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Isolation and Identification of *Helicobacter* spp. from Canine and Feline Gastric Mucosa

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It is known that virtually all healthy adult dogs and cats harbor spiral helicobacters in their gastric mucosa. Three species, *Helicobacter felis*, *Helicobacter bizzozeronii*, and *Helicobacter salomonis* have been isolated in vitro from the gastric mucosa of these animals. The aims of this study were to evaluate the efficacy of an isolation method for canine and feline gastric helicobacters that has been developed at the University of Helsinki; to estimate the prevalence and distribution of these taxa in the samples examined; and to assess the efficacy and validity of an extensive set of standardized conventional phenotypic tests, whole-cell protein profiling, and ultrastructural analysis in identifying the different species isolated from canine and feline gastric mucosa. We cultured 95 and 22 gastric mucosal biopsies from dogs and cats, respectively. Twenty-one *H. bizzozeronii* strains, 8 *H. felis* strains, 8 *H. salomonis* strains, 3 mixed cultures, 2 "*Flexispira rappini*"-like organisms, and 3 as yet uncharacterized strains were isolated from the dogs, and 3 *H. felis* strains were isolated from the cats. The methods used here yielded *Helicobacter* isolation rates of 51% from dogs and 13.6% from cats, which exceed those reported previously. The main difficulties were primary isolation, mixed cultures, and identification to the species level. In the species identification, a detailed morphological examination was found to yield important phenotypic characteristics. A large panel of biochemical and tolerance tests did not clearly differentiate the closely related species *H. bizzozeronii*, *H. felis*, and *H. salomonis*. Highly standardized whole-cell protein profiling was shown to be an excellent method for species identification. Improvements in culture conditions for these bacteria are still needed, especially for cats. A genetic identification method not requiring culture is needed for future studies of these very fastidious helicobacters, as the clinical significance and ecology of these species within the gastric mucosa of the domestic carnivores remain largely unknown.

The isolation of *Helicobacter pylori*, a spiral bacterium from the human gastric mucosa (54) and the subsequent recognition of its prevalence and clinical significance as a cause of gastric ulcers and related diseases in humans (10) led to an increased interest in similar organisms that had been observed in animals (notably domestic pets) over a century ago (49). As a consequence, several novel *Helicobacter* spp. from the gastric mucosa of various animals, including cheetahs (*Helicobacter acinonychis*, formerly *Helicobacter acinonyx*), ferrets (*Helicobacter mustelae*), monkeys (*Helicobacter nemestrinae*), and rodents (*Helicobacter muridarum*) have been described (5, 12, 15, 31). Moreover, three species from cats and dogs, *Helicobacter felis*, *Helicobacter bizzozeronii*, and *Helicobacter salomonis* (19, 26, 46) have been described, while "*Flexispira rappini*" (also called "*Helicobacter rappini*"), originally isolated from ovine abortions (28) and subsequently from humans with gastroenteritis (2) and laboratory mice (50), has also been found in canine gastric mucosa (11, 32).

The initial interest in animal helicobacters arose from the need for a suitable animal model for studying *H. pylori* infection, and subsequently from an ecological perspective (14, 29). However, there have been recent concerns regarding the potential of animals, notably domestic pets, to be a source of zoonotic *Helicobacter* infection. Cats used for biomedical research have been occasionally found to harbor *H. pylori* strains (18), while *H. felis* has been implicated as a potential human pathogen in a few cases (17, 57). In addition, the morphologically distinctive, tightly coiled bacteria (referred to as either "*Gastrospirillum hominis*" [34] or "*Helicobacter heilmannii*" [51]) observed in some cases of human gastritis are ultrastructurally indistinguishable from *H. bizzozeronii* (19) and also from atypical *H. felis* isolates from which cell surface periplasmic fibrils are absent (11). There is therefore a need to determine the relative prevalence of each species in domestic pets in order to evaluate the possible risk to human health, and also to that of the host animals, in which gastric and related complaints can occur.

Considerable difficulties in the isolation of these organisms from pet animals have been noted. A recent study yielded helicobacter isolation rates of just 11.1%, despite spiral bacteria being observed in 90.7% of the samples under study (11). In addition, there are significant problems in accurately identifying *Helicobacter* spp. (37), and for prevalence studies to be adequately informative, accurate and preferably simple identification methods should be available. In this regard, both conventional phenotypic tests (44) and whole-cell protein profiling (8) have previously been determined to be effective means for differentiating various *Helicobacter* spp., while ultrastructural differences between species may also provide important taxonomic data (26, 31).

The purpose of the present study was to evaluate the efficacy of an isolation method for canine and feline gastric helicobacters that has been developed at the University of Helsinki;

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to assess the efficacy and validity of conventional phenotypic characterization (by an extensive set of standardized tests), whole-cell protein profiling, and ultrastructural analysis in identifying the different species found in canine and feline gastric mucosa; and to estimate the prevalence and distribution of these taxa in the samples examined.

MATERIALS AND METHODS

Animals. During the period 1990 to 1997, the Faculty of Veterinary Medicine examined 95 canine gastric mucosal biopsies from 37 clinically healthy pet dogs, 23 patients with upper gastrointestinal signs (vomiting, nausea, or abdominal discomfort), 18 euthanized pet dogs (health status unknown), and 17 healthy experimental dogs. Feline gastric biopsies were taken from 22 cats, of which five were clinically healthy, two suffered from upper gastrointestinal problems, and 15 were euthanized pets (health status unknown). The biopsy samples were taken from the corpus (body) area under light anesthesia via endoscope from the live animals and immediately post mortem from the euthanized pets (22). The animals for endoscopic studies were not given food for 16 h prior to examination.

Primary microbial isolation. Up to four gastric biopsy samples were taken from each animal. One was used for rapid urease production testing (27); a positive result within 60 min was assumed to indicate the presence of *Helicobacter* spp., and culture was attempted with a second sample. The third sample was used for histology (unpublished data), and the fourth was used for electron microscopy. Biopsies for culture were handled as described earlier (19, 20). Both freshly prepared brain heart infusion (BHI) agar (Difco, Detroit, Mich.) and brucella agar (Oxoid, Basingstoke, United Kingdom), with cattle or horse blood and Skirrow's antibiotic supplement (Oxoid), were used for isolation as described earlier (20). Plates were regularly checked for bacterial growth, and a few drops of BHI broth were added to the plates during the incubation period to avoid the drying of the media.

