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Prevalence and Varieties of *Helicobacter* Species in Dogs from Random Sources and Pet Dogs: Animal and Public Health Implications

K. A. EATON,[1]* F. E. DEWHIRST,[2] B. J. PASTER,[2] N. TZELLAS,[2] B. E. COLEMAN,[2] J. PAOLA,[1] AND R. SHERDING[1]

Department of Veterinary Biosciences, Ohio State University, Columbus, Ohio 43210,[1] and Department of Molecular Genetics, Forsyth Dental Center, Boston, Massachusetts 02115[2]

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Gastric bacteria of a variety of ultrastructural morphologies have been identified in or isolated from domestic carnivores, but their prevalence in different populations of animals and their clinical significance are still unknown. The purposes of this study were (i) to evaluate the prevalence and morphologic types of gastric bacteria in three different populations of dogs; (ii) to determine which of the organisms were culturable, and if the cultured organisms were morphologically similar to the organisms seen in situ; (iii) to identify the isolated organisms; and (iv) to determine if gastric bacteria were associated with gastritis. Three groups of dogs were examined: healthy laboratory dogs, healthy dogs from an animal shelter, and pet dogs with various nongastric illnesses. Of these, 100% of laboratory and shelter dogs and 67% of pet dogs were colonized by large, tightly coiled gastric spiral bacteria morphologically similar to *Gastrospirillum hominis* or *Helicobacter felis* (referred to as gastrospirilla). Regardless of the presence or density of gastric bacteria, all of the dogs in the study except one had mild to moderate gastritis. *Helicobacter* spp. were isolated from only 6 of 39 stomachs cultured, and only three of the organisms isolated were morphologically similar to the bacteria seen in situ. Five helicobacters were identified by 16S rDNA (genes coding for rRNA) sequence analysis. Three were strains of *H. felis*, one was *H. bilis*, and one was a novel helicobacter morphologically similar to "*Flexispira rappini*." Gastrospirilla are almost universal in the stomachs of domestic dogs, and in most infected dogs, they do not appear to be associated with clinical signs or histologic lesions compared with uninfected dogs. Nongastrospirillum helicobacters are rare in dogs and are not histologically detectable. *Helicobacter pylori* was not isolated from domestic dogs.

The discovery of *Helicobacter pylori* in human beings (18) and its relationship to gastritis, peptic ulcer, and gastric neoplasia (22) has led to renewed interest in the incidence and clinical significance of gastric bacteria in domestic pets, specifically cats and dogs. The resulting research has focused on two specific areas of significance. First, it has been suggested that domestic animals could be a source of infection for human beings (21, 31, 32). Part of evaluating this possibility requires examination of the prevalence and types of gastric bacteria in dogs and cats. Second, the clinical significance of gastric helicobacters and related organisms in dogs and cats is of interest from a veterinary perspective as well as in terms of the development of animal models of human disease.

Research over the past 100 years has indicated that gastric bacteria are common in dogs, cats, and other mammals (6, 11, 12, 16, 21, 34 [for review, see reference 5]). The most common gastric organisms described are 7- to 10-μm-long tightly coiled spiral bacteria which live deep in the gastric glands and in the parietal cell canaliculi. These organisms, originally called gastric spirilla or spirilliform bacteria (11, 34), are now variously referred to as *Gastrospirillum* sp., *Gastrospirillum hominis*, and *Gastrospirillum suis* (19, 20, 30). Two unique human species were identified by 16S rRNA sequence analysis (30) and given the name "*Helicobacter heilmannii*." These species have been given the designations "*Gastrospirillum hominis* 1" (GenBank accession no. L10079) and "*G. hominis* 2" (GenBank accession number L10080). Recently, 10 strains were isolated from dogs and given the name *Helicobacter bizzozeronii* (8). It is probable that the large, tightly coiled gastric spiral bacteria seen in many mammalian species represent several different *Helicobacter* species. In this report, we will use the term gastrospirilla for this morphologically defined group of mammalian gastric organisms. Among the helicobacters, the gastrospirilla are most similar to *Helicobacter felis* in their 16S rRNA sequences, large size (7 to 10 μm), and habit of growth deep within the gastric glands and gastric parietal cell canaliculi (24, 30). Unlike *H. felis*, however, they lack superficial periplasmic fibers.

