Helicobacter heilmannii sp. nov., isolated from feline gastric mucosa

A. Smet, 1 B. Flahou, 1 K. D'Herde, 2 P. Vandamme, 3 I. Cleenwerck, 3 R. Ducatelle, 1 F. Pasmans 1† and F. Haesebrouck 1†

1Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium
2Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences, Ghent University, De Pintelaan 185, B-9000 Ghent, Belgium
3Department of Biochemistry and Microbiology, Faculty of Sciences, Ghent University, Ledeganckstraat 35, B-9000 Ghent, Belgium

Three Gram-negative, microaerophilic bacteria, strains ASB1 T, ASB2 and ASB3, with a corkscrew-like morphology isolated from the gastric mucosa of cats were studied using a polyphasic taxonomic approach. The isolates grew on biphasic culture plates under microaerobic conditions at 37 °C and exhibited urease, oxidase and catalase activities. They were also able to grow in colonies on dry agar plates. Based on 16S rRNA gene sequence analysis, ASB1 T, ASB2 and ASB3 were identified as members of the genus Helicobacter and showed 98 to 99 % sequence similarity to strains of Helicobacter felis, Helicobacter bizzozeronii, Candidatus Helicobacter heilmannii, Helicobacter cynogastricus, Helicobacter baculiformis and Helicobacter salomonis, six related Helicobacter species previously detected in feline or canine gastric mucosa. Sequencing of the partial hsp60 gene demonstrated that ASB1 T, ASB2 and ASB3 constitute a separate taxon among the feline and canine Helicobacter species. The urease gene sequences of ASB1 T, ASB2 and ASB3 showed approximately 91 % similarity to those of Candidatus Helicobacter heilmannii. Protein profiling, the absence of alkaline phosphatase activity and several other biochemical characteristics also allowed strains ASB1 T, ASB2 and ASB3 to be differentiated from other Helicobacter species of feline or canine gastric origin. The results of this polyphasic taxonomic study show that the cultured isolates constitute a new taxon corresponding to Candidatus Helicobacter heilmannii, which was previously demonstrated in the stomach of humans, wild felidae, cats and dogs. The name Helicobacter heilmannii sp. nov. is proposed for these isolates; the type strain is ASB1 T (=DSM 23983 T =LMG 26292 T).

1These authors contributed equally to this work.

Abbreviations: MALT, mucosa-associated lymphoid tissue; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of H. heilmannii ASB1 T, ASB2 and ASB3 are HM625820, HM625819 and HM625818, respectively; accession numbers for partial ureAB gene sequences of H. heilmannii ASB1 T, ASB2 and ASB3 are HM625826, HM625825 and HM625824, respectively; and accession numbers for hsp60 gene sequences of H. heilmannii ASB1 T, ASB2 and ASB3 are HM625823, HM625822 and HM625821, respectively.

A supplementary figure is available with the online version of this paper.

Long, spiral-shaped bacteria belonging to the genus Helicobacter have been demonstrated in the gastric mucosa of man and several animal species (Haesebrouck et al., 2009). Helicobacter pylori is the most common and best known gastric Helicobacter species in humans. Recently, a large number of gastric non-H. pylori Helicobacter species, provisionally named ‘Helicobacter heilmannii’ and naturally colonizing the stomach of animals, have also been described in humans. Sequence analysis of 16S rRNA genes detected in ‘H. heilmannii’-positive gastric biopsies revealed the presence of two sequence types. This has led to the subclassification of these non-H. pylori helicobacters into ‘Helicobacter heilmannii’ type 1 and ‘Helicobacter heilmannii’ type 2. ‘H. heilmannii’ type 1 represents a single Helicobacter species, namely Helicobacter suis. ‘H. heilmannii’ type 2 represents a group of species, including Helicobacter felis, Helicobacter bizzozeronii, Helicobacter salomonis and Candidatus Helicobacter heilmannii (Baele et al., 2008a; Haesebrouck et al., 2009).

