Helicobacter equorum sp. nov., a urease-negative *Helicobacter* species isolated from horse faeces

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Gram-negative, curved, motile bacteria (strains EqF1^T and EqF2) were isolated from faecal samples from two clinically healthy horses. Both strains possessed a single, monopolar, sheathed flagellum and were urease-negative. The novel strains grew at 37 °C under microaerobic conditions and were positive for oxidase, catalase and alkaline phosphatase activities. The isolates reduced nitrate to nitrite, but γ -glutamyl transpeptidase activity was not detected. The novel isolates did not grow at 42 °C or on media containing 1 % glycine. They were resistant to cephalotin and nalidixic acid and susceptible to metronidazole. Analysis of the 16S and 23S rRNA gene sequences of the two novel strains identified them as representing a single species within the genus *Helicobacter*. In terms of 16S rRNA gene sequence similarity, *Helicobacter pullorum* and *Helicobacter canadensis* were the most closely related species (98 % similarity). 23S rRNA gene sequence analysis also classified strains EqF1^T and EqF2 within the enterohepatic division of the genus *Helicobacter*, but only 94 % similarity was detected with *H. pullorum* and *H. canadensis*, which are helicobacters with unsheathed flagella. The most closely related species in terms of 23S rRNA gene sequence similarity was *Helicobacter canis* (95 %). Numerical analysis of whole-cell protein extracts by SDS-PAGE was performed and the novel isolates were clearly differentiated from *H. pullorum*, *H. canadensis*, *H. canis* and other species of the genus *Helicobacter*. This finding was also confirmed by sequence analysis of the *hsp60* gene. On the basis of these genetic, biochemical and protein data, the isolates are classified as representing a novel species, for which the name *Helicobacter equorum* sp. nov. is proposed (type strain EqF1^T=LMG 23362^T=CCUG 52199^T).

The genera *Helicobacter*, *Campylobacter*, *Arcobacter* and *Wolinella* belong phylogenetically to the *Epsilonproteobacteria*, formerly known as the ϵ -subclass of the *Proteobacteria* (Murray *et al.*, 1990). Over the last decade, species of the genus *Helicobacter* have been described in a wide variety of animal hosts, including wild birds, chickens, dogs, cats, cattle, sheep, swine, rodents, non-human primates, cheetahs, ferrets, rabbits, dolphins and whales (Whary & Fox, 2004; Van den Bulck *et al.*, 2005). Dimola &

Caruso (1999) reported the presence of rod-shaped organisms with a morphology similar to that commonly reported for *Helicobacter pylori* in the stomach of 15 horses. However, after this single preliminary report, horses have never been confirmed as hosts for *Helicobacter*-like organisms. Here we report the isolation of a novel *Helicobacter* species from horse faeces.

Two bacterial strains, designated EqF1^T and EqF2, were isolated from faecal samples from a 3- and a 4-year-old horse, respectively. Both horses were clinically healthy and were stalled at the Faculty of Veterinary Medicine, Ghent University, Belgium. Samples were inoculated on brain heart infusion (BHI) agar (Oxoid), supplemented with 10 % horse blood, 15 $\mu\text{g ml}^{-1}$ amphotericin B (Fungizone, Bristol-Myers Squibb) and Vitox (Oxoid) by using the filter technique of Steele & McDermott (1984). Plates were incubated, lids uppermost, at 37 °C under microaerobic conditions (5 % H₂, 5 % CO₂, 5 % O₂ and 85 % N₂) and examined daily. After 3–4 days incubation, growth

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, 23S rRNA and *hsp60* gene sequences of strain EqF1^T are DQ307735, DQ307737 and DQ888713, respectively. The accession numbers for the 16S rRNA, 23S rRNA and *hsp60* gene sequences of strain EqF2 are DQ307736, DQ307738 and DQ888714, respectively.

Figures showing transmission electron microscope images of cells of strains EqF1^T and EqF2 and phylogenetic trees based on 16S rRNA, 23S rRNA and *hsp60* gene sequences are available as supplementary material in IJSEM Online. Distance matrix analyses for the three genes are available as supplementary tables.

appeared as a thin, transparent, spreading film and microscopic examination revealed the presence of spiral, motile bacteria. In cultures of more than 5 days, coccoid forms predominated.

