International Journal of Systematic Bacteriology (1999), **49**, 1707–1715 Printed in Great Britain

Phylogenetic characterization of '*Candidatus* Helicobacter bovis', a new gastric helicobacter in cattle

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Recently helicobacter-like organisms have been reported in the pyloric part of the abomasum of calves and adult cattle. Cultivation of these spiral bacteria has not been successful to date. In the present study, comparative 16S rDNA sequence analysis was used to determine the taxonomic position of these bacteria. Seven abomasal biopsies of adult cattle were sampled from different Belgian and Dutch farms. In all samples the presence of helicobacter-like organisms was demonstrated by biochemical, immunohistochemical and electron microscopical data. Bacterial 16S rDNA was amplified by PCR and sequences were determined either by direct or indirect sequence analysis. Pairwise comparisons revealed all sequences to be more than 99% homologous. Phylogenetic analysis placed the organism, corresponding to the reference sequence R2XA, within the genus *Helicobacter*. A diagnostic PCR assay was designed, differentiating all of the bovine 16S rDNA sequences from *Helicobacter* and *Wolinella* species. The low similarity level towards *Helicobacter bilis* (92·8%), its closest validly named neighbour, indicates that this novel taxon is indeed a novel *Helicobacter* species. An *in situ* hybridization procedure associated the bovine sequences to the helicobacter-like organisms in the abomasum. The name '*Candidatus* Helicobacter bovis' is proposed for this new abomasal helicobacter from cattle.

Keywords: '*Candidatus* Helicobacter bovis', cattle, abomasum, 16S rDNA, taxonomy

INTRODUCTION

In the 19th century, gastric spiral organisms were described for the first time in different animals (Rappin, 1881; Bizzozero, 1893; Salomon, 1896). Salomon observed spiral organisms in the stomachs of dogs, cats and the brown Norwegian rat, but not in humans, monkeys, pigs, mice, pigeons, crows and cattle (Salomon, 1896). It was only in 1984 that a renewed interest emerged for similar organisms after the isolation of *Helicobacter pylori* from the human stomach (Marshall & Warren, 1984). The association of *H. pylori* with chronic gastritis, peptic ulceration and gastric cancer (Cover & Blaser, 1992; Blaser *et al.*, 1991; Parsonnet *et al.*, 1991) resulted in intensive research worldwide. Various *Helicobacter* species were isolated from the gastrointestinal tract of different animals. To date, the genus *Helicobacter* consists of 18 different species (On, 1996; Franklin *et al.*, 1996; Mendes *et al.*, 1996; Jalava *et al.*, 1997; Trivett-Moore *et al.*, 1997; Shen *et al.*, 1997) and constitutes, together with the genera *Wolinella*, *Campylobacter* and *Arcobacter*, the ε subclass of the *Proteobacteria*, also known as rRNA superfamily VI (Vandamme *et al.*, 1991).

In 1992, two different groups reported almost simultaneously the presence of helically shaped bacteria in the abomasum of calves and adult cattle based on histological data (Günther & Schulze, 1992; Haringsma & Mouwen, 1992). Both groups described large numbers of spiral-shaped bacteria in the gastric crypts of the pyloric region and considered them as

Abbreviation: APES, 3-aminopropyltriethoxysilane.

The GenBank accession number for the 16S rDNA sequence of '*Candidatus* Helicobacter bovis' is AF127027.

putative *Helicobacter* species. Further indirect evidence of the presence of helicobacter-like organisms in adult cattle and calves was given by serological studies. Seidel *et al*. (1996) found significant titres of antibodies against *H. pylori* epitopes in the serum of calves after absorption with *Campylobacter jejuni*, *Wolinella succinogenes*, *Escherichia coli* and *Proteus mirabilis* strains. One report described a bactericidal activity of bovine serum, colostrum and milk against *H. pylori* (Korhonen *et al*., 1995). *In vitro* isolation of these organisms has not been successful so far (Jelinski *et al*., 1995; Braun *et al*., 1997) and the taxonomic status of these putative helicobacter-like bacteria is unknown.

The pathogenic role of *H. pylori* led to speculations about the association of bovine helicobacter-like bacteria with abomasal ulcer disease, although no conclusive evidence has been provided to date (Günther & Schulze, 1992; Haringsma & Mouwen, 1992). Other bacteria such as *Campylobacter* species and *Clostridium perfringens* have also been studied in association with the occurrence of abomasal lesions (Al-Mashat & Taylor, 1980; Mills *et al*., 1990; Jelinski *et al*., 1995).