"*Flexispira rappini*" is a morphologic type of helicobacter characterized by fusiform shape, periplasmic fibers, and several amphitrichous flagella and found in rats, mice, sheep, and humans (5, 25, 29, 33). Examination of over a dozen "*F. rappini*" isolates indicates that there are at least six species with this morphology, including *Helicobacter bilis* (GenBank accession number U51873) and "*F. rappini*" (GenBank accession number M88138) (1). In this report, we will use the term flexispiras to refer to helicobacters with "*F. rappini*" morphology.

Despite the frequent occurrence of gastric bacteria in dogs and cats, there have been few studies examining the prevalence of these bacteria in different populations of animals, evaluating the presence of helicobacter-like bacteria other than gastrospirilla in domestic carnivores, or evaluating the pathogenicity of these species. Studies of laboratory cats and dogs with either naturally occurring (11, 34) or experimentally induced (2, 4, 7,

* Corresponding author. Mailing address: Department of Veterinary Biosciences, Ohio State University, 1925 Coffey Rd., Columbus, OH 43210. Phone: (614) 292-9667. Fax: (614) 292-6473. Electronic mail address: keaton@magnus.acs.ohio-state.edu.

TABLE 1. Oligonucleotide primers used for PCR amplification and sequencing of 16S rDNA

Primer no.	Identification	Type	Sequence (5′→3′)[a]	Position[b]	Orientation
1	C71	PCR	GAGAGTTTGATYMTGGC	7–23	Forward
2	C72	PCR	GYTACCTTGTTACGACTT	1492–1509	Reverse
3	B12	Sequencing	TGGCGCACGGGTGAGTAA	103–120	Forward
4	X88	Sequencing	GTATTAATCACCGTTTC	159–175	Reverse
5	B34	Sequencing	RCTGCTGCCTCCCGT	344–358	Reverse
6	B35	Sequencing	GTRTTACCGCGGCTGCTG	519–536	Reverse
7	B36	Sequencing	GGACTACCAGGGTATCTA	789–806	Reverse
8	C01	Sequencing	GGTTGCGCTCGTTGCGGG	1096–1113	Reverse
9	C31	Sequencing	GGAATCGCTAGTAATCG	1337–1353	Forward
10	X10	Sequencing	ACGGGCGGTGTGTRC	1392–1406	Reverse

[a] Base codes are standard International Union of Biochemistry codes for bases and ambiguity.
[b] Numbering based on the *E. coli* sequence.

15, 21, 26) infections with gastrospirilla, *H. felis*, or *H. pylori* demonstrated mild histologic lesions but no clinical signs. Some clinical studies with pet dogs and cats have suggested that gastrospirilla may be associated with clinical signs or histologic gastritis, but the almost universal presence of these bacteria in both sick and healthy animals precludes adequate evaluation of uninfected controls (6, 12).

The purposes of this study were first, to evaluate the prevalence and morphologic types of gastric bacteria in three populations of dogs; second, to determine what fraction of these organisms were culturable and whether the cultured organisms were morphologically similar to the organisms seen in situ; third, to identify the isolated organisms on the basis of morphology, urease and catalase testing, and sequence analysis of rDNA genes (genes coding for rRNA); and, finally, to determine if naturally occurring gastric bacteria were associated with gastritis in dogs.

MATERIALS AND METHODS

Animals. Dogs from three sources were evaluated. Group A consisted of 31 dogs from a commercial supplier of random-source laboratory dogs. These dogs were male and female mixed-breed dogs which ranged in age from 6 months to 4 years. They were all clinically healthy. Group B consisted of eight dogs from a local animal shelter (four males and four females). These dogs were all clinically healthy young and mature adults. Group C consisted of 15 male and female dogs which were presented to the pathology service of the Ohio State University School of Veterinary Medicine. These dogs ranged in age from 6 weeks to 16 years and had died or had been euthanatized for various nongastric illnesses.

Examination procedures. Group A dogs were examined endoscopically. Biopsy samples for histologic examination and culture were taken from cardiac, fundic, and antral areas of the stomach (one biopsy sample from each site). Group B and C dogs were examined by necropsy. Group B dogs were necropsied within 1 h of death, and group C dogs were necropsied within 24 h of death. Only tissue sections in which autolysis was absent or minimal (i.e., did not interfere with the interpretation of mild superficial gastritis) were included in group C. At necropsy, stomachs were removed, opened along the greater curvature, and examined. Samples from the cardia, fundus, and pyloric antrum were removed for urease testing, culture, and histopathologic examination. Mucosal samples for the urease test (2 to 4 mm in diameter) were immediately immersed in urease indicator medium (10). They were scored positive if the indicator turned red within 3 h and negative if there was no color change within that time. Samples for histologic examination were immersed in 10% neutral buffered formalin.

