

Isolation and Characterization of a *Helicobacter* sp. from the Gastric Mucosa of Dolphins, *Lagenorhynchus acutus* and *Delphinus delphis*

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Gastric ulcerations in dolphins have been reported for decades. Some of these lesions were associated with parasitic infections. However, cases of nonparasitic gastric ulcers with no clearly defined etiology also have been reported in wild and captive dolphins. Considerable speculation exists as to whether dolphins have *Helicobacter*-associated gastritis and peptic ulcer disease. The stomachs of seven stranded Atlantic white-sided dolphins, *Lagenorhynchus acutus*, and 1 common dolphin, *Delphinus delphis*, were assessed for the presence of *Helicobacter* species. Novel *Helicobacter* species were identified by culture in the gastric mucosa of two of the eight dolphins studied and by PCR in seven of the eight dolphins. The gram-negative organisms were urease, catalase, and oxidase positive. Spiral to fusiform bacteria were detected in gastric mucosa by Warthin Starry staining. Histopathology revealed mild to moderate diffuse lymphoplasmacytic gastritis within the superficial mucosa of the main stomach. The pyloric stomach was less inflamed, and bacteria did not extend deep into the glands. The lesions parallel those observed in *Helicobacter pylori*-infected humans. Bacteria from two dolphins classified by 16S rRNA analysis clustered with gastric helicobacters and represent a novel *Helicobacter* sp. most closely related to *H. pylori*. These findings suggest that a novel *Helicobacter* sp. may play a role in the etiopathogenesis of gastritis and gastric ulcers in dolphins. To our knowledge this represents the first isolation and characterization of a novel *Helicobacter* sp. from a marine mammal and emphasizes the wide host distribution and pathogenic potential of this increasingly important genus.

The genus *Helicobacter* has been expanded considerably in recent years, and several species have been isolated from the gastrointestinal tract of humans and a wide range of different animals (8–11, 14–16, 25, 28, 33, 34, 37). In humans, *Helicobacter pylori* is an important pathogen which causes chronic gastritis and peptic ulcers and has been associated with gastric adenocarcinoma and lymphoma (8–11, 25, 37). *H. pylori* has been identified worldwide and is considered to be responsible for the most common infection in humans (24, 27, 37). In animals, other *Helicobacter* species are linked to gastritis with and without ulcers in their respective hosts (11, 15, 34, 37).

Gastric ulcers have been reported for decades in wild and captive dolphins (1, 19, 20, 26, 31, 32, 36). Clinical signs include inappetence, anorexia, abdominal tenderness, depression, and occasional unresponsiveness (20, 36). Complete blood count may reveal a leukocytosis and anemia if bleeding is present. This clinical syndrome is consistent with gastritis and peptic ulcer disease (PUD).

The dolphin stomach has three divisions; forestomach, main stomach, and pylorus, which joins the duodenal ampulla. The forestomach has a keratinized stratified squamous epithelium, and it is the only nonglandular portion of the dolphin's stomach. The main and pyloric portions of the stomach are glandular. The main stomach consists of neck cells, chief cells, and parietal cells, whereas the pylorus has columnar mucous cells

and argentaffin cells. The duodenal ampulla is an extension of the duodenum from which the common bile duct exits from the liver (22, 31, 32).

Lesions in the forestomach and cranial portion of the main stomach can be visualized by endoscopy. However, the distal portions of the main stomach and pyloric stomach are not accessible by endoscopic examination. A number of cases of dolphins with gastric ulcers have been associated with parasitic infections and foreign bodies (6, 18, 23, 32, 35, 36). Reports of non-parasitic gastric ulcers with no clearly defined etiology also have been noted in the forestomach, main stomach, and pyloric stomach of dolphins (5, 20, 35, 36). As a consequence, considerable speculation exists as to whether dolphins have *Helicobacter*-associated gastritis and PUD.

White-sided dolphins, *Lagenorhynchus acutus*, are found in cool temperate waters in the northern Atlantic Ocean. Their distribution ranges from southern Greenland to northern Virginia, and they are commonly found in New England waters. They range in size from 6 to 9 ft and travel in pods of up to 1,000 individuals. They commonly strand themselves on the eastern seashores of the United States, but fortunately their species is not endangered or threatened (19). The common dolphin, *Delphinus delphis*, is seen worldwide in tropical, subtropical, and warm temperate waters. They are deep-water dolphins and travel in groups containing 20 to several thousand animals (4, 29).

The goal of this study was to determine if *Helicobacter* spp. could be isolated from the stomachs of stranded dolphins, as well as to determine whether the gastritis and ulcers that are noted in dolphins could be attributed to the presence of helicobacters.