In the present study, we propose a new candidate species, '*Candidatus* Helicobacter bovis', a gastric *Helicobacter* species from cattle.

METHODS

Samples. Seven abomasal stomachs from clinically healthy slaughterhouse cattle originating from different Belgian and Dutch farms, were selected. The stomachs were opened longitudinally along the greater curvatura and rinsed gently with tap water. Two small mucosal fragments were taken from each stomach, one near the torus pyloricus and one in the fundic region, and were tested for urease activity (CUTest; Temmler Pharma) for 2 h at 37 °C. Three mucosal biopsies from the pyloric region were taken for immunohistochemistry and *in situ* hybridization and placed into 4% buffered formalin for 24 h. For electron microscopy, a pyloric sample was taken from the same region and fixed in 0·1 M cacodylate buffer (pH 7·0) containing 5% glutaraldehyde and 0·15% (w/v) ruthenium red. From each stomach a mucosal fragment was also taken for PCR analysis, placed into sterile PBS (0·145 M NaCl, 0·15 M sodium phosphate) and frozen in liquid nitrogen. Special care was taken during sampling to avoid cross-contamination.

Reference strains. A total of 15 *Helicobacter* strains and one *W. succinogenes* strain were used to test the specificity of the '*Candidatus* Helicobacter bovis'-specific PCR (Table 1). Strains were grown on a 5% Mueller–Hinton blood agar and incubated at 37 °C in a microaerobic atmosphere containing approximately 5% O_2, 3·5% CO_2, 7·5% H_2 and 84% N_2. Absence of contaminants was checked by plating and Gram-staining.

Immunohistochemistry. Immunohistochemical staining was performed to assess the presence of helicobacter-like organisms. Formalin-fixed samples were dehydrated and paraffin-embedded. Sections of 4 μm were made of the paraffin-embedded tissues and were placed on 3-aminopropyltriethoxysilane-coated slides (APES; Sigma-Aldrich) and dried overnight at 60 °C. After de-waxing with xylene and rehydration in graded series with ethanol and distilled water, sections were placed in citrate buffer (0·1 M with 2% ureum) and heated in a microwave oven for antigen retrieval. Slides were then incubated with 12% hydrogen peroxide in methanol for 30 min in order to block endogenous peroxidase activity. Thereafter, the slides were pre-incubated with 30% normal goat serum in PBS for 30 min to reduce non-specific antibody binding. A mouse polyclonal antibody directed against *H. pylori* (DAKO), diluted 1/20 in PBS, was incubated overnight at 21 °C in a moist chamber. The sections were washed and incubated with biotinylated swine anti-rabbit immunoglobulin (DAKO) at 21 °C for 30 min and after rinsing covered with peroxidase-conjugated streptavidin–biotin complex. Peroxidase activity was de-

Table 1. Bacterial strains used for the evaluation of the '*Candidatus* Helicobacter bovis'-specific PCR

Taxon	Source	Strain no.
Helicobacter acinonychis	Cheetah gastric mucosa	LMG 12684^T
Helicobacter cinaedi	Human faeces	LMG 7543^T
Helicobacter sp. strain CLO-3	Human rectal swab	LMG 7792
Helicobacter fennelliae	Human faeces	LMG 11759
Helicobacter pametensis	Tern faeces	LMG 12678^T
Helicobacter sp. strain Bird B	Bird faeces	LMG 12679
Helicobacter sp. strain Bird C	Bird faeces	LMG 13642
Helicobacter hepaticus	Murine liver	LMG 16316^T
Helicobacter pullorum	Chicken lower bowel	LMG 16318
Helicobacter mustelae	Ferret gastric mucosa	LMG 18044^T
Helicobacter canis	Canine faeces	LMG 18086^T
Helicobacter muridarum	Murine intestinal mucosa	LMG 14378^T
Helicobacter bizzozeronii	Canine gastric mucosa	Strain 12A
Helicobacter salomonis	Canine gastric mucosa	CCUG 37845^T
Helicobacter felis	Feline gastric mucosa	CCUG 28539^T

Table 2. Oligonucleotide primers and probe used for PCR amplification, sequencing of genes encoding 16S rRNA and Southern blot hybridization

