays targeted at 16S rRNA gene sequences are available for the more recently described species such as *H. bilis*, *H. pullorum* and *H. trogontum*. A novel alternative approach to species identification is the use of PCR-based restriction fragment length polymorphism analysis of 23S rRNA genes and a scheme for *Helicobacter* has been developed[36].

• These molecular identification tools, which have yet to be fully evaluated against all member species of *Helicobacter*, will provide the most accurate approaches to identification in the future.

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