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TABLE 1. Culture and PCR results for the eight dolphins presented by animal number and sample site^a

Animal no. (group)	Species ^a	MIT accession no. ^b	Sample site ^c	Results ^d	
				Culture	PCR
1 (A)	WSD	99-5664	Forestomach	—	+
		99-5665	Main stomach	+	+
		99-5656	Main stomach	+	+
2 (A)	WSD	99-5658	Forestomach	—	+
		99-5657	Main stomach	+	+
3 (A)	WSD	99-5666	Forestomach	Mixed	+
		99-5659	Main stomach	—	+
4 (A)	WSD	99-5661	Forestomach	—	+
		99-5660	Main stomach	—	+
5 (A)	WSD	99-5662	Forestomach	—	—
		99-5663	Main stomach	—	+
6 (A)	WSD	99-5668	Forestomach	—	—
		99-5667	Main stomach	—	—
7 (B)	CD	99-7442-1	Jct fore- and main stomachs	Mixed	+
		99-7442-2	Main stomach	—	+
		99-7442-3	Jct main and pyloric stomachs	—	+
		99-7442-4	Pyloric stomach	Mixed	+
		99-7442-5	Jct pyloric and duodenal ampulla	Mixed	+
8 (B)	WSD	99-7443-1	Jct fore- and main stomachs	—	+
		99-7443-2	Main stomach	—	+
		99-7443-3	Jct main and pyloric stomachs	Mixed	—
		99-7443-4	Pyloric stomach	—	—
		99-7443-5	Jct pyloric and duodenal ampulla	—	+

^a WSD, Atlantic white-sided dolphin; CD, common dolphin.

^b MIT, Massachusetts Institute of Technology, Cambridge, Mass.

^c Jct, junction (of).

^d Mixed denotes mixed bacterial growth on artificial media that amplified a 1,200-bp *Helicobacter* species-specific PCR product.

MATERIALS AND METHODS

Animals. A total of eight stranded dolphins, all of which died on the beach, were evaluated. The animals were divided into two groups (A and B). Group A was comprised of six Atlantic white-sided dolphins (numbers 1 to 6), which stranded in March 1999 in Wellfleet Cape Cod, Mass. Group B consisted of two animals (one Atlantic white-sided dolphin and one common dolphin), numbered 7 and 8 (Table 1). Both groups consisted of dolphins that were stranded on the shores of Cape Cod in the fall of 1999.

Sample collection. Two stomach samples (forestomach and main stomach from the six Atlantic white-sided dolphins from Group A) were obtained for culture and PCR. No histological samples were collected from the animals in Group A. In the two animals from Group B, five gastric samples were collected for culture, PCR, and histology (junction of the forestomach and main stomach, main stomach, junction of the main stomach and pyloric stomach, pyloric stomach, and junction of the pyloric stomach and duodenal ampulla). Each sample measured 2 cm by 2 cm and was collected under field conditions.

Microaerobic culture and biochemical characterization. The specimens were gently rinsed with sterile physiological saline and placed in individual vials with 3 ml of 20% glycerol in brucella broth. The vials were maintained at -70°C prior to culture. Media used for culture were Trypticase soy agar with 5% sheep blood and TVP (trimethoprim, vancomycin, and polymyxin) and CVA (cefoperazone, vancomycin, and amphotericin B) antibiotic-impregnated media (Remel Laboratories, Lenexa, Kans.). In addition, selective antibiotic medium (ABM) contained the following components: blood agar base (Oxoid and Remel), 5% horse blood (Remel), 50 μg of amphotericin B per ml, 100 μg of vancomycin per ml, 3.3 μg of polymyxin B per ml, 200 μg of bacitracin per ml, and 10.7 μg of nalidixic acid per ml (Sigma Chemical Co., St. Louis, Mo.). A small amount of stomach tissue was homogenized in 1 ml of brucella broth (Difco Laboratories, Detroit, Mich.) containing 5% fetal calf serum (Summit Technologies, Fort Collins, Colo.) in a glass tissue grinder. Approximately 100 μl of sample was applied directly to TVP, CVA, and ABM media. Half of the remaining sample was filtered through a 0.45- μm -pore-size filter onto a blood agar plate. The plates were incubated at 37°C under microaerobic conditions for 2 to 4 weeks in vented jars containing N_2 , H_2 , and CO_2 (80:10:10). Biochemical and morphological analyses following a previously described protocol were performed on isolated bacteria (28).

Electron microscopy. Strain MIT 99-5665 was examined by electron microscopy. Cells grown on blood agar plates were centrifuged and suspended in 10 μM Tris-HCl buffer (pH 7.4) at a concentration of about 10^8 cells per ml. One percent (wt/vol) phosphotungstic acid (pH 6.5) was used to negatively stain the samples. The specimens were examined with a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV.