Growth of both strains was examined at 25, 37 and 42 °C under microaerobic conditions and at 37 °C under aerobic, microaerobic and anaerobic conditions. Tolerance of 1 % bile, 1 % glycine and 1.5 % NaCl was determined as previously described (On & Holmes, 1991). The isolates were Gram-stained and examined for catalase activity by standard microbiological methods (Wolf *et al.*, 1975). Oxidase activity was determined with Bactident oxidase strips (Merck). The following biochemical analyses were performed with the API-Campy identification system (bioMérieux): urease activity, reduction of nitrates, esterase activity, hydrolysis of hippurate, γ -glutamyl transferase activity, reduction of triphenyl tetrazolium chloride, alkaline phosphatase activity, production of H₂S, assimilation of glucose and pyrrolidonyl-, L-arginine- and L-aspartate arylamidase activities. Indoxyl acetate hydrolysis was determined as previously described by Mills & Gherna (1987). Susceptibility to cephalotin (30 µg per disc) and nalidixic acid (30 µg per disc) (Becton Dickinson) was examined by culturing the organisms in the presence of discs impregnated with the antimicrobial agent as described by Melito *et al.* (2001). Melito's technique was slightly modified in that plates were incubated for 72 h before examination instead of 48 h. Growth in the presence of metronidazole was determined with BHI agar (Oxoid) containing 10 % horse blood and 5 µg ml⁻¹ of the test compound. DNase activity was examined as described by Devriese & Oeding (1975). All tests were performed in two time-independent assays with freshly prepared media.

A detailed list of results is given in the species description below and a comparison of the most important phenotypic characteristics of the novel strains with those of other *Helicobacter* species is shown in Table 1.

The morphology of strains EqF1^T and EqF2 was studied by means of transmission electron microscopy after negative staining with 2 % uranyl acetate as described by Houf *et al.* (2005) and after ultrathin sectioning as described by Mast *et al.* (2005).

Cells appeared as slightly curved rods, 1.5–4 µm in length and 0.3 µm in width. As seen for *Helicobacter pullorum* and most of the *Helicobacter cinaedi* strains, cells of the novel strains carried a single flagellum. In contrast with *H. pullorum*, strains EqF1^T and EqF2 possess sheathed flagella. No periplasmic fibril was detected. Coccoid forms were also observed. Electron micrographs of cells of strain EqF1^T are available as Supplementary Fig. S1a–d in IJSEM Online.

DNA was extracted from a loopful of cells using the alkaline lysis method of Baele *et al.* (2000). To determine if the strains were members of the genus *Helicobacter*, bacterial DNA was amplified by PCR using the *Helicobacter* genus-specific

primers described by Germani *et al.* (1997) and analysed by gel electrophoresis. For both isolates, a 399 bp fragment was obtained, indicating that they are members of the genus *Helicobacter*.

PCR amplification of the 16S rRNA gene was performed using primers $\alpha\beta$ -NOT and ω_{MB} and amplicons were sequenced using primers pD, Gamma*, 3 and O* (Coenye *et al.*, 1999) as described previously (Baele *et al.*, 2001). The sequences were compared with entries in the NCBI GenBank using the BLAST search tool. Fragments of 1454 and 1343 bp were obtained that showed 98 % gene sequence similarity to *H. pullorum* and *Helicobacter canadensis*. Phylogenetic analysis was performed with KODON software (Applied Maths). A comparison of the 16S rRNA gene sequences of strains EqF1^T and EqF2 with those of other *Helicobacter* species is shown in a distance matrix (see Supplementary Table S1 in IJSEM Online). A phylogenetic tree obtained from this matrix using the neighbour-joining method is also available in IJSEM Online as Supplementary Fig. S2.

In the past, several studies have revealed that the comparison of 16S rRNA gene sequences alone is not sensitive enough to identify strains to the species level (Stackebrandt & Goebel, 1994; Jalava *et al.*, 1997; Vandamme *et al.*, 2000). Dewhirst *et al.* (2005) recently suggested that 16S rRNA gene sequence data do not always faithfully reflect phylogenetic relationships and that 23S rRNA gene sequence data are significantly more reliable for the identification and classification of helicobacters due to the threefold-higher number of informative bases. For strains EqF1^T and EqF2, *H. pullorum* and *H. canadensis* were the most closely related species in terms of 16S rRNA gene sequence similarity (98 %). However, strains EqF1^T and EqF2 possess a single, monopolar, sheathed flagellum, while *H. pullorum* and *H. canadensis* are unsheathed flagellum-bearing helicobacters (Dewhirst *et al.*, 2005). Therefore, we also amplified the 23S rRNA genes of strains EqF1^T and EqF2 using PCR primers O68 and M89; amplicons were sequenced by using the full set of 20 primers as described by Dewhirst *et al.* (2005). The sequences obtained were compared with sequences in the NCBI GenBank database using the BLAST search tool. Fragments of 2728 and 2712 bp were obtained which showed only 94 % gene sequence similarity to *H. pullorum* and *H. canadensis*. The most closely related species in terms of 23S rRNA gene sequence similarity was *Helicobacter canis* (95 %). A distance matrix calculated using KODON software (Applied Maths) is shown in Supplementary Table S2 in IJSEM Online. A phylogenetic tree obtained from this matrix using the neighbour-joining method is also available as Supplementary Fig. S3).