The first Helicobacter species isolated from the stomach of cats and dogs was H. felis (Lee et al., 1988). Subsequently, H. bizzozeronii, H. salomonis, Helicobacter baculiformis and Helicobacter cynogastricus were also isolated from feline and dog species previously detected in feline or canine gastric mucosa.
canine gastric mucosa (Baele et al., 2008b; Hänninen et al., 1996; Happonen et al., 1996; Jalava et al., 1998, 2001; Van den Bulck et al., 2006). H. cynogastricus and H. ballociforhmis have not yet been detected in the human gastric mucosa. A sixth long spiral-shaped Helicobacter species has been detected in wild feline and human, as well as in canine and feline, gastric biopsies (Neiger et al., 1998; Hwang et al., 2002; O’Rourke et al., 2004b); it could not be cultured in vitro and was provisionally named Candidatus H. heilmannii (O’Rourke et al., 2004b). Based on 16S rRNA gene sequence analysis, all these species are phylogenetically highly related to each other (Solnick et al., 1993). The similarity between their urease genes (ureAB) is, however, lower than 85 %, allowing discrimination between these species (O’Rourke et al., 2004b). The uncultured Candidatus H. heilmannii was found with a prevalence ranging from 20 to 100 % in the gastric mucosa of both cats and dogs (Haesebrouck et al., 2009; Hwang et al., 2002; Neiger et al., 1998; Van den Bulck et al., 2005). It was detected in 8–19 % of gastric biopsy samples of humans with histological evidence of a non-H. pylori Helicobacter infection (Haesebrouck et al., 2009; Trebesius et al., 2001; Van den Bulck et al., 2005). Moreover, Candidatus H. heilmannii has been propagated in mice for up to 28 months and was able to induce mucosa-associated lymphoid tissue (MALT) lymphomas in the stomachs of these animals (O’Rourke et al., 2004a).

In this study, we describe the successful isolation of Candidatus H. heilmannii in vitro and the characterization of this species using a polyphasic taxonomic study.

Strain ASB1T was isolated from the mucosa of the stomach of a cat euthanized at a shelter for homeless cats, Sint-Niklaas, Belgium. Two other strains, designated ASB2 and ASB3, were isolated from the mucosa of the stomachs of cats (positive for feline immunodeficiency virus) euthanized at the faculty of Veterinary Medicine, Ghent University, Belgium.

The stomachs of these three cats were submersed in a 1 % HCl bath for 1 h (Gruntar et al., 2003). The mucus was scraped off using a glass slide and collected in a sterile tube. The mucus was used to inoculate Brucella agar plates supplemented with 20 % (v/v) fetal calf serum, 5 mg amphotericin B 1 ml (Fungizone; Bristol-Myers Squibb), Campylobacter-selective supplement [Skirrow, containing (l−1) 10 mg vancomycin, 5 mg trimethoprim lactate and 2500 U polymyxin B; Oxoid], Vitox supplement (Oxoid), 0.1 % activated charcoal and approximately 0.05 % HCl to obtain a pH of 5. The mucus on these agar plates was scraped off using a glass slide and collected in a sterile tube. Growth of subcultures occurred as a spreading layer on moist agar plates. Bacterial cells were harvested in Brucella broth and stored at −70 °C in a medium consisting of 7.5 g glucose, 25 ml Brucella broth and 75 ml sterile inactivated fetal calf serum.

Genomic DNA of isolates ASB1T, ASB2 and ASB3 was extracted using PrepMan sample preparation reagent from Applied Biosystems as described by the manufacturer.

