Culture and Characteristics of Helicobacter bizzozeronii, a New Canine Gastric Helicobacter sp.

MARJA-LIISA HÄNNINEN,1* I. HAPPOinen,2 S. SAARI,3 AND J. KALAVA1
Department of Food and Environmental Hygiene,1 Department of Veterinary Clinical Sciences,2 and Institute of Pathology,3 Faculty of Veterinary Medicine, University of Helsinki, 00581 Helsinki, Finland

Organisms whose cells were large, tight spirals were isolated from gastric biopsies of dogs. Touch cytology samples from all of the dogs contained large spiral organisms. Characteristics of 10 strains are described. These organisms were 5 to 10 μm long by 0.3 μm wide, and each cell had 10 to 20 sheathed flagella at both ends of the cell. The cells did not have periplasmic fibrils. These organisms were microaerophilic and grew at 37 and 42°C but not at 25°C on brain heart infusion agar containing blood. They did not grow on brucella blood agar. They were catalase and oxidase positive, hydrolyzed urea but not hippurate, reduced nitrate, and were resistant to nalidixic acid but susceptible to cephalothin and metronidazole. In contrast to Helicobacter felis, they hydrolyzed indoxyl acetate. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles of all of the strains were similar, and the protein patterns of these organisms differed from those of other Helicobacter spp. Dot blot DNA-DNA hybridization experiments revealed that the new strains were closely related to each other but clearly different from H. felis, Helicobacter pylori, Helicobacter mustelae, and Campylobacter jejuni. The name Helicobacter bizzozeronii sp. nov. is proposed for these organisms. Our results suggest that other “uncultured” gastric helicobacters may be cultured if optimal culture conditions are found.

Since the end of the 19th century several authors have described spiral organisms that occur in the gastric mucosae of dogs and cats (1, 23, 25). Salmon found that such organisms colonize both the fundus and antrum areas of dogs, and he was able to infect mice with the organisms by feeding them pieces of gastrointestinal biopsies (25). The results of the electron microscopic studies of Weber and Schmittdiel (31) confirmed the spiral morphology of the organisms. Lockard and Boler (16) described three morphologically different types of spiral organisms that they found in gastric mucosae of dogs. Structurally, each cell of one of these organisms was a straight cylinder with a filament tightly coiled around its body. The cells of another type were large spirals with coiled helical filaments visible at the crest of each spiral. The cells of the third morphological type were tightly coiled and did not have visible filaments. Lockard and Boler (16) suggested that the three morphological forms represented various stages that occurred during movement of the same organism, and they suggested that the organism should be called Spiriochaetes instead of Spirillum rappini, as suggested previously by Rappin (2a, 24). The organisms could not be cultured.

Helicobacter pylori, a human-adapted gastric spiral organism, was cultured for the first time from a gastric biopsy about 10 years ago (18). During the last 10 years, there has been increasing interest in spiral organisms which colonize the gastrointestinal tracts of humans and animals (2, 3, 12, 13). The most important reason for the interest in gastrointestinal spiral organisms is the evident association of chronic Helicobacter pylori infections of humans with peptic ulceration and gastric cancer (2, 20).

In 1988 Lee et al. (14) cultured one of the spiral organisms that had been described morphologically previously from gastric biopsies of dogs and cats. This organism was later assigned to the genus Helicobacter and was named Helicobacter felis (21). H. felis is a long spiral organism, and it has typical periplasmic fibrils (21). The other spiral organism which resembles H. felis morphologically when light microscopy is used is also commonly found in gastric mucosae of dogs and cats. If electron microscopy is used, this organism can be differentiated from H. felis: the cells are more tightly coiled, and no periplasmic fibrils can be seen (3, 12, 16, 21). Attempts to culture this gastric spiral organism have been unsuccessful (3, 13). Both of these organisms have sheathed flagella at both ends of each cell (12, 21). Large, uncultured, morphologically similar organisms found in gastric mucosae of dogs, cats, pigs, and humans have been provisionally placed in the genus “Gastrospirillum” (10, 19, 23, 27).