Cultures. Only stomachs from dogs which had been dead for 1 h or less (that is, dogs in groups A and B) were cultured. Samples for culture were inoculated onto blood agar plates containing Skirrow's supplement (vancomycin, 0.01%; trimethoprim, 0.05%; and polymyxin B, 2.5 IU/ml). The biopsy samples were spread over the entire surface of the plate, and care was taken to ensure close contact of the mucosal surface with the agar. Plates were incubated in 5% O_2–10% CO_2–85% nitrogen at 37°C and 90% humidity and examined after 4 days. Growth suggestive of a *Helicobacter* sp. was identified either as small (1 mm or less in diameter), clear, dome-shaped colonies or as a fine, translucent lawn. Such growth was harvested with a sterile cotton-tipped applicator, tested for urease activity, subcultured onto fresh blood agar plates without an antibiotic supplement, and incubated for a further 4 days. Plates which showed no obvious growth were swabbed with a sterile cotton-tipped applicator, spread onto a fresh blood agar plate, and incubated as described above. Once pure cultures were achieved, they were examined for urease and catalase activity by Gram staining, by examination of wet mounts, and by electron microscopic examination.

Electron microscopy. Bacteria were harvested by scraping the surface of blood agar plates with a wire loop. They were suspended in 10% neutral buffered formalin and collected by centrifugation in a microcentrifuge for 1 min. Bacterial pellets were post-fixed in osmium tetroxide, embedded, stained with uranyl acetate, and examined with a Phillips 300 electron microscope (3).

Histologic examination. Formalin-fixed sections from the cardia, antrum, and fundus were paraffin embedded and cut in 6-μm sections. They were stained with hematoxylin and eosin and Warthin-Starry stains and examined for the presence of gastritis and bacteria. Hematoxylin-eosin-stained sections were scored on a six-point scale for severity of lymphoplasmacytic inflammation as follows: 0, no inflammation; 1, mild multifocal inflammation; 2, mild, widespread inflammation; 3, mild, widespread, and moderate multifocal inflammation; 4, moderate, widespread inflammation; 5, moderate, widespread, and multifocal severe inflammation; and 6, severe, widespread inflammation. Warthin-Starry-stained sections were scored for the density and distribution of bacteria with the same six-point scale. In addition, the presence or absence of mucosal lymphoid follicles, granulocytes, intraepithelial leukocytes, fibrosis, and tortuosity of gastric glands was recorded. All sections were scored blind, without knowledge of their source. Gastritis and density of bacterial colonization were scored separately for the cardia, fundus, and antrum. Unless otherwise noted, the scores reported for each dog are the highest of the three values.

Amplification of 16S rDNA cistrons. For isolation of DNA, bacteria were harvested from blood agar plates and resuspended in phosphate-buffered saline. DNA was extracted by protease digestion and phenol-chloroform extraction (28). The 16S rRNA cistrons were amplified with primers 1 and 2, shown in Table 1. PCRs were performed in thin-walled tubes with a Perkin-Elmer 480 thermal cycler. One microliter of DNA was combined with 0.6 μM primers and other reagents in the Hot Start protocol suggested by Perkin-Elmer. The following conditions were used for amplification: denaturation at 72°C for 45 s, annealing at 60°C for 45 s, and elongation at 72°C for 90 s, with 5 s added for each elongation step. A total of 30 cycles were performed, followed by a final elongation step at 72°C for 15 min. The purity of the product was determined by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide and viewed under long-wavelength UV light.

Purification of PCR products. The amplified DNA was purified by precipitation with polyethylene glycol 8000 (14). After removal of Ampliwax, 0.6 volume of 20% polyethylene glycol 8000 (Sigma, St. Louis, Mo.) 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 × *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.