DNA extraction and PCR analysis. DNA was extracted from bacteria with the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, Ind.) and from tissues by using modifications of the same method. *Helicobacter*-specific primer pairs C97 and C05 were used to generate 16S rRNA amplicons of 1,200 bases (16). Ten microliters of the DNA preparation was used for PCR. The PCR mixture contained $1 \times$ *Taq* polymerase buffer, 0.5 μM each of the two primers, 200 μM each deoxynucleotide, and 200 μg of bovine serum albumin per ml. The samples were heated at 94°C for 4 min, centrifuged briefly, and cooled to 58°C , and $0.5 \times$ *Taq* polymerase (Roche Molecular Biochemicals) was added. Amplification conditions were as follows: denaturation at 94°C for 1 min, annealing at 58°C for 2 min, and elongation at 72°C for 3 min. Thirty-five cycles were completed before a final elongation step at 72°C for 8 min. A 15- μl aliquot of the PCR product was electrophoresed through a 1% agarose gel separation matrix prior to ethidium bromide staining and viewing under a UV light.

Purification of PCR products for 16S rRNA sequencing from tissue samples. A 1.2-kb piece of amplified DNA from biopsy samples was purified by precipitation with polyethylene glycol 8000. After removal of Ampliwax, 0.6 volume of 20% polyethylene glycol 8000 (Sigma) in 2.5 M NaCl was added and the mixture was incubated at 37°C for 10 min. The sample was centrifuged for 15 min at $15,000 \times g$, and the pellet was washed with 80% ethanol and pelleted as described before. The pellet was air dried, and dissolved in 30 μl of distilled water, and used for cycle sequencing as described below.

Cloning and sequencing of 16S rRNA PCR products. A pGEM-T vector was used for cloning the PCR products (Promega, Madison, Wis.). The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, Calif.) from low-melting-point agarose gel. Fifty nanograms of purified PCR product was ligated with 50 ng of pGEM-T vector at 4°C overnight and then transferred into competent JM109 cells. Ampicillin plates with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and IPTG (isopropyl- β -D-thiogalactopyranoside) were used to select positive clones. Plasmid DNA was isolated from *E. coli* with a QIAprep Spin Miniprep Kit (Qiagen). The 1,200-base sequences of two PCR products from *Helicobacter*-specific primers were obtained by cycle sequencing (16).

Amplification of 16S rRNA cistrons by PCR and purification of PCR products from isolates. The 16S rRNA cistrons were amplified with bacterial universal primers F24 and F25 (7). PCR was performed in thin-walled tubes with a Perkin-Elmer 9700 Thermocycler. One microliter of the DNA template was added to a reaction mixture (50- μl final volume) containing 20 pmol of each primer, 40 nmol of deoxynucleoside triphosphates, and 1 unit of *Taq* 2000 polymerase (Stratagene, La Jolla, Calif.) in buffer containing Taqstart antibody (Sigma). In a hot-start protocol, samples were preheated at 95°C for 8 min

TABLE 2. Biochemical and phenotypic characteristics of the novel *Helicobacter* species from dolphins and other gastric *Helicobacter* species^a

Taxon	Catalase production	Nitrate reduction	Alkaline phosphatase hydrolysis	Urease activity	Indoxyl acetate hydrolysis	Gamma-glutamyl transpeptidase activity	Growth at 42°C	Growth with 1% glycine	Susceptibility to:		Periplasmic fibers	No. of flagella	Distribution of flagella	G+C content (mol%)
									Nalidixic acid (30-µg disc)	Cephalothin (30-µg disc)				
<i>Helicobacter</i> sp. from dolphin	+	-	-	+	-	+	+	+	S (3/3)	S (3/3)	-	4-8	Bipolar	35-37
<i>H. pylori</i>	+	-	+	+	-	+	-	-	R	S	-	4-8	Bipolar	24
<i>H. nemestrinae</i>	+	-	+	+	-	+	-	-	R	S	-	2-5	Bipolar	30
<i>H. acinonychii</i>	+	-	+	+	-	+	-	-	R	S	+	14-20	Bipolar	42
<i>H. felis</i>	+	-	+	+	-	+	-	-	R	S	+	4-8	Peritrichous	36
<i>H. mustelae</i>	+	+	+	+	+	+	+	-	S	R	-	4-8	Peritrichous	36

^a Symbols and abbreviations: +, positive reaction; -, negative reaction; S, susceptible; R, resistant; I, intermediate; ND, not determined. Numbers in parentheses are numbers of strains with the indicated result or number of strains tested.

followed by amplification under the following conditions: denaturation at 95°C for 45 s, annealing at 60°C 45 s, and elongation for 1.5 min with an additional 5 s for each cycle. A total of 30 cycles were performed, followed by a final elongation step at 72°C for 10 min. The results of PCR amplification were examined by electrophoresis in 1% agarose gel. DNA was stained with ethidium bromide and visualized under short-wavelength UV light.

16S rRNA sequencing. Purified DNA from PCR was sequenced with an ABI prism cycle-sequencing kit (BigDye Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS; Perkin-Elmer). The primers used for sequencing were the same ones previously described by us (7). Quarter-dye chemistry was used with 80 µM primers and 1.5 µl of PCR product in a final volume of 20 µl. Cycle sequencing was performed with an ABI 9700 DNA sequencer with 25 cycles of denaturation at 96°C for 10 s and annealing and extension at 60°C for 4 min. Sequencing reactions were run on an ABI 377 DNA sequencer.