A large, spiral organism has been found in a minority of human gastric biopsies in association with gastritis (10, 19, 32). Because this organism has not been cultured so far, its taxonomic description is based on the results of cloning and sequencing of its 16S rRNA gene after PCR amplification of samples obtained from infected gastric mucosae of mice fed gastric mucosa samples from two infected humans (27). The results of these studies suggested that there were probably two species of large spiral organisms (“Gastrospirillum” species 1 and 2) in the human gastric samples because there were two 16S rRNA sequences that differed by 3.5%. The 16S rRNA sequences obtained were most closely related to the 16S rRNA sequence of H. felis, and the organisms actually belonged to the genus Helicobacter (“Helicobacter heilmannii”). Human infections with large spiral organisms are thought to be zoonotic because the organisms found in gastric mucosae of humans and dogs are morphologically similar. Also, the results of epidemiological studies have suggested that infections may be transmitted from dogs to humans (27–29).

In this paper we describe the characteristics of large spiral organisms which we cultured from gastric mucosae of dogs. The results of electron microscopic studies, biochemical characteristics, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) protein profiles clearly showed that these organisms differed from H. felis, “Flexispira rappini,” and other previously cultured Helicobacter spp. that occur in gastric mucosae. The results of DNA-DNA hybridization stud-
ies confirmed that all 10 strains which we studied were closely related and that they differed from \textit{H. felis} and other related helicobacters. We propose the nomenclature \textit{Helicobacter bizzozeronii} sp. nov. for these organisms. Below we use the name \textit{H. bizzozeronii} for the large, canine, gastric, spiral organism which we studied.

**MATERIALS AND METHODS**

**Culture of samples.** Helicobacters were isolated from biopsy samples obtained from pet dogs after endoscopy or from gastric mucosa of euthanized dogs postmortem at the University of Veterinary Medicine in Helsinki, Finland. The samples were obtained from the antrum and fundus areas. The biopsy samples either were cultured directly within 1 h of sampling or were stored in Portagarm pylori transport medium (BioMerieux SA, Marcy l’Etoile, France) for 2 to 12 h before they were cultured. Direct Gram stain (mucus touch cytology) and rapid urease tests were performed with the biopsy samples, and histological samples were tested with 10% formaldehyde for later analysis.

The samples were crushed in 300- to 500-μl portions of brain heart infusion (BHI) (Difco) broth containing 7% horse serum, and the preparations were spread onto fresh BHI blood agar plates containing antibiotics (2.5 μg of trimethoprim per ml, 5 μg of vancomycin per ml, 1.25 IU of polymyxin B per ml, and 2 μg of amphotericin B per ml). These plates were incubated under microaerophilic conditions by using a model BR 56 gas-generating kit (Oxoid, Basingstoke, United Kingdom) with the lids uppermost at 37°C for 10 days. Usually growth occurred after 3 to 5 days of incubation as a thin spreading film.

**Method of incubation** has been described previously (9). The isolates that had the typical spiral morphology were isolated from different dogs with various degrees of gastritis between 1989 and 1994.

We used a Jeol Campylobacter jejuni strain. \textit{H. felis} (\textit{ATCC} 49179), \textit{H. pylori} (\textit{ATCC} 26695 and TX 30A), \textit{Helicobacter muselae} \textit{ATCC} 43772, and \textit{F. rappini} (a dog isolate) as controls.

**Ultrastructural studies.** The culture samples used for scanning electron microscopy were fixed with 10% formaldehyde for later analysis. The organisms were examined several times after several subcultures. The typical in vivo location of \textit{H. bizzozeronii} in a gastric gland is shown Fig. 1D.

**Isolation and growth characteristics.** Most of the strains (8 of 10 strains) were isolated from corpus samples. In two cases identical strains were isolated from corpus and antrum samples. We studied 30 dogs, and large spiral or helical organisms were found in all touch cytology samples. \textit{H. bizzozeronii} was isolated from 10 dogs, and \textit{H. felis} was isolated from two dogs. Differentiation between \textit{H. bizzozeronii} and \textit{H. felis} was originally based on the results of ultrastructural studies. During primary isolation the organisms grew after 5 to 10 days of incubation as thin, spreading nonhemolytic areas on moist BHI blood agar. In subsequent cultures the incubation time necessary for growth to become visible was between 3 and 5 days. Usually, \textit{H. felis} exhibited visible growth after a shorter incubation time than \textit{H. bizzozeronii}.

**Growth on different media and conditions.** The best growth was obtained on fresh BHI blood agar. The organisms grew also on Mueller-Hinton agar and nutrient agar supplemented with blood or horse serum. There was minimal growth on brucella blood agar. Only slight growth occurred in broth media, even though serum was added and a shallow medium was used (5).

