**Helicobacter cynogastricus** sp. nov., isolated from the canine gastric mucosa

K. Van den Bulck,† A. Decostere,† M. Baele,† P. Vandamme,‡ J. Mast,§ R. Ducatelle† and F. Haesebrouck†

†Department of Pathology, Bacteriology and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium
‡Department of Biochemistry, Physiology and Microbiology, Faculty of Sciences, Ghent University, Ledeganckstraat 35, B-9000 Ghent, Belgium
§Department of Pathology, Bacteriology and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, Grasestraat 6, B-9000 Ghent, Belgium

**Correspondence**
K. Van den Bulck
kvdbulck@prdbe.jnj.com

A Gram-negative, microaerophilic helical rod, isolated from the gastric mucosa of a dog and designated strain JKM4T, was subjected to a polyphasic taxonomic study. The tightly coiled organism, measuring 10–18 μm long and up to 1 μm wide, was motile by means of multiple sheathed flagella located at both ends of the helix and by a periplasmic fibril running along the external side of the helix. Strain JKM4T grew preferably on biphasic culture plates or on very moist agar. Coccoid forms predominated in cultures older than 4 days as well as in growth obtained on dry agar plates. The strain grew at 30 and 37 °C, but not at 25 or 42 °C and exhibited urease, oxidase and catalase activities. On the basis of 16S rRNA gene sequence analysis, the novel isolate was identified as a member of the genus *Helicobacter* and showed > 97% similarity to *Helicobacter felis*, *Helicobacter bizzozeronii* and *Helicobacter salomonis*, three species previously isolated from the canine gastric mucosa. Protein profiling of strain JKM4T using SDS-PAGE revealed a pattern different from those of other *Helicobacter* species of mammalian gastric origin and from *Helicobacter canis*. Additionally, the urease gene sequence of strain JKM4T was different from those of urease genes of *H. felis*, *H. bizzozeronii*, *H. salomonis* and *Candidatus Helicobacter heilmannii*. It is thus proposed that strain JKM4T (=LMG 23188T) represents a novel species within this genus, *Helicobacter cynogastricus* sp. nov.

*Helicobacter* species are fastidious and slow-growing organisms with specific nutritional and environmental requirements. Three *Helicobacter* species have previously been cultured from the gastric mucosa of cats and dogs, namely *Helicobacter felis*, *Helicobacter bizzozeronii* and *Helicobacter salomonis* (Hänninen et al., 1996; Jalava et al., 1997; Lee et al., 1988). These three organisms are highly related to each other, both genetically and morphologically, although small morphological differences are perceptible through electron microscopic examination. The thicker and medium-length organism *H. felis* possesses two or three periplasmic fibrils wrapped around the cell body, while both *H. bizzozeronii*, thinner and longer, and *H. salomonis*, thick, short and less spiralled, lack such appendages (Utriainen et al., 1997). Recently, an uncultured spiral organism, *Candidatus Helicobacter heilmannii* was described as occurring in the gastric mucosa of human and several wild felines (O’Rourke et al., 2004) and it is most probably also present in the gastric mucosa of pet dogs and cats (Van den Bulck et al., 2005a). In addition, less spiralled bacteria with numerous periplasmic fibrils, tentatively named *Flexispira rappini*, have been isolated from the stomachs of various dogs (Hänninen et al., 2003).

A study set up to determine the prevalence of each individual *Helicobacter* species in the canine and feline stomach led to the isolation of a large spiral organism from the stomach of a dog. In this study, it is suggested that this isolate represents a novel species of the genus *Helicobacter*.

The strain, designated JKM4T, was isolated from the antrum region of the stomach of a euthanized dog at the Faculty of Veterinary Medicine, Ghent University, Belgium. Tissue samples were handled as described by Gruntar et al. (2003). Bacteria were grown on brain heart infusion (BHI; Oxoid) agar plates. The strain grew at 30 and 37 °C, but not at 25 or 42 °C and exhibited urease, oxidase and catalase activities. On the basis of 16S rRNA gene sequence analysis, the novel isolate was identified as a member of the genus *Helicobacter* and showed > 97% similarity to *Helicobacter felis*, *Helicobacter bizzozeronii* and *Helicobacter salomonis*, three species previously isolated from the canine gastric mucosa. Protein profiling of strain JKM4T using SDS-PAGE revealed a pattern different from those of other *Helicobacter* species of mammalian gastric origin and from *Helicobacter canis*. Additionally, the urease gene sequence of strain JKM4T was different from those of urease genes of *H. felis*, *H. bizzozeronii*, *H. salomonis* and *Candidatus Helicobacter heilmannii*. It is thus proposed that strain JKM4T (=LMG 23188T) represents a novel species within this genus, *Helicobacter cynogastricus* sp. nov.
Plates were incubated with lids uppermost at 37°C under humidified microaerobic conditions in a closed circuit that was created by evacuating 80% of the normal atmosphere and introducing a gas mixture of 8% CO2, 8% H2 and 84% N2.