16S rRNA data analysis. Sequence data were entered into RNA, a program set for data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation, and dendrogram construction for 16S rRNA in Microsoft QuickBasic for use with personal computers and were aligned as previously described (30). Our database contains over 1,000 sequences obtained in our laboratory and over 500 obtained from GenBank. Dendrograms were constructed by the neighbor-joining method.

Southern blot analysis. Southern blot analysis was performed with a 16S ribosomal DNA *Helicobacter* probe. Fifteen microliters of amplicons was electrophoresed through a 1% agarose gel and transferred onto a Hybond N nylon membrane as outlined by the manufacturer (Amersham, Arlington Heights, Ill.). DNA was then UV cross-linked. The fixed DNA was then hybridized with a *Helicobacter* probe generated by PCR amplification of *H. pylori* DNA using primers C97 and C05. The probe was labeled with horseradish peroxidase, hybridized overnight to the nylon membrane at 42°C, and exposed in the presence of luminol to Hyperfilm-ECL as outlined by the manufacturer (Amersham).

RFLP analysis. DNA fragments of 1.2 kb from the bacterial isolates were subjected to restriction fragment length polymorphism (RFLP) analysis. DNA digestion was accomplished by addition of 10 U of restriction endonucleases *AhaI* and *HhaI* (New England Biolabs, Beverly, Mass.) and 2 µl of restriction buffer (New England Biolabs) to 16 µl of DNA and incubation at 37°C for 2 h. The samples were then electrophoresed through a 6% Visigel separation matrix followed by ethidium bromide staining and viewed by UV illumination.

Histopathology. The tissue was fixed in neutral buffered 10% formalin, processed by standard methods, and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin and Warthin-Starry silver stains. These sections were examined by light microscopy for evidence of lesions and for the presence of a bacterium with morphology consistent with members of the genus *Helicobacter*.

RESULTS

Biochemical characterization of *Helicobacter* species. *Helicobacter* species were cultured from the glandular mucosa in two of the eight animals (Table 1). *Helicobacter* spp. were not isolated by culture from the forestomach. A total of three pure cultures of *Helicobacter* spp. were isolated from the main stomach of two different Atlantic white-sided dolphins from Group A; two cultures were isolated from animal 1 and one culture was isolated from animal 2 (99-5656, 99-5657, and 99-5665, respectively). The biochemical characteristics were determined for the three pure cultures from Group A (Table 2). The bacteria were oxidase, catalase, and urease positive. The bacteria were negative for nitrate reduction, alkaline phosphatase hydrolysis, and indoxyl acetate hydrolysis. All isolates grew at 37°C and 42°C and were susceptible to both cephalothin and nalidixic acid (Table 2). In Group B, no pure cultures of *Helicobacter* spp. were isolated. However, four mixed cultures containing *Helicobacter* spp. were isolated as ascertained by PCR analysis (Table 1).

Ultrastructure. The bacteria isolated from animal 1 (99-5665) measured 0.6 µm by 4 µm and are slightly spiral with bipolar sheathed flagella, which are laterally located at the end of the bacteria (Fig. 1).

Phylogenetic analysis. Full 16S rRNA sequences of about 1,490 bases were obtained for the three bacterial isolates from two dolphins in Group A (99-5657, 99-5656, 99-5665), and partial sequences of two 1,200-base PCR products were obtained from dolphin stomach tissue. A neighbor-joining tree for these sequences is shown in Fig. 2. The branch position and