**Biochemical and tolerance tests.** A total of 24 tests were performed in order to study the characteristics of 10 \textit{H. bizzozeronii} isolates obtained from dog gastric mucosa. Typical biochemical characteristics and the results of the tolerance tests are shown in Table 1. All of the strains were oxidase, catalase, and urease positive. They reduced nitrate and TTC, they were positive in the indoxyl, acetate, gamma-glutamyl transferase, and alkaline phosphatase tests, and they were negative in the hippurate hydrolysis, pyridoxal arylamidase, t-arginine arylamidase, and L-aspartate arylamidase tests. They were resistant to nalidixic acid and susceptible to cephalothin, ce-

**DNA-DNA hybridization.** Chromosomal DNA was isolated by the modified method of Pitcher et al. (22). Phenol-chloroform was used for DNA extraction instead of chloroform, and an RNase treatment was used to remove the RNA.

**RESULTS**

**Ultrastructure.** Our ultrastructural studies revealed that the organisms which we studied were similar to the organisms found previously in gastric mucosa of dogs (12, 16) and resembled an organism called \textit{‘H. helimannii’} found in human gastric biopsy samples (10). The cells were large spirals that were 5 to 10 μm long and 0.3 μm wide and had three to eight spirals per cell and no periplasmic fibers (Fig. 1A and B).

There were multiple sheathed flagella at both ends of each cell (Fig. 1B); these flagella were slightly off center at the ends of the cells (Fig. 1C). Cocccoid forms were observed in older cultures (Fig. 1A). The ultrastructural characteristics of the organisms were examined several times after several subcultures and were the same in all studies.

The typical in vivo location of \textit{H. bizzozeronii} in a gastric gland is shown Fig. 1D.

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FIG. 1. (A) Scanning electron micrograph of *H. bizzozeronii* sp. nov. on a nylon membrane after growth on BHI blood agar, showing the characteristic spiral morphology. No periplasmic fibrils are visible. Coccolid forms are evident. Bar = 5 \(\mu\)m. (B) Transmission electron micrograph of a negatively stained cell of *H. bizzozeronii* sp. nov., showing the spiral morphology and the multiple sheathed flagella at both ends of the cell. Bar = 0.5 \(\mu\)m. (C) Transmission electron micrograph of a negatively stained *H. bizzozeronii* sp. nov. cell, showing the position of flagella slightly off center. Bar = 0.5 \(\mu\)m. (D) Presence of *H. bizzozeronii* sp. nov. in vivo in the gastric gland lumen of a dog. Abbreviations: P, parietal cell; C, chief cell. The arrow indicates sheathed flagella. Bar = 1 \(\mu\)m.
foperazone, and metronidazole. They did not grow on medium containing 1% ox bile, 1% glycine, or 1.5% NaCl. They grew at 37 and 42°C but not at 25°C. All of the characteristics studied except indoxyl acetate hydrolysis were similar to the characteristics found for *H. felis* CS1. All *H. bizzozeronii* strains and *H. felis* CS1 produced DNase.

**SDS-PAGE protein profile patterns.** The total-protein profile patterns of the *H. bizzozeronii* strains were similar (Fig. 2).
The major bands were bands at 30 and 42 kDa, a double band at 50 to 55 kDa, bands at 59 and 62 kDa, and two double bands at 72 to 74 and 84 to 88 kDa. In contrast, the protein profile of *H. felis* CS1 differed from the *H. bizzozeronii* profiles. *H. felis* produced major bands at 40, 53, 56, 59, 66, 72, and 105 kDa. In particular, the strong bands at 56 and 105 kDa found in the *H. felis* profile were not present in the *H. bizzozeronii* profile. The bands at 62 and 66 kDa represented urease B subunits in *H. bizzozeronii* and *H. felis*, respectively (7). The intensities of the bands at 59, 62, and 66 kDa varied according to the age of the culture. In young cultures (cultures that were 3 to 5 days old) the band at 59 kDa was strong, and in cultures that were 5 to 10 days old the urease B subunit bands predominated.

DNA-DNA hybridization. The relative levels of hybridization were assessed by comparing the intensities of the color that developed with the three concentrations of DNA added to the membrane. The DNA-DNA hybridization results showed that all of the *H. bizzozeronii* strains hybridized intensively with the two *H. bizzozeronii* probe strains used. The probes hybridized weakly with the DNA of *H. felis* CS1. No reaction was observed with *C. jejuni*, *H. mustelae*, or *H. pylori*. The *H. felis* CS1 probe DNA hybridized weakly with the *H. bizzozeronii* strains. "*F. rappini*" did not hybridize with the *H. bizzozeronii* strains.