Primer	Sequence (5′ → 3′)	*E. coli* 16S rRNA position
H33f	ACG CTG GCG GCG TGC CTA ATA CAT GCA AGT CG	33–64
H1368r	GGT GAG TAC AAG ACC CGG GAA CGT ATT CAC CG	1368–1388
H390f	GCA GCA ACG CCG CGT GGA GGA TGA	390–413
H1053r	ACG AGC TGA CGA CAG CCG TG	1053–1072
R574f	AGA GCG TGT AGG CGG AAT GAT	574–593
R628f	AAC TGC GTT TGA AAC TAT CAT T	628–649
R832r	CGA GGA GAC AAG CCC CCC GA	832–851

Table 3. Sources and accession numbers of strains used for phylogenetic analysis

Taxon	Source	GenBank no.	Strain/clone no.
'*Gastrospirillum hominis*' type 1	Human gastric mucosa	L10079	Clone G1A1
'*Gastrospirillum hominis*' type 2	Human gastric mucosa	L10080	Clone G2A9
Helicobacter acinonychis	Cheetah gastric mucosa	M88148	Strain Eaton 90-119-3
Helicobacter bilis	Murine liver	U18766	Strain MIT 93-1909
Helicobacter bizzozeronii	Canine gastric mucosa	Y09404	CCUG 35045
Helicobacter canis	Canine faeces	L13464	NCTC 12739
Helicobacter cholecystus	Murine liver	U46129	Strain Hkb-1
Helicobacter cinaedi	Human faeces	M88150	CCUG 18818
Helicobacter felis	Feline gastric mucosa	M57398	Strain CS1
Helicobacter fennelliae	Human faeces	M88154	CCUG 18820
Helicobacter hepaticus	Murine liver	U07574	Strain FRED1
Helicobacter muridarum	Murine intestinal mucosa	M80205	Strain ST1
Helicobacter mustelae	Ferret gastric mucosa	M35048	ATCC 43772
Helicobacter nemestrinae	Macaque gastric mucosa	X67854	ATCC 49396^T
Helicobacter pametensis	Swine faeces	M88155	Strain M17 Seymour
Helicobacter pullorum	Broiler chicken caecum	L36141	NCTC 12824
Helicobacter pylori	Human gastric mucosa	M88157	ATCC 43504
Helicobacter salomonis	Canine gastric mucosa	Y09405	CCUG 37845^T
Helicobacter trogontum	Rat colon mucosa	U65103	LRB 8581
Helicobacter rodentium	Murine intestinal mucosa	U96297	CCUG 10373
Arcobacter butzleri	Human	L14626	CCUG 10373
Campylobacter jejuni	Human faeces	L14630	CCUG 24567
Wolinella succinogenes	Cattle abomasal mucosa	M88159	ATCC 29543

veloped using H_2O_2 with diaminobenzidine (DAB) as a chromogen (Fast DAB Tablet Set; Sigma-Aldrich). Subsequently, the sections were counterstained with Mayer's hematoxylin and mounted. As a negative control, the primary antibody was replaced with foetal calf serum in Tris/HCl buffer. As a positive control, a section of a mouse stomach experimentally infected with *Helicobacter pylori* LMG 7539^T was used.

Transmission electron microscopy. Three different pyloric samples were selected for electron microscopic evaluation based upon the high presence of helicobacter-like organisms in the corresponding immunostained sections.

After dehydration in a graded series of acetone washes, the samples were embedded in Spurr low-viscosity resin. Ultrathin sections were poststained with uranyl acetate and lead citrate and examined with an electron microscope (Phillips 201 TEM) at an accelerating voltage of 60 kV.

DNA extraction. DNA was isolated from the scrapings of the gastric biopsies and from the reference strains by lysis with guanidinium isothiocyanate and DNA was bound to silica particles according to the method of Boom *et al.* (1990).

Primers and PCR amplification of 16S rDNA. Broadrange primers *H33f* and *H1368r* were selected from rRNA superfamily VI (*Helicobacter*, *Campylobacter*, *Arcobacter*, *Wolinella*) specific regions of the 16S rRNA gene (Table 2). PCR reactions were performed in a volume of 50 µl

containing 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 3·5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 1·5 U AmpliTaq Gold polymerase (Perkin-Elmer, Roche Molecular Systems) and 25 pmol of both forward and reverse primer (Eurogentec). Samples were covered with mineral oil and PCR was performed in a Biomed-60 thermocycler under the following conditions: 9 min preincubation at 94 °C to activate AmpliTaq Gold, followed by 50 cycles of 30 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C. Final extension was performed for 5 min at 72 °C. DNA extracts of *Helicobacter acinonychis* LMG 12684^T and *Helicobacter mustelae* LMG 8776 were used as positive controls.