Primary identification of field isolates. Strains were characterized by determining colonial and cellular morphology and the type of movement and by the urease reaction as described earlier (19, 26). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) whole-cell protein profiles were prepared and electrophoresed with a minigel system (running length, 4 cm) as described before (19), and the patterns were visually compared with those of type strains of relevant *Helicobacter* species.

In vivo electron micrographs. Biopsy samples for electron micrographs were taken from 52 animals. The samples were fixed in 2.5% glutaraldehyde in Sörensen buffer containing 0.1 mmol of phosphate per liter (pH 7.3). After dehydration in acetone, the samples were embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined under a JEOL model 1200 EX electron microscope.

Extended phenotypic characterization. Thirty-four representative strains of *H. felis* ($n = 16$; including three atypical *H. felis* strains), *H. bizzozeronii* ($n = 10$), *H. salomonis* ($n = 6$), and "*F. rappini*" ($n = 2$) (Table 1) were characterized with 65 phenotypic tests listed in a probability matrix for identifying campylobacters, helicobacters, and related taxa (44). Growth at 30°C and on buffered charcoal-yeast agar was not determined. In addition, spiral cell morphology (as determined by light-microscopic examination of Gram-stained bacterial films) was further distinguished into tightly or loosely coiled helical forms. Tests were performed with the recommended media by methods described previously (25, 39–43), with the following amendments. Nalidixic acid-, cephalothin-, metronidazole-, and carbenicillin-containing media were prepared by using filter-sterilized solutions prepared from the native antibiotic (obtained from Sigma Chemical Co. Ltd., Poole, England). Conditions of anaerobiosis, where needed, were produced in an anaerobic jar containing a palladium catalyst (Struers Kebo Lab, Copenhagen, Denmark) by applying four consecutive treatments of the gas replacement (anaerobic) method described by On and Holmes (41). All tolerance (growth) tests were performed with an inoculum size of ca. 10^9 CFU/ml and read after 3 and 7 days of incubation. The quality of all tests was examined by using appropriate control strains (37a).

Ultrastructural analysis. Electron microscopic examination of bacterial cell morphology was performed on 26 selected isolates (Table 1) by methods described previously (52).

Extraction, separation, and numerical analysis of whole-cell proteins. The strains included in the highly standardized SDS-PAGE analysis are shown in Table 1. The strains were grown on Mueller-Hinton agar with 5% horse blood at 37°C in a microaerobic atmosphere for 72 h. Protein samples were prepared, separated by PAGE (running length, 9 cm), digitized, and subjected to comparative numerical analysis as described previously (47). Protein extraction and electrophoresis were performed as described earlier (47). Numerical analysis of the protein profiles was performed by using the GelCompar system, version 4.0 (Applied Maths, Kortijk, Belgium). The profiles were recorded and stored on a personal computer. The similarities between all pairs of traces were expressed by Pearson product moment correlation coefficients converted for convenience to percent values.

RESULTS

Primary isolation. Fifty-one gastric *Helicobacter* isolates were obtained, of which 48 were of canine origin and 3 were isolated from cats. Three of the dog strains were lost during subcultivation and thus could not be examined in detail. The isolation rates of the canine and feline biopsies (as indexed to urease-positive biopsy samples) were 51 (48 of 95) and 14% (3 of 22), respectively. The primary growth appeared after 3 to 12 days of incubation as a thin spreading film; no single colonies were formed, and the primary area of growth was often very small. However, if air-dried plates were used, colonies could be observed, although the bacterial growth was of a poor quality. It was also noted that BHI, brucella, and Mueller-Hinton base agar media supported growth provided either cattle or horse blood was added (25a). The isolation rate was approximately the same throughout the study period (data not shown).

Of the taxa encountered in this study, *H. felis* proved the easiest to isolate. Relatively profuse growth was obtained after 4 to 7 days of incubation, and strains were readily subcultured. Similarly, *H. salomonis* also grew comparatively quickly and profusely on the primary plates, although examination of Gram-stained bacterial films indicated that cells transformed rapidly to coccoid forms and maintenance by subculture proved difficult. *H. bizzozeronii* isolates grew slowly, with primary growth observed within 4 to 12 days of incubation. The subculture of *H. felis*, *H. bizzozeronii*, and *H. salomonis* was expedited by the addition of a few drops of BHI broth to plate cultures. All commercial microaerobic atmospheres tested (gas generating kit model BR 56 with a palladium catalyst [Oxoid] and gas generating kit model BR 38 without the catalyst, [Oxoid]) as well as an evacuation and gas exchange system supported growth of these bacteria when the aforementioned subculturing procedures were employed. Additional hydrogen was not essential in order to obtain growth.

Four mixed cultures with two different *Helicobacter* spp. and one mixed culture with a *Helicobacter* sp. and a *Campylobacter* sp. were obtained. Two of these mixed cultures were recognized by examination of Gram-stained slide preparations and were subsequently purified. The first purified *Helicobacter* strain was contaminated with a *Campylobacter*-like organism, and the pure culture (*H. bizzozeronii* 13) was obtained after several subcultures on plates containing 100 IU of polymyxin B per ml (6). The second mixed isolate was originally "*F. rappini*"-*H. bizzozeronii*. As "*F. rappini*" strains show faster growth, the pure "*F. rappini*" strain (strain 19) was obtained from the edge of the culture. These two strains were then handled as pure cultures in the present study. The remaining three mixed cultures were revealed by the comparison of the protein pattern of the early culture with that of the later culture and were not successfully separated into pure cultures of the respective taxa (strain Jutta, *H. bizzozeronii*-*H. felis*; strain Tuohimetsa, *H. bizzozeronii*-*H. felis*; and strain Loko 18, *H. bizzozeronii*-*H. salomonis*).