**DISCUSSION**

The number of new species in the genus *Helicobacter* has expanded greatly since the first two species, *H. pylori* and *H. mustelae* (6), were described. Twelve *Helicobacter* species have been described in recent papers, and at least six new *Helicobacter* species are waiting for more definite taxonomic descriptions (4). In this paper we describe a new *Helicobacter* species which has been studied ultrastructurally by using histological tissue samples from dogs for more than 100 years but which has not been cultured previously (2a, 12, 16, 24). The morphology of this bacterium is similar to the morphology of organisms provisionally called "*H. helmannii*" and "*Gastrospirillum suis*" and found in histological specimens from humans (10, 19) and pigs (23), respectively. *H. bizzozeronii* is a 5- to 10-μm-long, spiral bacterium that has multiple sheathed flagella at both ends of each cell. The most significant morphological difference between this organism and *H. felis* is the lack of periplasmic fibrils in the former. When light microscopy is used, reliable differentiation of *H. bizzozeronii* and *H. felis* is not possible because both organisms have a spiral morphology and their sizes are similar. Although *H. bizzozeronii* was more common than *H. felis* in gastric biopsy samples from dogs, *H. felis* was also detected. On BHI blood agar the growth of *H. bizzozeronii* and the growth of *H. felis* were similar. After primary incuba-

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**TABLE 1. Characteristics of *H. bizzozeronii* and related gastric helicobacters**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>Helicobacter bizzozeronii</em></th>
<th><em>Helicobacter felis</em></th>
<th><em>Helicobacter pylori</em></th>
<th><em>Helicobacter acinonyx</em></th>
<th>“Flexispira rappini”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periplasmic fibrils</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Location of flagella</td>
<td>10-20</td>
<td>14-20</td>
<td>4-8</td>
<td>2-5</td>
<td>10-20</td>
</tr>
<tr>
<td>No. of flagella</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flagellar sheath</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase activity</td>
<td>+ (10/10)°</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase activity</td>
<td>+ (10/10)°</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease activity (rapid)</td>
<td>+ (10/10)°</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Hippurate hydrolysis</td>
<td>− (10/10)°</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Indoxyl acetate hydrolysis</td>
<td>+ (10/10)°</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>γ-Glutamyl transpeptidase activity</td>
<td>+ (10/10)°</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TTC reduction</td>
<td>+ (10/10)°</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Alkaline phosphatase activity</td>
<td>+ (10/10)°</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNase activity</td>
<td>+ (10/10)°</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Growth at:</td>
<td>− (10/10)°</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>25°C</td>
<td>− (10/10)°</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>37°C</td>
<td>+ (10/10)°</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>42°C</td>
<td>+ (10/10)°</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Tolerance to 1% ox bile</td>
<td>− (10/10)°</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Susceptibility to:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nalidixic acid (30 μg disk)</td>
<td>R (10/10)°</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Cephalothin (30 μg disk)</td>
<td>S (10/10)°</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Cefoperazone (60 μg disk)</td>
<td>S (10/10)°</td>
<td>S</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Metronidazole (50 μg disk)</td>
<td>S (8/10)°</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

*−*, negative or not present; +, positive or present; ND, not determined; R, resistant; S, susceptible.
° The data in parentheses are number of strains that have the characteristic indicated/number of strains tested.
tion, both organisms grew as thin, spreading nonhemolytic films on the agar. Both organisms produced cocoid forms in older cultures, as described previously for campylobacters and other helicobacters (5).

**Helicobacter** spp. can be divided into two groups on the basis of their primary ecological niches (either the gastric mucosa or the lower gastrointestinal tract) (26). *H. bizzozeronii* is a species that is adapted to gastric areas; *H. bizzozeronii* cells are found superficially in mucus and in gastric glands, as well as inside parietal cells (3, 12, 16). All known primary gastric *Helicobacter* spp., including *H. bizzozeronii*, contain rapidly preformed urease, which probably plays a role during colonization (2, 3, 7). One other typical characteristic of the gastric helicobacters is their sensitivity to bile and bile salts (5, 8) compared with the intestinal helicobacters, which can tolerate bile salts at concentrations of up to 20% (4). *H. bizzozeronii* is sensitive to bile.