Analysis of amplified samples. PCR products were separated on 2% agarose gels and stained with ethidium bromide. In order to determine whether PCR products were derived from helicobacter-like organisms, the desired DNA bands were cut from the gels, diluted in an equal volume of distilled water and sequenced using the *H33f* and *H1368r* 5′-Indocarbocyanin (Cy5) labelled primers. Partial sequences were screened for homologous sequences using the NCBI GENINFO BLAST network service (http://www.ncbi.nlm.nih.gov/BLAST/) (Altshul *et al.*, 1997).

DNA cloning and sequence analysis. PCR amplimers comprising the 16S rDNA sequences derived from four different stomach samples (R2, R3, R5, R6) were each cloned into plasmid vector pGEM-T (Promega Biotech) according to the manufacturer's instructions and transformed into *E. coli* JM109 using standard procedures. Plasmids were purified using the Easy Prep Plasmid Preparation Kit (Pharmacia Biotech). Sequences were determined using the T7-sequencing kit (Pharmacia Biotech) in combination with an ALFexpress DNA sequencer (Pharmacia Biotech), according to the manufacturer's guidelines. Two primers flanking the multiple cloning sites (T7, SP6) as well as internal primers *H390f* and *H1053r* were used (Table 2). The sequence derived from the clone of the R2 sample (R2XA) was used as reference sequence. Sequence analysis was performed with the PCGene software (IntelliGenetics).

PCR amplicons of three other gastric samples (R13, R27, R28) were sequenced without prior cloning (referred to below as direct sequence analysis).

Phylogenetic analysis. Phylogenetic analysis was performed using the GeneCompar 2.0 software package (Applied Maths). Sequences of strains belonging to the ε subclass (Table 3) were retrieved from the EMBL database and were aligned with reference sequence R2XA. A similarity matrix was constructed from the aligned sequences and was corrected for multiple base changes by the method of Jukes & Cantor (1969). Unknown bases and gaps were not considered in the numerical analysis. A phylogenetic tree was constructed using the neighbour-joining method of Saitou & Nei (1987).

'*Candidatus* Helicobacter bovis'-specific PCR assay. '*Candidatus* Helicobacter bovis'-specific oligonucleotides *R574f* and *R832r* (Table 2), were selected from variable rDNA regions of the sequences determined by direct and indirect sequence analysis. These primers comprised a 259 bp 16S rDNA fragment and were used to develop a specific PCR and an *in situ* hybridization procedure. Within this fragment an internal '*Candidatus* Helicobacter bovis'-specific probe *R628f* (Table 2) was selected for Southern blot hybridization purposes.

PCR reactions were performed in a volume of 50 μl containing 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 2·5 mM $MgCl_2$, 200 μM each dNTP, 1·5 U of AmpliTaq Gold polymerase, and 25 pmol of both forward and reverse primer. PCR amplification was performed under the following conditions: 9 min preincubation at 94 °C to activate AmpliTaq Gold, followed by 40 cycles of 30 s at 94 °C, 45 s at 60 °C and 45 s at 72 °C. Final extension was performed for 5 min at 72 °C. All gastric DNA extracts were tested with this PCR. For positive controls, plasmid DNA was used from the cloned 16S rDNA fragments (R2XA). As a negative control a DNA extract was used from an abomasum lacking helicobacter-like organisms.

Specificity of the '*Candidatus* Helicobacter bovis'-specific oligonucleotides *R574f* and *R832r* was tested by PCR using DNA extracts of 15 different *Helicobacter* strains and a *W. succinogenes* strain (Table 1).

PCR products were separated on 2% agarose gels, stained with ethidium bromide and transferred to Hybond-N+ (Amersham) by electroblotting. Southern blot hybridization was performed with the [γ^{32P}]ATP-labelled probe *R628f* (Table 2) according to standard procedures (Amersham Pharmacia Biotech). In order to ensure the specificity of the probe hybridization, blots were washed twice with 0·1 × SSC + 0·1% SDS at 55 °C.

***In situ* hybridization.** In order to make the link between the '*Candidatus* Helicobacter bovis'-specific probe and the bacterial spiral cells observed in the tissue sections, an *in situ* hybridization procedure was performed on the formalin-fixed and paraffin-embedded pyloric samples of each animal. A 259 base digoxigenin-labelled probe was synthesized using the PCR Dig Probe Synthesis Kit (Boehringer Mannheim) in combination with the '*Candidatus* Helicobacter bovis'-specific primers *R574f* and *R832r* (Table 2). PCR conditions were adapted from those described in the diagnostic PCR assay. The resulting PCR product was purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim) following manufacturer's instructions.