Plates were checked every 2 days and BHI broth was added to the agar surface to ensure that the plates did not dry out. Primary growth occurred after 10 days of incubation as an oily aspect on the broth covering the agar medium. Examination of the broth by light microscopy revealed the presence of spiral-shaped, motile cells. Gram-staining revealed that the isolate was Gram-negative and had a helical shape. Growth of subcultures occurred as a spreading layer on moist agar plates. Pinpoint colonies were observed when an abundant amount of bacteria was inoculated onto a dry agar surface; however, bacteria grown on such a medium mostly lost their spiral morphology and transformed to coccoid forms.

Bacteria with typical spiral morphology were harvested in BHI broth and stored at −70°C in a medium consisting of 7.5 g glucose, 25 ml BHI and 75 ml sterile inactivated horse serum.

Genomic DNA of strain JKM4T was extracted using the DNeasy tissue kit (Qiagen) according to the manufacturer’s instructions. To determine whether the novel isolate was a member of the genus Helicobacter, a Helicobacter genus-specific PCR was applied, as described previously by Germani et al. (1997). The positive result in this PCR demonstrated that strain JKM4T represents a Helicobacter species.

To identify strain JKM4T, a multiplex PCR was performed that enables the identification of Helicobacter species previously detected in the gastric mucosa of cats and dogs, namely H. felis, H. bizzozeronii and H. salomonis (Jalava et al., 1998; Van den Bulck et al., 2005a). This PCR is based on a part of the tRNA intergenic spacer of Helicobacter species, amplified with TET-labelled primers, and on the urease gene of H. felis (NED-labelled primers) and H. bizzozeronii (HEX-labelled primers), as described earlier (Baele et al., 2004). DNA extracted from pure cultures of H. felis, H. salomonis and H. bizzozeronii served as positive controls, while highly purified water was included as a negative control. Fluorescently labelled PCR products were separated by means of capillary electrophoresis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Product lengths were determined by interpolation with an internal size standard mixture of GeneScan 500 ROX (Applied Biosystems) and GeneScan 400-HD ROX (Applied Biosystems) using GeneMapper (Applied Biosystems).

Analysis of the PCR products produced from the DNA of strain JKM4T in the multiplex PCR consistently revealed a TET-labelled amplicon of 136-6 bp, which differed from the TET-labelled amplicons of H. felis (137 bp), H. bizzozeronii (136 bp) and H. salomonis (134 bp). Additionally, no amplicons were obtained with the NED- or HEX-labelled primers for the urease gene of H. felis or H. bizzozeronii, respectively. These results indicated that strain JKM4T is different from H. salomonis, H. felis and H. bizzozeronii.

‘Candidatus H. heilmannii’ is a hitherto unculturable spiral organism that is highly related phylogenetically to H. felis, H. salomonis and H. bizzozeronii and may occur in the gastric mucosa of wild carnivores. To exclude the possibility that strain JKM4T was identical to ‘Candidatus H. heilmannii’, DNA of strain JKM4T was subjected to a PCR which specifically amplifies a part of the urease gene of this Candidatus organism (O’Rourke et al., 2004). DNA from ‘Candidatus H. heilmannii’ served as positive control, while highly purified water was included as a negative control. PCR products were separated by gel electrophoresis as described by Baele et al. (2004). PCR products obtained for ‘Candidatus H. heilmannii’ and strain JKM4T were sequenced using the BigDye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems) on the Genetic Analyzer. The electropherograms were exported and converted to KODON (Applied Maths) and sequences were aligned with GenBank using BLAST.

The ‘Candidatus H. heilmannii’-specific PCR resulted in the production of a 320 bp fragment for strain JKM4T which consistently differed from the 380 bp fragment produced from DNA of ‘Candidatus H. heilmannii’. Sequence analysis of the 320 bp fragment of strain JKM4T revealed a unique sequence bordered by the primer pair and the sequence obtained was not related to any sequence in GenBank.

The 16S rRNA gene of strain JKM4T was amplified using primers complementary to the conserved edges. Consensus primers 5′-…TACCTTGTTACTTCACCCCA (O*, as described elsewhere (Coenye et al., 1999). Sequence analysis was performed using the Genetic Analyzer and sequences were aligned with GenBank using BLAST. Phylogenetic analysis was performed using the KODON program. Pairwise alignment similarities were calculated and a dendrogram was constructed using the neighbour-joining method.