Primary identification. All fresh isolates were urease positive when first tested. Light-microscopic examination of Gram-stained bacterial films revealed that the isolates belonged to one of three morphological categories, corresponding to the taxa *H. felis* and *H. bizzozeronii* (tightly helical cells), *H. salomonis* (plump, less tightly coiled cells), and "*F. rappini*" (straight, cigar-shaped cells with tapering ends). Furthermore, two forms of motility were observed; a rapid, screw-like motion proved typical for *H. felis*, *H. bizzozeronii*, and "*F. rappini*" (with the unique cellular morphology of the last clearly distinct), while the movement of cells of *H. salomonis* strains was relatively slow and wavy. The visual examination of SDS-

TABLE 1. Strains isolated in this study and reference strains

Species	Strain[a]	Received from[b]	Source	Analysis performed[c]	Note
H. acinonychis	LMG 12684^T, CCUG 29263^T	CCUG	Canine gastric mucosa	c*, e	
H. bizzozeronii	CCUG 35545^T, Storkis		Canine gastric mucosa	c*, d, e, f	
H. bizzozeronii	CCUG 35546, 14		Canine gastric mucosa	c*, d, e, f	
H. bizzozeronii	Wiberg		Canine gastric mucosa	c*, d, e, g	
H. bizzozeronii	5F		Canine gastric mucosa	c*, d, e, f	
H. bizzozeronii	10		Canine gastric mucosa	c*, d, e, f	
H. bizzozeronii	12A		Canine gastric mucosa	c*, d, e	
H. bizzozeronii	Heydar		Canine gastric mucosa	c, d, e, g	
H. bizzozeronii	Yrjala		Canine gastric mucosa	c, d, e, f	
H. bizzozeronii	Emo		Canine gastric mucosa	d, e, f	
H. bizzozeronii	Loko 21		Canine gastric mucosa	c, d, e	
H. bizzozeronii	11AM		Canine gastric mucosa	d, e	
H. bizzozeronii	Moppi		Canine gastric mucosa	e, g	
H. bizzozeronii	Pinky		Canine gastric mucosa	e, g	
H. bizzozeronii	Renny		Canine gastric mucosa	e, g	
H. bizzozeronii	Bertta		Canine gastric mucosa	e, g	
H. bizzozeronii	Roope		Canine gastric mucosa	e	
H. bizzozeronii	Nummijärvi		Canine gastric mucosa	c, g	
H. bizzozeronii	9F		Canine gastric mucosa	c, f	
H. bizzozeronii	13		Canine gastric mucosa	c	
H. bizzozeronii	Loko 20		Canine gastric mucosa	c, f, g	
H. bizzozeronii	Rinne		Canine gastric mucosa	c, g	
H. felis	CS 1, CCUG 28539^T	CCUG	Feline gastric mucosa	c*, e, f	
H. felis	CS 5	O'Rourke	Feline gastric mucosa	d, e	
H. felis	CS 6	O'Rourke	Feline gastric mucosa	d, e	
H. felis	CS 7	O'Rourke	Feline gastric mucosa	d, e	
H. felis	CS 8	O'Rourke	Feline gastric mucosa	d, e	
H. felis	DS 1	O'Rourke	Canine gastric mucosa	d, e	
H. felis	DS 3	O'Rourke	Canine gastric mucosa	c*, d, e	
H. felis	DS 4, CCUG 28540	CCUG	Canine gastric mucosa	c*, d, e, f	
H. felis	DS 5	O'Rourke	Canine gastric mucosa	d, e	
H. felis	Dog 1, 1602	Eaton	Canine gastric mucosa	d, e, f	
H. felis	Dog 2, 2301	Eaton	Canine gastric mucosa	d, e	
H. felis	Dog 3, Dog 7	Eaton	Canine gastric mucosa	c*, d, e, f	
H. felis	Into		Canine gastric mucosa	c, d, e, f	
H. felis	Hellu		Canine gastric mucosa	c, d, e	
H. felis	Kukka		Canine gastric mucosa	e, g	
H. felis	Teppo		Canine gastric mucosa	e	
H. felis	Loko 14		Canine gastric mucosa	c, g	
H. felis	Solanti		Canine gastric mucosa	c	
H. felis	Tellu		Canine gastric mucosa	c	
H. felis	Vilma		Canine gastric mucosa	c	
H. felis	Cintti		Feline gastric mucosa	c, d, e, g	
H. felis	Loki 13		Feline gastric mucosa	c, f, g	
H. felis	Fievel		Feline gastric mucosa	c, f, g	
H. felis-H. bizzozeronii	Tuohimetsa		Canine gastric mucosa	c, d, e, f, g	Mixed culture
H. felis-H. bizzozeronii	Jutta		Canine gastric mucosa	c, f, g	Mixed culture
H. salomonis	Inkinen^T, CCUG 37845^T		Canine gastric mucosa	c*, d, e, f, g	
H. salomonis	06A, CCUG 37848		Canine gastric mucosa	c, e, f	
H. salomonis	Alma		Canine gastric mucosa	c, f	
H. salomonis	Elviira		Canine gastric mucosa	c, f	
H. salomonis	Vilho		Canine gastric mucosa	c, d, e, f	
H. salomonis	Mini		Canine gastric mucosa	c*, d, e, f, g	
H. salomonis	Ko.K. III		Canine gastric mucosa	c, d, e, f	
H. salomonis	Remu		Canine gastric mucosa	c, d, e, f, g	
H. salomonis-H. bizzozeronii	Loko 18		Canine gastric mucosa	c, d, e, f, g	Mixed culture
"F. rappini"	CCUG 23435, ATCC 43879	ATCC	Aborted sheep fetus	e	
"F. rappini"	Hilli		Canine gastric mucosa	c*, d, e, f	
"F. rappini"	19		Canine gastric mucosa	c, d, e, f	
H. mustelae	CCUG 25715^T	CCUG	Ferret gastric mucosa	c*, e	
H. muridarum	CCUG 29262^T, ATCC 49282^T	CCUG	Murine intestinal mucosa	e	
H. nemestrinae	CCUG 32350^T, ATCC 49396^T	CCUG	Macaque gastric mucosa	e	
H. pylori	CCUG 17874^T, ATCC 43504^T	CCUG	Human gastric mucosa	e	

[a] ATCC, American Type Culture Collection, Manassas, Va.; CCUG, Culture Collection, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden.
[b] Own isolate unless indicated otherwise. Eaton, K. A. Eaton, Department of Veterinary Pathobiology, Ohio State University, Columbus; LMG, Culture Collection, Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium; O'Rourke, J. L. O'Rourke, School of Microbiology and Immunology, University of New South Wales, Sydney, Australia.
[c] c, DNA-DNA hybridization (an asterisk indicates that the analysis was done as part of a previous study [19, 26]); d, phenotypic characterization; e, highly standardized protein profile analysis; f, electron micrographic analysis; g, in vivo electron micrographic analysis of biopsy sample.