Other biochemical characteristics of *H. bizzozeronii*, including utilization of glycine, tolerance to NaCl, susceptibility to cephalothin, cepofarzone, and metronidazole, and resistance to nalidixic acid, were similar to characteristics of *H. felis*. Both species also produced DNase. *H. bizzozeronii* differed from *H. felis* by reacting positively in indoxyl acetate hydrolysis and TTC reduction tests; *H. felis* is negative in these tests (21).

Our SDS-PAGE profile analysis of whole-cell proteins produced reliable, reproducible results which could be used to differentiate *H. bizzozeronii* from *H. felis*. *H. felis* produced major protein bands at 56, 72, and 105 kDa which were not found in *H. bizzozeronii*. These proteins might be associated with the periplasmic fibrils observed on the surfaces of *H. felis* cells. The ureases of helicobacters are visible on denatured gels as two subunits, one at about 62 to 66 kDa and one at 30 kDa (7). The *H. felis* urease had two subunits, which had molecular weights of about 66,000 and 30,000 (7). The molecular weight of the putative *H. bizzozeronii* urease B subunit (62,000 to 63,000) was lower than the molecular weight of the urease B subunit of *H. felis*. A more detailed study of the membrane proteins of canine gastric helicobacters is in progress.

Our DNA-DNA hybridization data confirmed that all of the large, canine, spiral organisms were closely related to each other and were clearly different from *H. pylori* and *H. mustelae*. There was some hybridization between *H. bizzozeronii* and *H. felis* strains; a weak color developed when *H. bizzozeronii* strains were hybridized with the *H. felis* probe DNA. Because *H. bizzozeronii* has not been cultured previously, there have been suggestions that *H. felis* and *H. bizzozeronii* may represent different forms of the same organism (16, 27). Our data confirmed that these two large, gastric, spiral organisms differ morphologically, phenotypically, and genetically and that they are two different *Helicobacter* spp. which both colonize the gastric mucosa of dogs.

It was not possible to study the relationship between the human parasite “*H. heilmannii*” and the canine parasite *H. bizzozeronii* because “*H. heilmannii*” has not been cultured yet. Morphological and 16S rRNA sequence data are the only data for “*H. heilmannii*” that are available. Morphologically, the canine and human organisms are similar. A 16S rRNA sequence analysis of canine *H. bizzozeronii* strains is in progress in our laboratory. A comparison of the 16S rRNA sequences should provide more information concerning the possible relationship between the canine and human organisms. However, in order to confirm the possible identity of *H. bizzozeronii* and “*H. heilmannii*,” it will be necessary to compare phenotypic and genetic characteristics of cultured organisms.

**Taxonomic description of Helicobacter bizzozeronii** sp. nov.

*Helicobacter bizzozeronii* (biz.zo.ze.ro.ni.i. L. gen. n. bizzozeroni, of Bizzozeron, referring to Giulio Bizzozeron, an Italian pathologist who was one of the first scientists to describe canine gastric spiral organisms [1]); The cells are spirals that are 0.3 by 5 to 10 μm. They do not have periplasmic fibrils. In older cultures cocoid forms predominate. The cells are gram negative and nonsporulating. They are motile by means of tufts of 10 to 20 sheathed flagella at both ends of each cell. Individual colonies are not usually produced on agar media, but cultures grow as spreading films on fresh moist agar media. All strains are oxidase, catalase, and urease positive. They reduce nitrate and TTC, and they are positive in indoxyl acetate, gamma-glutamyl transpeptidase, and alkaline phosphatase tests. They are negative in hippurate hydrolysis, pyrrolidonyl arylamidase, l-arginine arylamidase, and l-aspartate arylamidase tests.

They are resistant to nalidixic acid and susceptible to cephalothin, cefoperazone, and metronidazole. They do not grow on medium containing 1% ox bile, 1% glycine, or 1.5% NaCl. They grow at 37 and 42°C but not at 25°C. All of the biochemical and tolerance characteristics which we studied except indoxyl acetate hydrolysis are similar to the characteristics of *H. felis* CS1. All *H. bizzozeronii* strains and *H. felis* CS1 produce urease. Two strains have been deposited in the culture collection of the University of Göteborg, Sweden: CCUG 35045 and CCUG 35046.

**REFERENCES**