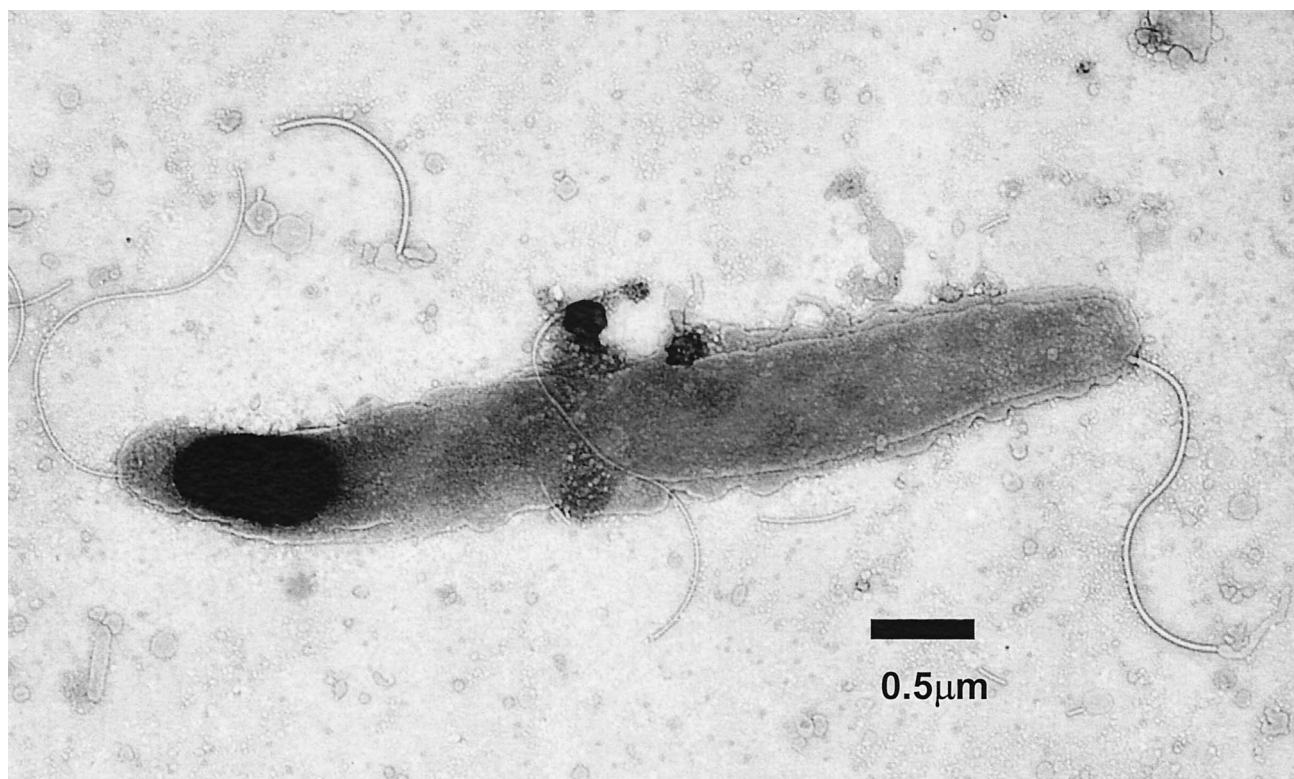


FIG. 1. Transmission electron micrograph of the novel *Helicobacter* species (MIT 99-5665) isolated from an Atlantic white-sided dolphin, *Lagenorhynchus acutus*. The bacterium is fusiform to slightly spiral and has several sheathed flagella at each end. Bar, 0.5 μm .

branch length for the two partial sequences (99-5660-6 and 99-5659-3) are probably distorted (due to the 290 bases of missing data); however, it is highly probable that full sequences would have fallen in nearly the same position in the tree. The dolphin sequences cluster with the gastric helicobacters (*H. pylori*, *H. nemestrinae*, *H. acinonychis*, *H. felis*, *H. salmonis*, *H. bizzozeronii*, and the "gastrospirillia"). The dolphin isolates, except MIT 99-5659-3, are most closely related to *H. pylori*, differing by 2 to 3%. Strain MIT 99-5659-3 may be closest to the "gastrospirillum" side of the gastric cluster, but its branching is slightly uncertain due to its being a partial sequence. These isolates and PCR products represent novel *Helicobacter* species.

PCR identification of *Helicobacter* spp. in tissue. DNA from stomach tissue of eight dolphins from Groups A and B was amplified with a *Helicobacter*-specific primer set (Roche Molecular Biochemicals). Of the 23 samples analyzed, 18 were positive on the basis of confirmation by Southern blot analysis (Table 1 and Fig. 3). In Group A, animals 1, 2, 3, and 4 were positive for the presence of *Helicobacter* spp. in both the forestomach and the main stomach. In samples from animal 5, *Helicobacter* spp. were identified by PCR in the main stomach but not in the forestomach. Animal 6 was negative in both the main stomach and the forestomach. In Group B, animals 7 and 8 were positive in all five parts of the stomach with the exception of animal 8, which was negative in the pyloric stomach (Table 1).

RFLP. Five 1,200-bp *Helicobacter*-specific PCR products from four dolphins in Group A (99-5656, 99-5657, 99-5659, 99-5660, and 99-5665) were subjected to RFLP analysis by *Hha*I and *Alu*I digestion. Two RFLP patterns were observed. Strains MIT 99-5656 and MIT 99-5665 were isolated from the

same dolphin (animal 1) and had identical RFLP patterns. The RFLP patterns of the dolphin PCR products corresponded with the 16S rRNA sequence data (Fig. 4).

Histopathology. Within the superficial lamina propria of the main stomach of the Group B common dolphin, there was a mild to moderate diffuse lymphoplasmacytic infiltrate with occasional focal aggregates (Fig. 5). Many small (<5 μm), rod- to comma-shaped, silver-positive bacteria were closely associated with the epithelial lining of the gastric pits and, to a lesser extent, superficial areas of the fundic glands (Fig. 6). Similar infiltrates and organisms were observed in the main stomach of the Group B white-sided dolphin, although the tissues were considerably more autolyzed. The pyloric stomachs of both dolphins had minimal lymphoplasmacytic infiltrates in the superficial lamina propria and occasional silver-positive bacteria closely associated with the epithelium lining the stomach lumen and the apical portions of the gastric pits. In addition, the white-sided dolphin had multifocal moderately sized oval lymphocytic aggregates within the deep lamina propria and submucosa, where they were often perivascular. Rare trematodes observed in the main deep stomach mucosa of the common dolphin were not associated with inflammation.