The 16S rRNA gene was amplified using the commercially available Qiagen Taq Mastermix and primers 5'-NOT (5'-TCAACTAGGACCGAGTC-3') and oMB (5'-TACCTTGGTACTTCAACCCA-3') as described by Baele et al. (2001). PCR products were sequenced using the BigDye Terminator sequencing kit (Applied Biosystems) and primers pD, Gamma*, 3 and O* (Coenye et al., 1999). Sequences were determined on an automatic DNA sequencer (ABI Prism 3100 Genetic analyser; Applied Biosystems) and electropherograms were exported and converted to the VectorNTI software (Invitrogen). Sequences were compared with those in NCBI/GenBank by using the BLAST search tool. All Helicobacter species with validly published names (http://www.bacterio.cict.fr/h/helicobacter.html) were included for phylogenetic analysis. Phylogenetic analysis was performed using CLUSTAL W, BioEdit and Jalview software tools. Multiple alignment was determined using CLUSTAL W with an open gap penalty of 100 % and a unit gap penalty of 0 %. A phylogenetic tree, with H. pylori as outgroup, was constructed using the neighbour-joining method (Fig. 1).

The 16S rRNA gene sequences of strains ASB1T, ASB2 and ASB3 showed more than 98 % sequence similarity with each other and with a sequence from GenBank (accession no. AF506786) originating from Candidatus H. heilmannii detected in human gastric mucosa (O’Rourke et al., 2004b). The most closely related organisms were strains of Candidatus H. heilmannii, H. felis, H. bizzozeronii, H. salomonis and H. suis, with sequence similarities ranging from 93 to 98 % to the novel strains.

Sequence analysis of the urease gene has been found to be more discriminatory than the 16S rRNA gene for species differentiation between gastric Helicobacter species of animal origin (O’Rourke et al., 2004b). Therefore, the sequences of partial fragments of the ureA and ureB genes, including a spacer region, were determined after amplification using primers U430F and U1735R (1224 bp amplicon) (O’Rourke et al., 2004b). Sequences were compared with those in...
NCBI/GenBank by using the BLAST search tool. Based on the phylogenetic tree, reconstructed from genetic distances, strains of the most closely related *Helicobacter* species were included for phylogenetic analysis of the *ureAB* gene. Phylogenetic analysis was performed using the same software tools as described for the 16S rRNA gene. A phylogenetic tree, with *H. pylori* as outgroup, was constructed using the neighbour-joining method (Fig. 2).

Isolates ASB1<sup>T</sup>, ASB2 and ASB3 showed 98 % similarity with each other and 91 % similarity with *ureAB* gene sequences from ‘*Candidatus* H. heilmanni’ strains, detected in human and wild feline gastric mucosa and previously deposited in GenBank (O’Rourke et al., 2004b). Moreover, these three isolates clustered with the ‘*Candidatus* H. heilmanni’ strains (Fig. 2). Other phylogenetic neighbours were strains of *H. bizzozeronii* (about 84 % similarity), *H. suis* (about 82 % similarity), *H. felis* (about 76 % similarity), *H. cynogastricus* (about 76 % similarity) and *H. salomonis* (about 75 % similarity).

Mikkonen et al. (2004) showed that conserved partial 60 kDa heat-shock protein (HSP60) gene sequences give additional phylogenetic information that is useful for differentiating *Helicobacter* species. The *hsp60* gene sequences of the ‘*Candidatus* H. heilmanni’ strains described by O’Rourke et al. (2004b) are not available from GenBank. A 550 bp sequence was obtained for strains ASB1<sup>T</sup>, ASB2 and ASB3 using the methodology as described by Mikkonen et al. (2004). Sequences were compared with those in NCBI/GenBank by using the BLAST search tool. Based on the phylogenetic tree, reconstructed from genetic distances, the most closely related *Helicobacter* species were included for phylogenetic analysis of the *hsp60* gene. Phylogenetic analysis was performed using the same software tools as described for the 16S rRNA gene. A phylogenetic tree, with *H. pylori* as outgroup, was constructed using the neighbour-joining method (Fig. 3).

The partial *hsp60* gene sequences of ASB1<sup>T</sup>, ASB2 and ASB3 showed approximately 95 % sequence similarity with each other. Gene sequence similarities of 85–86, 84–86, 84, 83 and 81 % were obtained with strains of *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis* and *H. suis*, respectively, yielding sufficient difference to consign isolates ASB1<sup>T</sup>, ASB2 and ASB3 to a new taxon.