Sequencing methods. The DNA sample from the PCR was directly sequenced with a cycle sequencing kit (*fmol* DNA Sequencing System; Promega Corp., Madison, Wis.). The manufacturer's protocol was followed. The eight sequencing primers are given in Table 2. Primers 3 and 4 were used for sequencing only those strains which possessed an intervening sequence inserted into the 16S rDNA at *Escherichia coli* position 210. Primers were end labeled with ^{33}P (NEN/Dupont) according to the manufacturer's protocol. Approximately 100 ng of purified DNA from the PCR was used for sequencing. Reaction products were loaded onto 8% polyacrylamide–urea gels, electrophoresed, and detected by exposure to X-ray film for 24 h.

16S rRNA data analysis. A program set for data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation, and dendrogram construction for 16S rRNA data was written in Microsoft Quick BASIC for use on IBM PC-AT and compatible computers (23). RNA sequences were entered and aligned as previously described (23). Our sequence database contains approximately 500 sequences determined in our laboratory and another 400 obtained from GenBank or the Ribosomal Database Project (17). Similarity matrices were constructed from the aligned sequences by using only those se-

TABLE 2. Morphologic and biochemical features of bacteria isolated from dog stomachs

Bacterial strain	Source	Activity of:		Morphology	
		Urease	Catalase	Colony	Bacterial
Dog-1	Group A	+	+	Pinpoint colonies	*H. felis*-like without periplasmic fibers
Dog-2	Group A	+	+	Pinpoint colonies	*H. felis*-like without periplasmic fibers
Dog-3	Group B	+	+	Pinpoint colonies	*H. felis*-like without periplasmic fibers
Dog-4	Group A	+	−	Lawn	Flexispira
Dog-5	Group A	+	−	Lawn	Flexispira

quence positions from which 90% of the strains had data. The similarity matrices were corrected for multiple base changes at single positions by the method of Jukes and Cantor (13). Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei (27).

Nucleotide sequence accession number. The GenBank accession numbers of all strains examined in this study are included in Fig. 3.

RESULTS

Prevalence and type of bacteria. The histologic examination and urease test revealed gastric bacteria in 100% of the dogs in groups A (31 of 31) and B (8 of 8) and 67% of the dogs in group C (10 of 15). In all cases, the bacteria were large, tightly coiled spiral organisms which were morphologically consistent with gastrospirilla. They were present in the surface mucus, gastric pits, gastric glands, and parietal cells and in cardiac, fundic, and antral regions of the stomach. Bacteria morphologically consistent with *H. pylori*, flexispiras, or other nongastrospirilla were not seen in any dog.

Culture of gastric helicobacters, as determined by the presence of urease-positive, slowly growing colonies or lawns, was successful for only six dogs: five from group A and one from group B. Of these, only five strains could be isolated in pure culture. All organisms cultured were urease positive and grew either as small, translucent colonies or as a translucent lawn on blood agar plates, and all were obligate microaerophiles. Catalase activity, colony morphology, and bacterial morphology varied, however (Table 2 and Fig. 1). Three were long, loosely coiled spiral organisms (7 to 10 μm long) with bipolar flagellar tufts. These bacteria most resembled *H. felis*, except that two isolates did not contain periplasmic fibers. Subculture of the isolate with periplasmic fibers resulted in loss of the fibers (Fig. 1). The two remaining isolates were long, straight rods with numerous periplasmic fibers, characteristic of flexispiras.

Most dogs had moderate to large numbers of bacteria in the gastric glands and on the epithelial surface (bacterial scores of 4 to 6). Group C was the only group in which there were uninfected dogs (5 of 15 dogs) and the only group in which there were dogs with bacterial scores of 1 (2 of 15 dogs). Otherwise, there were no differences in bacterial density between the groups (Fig. 2).

Histologic examination. Most of the dogs had mild to moderate lymphocytic gastritis (scores of 1 to 3) consisting of scattered lymphocytes and a few plasma cells in the superficial mucosa (Fig. 2). One dog (group C) had no evidence of gastritis. Five dogs (two in group A, one in group B, and two in group C) had gastritis scores of 4 or 5. None of the dogs had widespread severe gastritis. Inconsistent findings were mucosal lymphoid follicles (21 of 54 dogs), increased tortuosity of glands (9 of 54 dogs, all in group A), and microerosions (1 dog in group A). The gastritis scores did not differ among the cardia, fundus, or antrum (not shown).