DISCUSSION

In this study we have isolated and identified novel a *Helicobacter* species from the main stomachs of two stranded Atlantic white-sided dolphins. In addition, we have identified by PCR, using *Helicobacter*-specific primers, gastric helicobacters in seven dolphin stomachs. It is not surprising that we were unable to grow organisms from the nonglandular portion of the stomach (forestomach) given the fact that gastric helicobacters

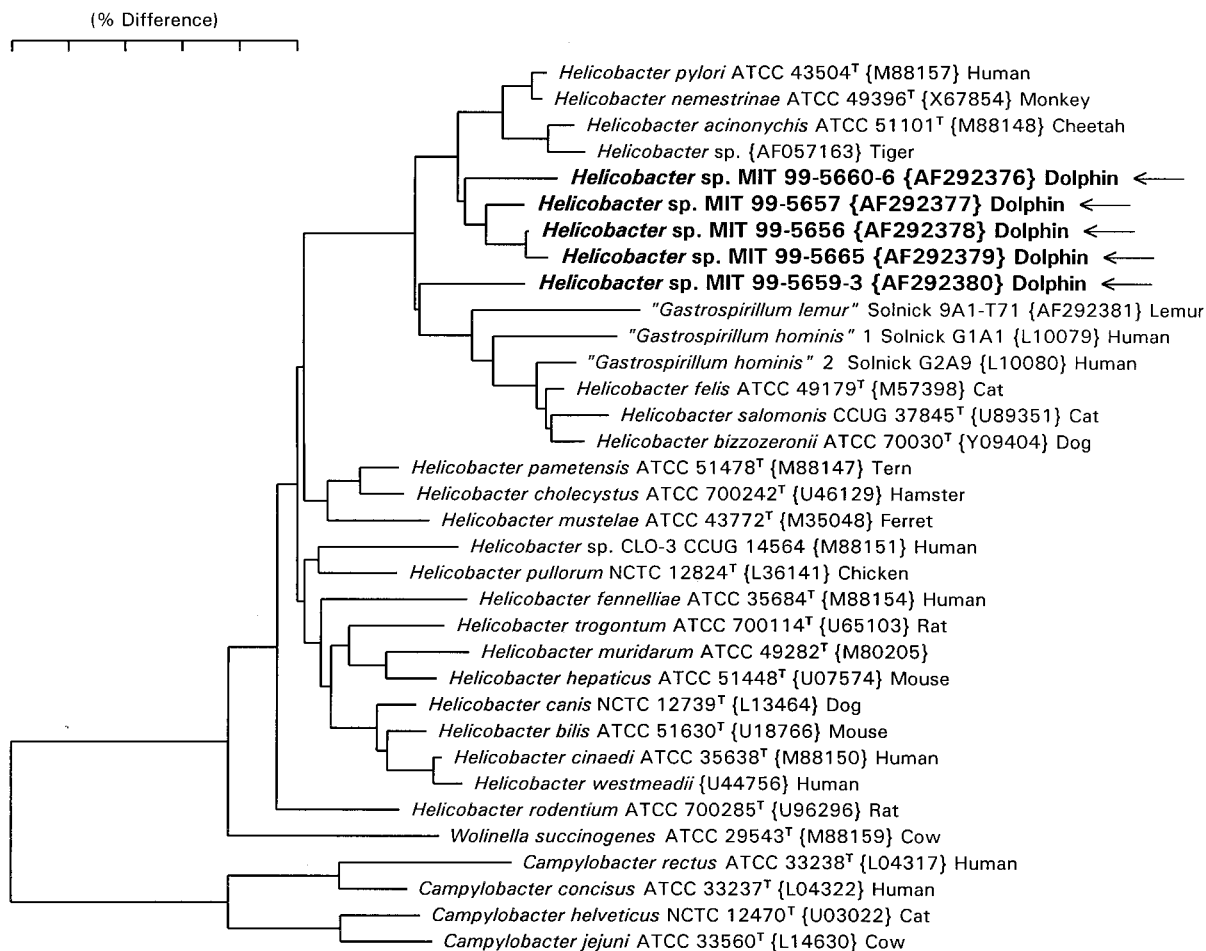


FIG. 2. Phylogenetic tree depicting the phylogenetic location of the novel *Helicobacter* sp. constructed on the basis of 16S rRNA sequence similarity values. The scale bar is equal to a 5% difference in nucleotide sequences as determined by measuring the lengths of the horizontal lines connecting two species.

colonize only glandular tissue. However, we identified by PCR the presence of *Helicobacter* spp. in both the glandular and nonglandular stomachs; the latter probably reflects gastric reflux into the more proximal forestomach. The *Helicobacter* spp. isolated from the dolphins' glandular stomachs were urease, catalase, and oxidase positive, which is biochemically consistent with the properties of other gastric helicobacters. Ultrastructurally, helicobacters are typically fusiform to spiral and possess various numbers of sheathed flagella, which can be distributed in a polar or bipolar manner. The novel dolphin

Helicobacter sp. is slightly spiral with bipolar sheathed flagella. Phylogenetically, the bacteria cluster with *H. pylori* differing by only 2 to 3%.