TABLE 2. Dogs used in this study and the strains isolated[a]

Type of dog	No. of dogs	No. of strains	% Isolation	No. of strains identified as:					No. of strains that were:	
				H. felis	*H. bizzozeronii*	*H. salomonis*	"*F. rappini*"	Mixed culture	Unidentified	Lost
Euthanized pet	18	10	55	1	7	0	1	1	0	0
Healthy pet	37	16	43	1	7	3	0	2	2	1
Gastrointestinal patient	23	12	52	2	6	0	1	0	1	2
Experimental	17	10	59	4	1	5	0	0	0	0
Total	95	48	51	8	21	8	2	3	3	3

[a] All biopsy samples were urease positive.

PAGE protein profiles with the minigel system clearly differentiated *H. felis*, *H. bizzozeronii*, and "*F. rappini*". The differentiation of *H. salomonis* from *H. bizzozeronii* was more difficult with this method, and the final species designation was done with dot blot and quantitative DNA-DNA hybridization (26). The species distribution of canine isolates is shown in Table 2. Three strains were isolated from cats (two from healthy pets and one from a euthanized pet); these were all shown to be *H. felis* by the minigel SDS-PAGE system.

Ultrastructure of cultured strains. No significant infraspecific variation in cellular ultrastructure was observed in pure cultures of *H. bizzozeronii* ($n = 8$), *H. salomonis* ($n = 8$), or "*F. rappini*" ($n = 2$). Only the *H. felis* strains that were previously described as atypical by virtue of the absence of periplasmic fibrils (11) differed from other strains of this species. Typical examples of ultrastructural cell morphology of each species, and of the atypical *H. felis* strains, are reproduced in Fig. 1 and conform to descriptions given previously (11, 19, 26, 28, 29). In brief, typical *H. felis* cells were corkscrew-like, possessing one or two periplasmic fibrils; *H. bizzozeronii* strain cells were similar but slightly more helical, and no periplasmic fibrils were seen; *H. salomonis* cells were thicker and only slightly curved, while "*F. rappini*" cells were cigar-shaped organisms with tapering ends and had a remarkable net-like ultrastructure on the surface. The atypical *H. felis* strains closely resembled *H. bizzozeronii* in ultrastructure.

In vivo electron micrographs. Relatively few bacteria could be seen in electron micrographs prepared from tissue samples. Furthermore, these results did not always correlate with corresponding ultrastructural examinations of cultured bacteria. Moreover, only two types of organisms could be seen: either tightly coiled organisms without the periplasmic fibrils, resembling *H. bizzozeronii* or atypical *H. felis* (see above), or similar bacteria with the periplasmic fibrils, thus resembling *H. felis*. Thus, organisms resembling *H. salomonis* or "*F. rappini*" organisms were not detected. For seven animals (13%) where organisms resembling *H. felis* were seen, no culture was obtained. Also, for two animals *H. felis* cells were seen in tissue samples, yet *H. bizzozeronii* growth was obtained. *H. felis*-like cells were seen only in 12 samples (23%) and only as a small proportion of spiral organisms, most of which resembled *H. bizzozeronii*. In 36 animals (64%) *H. bizzozeronii*-like organisms were seen as the only colonizers. Conversely, although *H. bizzozeronii*-like strains were observed to be the sole colonizers of these animals, typical *H. felis* strains were isolated from four (7%) of these samples of which two were from cats.

Phenotypic characteristics. Considerable difficulties in culturing bacteria for phenotypic testing were encountered with the methods used; consequently, two strains (strain 10 [*H. bizzozeronii*] and strain 06A [*H. salomonis*]) could not be tested. Similar problems were encountered when determining phenotypic properties of the cultured isolates. Initial studies using the recommended inoculum size for tolerance testing (10^6 CFU/ml [40]) proved unsuccessful, since many strains failed to grow even on the control medium (5% blood agar) with the aforementioned inoculum size (data not shown). Although results could be obtained by employing a significantly greater inoculum size (10^9 CFU/ml) and extending the incubation period for up to 7 days, the strains proved to be notably unreactive. No growth was observed on the following test media: unsupplemented nutrient agar; unsupplemented "Preston" (campylobacter charcoal-deoxycholate) medium; minimal medium; MacConkey agar; tyrosine and casein agars; and media containing 2, 3.5, or 4.0% NaCl, 0.1% $KMnO_4$, 0.001% $NaASO_2$, 0.02% or 0.05% safranin, 0.0005% crystal violet, 0.01% Janus green, 0.005% basic fuchsin, 0.1% sodium deoxycholate, and 0.02% pyronine. No growth on nutrient agar media containing 4 mg of metronidazole, 64 mg of cefoperazone, or 32 mg of carbenicillin per liter was noted. Strains did not grow at room temperature (18 to 22°C) or 25°C under microaerobic conditions or at 25 or 37°C aerobically. Strains did not hydrolyze hippurate or DNA, and reduction of selenite was not detected. Neither hydrogen sulfide nor acid in triple-sugar iron agar was produced. Bacterial growth did not exhibit any pigmentation, and pitting of agar media was not observed. All strains produced catalase. As a consequence of the difficulties encountered with culturing strains, nitrate reduction and α-hemolysis results could not be reliably determined for all isolates and are not presented here. Table 3 summarizes the results of those tests for which some differences between strains were noted. Tests clearly distinguishing *H. bizzozeronii*, *H. felis*, and *H. salomonis* were not identified in the scheme applied, although certain traits (namely distinctions in cell morphology, growth at 37°C on unsupplemented blood agar, and resistance to 5-fluorouracil [100 mg/liter]) provided a broad distinction between *H. bizzozeronii* or *H. felis* and *H. salomonis*. "*F. rappini*"-like strains were readily distinguished from the other taxa by being tolerant to 1.0, 1.5, and 2.0% bile, with other traits (elongated), loosely spiral cell morphology, alkaline phosphatase production, reduction of and growth on triphenyl-tetrazolium chloride medium, growth on potato starch medium) providing some additional discrimination from the other *Helicobacter* species isolated. In contrast with results obtained from freshly isolated strains, no urease activity was detected in four strains ("*F. rappini*"-like Hilli and *H. bizzozeronii* 11AM, Loko 21, and 12A), despite repeated examination and overnight incubation in the urease test medium.