The five dogs with the highest gastritis score (4 to 5) had bacterial scores of 5 to 6, and the dog with the lowest gastritis score (0) had no bacteria (Fig. 2). In the other dogs, there was no correlation between the bacterial density score and the gastritis score. Most of the dogs had gastritis scores of 2 or 3 and corresponding bacterial scores of 4 or 5, but 11 dogs had minimal gastritis (score of 1 or 2) with large numbers of bacteria (scores of 5 to 6), and 4 dogs had few bacteria (scores of 0 to 2) but moderate gastritis (score of 3). The gastritis scores of the five dogs with no bacteria ranged from 0 to 3. Finally, there was no correlation between the presence or density of bacteria and the presence of mucosal lymphoid follicles, tortuous glands, or other epithelial changes.

16S rDNA sequence analysis. The full sequences (bases 24 to 1491 according to *E. coli* numbering) were determined for each of the five dog isolates. Analysis of 16S rDNA sequences allowed placement of all five isolates into the genus *Helicobacter*. A similarity matrix was constructed by using the sequences of the 5 dog strains, 16 reference helicobacter strains, 1 reference campylobacter strain, and 1 reference arcobacter strain. A phylogenetic tree constructed from this matrix is shown in Fig. 3. Dog strains Dog-1, Dog-2, and Dog-3, which were morphologically similar to *H. felis*, were identified as *H. felis* on the basis of sequence analysis. The sequences of each of the dog strains isolated in this study were more closely related to the previously described dog strain sequence than to the cat strain sequence (24). This may reflect slight genotypic differences between *H. felis* strains isolated from cats and those isolated from dogs. The two strains which had flexispira morphology represent two species. Strain Dog-5 was identified as *H. bilis* (99.3% similarity). Thus, *H. bilis* can infect both mice and dogs. The sequence of strain Dog-4 differs from the other helicobacter sequences in our database by about 2%, indicating that it represents a novel species.

DISCUSSION

The results of this study confirm those of other studies suggesting that the presence of gastrospirilla is almost universal in dogs (6, 11, 12, 34). In addition, we found, as did other investigators, that these large gastric spiral bacteria are the most common type of organism in dogs. Even when flexispiras were cultured from dog stomachs, gastrospirilla predominated on the basis of histologic examination. Organisms with flexispira morphology were not seen in the stomach of any dog.

It is interesting that of the 39 stomachs cultured, helicobacters were recovered from only 6, and helicobacters morphologically similar to those seen histologically were isolated from only 3. This finding is consistent with previous studies suggesting that in most cases, gastrospirilla are not culturable by routine laboratory methods (16, 19, 30), although some species or strains may be recovered in culture (8, 9). The culture results suggest that in this study, the overwhelming majority of the gastric organisms in these dogs were unculturable gastrospirilla.

The culture of two strains of *H. felis* lacking in periplasmic fibers somewhat blurs the distinction between *H. felis* and gas-