The microbial floras of human and animal stomachs have been the focus of considerable research since the discovery that *H. pylori* in humans caused a variety of gastric diseases (8–13, 15, 25, 27, 34). *Helicobacter* spp. have been isolated from the stomachs of a wide variety of mammals, including dogs, cats, ferrets, pigs, monkeys, and cheetahs, all of which have been associated with various degrees of gastritis in their hosts (9, 11, 37). *Helicobacter* species also have been isolated from the intestinal tracts of humans, other animals, and birds. There are currently at least 30 formally named species of the genus *Helicobacter*, and the majority have been proven to be or are suspected to be gastrointestinal pathogens (37). Furthermore, several of these organisms have zoonotic potential (9). Every year additional *Helicobacter* species are identified, some of which have been determined to be important pathogens in humans and animals.

Most of the reports on the epizootiology of helicobacter infections in animals have been done with *H. mustelae* in ferrets. These studies have focused on assessing the age of acquisition, mode of transmission, and susceptibility to natural or experimental reinfection following eradication (15). These data support the concept that *H. mustelae* (and perhaps other

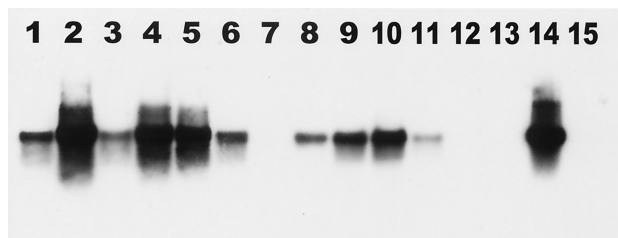


FIG. 3. Southern blot hybridization of the 1,200-bp PCR products of *Helicobacter*-specific primers hybridized with helicobacter 16S rRNA gene PCR product. Lanes 1 to 13, DNA extracted from stomach tissue of six dolphins (animals 1 to 6, accession numbers 99-5656 to 99-5668); lane 14, *Helicobacter* positive control; lane 15, reagent control.

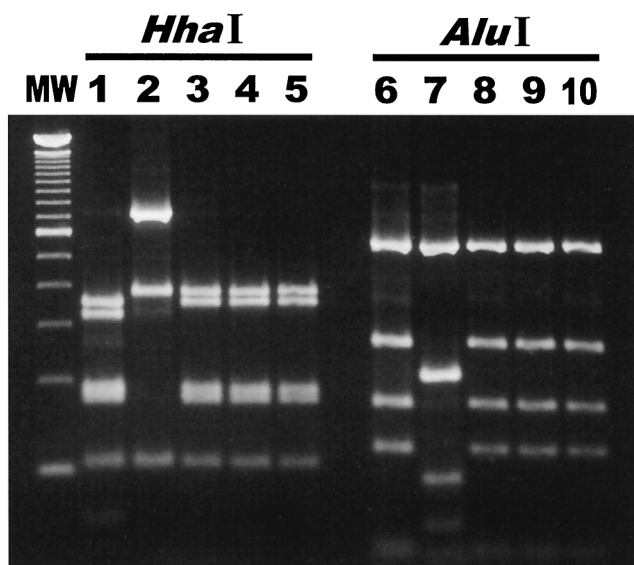


FIG. 4. PCR-RFLP patterns of the 1,200-bp species-specific *Helicobacter* PCR product from the dolphin isolates and from stomach tissue. Lanes 1 and 2, 99-5660 and 99-5659 DNA from dolphin stomach tissue digested by *HhaI*; lanes 3 to 5, 99-5656, 99-5657, and 99-5665 DNA from dolphin isolates digested by *HhaI*; lanes 6 and 7, 99-5660 and 99-5659 DNA from dolphin stomach tissue digested by *AluI*; lanes 8 to 10, 99-5656, 99-5657, and 99-5665 DNA from dolphin isolates digested by *AluI*. Two patterns were observed in the *HhaI* digests, and two patterns were observed in the *AluI* digests.

gastric helicobacters) transmission is fecal-oral (3, 12, 13). *H. pylori* also has been cultured from feces and may survive in water in a nonculturable but viable coccoid form. The other proposed route is oral-oral (27, 37), which is supported by the observation that *H. pylori* has been cultured from saliva and