Numerical analysis of protein profiles. Duplicate protein extracts were prepared to check the reproducibility of the growth conditions and the preparation of the extracts. The correlation level between duplicate protein patterns was above 93% (data not shown). The whole-organism protein patterns

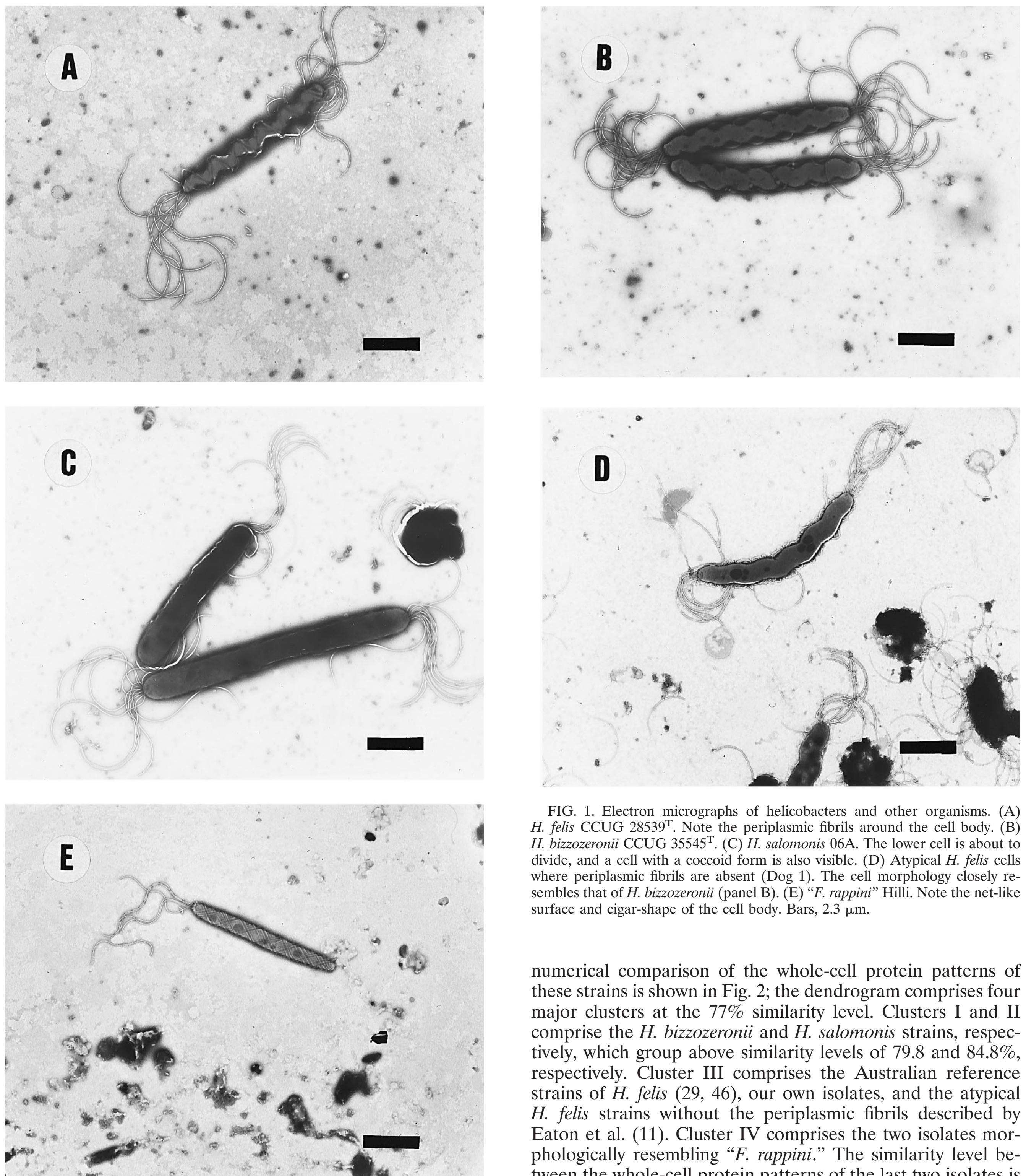

FIG. 1. Electron micrographs of helicobacters and other organisms. (A) *H. felis* CCUG 28539^T. Note the periplasmic fibrils around the cell body. (B) *H. bizzozeronii* CCUG 35545^T. (C) *H. salomonis* 06A. The lower cell is about to divide, and a cell with a coccoid form is also visible. (D) Atypical *H. felis* cells where periplasmic fibrils are absent (Dog 1). The cell morphology closely resembles that of *H. bizzozeronii* (panel B). (E) "*F. rappini*" Hilli. Note the net-like surface and cigar-shape of the cell body. Bars, 2.3 μm.

of *H. felis* reference strains and of all of the strains isolated in the present study were compared with those of reference strains of other gastric helicobacters (i.e., *H. acinonychis*, *H. mustelae*, *H. nemestrinae*, and *H. pylori*) and of "*F. rappini*" CCUG 23435. A dendrogram illustrating the results of the numerical comparison of the whole-cell protein patterns of these strains is shown in Fig. 2; the dendrogram comprises four major clusters at the 77% similarity level. Clusters I and II comprise the *H. bizzozeronii* and *H. salomonis* strains, respectively, which group above similarity levels of 79.8 and 84.8%, respectively. Cluster III comprises the Australian reference strains of *H. felis* (29, 46), our own isolates, and the atypical *H. felis* strains without the periplasmic fibrils described by Eaton et al. (11). Cluster IV comprises the two isolates morphologically resembling "*F. rappini*." The similarity level between the whole-cell protein patterns of the last two isolates is 91.6%, and this cluster is linked at the "*F. rappini*" reference strain, LMG 8738, at a similarity level of 77.9%. The type strains of *H. acinonychis*, *H. mustelae*, *H. nemestrinae*, and *H. pylori* each occupy distinct positions in the dendrogram. Figure 3 illustrates representative whole-cell protein patterns of the different groups of gastric isolates examined.