The sequence of the 16S rRNA gene of strain JKM4T showed > 97% similarity to those of H. felis, H. bizzozeronii, H. salomonis and ‘Candidatus H. heilmannii’ and the sequence differed by more than 3% from that of ‘Candidatus Helicobacter suis’ (see Supplementary Table S1 in IJSEM Online). A phylogenetic tree based on the 16S rRNA gene sequences revealed clustering of the novel isolate among Helicobacter species of carnivore origin (see Supplementary Fig. S1 in IJSEM Online).
Analysis of the urease gene has recently been found to be more discriminatory for species differentiation within gastric Helicobacter species of carnivore origin than 16S rRNA gene sequence analysis (O’Rourke et al., 2004). Therefore, a PCR with primers U430f and U1735r was performed to amplify a part of the urease gene, as described previously (O’Rourke et al., 2004).

For H. felis, H. bizzozeronii, H. salomonis and ‘Candidatus H. heilmannii’, a PCR amplicon of 1224 bp was obtained, while genomic DNA of strain JKM4T produced a series of aspecific fragments instead of the expected 1224 bp fragment. The result confirms that strain JKM4T is different from H. felis, H. salomonis, H. bizzozeronii and ‘Candidatus H. heilmannii’.

PAGE of whole-cell proteins of strain JKM4T was performed in order to establish its relationship with other cultured Helicobacter species. For this purpose, strain JKM4T was grown on Mueller–Hinton agar supplemented with 5% (v/v) horse blood and incubated at 37 °C in a microaerobic atmosphere as described above. A whole-cell protein extract was prepared and SDS-PAGE was performed as described previously (Pot et al., 1994). Whole-cell protein profiles of reference strains of H. bizzozeronii, H. salomonis and H. felis and of type and reference strains of other Helicobacter species were available from previous studies (Jalava et al., 1998, 2001). Densitometric analysis, normalization and interpolation of the protein profiles and numerical analysis were performed using the GelCompar software package version 4.2 (Applied Maths). The similarity between all pairs of traces was expressed by the Pearson product-moment correlation coefficient, presented as percentages of similarity.

The whole-cell protein profile of strain JKM4T differed considerably from those of the reference strains of other Helicobacter species. Correlation levels to the protein profiles of other Helicobacter reference strains were all below 80%. Therefore, we conclude that strain JKM4T represents a novel Helicobacter species. Whole-cell protein electrophoresis is a taxonomic tool that generally allows closely related species to be distinguished (Vandamme et al., 1996). For many Campylobacter and Helicobacter species, the correlation between whole-cell protein pattern similarity and the level of DNA–DNA hybridization has been documented (Costas et al., 1990; Jalava et al., 1998, 2001; On et al., 1995; Vandamme et al., 1989, 1990, 1991, 1992a, b, 1997, 2000), even for species such as Helicobacter cinaedi and Campylobacter hyointestinalis, where substantial 16S RNA gene sequence diversity has been observed (Vandamme et al., 2000). The correlation between protein pattern similarity and the level of DNA–DNA hybridization has also been demonstrated for the phylogenetic cluster comprising H. bizzozeronii, H. felis and H. salomonis (Jalava et al., 1998, 2001). Whole-cell protein electrophoresis has therefore been recommended as an alternative method for taxonomic discrimination at the species level in the recommended minimal standards for describing novel Helicobacter species (Dewhirst et al., 2000). The result of the numerical analysis of the protein profiles of strain JKM4T and its nearest cultured phylogenetic neighbours H. bizzozeronii, H. salomonis and H. felis is shown in Fig. 1.

The morphology of strain JKM4T was studied by means of scanning and transmission electron microscopy (SEM, TEM). For these examinations, bacterial cultures were negatively stained (Houf et al., 2005) or they were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer (0.1 M, pH 7.3). They were post-fixed in 1% (w/v) osmium tetroxide in distilled water. Samples for SEM were dehydrated in alcohol and acetone for subsequent drying.
Critical-point drying in liquid CO$_2$, glued with carbon cement on to aluminium stubs, sputtered with a gold layer and examined with a Philips 501 SEM. Samples for TEM were block-stained with 2 % (w/v) uranyl acetate in distilled water and dehydrated in ethanol. They were embedded in Epon–Spurr’s (1 : 1) medium. Ultrathin sections were cut from samples in which bacteria were demonstrated, stained with lead citrate and examined with a Philips EM 208S.

Cells of strain JKM4$^T$ were large spiral cells, 10–18 $\mu$m long and approximately 1 $\mu$m wide, with three to eight spirals per cell (see Supplementary Fig. S2 in IJSEM Online). The presence of sheathed flagella located slightly off-centre at both ends of the cells demonstrated the morphological resemblance of strain JKM4$^T$ to H. *salomonis*, H. *bizzozeronii* and H. *felis* (Utriainen et al., 1997). The flagella of strain JKM4$^T$ were blunt-ended and the terminal diameter was wider than the mean diameter of the flagellar body. Whereas periplasmic fibrils are thought to be a characteristic feature of H. *felis* (Utriainen et al., 1997), a periplasmic fibril running along the external side of the helix was detected on every cell of strain JKM4$^T$. Coccolid forms were observed in cultures older than 4 days, as are also found in older cultures of H. *salomonis*, H. *bizzozeronii* and H. *felis*. Ultrastructural characteristics were examined several times after several subcultures and were the same in all studies.