**Fig. 2.** A phylogenetic tree, reconstructed from genetic distances, based on partial *ureA* and *ureB* gene sequences of *H. heilmanni* sp. nov. and other urease-positive gastric *Helicobacter* species. Bootstrap values are indicated. *H. pylori* strains DA, 26695 and OB were used as the outgroup. Bar, 0.1 substitutions per position.
PAGE of whole-cell proteins of strains ASB1T, ASB2 and ASB3 and of H. pylori, H. bizzozeronii, H. felis, H. salomonis, H. cynogastricus, H. baculiformis and H. suis reference strains (Jalava et al., 1998, 2001; Van den Bulck et al., 2006) was performed in order to establish the distinct taxonomic status of the novel isolates among cultured species of the genus Helicobacter. For this purpose, strains were grown on Brucella agar supplemented with 20% fetal calf serum, 5 mg amphotericin B l−1, Campylobacter-selective supplement, Vitox supplement and approximately 0.05% HCl to obtain a pH of 5. Plates were incubated at 37 °C in a microaerobic atmosphere as described above. Whole-cell protein extracts were prepared and SDS-PAGE was performed as described previously (Pot et al., 1994). Sample preparation and SDS-PAGE using Criterion XT 12% (w/v, acrylamide) Bis-Tris precast gels with XT MOPS denaturing running buffer were carried out according to the manufacturer’s instructions (Bio-Rad), but without heating the samples before loading. Staining was performed with Bio-Safe Coomassie stain, according to the manufacturer’s instructions (Bio-Rad).

Visual and numerical analysis of the protein profiles demonstrated that strains ASB1T, ASB2 and ASB3 can be clearly distinguished from strains of their closest phylogenetic neighbours (Supplementary Fig. S1, available in IJSEM Online). These differences are not limited to one or a few bands, but are apparent in the entire profile.

The morphology of strains ASB1T, ASB2 and ASB3 was studied by means of transmission electron microscopy (TEM) as described by Houf et al. (2005) and Mast et al. (2005) (Fig. 4).

Fig. 3. A phylogenetic tree, reconstructed from genetic distances, based on partial hsp60 gene sequences of H. heilmannii sp. nov. and other gastric Helicobacter species. H. pylori strains Tx30a and 7546 were used as the outgroup. Bootstrap values are indicated. Bar, 0.1 substitutions per position.
Isolates ASB1ᵀ, ASB2 and ASB3 presented tightly coiled spiral-shaped cells with up to nine turns that were approximately 3.0–6.5 μm long and 0.6–0.7 μm wide (Fig. 4). The length and width were variable depending on the state of contraction. No periplasmic fibrils were observed and coccoid cells predominated in older cultures. Up to 10 sheathed blunt-ended flagella were found at both ends.

According to the recommendations of Dewhirst et al. (2000), biochemical and tolerance tests were carried out. Growth of strains ASB1ᵀ, ASB2 and ASB3 was determined on Brucella agar plates supplemented with 20% fetal calf serum, 5 mg amphotericin B l⁻¹, Campylobacter-selective supplement, Vitox supplement and 0.05% HCl to obtain a pH of 5 at 25, 37 and 42 °C under microaerobic conditions and at 37 °C under aerobic, anaerobic and microaerobic conditions. Tolerance to 1% bile, 1% glycerol and 1.5% NaCl was determined on Brucella agar plates with the same supplements as described above. Growth was also studied on BHI agar, Brucella agar and Mueller–Hinton agar (Oxoid), supplemented with 20% fetal calf serum or 10% defibrinated horse blood, Vitox and Skirrow supplements, amphotericin B and HCl to give a pH of 5. Plates were incubated for several days in a microaerobic atmosphere at 37 °C. Cells were also able to grow in colonies on dry agar plates.