The whole-cell protein patterns of the *H. bizzozeronii* strains are fairly homogeneous, with some variability primarily in the

TABLE 3. Differential phenotypic characteristics of *H. bizzozeronii*, *H. felis*, *H. salomonis*, and "*F. rappini*"-like strains examined

Characteristic[a]	% of strains of indicated species positive for the characteristic			
	H. bizzozeronii (n = 10)	*H. felis* (n = 16)	*H. salomonis* (n = 6)	"*F. rappini*"-like (n = 2)
Cell morphology				
Tightly coiled spiral	78	67	0	0
Loosely coiled spiral	33	7	17	100
Vibrioid/coccoid forms	20	40	83	0
Oxidase	90	100	100	100
Urease	80	94	100	50
Alkaline phosphatase	50	62.5	50	100
Indoxyl acetate hydrolysis	20	25	17	0
TTC reduction	0	12.5	0	100
Anaerobic growth on BA	30	56	67	100
Anaerobic growth on TMAO	0	25	33	100
Growth at 37°C	90	100	67	100
Growth at 42°C	60	50	0	0
Growth on:				
Potato starch medium	0	6	17	99
Lecithin medium	0	12.5	0	0
Tolerance to:				
1, 1.5, and 2% bile	0	0	0	100
1% glycine	10	6	0	0
0.04% TTC	0	12.5	0	100
Nalidixic acid, 32 mg/liter	0	6	0	0
Cephalothin, 32 mg/liter	10	19	50	100
Metronidazole, 4 mg/liter	0	12.5	17	0
Carbenicillin, 32 mg/liter	10	19	33	0
Cefoperazone, 64 mg/liter	0	6	0	0
5-Fluorouracil, 100 mg/liter	80	75	17	100
0.05% sodium fluoride	20	31	33	0

[a] BA, blood agar; TMAO, trimethylamine *N*-oxide medium; TTC, triphenyl tetrazolium chloride.

high-molecular-weight region (molecular weight > 60,000). The majority of the *H. felis* strains have very similar whole-cell protein patterns (Fig. 3). However, the strains without the periplasmic fibrils are characterized by the absence of two prominent bands which are present in all other strains (estimated molecular weights: 80,000 and 54,000; cf. the patterns of strains CCUG 28539^T, Hellu, and Into with the pattern of strain Dog 1 in Fig. 3). Two additional strains have slightly aberrant patterns, characterized by an additional low-molecular-weight protein band of approximately 33,000 (Into) or by a slightly different size of the prominent protein band with an approximate molecular weight of 80,000 (DS 3). The patterns of the *H. salomonis* strains are very homogeneous and are typically characterized by an unusual prominent high-molecular-weight protein band (approximate size: 95,000; Fig. 3). Finally, the whole-cell protein patterns of the two "*F. rappini*"-like strains are similar to, but clearly different from, that of the "*F. rappini*" reference strain (LMG 8738) included in the analysis.

Two subcultures of the mixed culture of strain Loko 18 were included in the analysis. As illustrated in Fig. 2, the 1995 subculture of this strain (listed as Loko 18-95) was identified as *H. bizzozeronii*, while the 1996 subculture (listed as Loko 18-96) was identified as *H. salomonis* (Fig. 2). The species in the subculture of strain Tuohimetsa included in the present analysis was identified as *H. felis* (Fig. 2). It seems logical that the initial mixed cultures, which were dominated by the slow-growing *H. bizzozeronii* strains, were later dominated (after a considerable number of subcultivations in vitro) by the faster-growing *H. salomonis* and *H. felis* components of the mixed populations.

DISCUSSION

Isolation of canine and feline gastric *Helicobacter* spp. Previous studies have demonstrated the presence of *Helicobacter*-like organisms in the gastric mucosa of virtually all adult cats and dogs (11, 22, 24, 36, 45). We have demonstrated that a variety of different *Helicobacter* species can be cultured from gastric biopsy samples of animals, particularly dogs, provided adequate isolation procedures are used. We attained *Helicobacter* isolation rates of 51% from dogs and 13.6% from cats, which exceed those reported previously (11, 36), and were successful in acquiring a diverse range of taxa, in contrast with other studies where only *H. felis* (7, 11, 23, 29), "*F. rappini*" (11), or intestinal helicobacters such as *Helicobacter bilis* (11) or *Helicobacter pametensis* (36) have been isolated in vitro. Clearly, the method used for primary isolation is critical. We examined only biopsies giving a positive urease reaction within 60 min, since there is considerable correlation between this and the actual number of helicobacters in the gastric mucosal biopsy (21, 30).

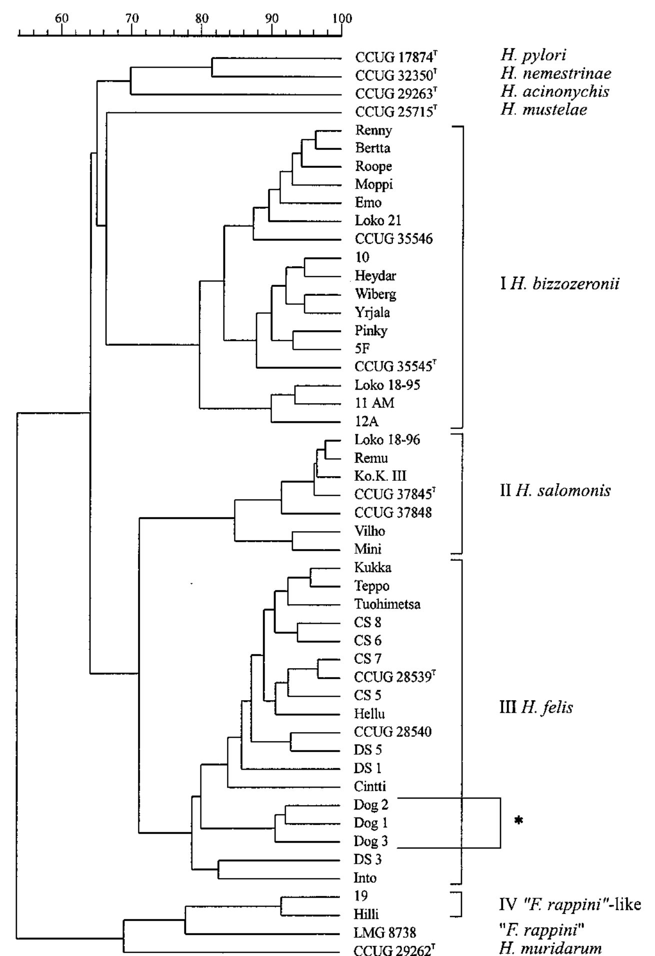

FIG. 2. Dendrogram derived from the numerical analysis of the whole-cell protein patterns of all of the strains examined. Strains marked Loko 18-95 and Loko 18-96 represent the 1995 and 1996 subcultures of strain Loko 18. *H. felis* strains marked by an asterisk lack periplasmic fibrils. Roman numerals I through IV are the cluster numbers discussed in the text.