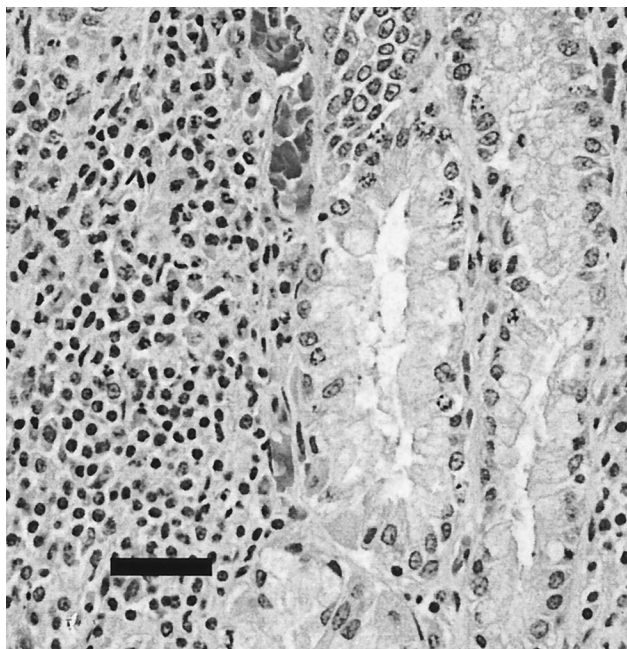


FIG. 5. Common dolphin (MIT 99-7442), main stomach, hematoxylin and eosin stain. Within the superficial lamina propria of the main stomach there is a focus of lymphoplasmacytic infiltration, and bacteria are associated with the epithelial surfaces of adjacent gland lumens. Bar, 20 μ m.

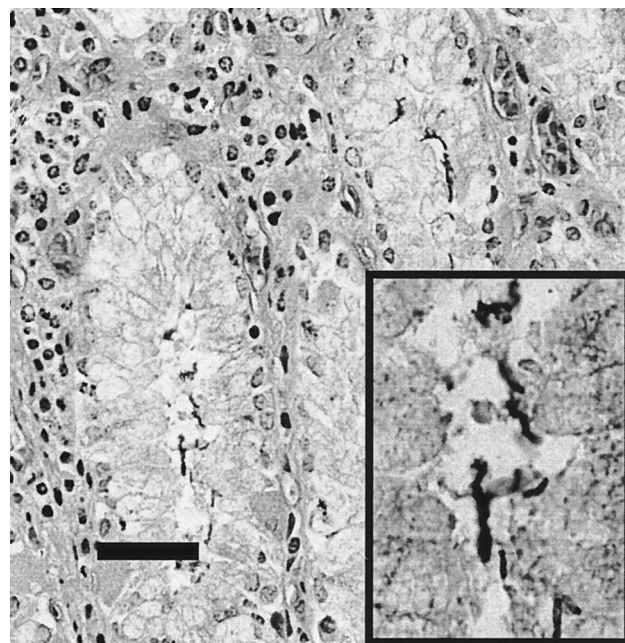


FIG. 6. Common dolphin (MIT 99-7442), main stomach, Warthin-Starry stain. Plump comma-shaped bacteria that are positive with Warthin-Starry stain are closely associated with the epithelial surfaces lining gastric lumens within the main stomach. The inset provides an additionally magnified image of bacteria. Bar, 20 μ m.

dental plaque in humans and from the saliva of *H. pylori*-infected cats (2, 17, 21, 27).

Gastric ulcers have been reported in wild and captive cetaceans. In certain cases the ulcerations were reported in dolphins and whales, such as *Delphinus delphis*, *Stenella coeruleoalba*, *Tursiops truncatus*, *Phocoena phocoena*, and *Globicephala mela* (1, 5, 23, 32, 35). Many of these cases were associated with parasitic infections, e.g., *Anisakis simplex*, *Pholeter gastrophilus*, and *Braunina cordiformis* (1, 5, 23, 26). The lesions were noted in the forestomach and the main stomach. However, incidences of nonparasitic gastric ulcers with no clearly defined etiology have also been noted in various species such as *Delphinapterus leucas*, *Tursiops truncatus*, and *Phocoena phocoena* (6, 20, 35, 36). However, there are shortcomings in the published reports regarding cetacean gastric ulcers. First, there was a lack of microbiological analysis associated with the lesions, and in many instances the poor quality of the tissue available precluded any in-depth histological analysis. Furthermore, most of the gastric lesions were defined primarily by gross observation.

Histological samples were unfortunately not taken from animals in Group A due to circumstances associated with the timing and location of the stranding. The histological sections analyzed in the two dolphins from Group B had gastric lesions which parallel those observed in *H. pylori*-infected humans, including multifocal lymphoplasmacytic gastritis. The Warthin-Starry stain clearly showed fusiform to slightly spiral bacteria closely associated with gastric epithelia and in areas of gastritis.

Our findings suggest that a novel *Helicobacter* sp. plays a role in the etiopathogenesis of gastritis in the main and pyloric stomachs of dolphins and may play a role in the development of gastric ulcers. To our knowledge this represents the first isolation and characterization of a novel *Helicobacter* sp. from a marine mammal and emphasizes the wide host distribution and pathogenic potential of this increasingly important genus.

Further work is required to confirm whether these microorganisms play a role in etiopathogenesis of PUD in wild and captive dolphins.

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