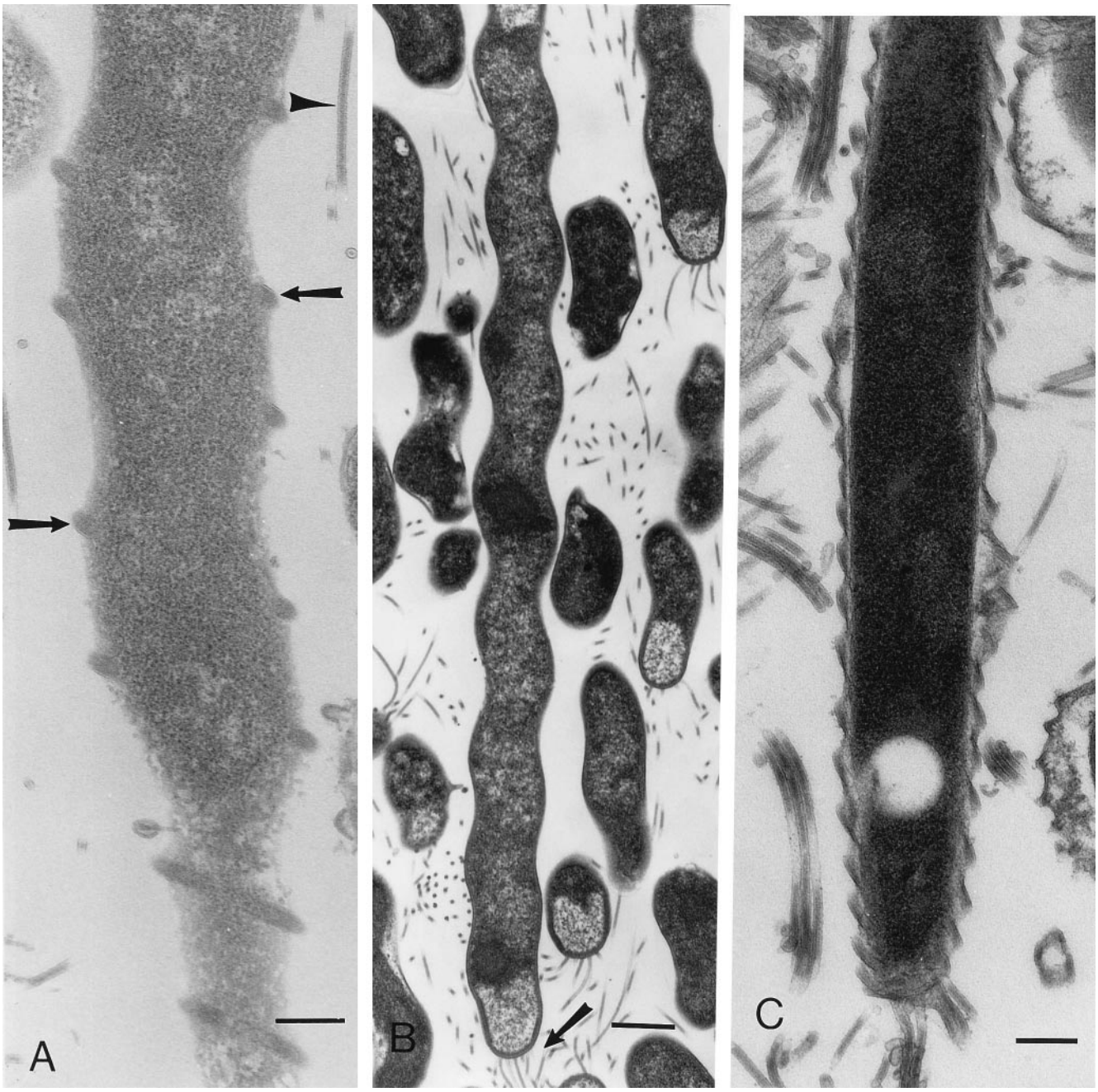

FIG. 1. Transmission electron micrograph of gastric bacteria cultured from dog stomachs. (A) *H. felis*-like organism with spiral morphology and periplasmic fibers (arrows). Cross-sections of flagella are visible (arrowhead). Bar, 0.25 μm. (B) *H. felis*-like organism without periplasmic fibers. Note polar tuft of flagella (arrow). Bar, 0.35 μm. (C) Flexispira-like organism with rod morphology, numerous periplasmic fibers, and polar flagella. Bar, 0.2 μm.

trospirilla such as *H. bizzozeronii*, "*G. hominis* 1," and "*G. hominis* 2." It is usually thought that *H. felis* and other gastrospirilla may be distinguished by culturability and the presence or absence of periplasmic fibers. However, in this study, two strains of *H. felis* with no periplasmic fibers were isolated, suggesting that this anatomic feature is not necessarily characteristic of *H. felis*. The subsequent loss of periplasmic fibers from one isolate further suggests that the presence of these fibers may not be useful for identification of these bacteria.

Strikingly, *H. pylori* or *H. pylori*-like organisms were not cultured or identified histologically in any of the 54 dogs in this study. This is consistent with other studies in which *H. pylori* was not described for dogs (6, 11, 12, 34). These findings are important, because they indicate that pet dogs do not represent a source of *H. pylori* for the human population, at least in central Ohio. Cats were not examined in this study, but other studies suggest that the prevalence of gastrospirilla and the absence of *H. pylori* in cats are similar to the findings for dogs (6, 12, 21). The only description of *H. pylori* in cats or dogs was of a single specific-pathogen-free laboratory cat colony in which gastrospirilla were absent (7). It is most likely that these