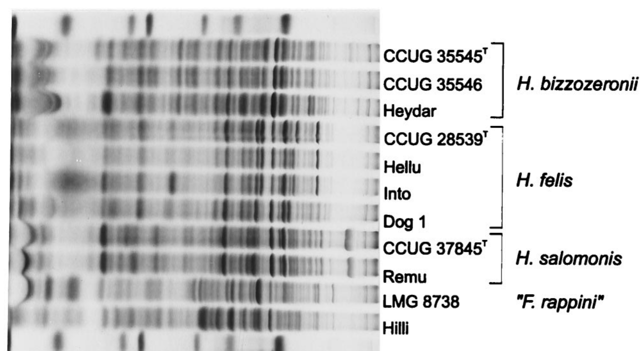

FIG. 3. Whole-cell protein profiles of a representative selection of the strains examined. The molecular mass markers used (indicated in the bottom and top lanes) are (from left to right) lysozyme (14,500 Da), trypsin inhibitor (20,100 Da), trypsinogen (24,000 Da), carbonic anhydrase (29,000 Da), glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), egg albumin (45,000 Da), and bovine albumin (66,000 Da).

The main difficulties in obtaining bacterial growth were the long incubation period, high atmospheric humidity, and the use of moist, freshly prepared media, each of which contributed to the high level of contaminants encountered. We consider that fasting animals prior to biopsy is important in order to decrease the number of contaminating organisms, particularly since helicobacter growth was often first seen on the plate in a relatively small area, making subculture difficult. In addition, contamination of the primary plates of the cat biopsies was more severe than that found in comparable investigations of canine samples. This may partly explain the high proportion of culture failures for cat biopsies. However, *H. pylori* cells are often distributed unevenly within the human gastric mucosa (4), and it is conceivable that some of the culture failures encountered here were also due to similar phenomena, since only one biopsy sample was used for this purpose. Moreover, the differences noted here between the cell morphologies of the bacteria observed in vivo and those obtained in vitro may suggest that mixed populations of different taxa are much more common than the data allow us to conclude. However, it is equally possible that the cellular forms of some species cultured on artificial media differ from those the species display in the host environment, and such phenomena have been described for *H. felis* (29) and *H. salomonis* (26).

Although the methods used here yielded a considerably increased success rate of isolation (43.6% overall) compared with that of a previous study (10.2%) (11), 56.4% of biopsies in which helicobacters were indicated (i.e., rapidly urease positive) or observed failed to give positive results for culture. While the methods described here for isolation of these bacteria represent a considerable advance over those used previously, further improvements in culture conditions for these bacteria are still required. The development of noncultural methods of detection such as species-specific PCR tests would also be of benefit. Such methods were employed by Neiger et al. to infer that 78% of cats harbored "*H. heilmannii*" and none harbored *H. felis* (36). The suitability of genes such as the urease genes used by Neiger et al. for the differentiation of gastric helicobacters has not been determined, and therefore the sensitivity and specificity of such approaches are rightfully questioned. It is thus conceivable that the "*H. heilmannii*" isolates detected by Neiger et al. (36) represent misidentified strains of any of the other three taxa mentioned. Unfortunately, recent data show that the levels of 16S rDNA sequence similarity between *H. felis*, *H. bizzozeronii*, *H. salomonis*, and "*H. heilmannii*" are extremely high (26), and this widely used gene does not seem suitable as a target for species-specific PCR. In any case, available data strongly indicate the need for further work concerning the specificity and application of PCR assays for detecting the various *Helicobacter* spp. known to inhabit the gastric mucosa of domestic pets and humans (1, 17, 19, 26, 29).

Only three strains were isolated from cats, and all these were shown to be *H. felis* strains. It has been clearly observed in the early morphological studies comparing canine and feline gastric spiral organisms that the majority of feline gastric helicobacters are more tightly spiraled, thinner, and longer organisms than those from the dogs (55, 56). It may be that the tightly spiraled organisms without the periplasmic fibrils in cats represent a species different from *H. bizzozeronii* and our method is not adequate for isolating it. Further studies are needed to clarify this matter.

Identification of gastric *Helicobacter* spp. The accurate identification of helicobacters and related organisms is essential in order to determine the prevalence and clinical significance of all taxa, although there are considerable difficulties associated with this process (37). Certainly the identification of strains to the species level was challenging in the present study. In primary identification tests, "*F. rappini*"-like isolates were readily distinguished from the other taxa encountered by virtue of their distinctive morphology, which was evident from both light and electron microscopy. Furthermore, *H. salomonis* isolates could be presumptively identified by their less helical cell morphology and unusually slow and sporadic motility (in contrast to *H. bizzozeronii* and *H. felis*), although the visual examination of protein patterns by using the minigel system proved a less useful means of identification. The unequivocal differentiation of *H. bizzozeronii* from *H. felis* was especially problematic. No clear differences were noted in primary identification tests. Moreover, the description of atypical *H. felis* strains lacking cellular periplasmic fibrils (11) invalidated this characteristic as an unequivocal means of distinguishing these two species, although all Finnish *H. felis* isolates proved typical in that respect. While useful, ultrastructural differences could not therefore be relied upon as a wholly accurate means of speciation, and the complex nature of the technique is not suited to routine use.

The phenotypic identification scheme used here has been found to provide effective discrimination between 11 of the 12 *Helicobacter*/"*Flexispira*" spp. tested (43, 44) and has also been used to identify field strains of the enteric species *Helicobacter canis* and *Helicobacter pullorum* (3, 38). In this study, considerable difficulties were encountered with simply cultivating the strains for further characterization, and these problems were reflected in the results obtained, since all strains were typified by their unreactivity. These data are consistent with the general difficulties associated with the isolation and culture of gastric helicobacters from domestic pets (11; this study). Nonetheless, it proved impossible to clearly differentiate the closely related species *H. bizzozeronii*, *H. felis*, and *H. salomonis*, although some useful traits for broadly distinguishing the last species from the first two taxa were noted (Table 3). Interestingly, urease was not detected in one "*F. rappini*"-like strain and three *H. bizzozeronii* isolates, suggesting a spontaneous loss of enzyme activity. This phenomenon has been described before for *H. pylori* (33) and *H. mustelae* (9).