To avoid RNase activity, all glassware was heated at 180 °C for 3 h. Further precautions included the use of RNase-free water, and the use of sterile disposable materials whenever possible. Sections of the paraffin-embedded tissues (4 μm thick) were mounted on RNase-free, APES-coated slides (Sigma-Aldrich) and fixed by heating for 1 h at 60 °C. The sections were deparaffinized in xylene (2 × 5 min), rehydrated through graded ethanol, and washed twice in PBS for 5 min each. Sections were then treated with proteinase K (DAKO) for 15 min each at 37 °C in a humidified chamber. The enzyme was inactivated by treatment with 0·2% glycine in PBS for 3 min. Sections were washed twice in PBS for 5 min each, dehydrated in graded ethanol and air-dried. Tissues were circumlined with a DAKO Pen to avoid liquid spillage during further processing and to ensure an efficient sealing of the coverslip. For the hybridization step, sections were covered with 5–15 μl solution, containing 5 ng μl^{-1} labelled probe in 50% deionized formamide, 2 × SSC, 10% dextran sulfate, 0·25 μg yeast tRNA μl^{-1}, 0·5 μg heat-denatured salmon sperm DNA μl^{-1}, and 1 × Denhart's solution. Sections were covered with a piece of coverslip to avoid evaporation. To denature the probe, sections were heated for 10 min at 95 °C and chilled on ice for 10 min. Slides were then incubated overnight at 37 °C in a humidified chamber. To remove the unbound probe, the coverslips were removed and the sections were washed in 2 × SCC and 1 × SCC at room temperature for 10 min each followed by two

washes of 0·3× SCC at 40 °C for 10 min and at room temperature for 10 min, respectively.

All steps involving the immunological detection of the hybridized probe were performed at room temperature. The sections were treated first for 30 min in Buffer 1 (100 mM Tris/HCl, 150 mM NaCl, pH 7·5) containing 2% normal goat serum and 0·3% Triton X-100. An incubation step followed for 3 h with diluted (1:30 in the same solution) anti-digoxigenin antibodies conjugated to horseradish peroxidase (DAKO). Unbound antibodies were washed gently on a shaker with Buffer 1 followed by Buffer 2 (100 mM Tris/HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9·5) for 15 min each. To optimize the detection level, the Tyramid Signal Amplification System (NEN Life Science Products) was applied on each section, following manufacturer's instructions. The hybridized probe was then visualized, using H_2O_2 with diaminobenzidine as a chromogen (Fast DAB Tablet Set; Sigma-Aldrich). Thereafter the sections were counterstained with Mayer's hematoxylin and mounted.

Nucleotide sequence accession numbers. Accession numbers of the 16S rDNA gene sequences used for the phylogenetic analysis are listed in Table 3.

RESULTS

Urease activity and immunohistochemical evaluation

Urease activity was observed in all pyloric samples (7/7). In the fundic samples, urease activity was absent (0/7). Spiral immunostained organisms were observed in the pyloric samples of all animals (Fig. 1). The highest concentration was seen in the most distal pyloric samples. Bacteria were mostly situated in the mucus layer and in the lumen of the proximal part of the gastric crypts where they formed small clusters. In some samples, coccoid organisms were observed between the spiral bacteria, which also cross-reacted with the *H. pylori* polyclonal antibodies. In the positive control only *Helicobacter pylori*-like bacteria were stained, while in the negative controls no staining was observed.

Transmission electron microscopy

Large groups of multiple spiral bacteria were seen within the crypts of the gastric mucosa (Fig. 2). There was no obvious association between the bacteria and the gastric cells, neither were there any intracellular bacterial inclusions. The bacteria were helical-shaped and had 1–3 complete spiral turns per cell with a wavelength of approximately 750 nm. Cells were 1–2·5 μm long and 0·3 μm wide. At least four flagellae were seen at one end. It was unclear whether these flagellae were uni- or bipolar, neither could the presence or absence of a flagella sheath be noted.