Catalase activity of the isolates was examined by adding a 3% H₂O₂ solution and observing the reaction within 5 s. Oxidase activity was performed with Bactident Oxidase strips (Merck). The following characteristics were studied using the API Campy identification system (bioMérieux): urease activity, reduction of nitrate, esterase activity, hydrolysis of hippurate, γ-glutamyltransferase activity, reduction of triphenyl-tetrazolium chloride, alkaline phosphatase activity, and pyrrolidonyl-L-arginine and L-aspartate arylamidase activities. Tests were read after 24 h of incubation at 37 °C in an aerobic atmosphere.

Growth of the three isolates was observed on Mueller–Hinton II agar plates supplemented with 5 μg metronidazole ml⁻¹ and 10% horse blood. Conditions for growth are listed in the species description below and a comparison of the most important phenotypic characteristics of strains ASB1ᵀ, ASB2 and ASB3 with those of other gastric species of the genus Helicobacter is shown in Table 1. The absence

**Fig. 4.** TEM images of cells of *H. heilmannii* strain ASB1ᵀ. (a) Negatively stained cell; (b) negatively stained cell showing bipolar flagella; (c) TEM image showing an unusually long cell with up to nine turns; (d) negatively stained cell with blunt-ended flagella; (e) TEM image showing a cross section of the flagella (arrow); (f) negatively stained cell showing sheathed flagella. Bars: (a) 2 μm; (b, c, f) 1 μm; (d) 500 nm; (e) 200 nm.
of alkaline phosphatase activity and several other characteristics allowed strains ASB1T, ASB2 and ASB3 to be differentiated from their closest phylogenetic neighbours.

In conclusion, phylogenetic analysis of the 16S rRNA, ureAB and hsp60 genes and whole-cell protein electrophoresis revealed that strains ASB1T, ASB2 and ASB3 represent a novel species within the phylogenetic lineage that currently consists of \(H. \) felis, \(H. \) bizzozeronii, \(H. \) salomonis, \(H. \) baculiformis, \(H. \) cynogastricus and \(H. \) suis.

**Description of Helicobacter heilmannii sp. nov.**

*Helicobacter heilmannii* [heilmann’ni. N.L. gen. masc. n. heilmannii of Heilmann, in honour of Konrad Heilmann who described the first large case study of gastrospirilla infections in humans (Heilmann & Borchard, 1991)].

Cells are tightly coiled spirals with up to nine turns, approximately 3.0–6.5 \(\mu\)m long and 0.6–0.7 \(\mu\)m wide. No periplasmic fibrils are observed and cocoid cells predominate in older cultures. Cells are motile by means of tufts of up to 10 sheathed blunt-ended flagella at both ends of the cells. Cells are Gram-negative and non-sporulating. Growth is observed on BHI agar, on *Brucella* agar and on Mueller–Hinton agar supplemented with 20% fetal calf serum or 10% defibrinated horse blood. Cells are also able to grow in colonies on dry agar plates. Growth is detected at 37 °C, but not at 25 or 42 °C. No growth on media supplemented with 1% bile, 1.5% NaCl or 1% glycine. Oxidase-, catalase- and urease-positive. Reduces triphenyl-tetrazolium chloride and nitrate, hydrolyses hippurate and is positive for esterase, \(\gamma\)-glutamyltransferase and L-arginine arylamidase. Pyrrolidonyl arylamidase, L-aspartate arylamidase, indoxyl acetate hydrolysis and alkaline phosphatase are not detected. Its clinical significance in cats is unknown. *H. heilmannii*, as well as other gastric non-*H. pylori* Helicobacter species, has been associated with gastritis, gastric and duodenal ulcers and low grade MALT lymphoma of the stomach in humans (*Haesebrouck et al., 2009*). *H. heilmannii* has been shown to induce MALT lymphomas when propagated in mice for up to 28 months (*O’Rourke et al., 2004a*).

The type strain is ASB1T (=DSM 23983T=LMG 26292T), isolated from the gastric mucosa of a cat.

**Acknowledgements**

This work was supported by the Research Fund of Ghent University, Belgium, Code GOA01G00408. The authors are very grateful to Sofie De Bruyckere and Dominique Jacobus for their excellent technical assistance.

**References**