The efficacy of highly standardized whole-cell protein analysis for identifying helicobacters and related organisms is well established (8, 37, 53) and was confirmed in the present study. All strains identified by quantitative or dot-blot DNA-DNA hybridization methods to the species level (26) were found to

form discrete clusters after numerical analysis of the data. *H. salomonis*, *H. bizzozeronii*, and *H. felis* were all readily recognizable. The three *H. felis* strains without periplasmic fibrils clustered among the other *H. felis* strains, although two prominent protein bands were absent. The whole-cell protein analysis confirms the identification of these strains as *H. felis* (11, 26) but highlights their aberrant nature. Therefore, we strongly recommend the use of highly standardized SDS-PAGE as the means of species identification for gastric spiral *Helicobacter* spp.

The identity of the "*F. rappini*"-like strains (strains 19 and Hilli) is undetermined. Both strains have a typical *Helicobacter* whole-cell protein pattern which shares some similarities with that of the "*F. rappini*" reference strain examined (LMG 8738; also ATCC 43879). Strain LMG 8738 is a human "*F. rappini*" reference strain isolated by Archer et al. in 1988 (2). Later studies revealed significant heterogeneity among isolates tentatively classified as "*F. rappini*," and novel *Helicobacter* species with similar ultrastructural features have been described (*H. bilis* and *H. trogontum* [16, 35]). *H. bilis* and *H. trogontum* are, however, intestinal helicobacters isolated from rodents, while strains 19 and Hilli are canine gastric strains. Our data indicate that strains 19 and Hilli do not belong to the same species as strain LMG 8738; several phenotypic differences (namely growth at 42°C, anaerobic growth, and growth on starch and media containing bile or triphenyltetrazolium chloride) are also evident (this study; 44). The relationship of strains 19 and Hilli to *H. bilis*, *H. trogontum*, and other helicobacters is under investigation.

The prospect of strain misidentification due to mixed cultures is evident from the examples described in this study (Fig. 2), although this problem is not easily solved. Several species may coexist in the gastric tracts of domestic pets (19, 26, 29; this study), and the bacterial growth of many of these helicobacters shows a tendency to swarm. This is strikingly illustrated by the differences in morphology between the organisms seen in in vivo electron micrographs and the culture isolates. *Campylobacter* contaminants have been noted in primary cultures of *H. felis* (29), although the differential susceptibilities of helicobacters and campylobacters to polymyxin B (6, 25a) can be exploited to eliminate campylobacter contamination. Conversely, no tolerance tests to differentiate between the *Helicobacter* spp. studied were found in the present study, and no recommendations for selective media can be made at present. Logically, repeated subcultivation of these mixed cultures and transfer of the strains after a relatively short incubation period will favor growth of the less fastidious strains. In our experience, an apparently pure "*F. rappini*"-like strain was recovered from a mixture of "*F. rappini*" and *H. bizzozeronii* strains and *H. salomonis* or *H. felis* strains seemed to become the dominant strains in mixtures which were initially dominated by *H. bizzozeronii* strains (strains Jutta, Tuohimetsa, and Loko 18). The prospect of a mixed culture must be considered when examining suspect helicobacter growth from a canine or feline gastric biopsy. Consequently, the cell morphology, motility, and protein profile of any isolate should be carefully scrutinized for anomalies.

Prevalence and significance of gastric helicobacters in domestic pets. We isolated only *H. felis* from feline gastric biopsies. By contrast, *H. bizzozeronii*, *H. felis*, *H. salomonis*, and "*F. rappini*" were obtained in 55.6, 22.2, 22.2, and 4.4%, respectively, of canine biopsies; mixed cultures were obtained in a total of 8.8% of these biopsies. Previous studies of the ecology of different *Helicobacter* species in the gastric mucosa of domestic carnivores are difficult to interpret, especially in view of recent developments in our understanding of the taxonomic diversity of these bacteria (11, 19, 26). Our data clearly indicate that several distinct species colonize the canine gastric mucosa and may also coexist with related taxa.

However, the clinical significance of these bacteria is unclear. Mild-to-moderate gastritis in infected animals is seen without any gastrointestinal signs (11, 22), and gastritis has also been observed without bacterial association (36). Moreover, it has been difficult to draw any conclusions from these studies as almost all dogs and cats are infected and the number of negative controls is low in a natural population (11, 22). However, an experimental infection of gnotobiotic beagles with *H. felis* caused gastritis in the animals (30). Similarly, natural *H. felis* infection has been suspected as a cause of severe gastrointestinal signs (48). No such data are available regarding *H. bizzozeronii*, *H. salomonis*, or "*F. rappini*," but it is feasible that intraspecific variation in pathogenic potential might explain the variance in the severity of gastritis seen among animals. Further work is required to clarify this issue.

The zoonotic potential of gastric helicobacters has been the subject of considerable interest, especially since the isolation of *H. pylori* from cats (18). Although subsequent studies have indicated that cats do not represent a significant reservoir for human *H. pylori* infection (11, 13), the issue concerning "*H. heilmannii*" (also known as "*G. hominis*") is far less certain. Several reports describe "*H. heilmannii*" infection as a possible zoonosis (34, 51). However, "*H. heilmannii*" is ultrastructurally indistinguishable from *H. bizzozeronii* and the atypical *H. felis* strains described by Eaton et al. (11) in vivo, and all these taxa have a complex relationship by 16S rRNA sequence analysis (26). Clearly, it is crucial to accurately determine the taxonomic position of "*H. heilmannii*" to properly evaluate its zoonotic potential, but such studies have been hindered by the failure of most workers to culture the organism (34, 51). It is possible that the methods described in this report for the culture of canine and feline helicobacters could be used to isolate human "*H. heilmannii*" strains, facilitating the necessary investigations. In this respect, it is encouraging to note that one such strain has been described (1), and we are pursuing comparative taxonomic studies to clarify this important issue.

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