Since the 16S and 23S rRNA gene sequence phylogenies for strains EqF1^T and EqF2 were contradictory, a third phylogenetically informative gene, the *hsp60* gene, was sequenced as described previously (Mikkonen *et al.*, 2004). The *hsp60* sequences were compared with those in the NCBI GenBank database using the BLAST search tool. Fragments of 518 bp were obtained which showed only 74 % and 78 %

Table 1. Phenotypic characteristics that differentiate *Helicobacter equorum* sp. nov. from other *Helicobacter* species

Taxa: 1, *H. equorum* sp. nov.; 2, *H. pullorum*; 3, *H. canadensis*; 4, *H. pylori*; 5, *H. mustelae*; 6, *H. felis*; 7, *H. fennelliae*; 8, *H. cinaedi*; 9, *H. nemestrinae*; 10, *H. muridarum*; 11, *H. acinonychis*; 12, *H. canis*; 13, *H. hepaticus*; 14, *H. pametensis*; 15, *H. bilis*; 16, *H. bizzozeronii*; 17, *H. trogonum*; 18, *H. cholecystus*; 19, *H. rodentium*; 20, *H. salomonis*; 21, *H. mesocricetorum*; 22, *H. ganmani*; 23, *H. aurati*; 24, *H. typhlonius*; 25, *H. cynogastricus*. Data were obtained from this study, Dewhirst *et al.* (2000), Simmons *et al.* (2000), Fox *et al.* (2000), Patterson *et al.* (2000), Robertson *et al.* (2001), Franklin *et al.* (2001) and Van den Bulck *et al.* (2006). +, 100% strains positive; –, 100% strains negative; (+), 80–94% strains positive; v, 42–66% strains positive; (–), 7–33% strains positive; S, sensitive; R, resistant; I, intermediate; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Catalase production	+	+	+	+	+	+	(+)	(+)	+	+	+	–	+	(+)	+	+	+	+	+	+	+	(–)	+	+	+
Nitrate reduction	+	+	v	–	+	+	–	+	–	–	–	–	+	+	+	+	+	+	+	+	(+)	+	–	v	+
Urease	–	–	–	+	+	(+)	–	–	+	+	+	–	+	–	+	(+)	+	–	–	+	–	–	+	–	+
Alkaline phosphate hydrolysis	+	–	–	+	+	v	(–)	(–)	+	+	+	+	–	(+)	–	v	–	+	–	v	+	–	–	–	+
γ-Glutamyl transpeptidase	–	ND	–	+	+	+	–	–	ND	+	+	+	–	–	+	+	+	–	–	+	–	ND	+	–	+
Indoxyl acetate hydrolysis	–	–	+	(–)	+	(–)	+	(–)	–	–	(–)	+	+	–	–	(–)	–	–	–	(–)	ND	–	+	–	–
Growth at 42 °C	–	+	+	(–)	v	v	(–)	v	+	–	(–)	+	–	+	+	v	+	+	+	–	+	–	+	+	–
Growth on 1% glycine	–	–	+	–	–	–	–	–	–	–	–	–	+	v	+	(–)	ND	+	+	–	–	–	–	+	–
Susceptibility to:																									
Nalidixic acid (30 µg)	R	R	R	R	S	R	S	S	R	R	R	S	R	S	R	R	R	I	R	R	S	S	S	S	ND
Cephalotin (30 µg)	R	S	R	S	R	S	S	I	S	R	S	I	R	S	R	S	R	R	R	S	R	R	R	R	ND
Periplasmic fibril	–	–	–	–	–	+	–	–	–	+	–	–	–	–	+	–	+	–	–	–	–	–	+	–	+
No. flagella per cell	1	1	1–2	4–8	4–8	14–20	2	1–2	4–8	10–14	2–5	2	2	2	3–14	10–20	5–7	1	2	10–23	2	2	7–10	2	6–12
Sheathed flagella	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No	Yes	Yes	Yes
Distribution of flagella*	M	M	B	B	P	B	B	B	B	B	B	B	B	B	B	B	B	M	B	B	B	B	B	B	B

*B, Bipolar; M, monopolar; P, peritrichous.

Fig. 1. Dendrogram derived from the numerical analysis of the whole-cell protein profiles of strains EqF1^T and EqF2 and other *Helicobacter* reference strains.

gene sequence similarity to *H. pullorum* and *H. canis*, respectively. The most closely related species in terms of *hsp60* gene sequence similarity was *Helicobacter hepaticus* (85 %). A distance matrix, calculated using KODON software, is shown in Supplementary Table S3 in IJSEM Online. A phylogenetic tree obtained from this matrix using the neighbour-joining method is also available as Supplementary Fig. S4.