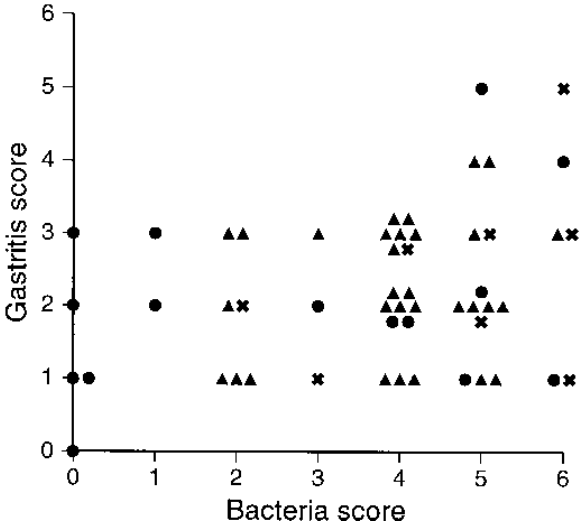

FIG. 2. Relationship between presence and density of bacteria and severity of gastritis. Each point represents a single dog in group A (▲), B (X), or C (●).

animals were susceptible to *H. pylori* because of the lack of normal gastric flora and that they became colonized by contact with a human caretaker.

The risk of transmission of gastrospirilla between pets and owners in the developed world is also likely to be low. Gastrospirilla are found in 0.3% or less of gastroscopies, compared with *H. pylori*, which is found in between 30 and 100% of gastroscopies (31). This is in spite of the frequent occurrence of these organisms in dogs and cats, the large number of pets, and their close contact with their human companions. Thus, there is a large reservoir of gastrospirilla in close contact with the human population, but human infection remains extremely rare.

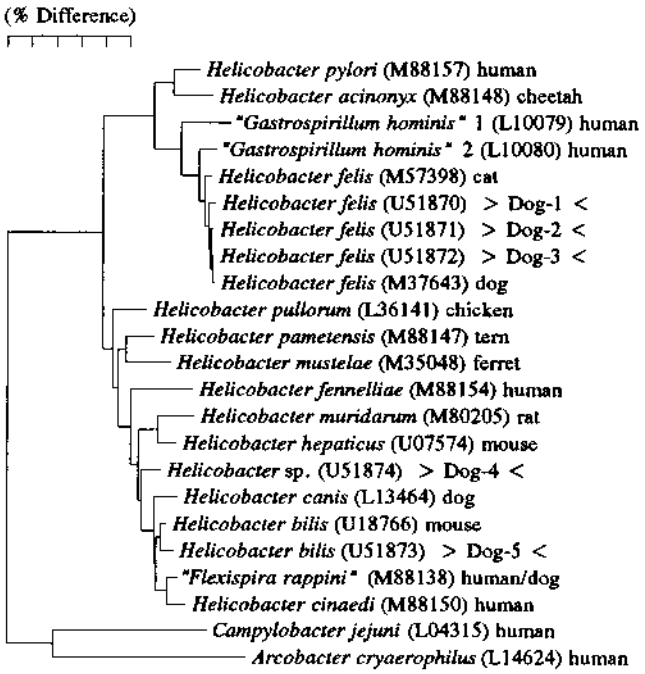

FIG. 3. Phylogenetic tree showing the genetic relationships between the bacteria isolated in this study and closely related helicobacter species.

The clinical significance of gastrospirilla in dogs was not determined with certainty by this study. Almost all of the animals studied were infected, and therefore, the number of negative controls was insufficient to allow comparison. This is consistent with other published studies which suggest that gastrospirilla are almost universally present in cats and dogs (6, 11, 12, 16, 21, 34). The dogs in this study demonstrated histologic gastritis consistent with similar descriptions for dogs and cats in other studies (6, 11, 12, 21, 34). The lesions were similar to those described for cats experimentally infected with gastrospirilla from cheetahs (2). It is important to note, however, that in most dogs, the lesions were mild despite the very large number of gastrospirilla in some animals. The five dogs in this study with moderate to severe gastritis also had large numbers of gastrospirilla. Thus, it is possible that these organisms induce gastritis in some individual animals or that certain bacterial strains induce gastritis in dogs. However, most dogs had many bacteria and only mild gastritis, suggesting that in these animals, bacteria did not induce histologically evident disease. Finally, all of the dogs in this study either were clinically healthy or had clinical conditions which were unrelated to gastric disease. Thus, whether or not the bacteria were responsible for the mild gastritis seen, it is likely that large numbers of gastrospirilla are not clinically significant in healthy dogs without underlying disease.

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