Amplification, cloning and sequencing of helicobacter-like 16S rDNA fragments

PCR amplification of the 16S rRNA gene using the *H33f* and *H1368r* primers, produced a fragment of the expected size range (±1·3 kbp) in all seven samples examined. Partial direct sequence analysis of four of these bands (R2, R3, R5, R6) and subsequent database comparison (BLAST) confirmed the PCR products to be helicobacter-like 16S rDNA fragments. Four PCR products (R2, R3, R5, R6) were cloned followed by partial sequencing. In one clone a *Clostridium*-like 16S rDNA fragment was found. All other clones contained helicobacter-like inserts. The 16S rDNA sequences of four clones derived from different animals (R2XA, R3XA, R5XE, R6XA), were determined. Additional sequences of three other samples (R13, R27, R28) were characterized by direct sequence analysis using the primers *H33f*, *H1368r*, *H390f* and *H1053r*.

Sequences and phylogenetic analysis

Sequence length varied from 1267 to 1335 bp. Pairwise comparisons between these seven sequences revealed a sequence homology of more than 99%. One reference sequence (R2XA) of 1335 bp was selected for phylogenetic evaluation. A similarity matrix based on comparisons of 16S rRNA sequences of 23 strains representing all validly named *Helicobacter* species, '*Helicobacter heilmannii*' (type 1, type 2), *Campylobacter jejuni*, *Arcobacter cryaerophilus* and *Wollinella succinogenes* was calculated. By this analysis it was shown that the sequences of the bovine helicobacter-like organisms form a distinct group within the genus *Helicobacter* with *Helicobacter bilis* as closest taxonomic relative (level of similarity 92·8%). The reference sequence was clearly distinct from sequences belonging to other superfamily VI genera, as shown by a 85·6, 85·1% and 89·7% homology with *Campylobacter jejuni*, *Arcobacter butzleri* and *W. succinogenes*, respectively. A phylogenetic tree based on this analysis is shown in Fig. 3.

Diagnostic PCR assay

A 259 base fragment was produced for all seven stomach samples with primer pair *R574f-R832r*. All PCR products hybridized with the *R628f* probe after Southern blot hybridization. No amplification product was obtained using DNA preparations from any of the *Helicobacter* strains, nor from the bovine *W. succinogenes* strain (Table 1). The positive control yielded a ~0·3 kb product as expected. There was no DNA amplification using the negative control material.

In situ hybridization

In situ hybridization of the bovine helicobacter-like bacteria with the '*Candidatus* Helicobacter bovis'-specific probe was observed in sections from all (7/7) stomachs. These bacteria were observed as darkbrown spiral organisms, organized in small clusters, situated in the gastric crypts of the pyloric part of the abomasal stomach. Not all spiral bacteria were stained. Sometimes a faint background, seen as fine stained strings,

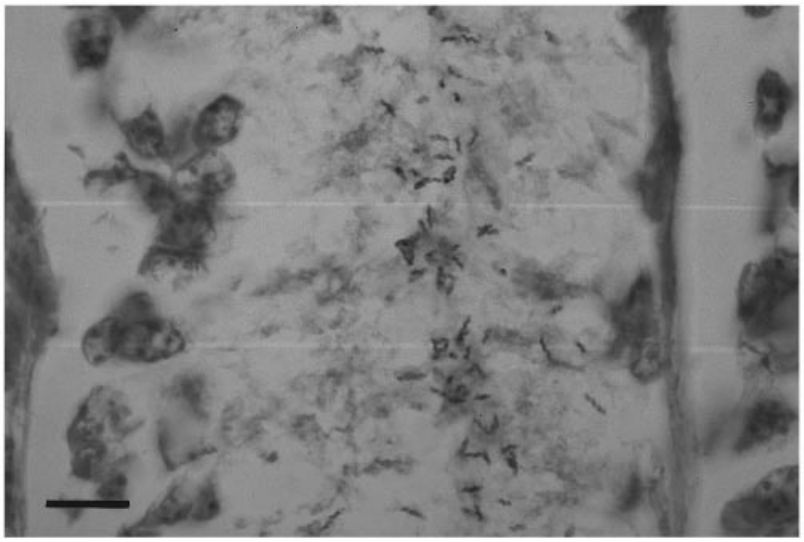

Fig. 1. Immunohistochemical staining of a helicobacter-like organism in the gastric crypts of the abomasum showing a spiral morphology. Bar, 10 μm.