The taxonomic position of strains EqF1^T and EqF2 was further investigated by whole-cell protein profiling, a technique that is considered a good alternative for DNA–DNA hybridization experiments in the genus *Helicobacter* (Dewhirst *et al.*, 2000; Vandamme *et al.*, 2000). Whole-cell protein extracts of strains EqF1^T and EqF2 were prepared and one-dimensional SDS-PAGE was performed as described previously (Pot *et al.*, 1994). Whole-cell protein profiles of *H. pullorum*, *H. canadensis*, *H. canis* and other *Helicobacter* species were available from previous studies. Densitometric analysis, normalization and interpolation of the protein profiles and numerical analysis were performed using GelCompar version 4.2 software (Applied Maths). The similarity between all pairs of traces was expressed by using the Pearson product-moment correlation coefficient presented as percentages of similarity. A dendrogram derived from the numerical analysis of the protein profiles of both novel strains and of *Helicobacter* reference strains is shown in Fig. 1. The whole-cell protein profile of strains EqF1^T and EqF2 differed significantly from those of *H. pullorum*, *H.*

canadensis, *H. canis* and other *Helicobacter* reference strains (the profiles of the *H. equorum* strains cluster at a correlation level of about 71 % with those of several other *Helicobacter* species).

To determine the DNA G + C content of strain EqF1^T, DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated using a Waters Breeze HPLC system and XBridge Shield RP18 column thermostabilized at 37 °C. The solvent was 0.02 mol l⁻¹ NH₄H₂PO₄ (pH 4.0) with 1.5 % (v/v) acetonitrile. Non-methylated λ-phage (Sigma-Aldrich) and *Escherichia coli* DNA were used as the calibration reference and control, respectively. The DNA G + C content was determined as 38.5 mol%.

Both novel strains were tested for the presence of a cytolethal distending toxin (*cdtB*) encoding gene by using the degenerative primers VAT2, WMI1 and DHF1 as described by Chien *et al.* (2000). DNA from *H. hepaticus* LMG 16316^T was used as a positive control. For strains EqF1^T and EqF2, the PCR did not produce the amplicons expected.

The data from the present study indicate that strains EqF1^T and EqF2 represent a single novel taxon within the genus *Helicobacter*, for which the name *Helicobacter equorum* sp. nov. is proposed. Analysis of three phylogenetic markers, 16S and 23S rRNA and *hsp60* genes, revealed no specific relationship between this taxon and any of the recognized

Helicobacter species. Whole-cell protein electrophoresis, a technique known to correlate with whole genome DNA–DNA hybridization, further demonstrated that there is no specific resemblance between the profiles of these strains and those of established *Helicobacter* species, indicating that the isolates represent a novel species. Biochemically, this novel species is most similar to *H. cinaedi* with hydrolysis of alkaline phosphatase being the best test to differentiate these species (Table 1). This is the first formal description of a *Helicobacter* species isolated from horse faeces. Further studies are necessary to define the natural habitat and host range of *Helicobacter equorum* sp. nov. and to determine its clinical significance and zoonotic potential.

Description of *Helicobacter equorum* sp. nov.

Helicobacter equorum (eq.uo'rum. L. gen. pl. n. *equorum* of horses, from where the type strain was isolated).

Cells are motile, non-spore-forming, curved, Gram-negative rods (1.5–4 µm × 0.3 µm) and carry a single, sheathed monopolar flagellum. Cells do not possess a periplasmic fibril. A thin, transparent spreading film is seen after 3–4 days growth on 10 % horse blood agar. Single colonies are not seen. Coccoid cells predominate in older cultures. Grows microaerobically at 37 °C; no growth is obtained either in aerobic or anaerobic conditions at 37 °C, or at 25 °C or 42 °C under microaerobic conditions. Produces oxidase, catalase, alkaline phosphatase, esterase and L-arginine arylamidase. Reduces nitrate. No urease, DNase, γ-glutamyl transferase, hippuricase, pyrrolidonyl arylamidase or L-aspartate arylamidase activity is detected. Indoxyl acetate is not hydrolysed. Triphenyl tetrazolium chloride is not reduced. Neither hydrogen sulphide nor acid are produced from glucose fermentation. No growth is obtained on media containing 1 % glycine, 1 % ox bile, 1.5 % NaCl or 5 µg metronidazole ml⁻¹. Resistant to cephalotin and nalidixic acid. The G + C content of the genomic DNA is 38.5 mol%.

The type strain, EqF1^T (=LMG 23362^T=CCUG 52199^T), was isolated from horse faeces in Merelbeke, Belgium. The clinical significance of *H. equorum* is unknown.

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