Biochemical and tolerance tests were carried out as recommended by Dewhirst et al. (2000). The novel isolate was tested for oxidase, catalase (with 3 % hydrogen peroxide) and rapid urease activities and for hydrolysis of indoxyl acetate. The strain was also subjected to API Campy test strips (bioMérieux), which include tests for urease activity, nitrate reductase activity, esterase activity, hippurate hydrolysis, $\gamma$-glutamyl transeptidase activity, alkaline phosphatase activity, triphenyltetrazolium chloride (TTC) reduction, pyrrolidonyl arylamidase and l-arginine and l-aspartate arylamidase activities. Tolerance of 1 % glycine (Merck) and 1·5 % NaCl (Merck) was tested on tryptic blood agar base (Oxoid) supplemented with 10 % horse blood, as recommended by the Cape Town protocol for Campylobacteriae and helicobacters. Tolerance of ox bile was tested by plating the bacteria on unsalted MacConkey agar (Oxoid). Growth was tested on BHI blood agar, *Brucella* blood agar (Oxoid) and Mueller–Hinton blood agar. Growth at 25, 30, 37 and 42 $^\circ$C was determined on BHI blood agar. All media were incubated for 7 days in a microaerobic atmosphere at 37 $^\circ$C. In addition, growth on blood-supplemented BHI agar was tested in aerobic, aerobic with 5 % CO$_2$, microaerobic and anaerobic atmospheres.

The salient tests that distinguish the novel isolate from other gastric helicobacters and from H. *canis* are listed in Table 1, while the other biochemical and growth characteristics are given in the description below.

Susceptibility to metronidazole, ampicillin, clarithromycin, tetracycline, enrofloxacin, lincomycin, tylosin, neomycin, spectinomycin and gentamicin was tested on Mueller–Hinton agar (Oxoid) supplemented with 10 % horse blood using the agar dilution method as previously described (Van den Bulck et al., 2005b). All antimicrobials were supplied by Sigma as standard powders with known potencies, except for enrofloxacin which was purchased from Bayer. These tolerance test preparations were incubated for 7 days in a microaerobic atmosphere at 37 $^\circ$C. The novel isolate was susceptible to all antimicrobials tested, as indicated by low MIC values, ranging from 0·03 to 0·25 $\mu$g ml$^{-1}$.

Although until now only H. *felis*, H. *bizzozeronii* and H. *salomonis* have been isolated from the gastric mucosa of dogs, evidence has been presented previously that indicates that a significant proportion of the spiral organisms in the canine and feline stomach is unculturable (Cattoli et al.,...
1999; Jalava et al., 1998). The present study demonstrates the existence of a fourth cultured Helicobacter species able to colonize the canine stomach, for which we propose the name Helicobacter cynogastricus sp. nov.

Following discussions held at the meetings of the International Committee on Systematics of Prokaryotes (ICSP) and its Judicial Commission (JC) in San Francisco in 2005, and in anticipation of the published minutes of the JC and ICSP, a committee consisting of the Chairman of the ICSP, the Chairman of the JC of the ICSP and the Editor of the International Journal of Systematic and Evolutionary Microbiology has granted an exception in this instance to Rule 27(3) of the Bacteriological Code governing the deposit of type material in two different collections in two different countries. Helicobacter cynogastricus sp. nov. IJM4T has been submitted to the CCUG and NCTC culture collections but, due to the extremely fastidious nature of Helicobacter species, the strain has not been able to be revived or stored after lyophilisation. Attempts are still underway to complete the deposition of the type strain in a second culture collection.

Description of Helicobacter cynogastricus sp. nov.

Helicobacter cynogastricus (cy.no.gas’tri.cus. N.L. gen. n. cyno dog, from Gr. n. kyon kyno dog; N.L. masc. adj. gastricus of the stomach, from Gr. n. gaster stomach; cynogastricus N.L. masc. adj. of a dog’s stomach).

Cells are tightly coiled spirals that are up to 1 μm wide by 10–18 μm long. They possess one periplasmic fibril running along the external side of the helix. In older cultures, coccoid cells predominate. Cells are Gram-negative and non-sporulating. They are motile by means of tufts of 6–12 sheathed flagella at one or both ends of the cell with a movement similar to that of H. felis and H. bizzozeronii. Growth on moist agar plates occurs as a spreading film or as small colonies. The type strain, IJM4T (= LMG 23188T), was isolated from the gastric mucosa of a dog.

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References