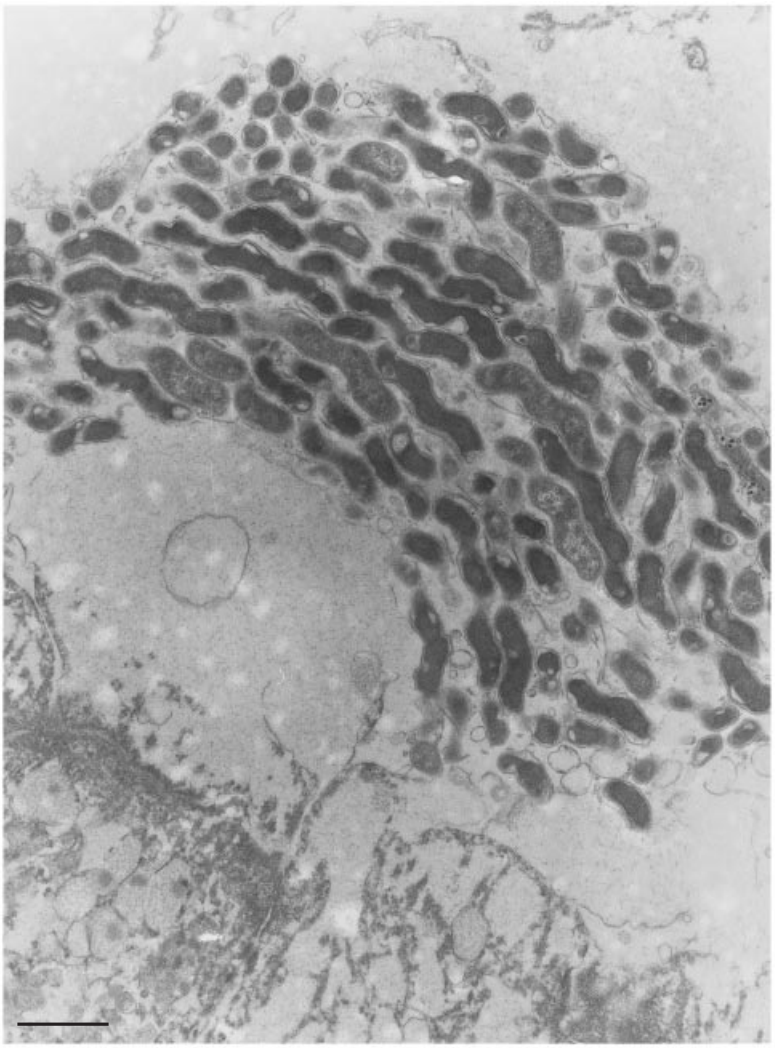

Fig. 2. Transmission electron micrograph showing numerous sections of spiral-shaped bacteria in the gastric crypts of the pyloric region of the abomasum. Bar, 1 μm.

was observed in the surrounding cells. This background staining was also observed in the *H. pylori*-infected mouse stomach which was used as a negative control. The *H. pylori* cells in this control did not hybridize with the '*Candidatus* Helicobacter suis'-specific probe.

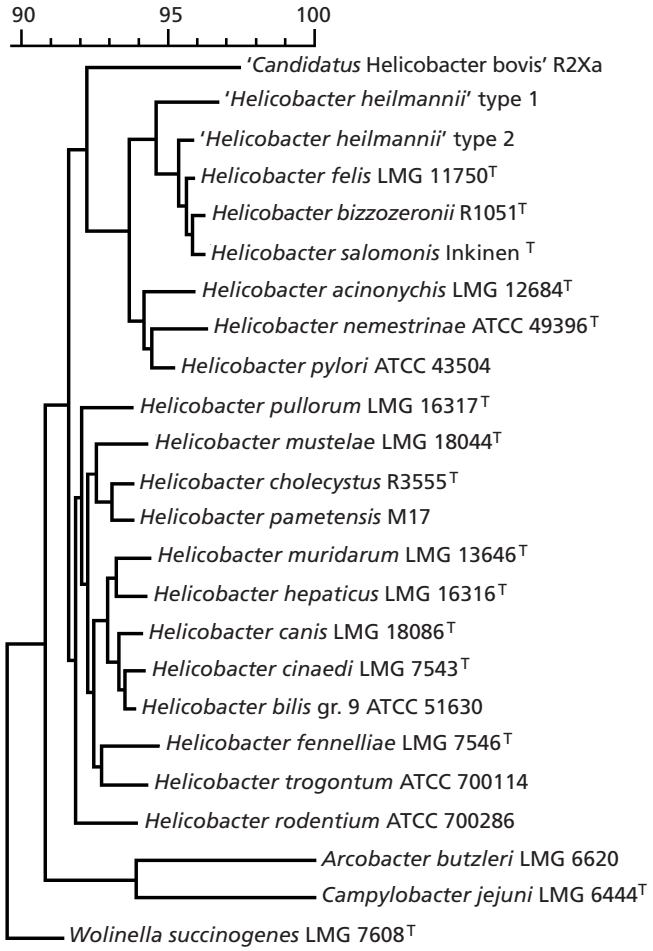

Fig. 3. Phylogenetic tree for 23 strains of *Helicobacter*, *Campylobacter*, *Arcobacter* and *Wolinella* species on the basis of 16S rDNA sequence similarity. The scale bar represents a 1% difference in nucleotide sequence as determined by measuring the length of horizontal lines connecting any two species.

DISCUSSION

The spiral helicobacter-like organisms described in this study, were clearly distinct from other helically shaped bacteria observed in the rumen of cattle, including *W. succinogenes*, *Treponema saccharophilum* and *Treponema bryantii* (Wolin & Jacobs, 1961; Paster & Canale-Parola, 1985; Stanton & Canale-Parola, 1980). Our electron microscopical data also showed that they do not possess the tightly coiled appearance of gastrospirillum-like organisms, often described in the stomachs of other animals (Lee *et al.*, 1988; O'Rourke *et al.*, 1992; Hänninen *et al.*, 1996; Jalava *et al.*, 1997; Queiroz *et al.*, 1990; Eaton *et al.*, 1993; Jakob *et al.*, 1997). To date these bovine helicobacter-like organisms have not been cultivated nor have they been phylogenetically characterized. One report claims the isolation of three *Helicobacter cinaedi* strains, identified by using the API-Campy system (Bio-Mérieux), from the abomasum of cattle (Braun *et al.*, 1997). Unfortunately, these strains are no longer available for comparative studies (L. Corboz, personal communication).

In the present study, seven 16S rDNA sequences were determined either by direct or indirect sequence analysis. Each sequence was derived from an abomasal stomach biopsy, sampled from adult cattle on seven different farms in Belgium and The Netherlands. Pairwise comparisons revealed these sequences to be more than 99% homologous, suggesting them to originate from bacterial strains belonging to a single species. Phylogenetic analysis of the reference sequence R2XA, placed the corresponding organism within the genus *Helicobacter*. Immunohistochemistry revealed these bacteria to be antigenetically related to *Helicobacter pylori*. A strong urease activity, characteristic for the presence of gastric helicobacters, was observed in all samples. A diagnostic PCR assay was developed, differentiating the reference DNA R2XA from all *Helicobacter* and *Wolinella* species. A 259 bp fragment was produced from all seven stomach samples with this test. *In situ* hybridization associated a R2XA-specific probe with spiral bacteria situated in the gastric crypts of the pyloric samples. These results indicate that the bovine sequences represent a single taxon corresponding to a helicobacter-like organism that is present in the pyloric part of the abomasum of cattle. The low similarity level towards *Helicobacter bilis* (92·8%), its closest validly named neighbour, indicates that this novel taxon is indeed a novel helicobacter species.

According to the suggestions of Murray & Stackebrandt (1995) we propose to assign the bovine taxon to the category *Candidatus* as '*Candidatus* Helicobacter bovis' pending further characterization of this bacterium after *in vitro* cultivation of strains.

On this basis we propose for the bovine gastric helicobacter-like organisms the designation '*Candidatus* Helicobacter bovis', bovis from the latin bos which means cow. The description is as follows: '*Candidatus* Helicobacter bovis' [(*ε-Proteobacteria*), genus *Helicobacter*; NC; Gram-negative; helical; NAS (GenBank no. AF127027), oligonucleotide sequence complementary to unique region of 16S rRNA gene 5′-AAC TGC GTT TGA AAC TAT CAT T-3′; morphology 1·5–2·5 μm long, 0·3 μm wide, 1–3 complete spiral turns with a wavelength of ±750 nm, multiple flagellae; symbiotic (*Bos*, abomasum); strong urease activity, cross-reaction with polyclonal *Helicobacter pylori*-derived antibodies].

ACKNOWLEDGEMENTS

D.D.G. is indebted to the Flemish Institute for the Encouragement of Research in the Industry (Grant SB/951027). P.V. is indebted to the Scientific Research Fund-Flanders (Belgium) for a position as postdoctoral research fellow. We thank Drs G. Charlier and S. Roels of the Centre

for Research in Veterinary Medicine and Agrochemistry (CODA) for their help with the electron microscopy. We are also grateful to Messrs C. Puttevils and J. P. Logghe for their technical assistance.

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