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Identification of Non-*Helicobacter pylori* Spiral Organisms in Gastric Samples from Humans, Dogs, and Cats

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Tightly coiled bacteria are a rare cause of gastric pathology in humans and represent a mixture of species for which a zoonotic origin is suspected. Similar organisms are common inhabitants of the gastric mucosae of carnivores and pigs. It was the goal of the present study to determine the actual occurrence of each individual *Helicobacter* species in human, canine, and feline stomachs in order to better understand the possible zoonotic significance. Gastric biopsy samples from humans with histological evidence of non-*Helicobacter pylori* spiral bacteria ($n = 123$) and samples from the gastric antrum, corpus, and cardia from dogs ($n = 110$) and cats ($n = 43$) were subjected to a multiplex PCR, enabling the identification of *Helicobacter felis*, *Helicobacter bizzozeronii*, *Helicobacter salomonis*, and "*Candidatus* Helicobacter suis." A PCR for detecting *H. pylori* was applied to all human samples. Single infections with "*Candidatus* Helicobacter suis," *H. felis*, *H. bizzozeronii*, *H. salomonis*, a hitherto unknown genotype of a non-*H. pylori* spiral organism (*Helicobacter*-like organism 135 [HLO135]), and *H. pylori* were identified in 30.9%, 8.9%, 2.4%, 11.4%, 7.3%, and 8.9% of the human biopsy samples, respectively. Mixed infections (16.3%) with two or even three of these were also found. In the canine stomach, *H. bizzozeronii* (70.0%) was encountered as the main spiral organism, while *H. felis* (62.7%) and HLO135 (67.4%) were the predominant *Helicobacter* species found in the feline gastric mucosa. Although the majority of human non-*H. pylori* organisms are *Helicobacter* species naturally occurring in the stomachs of pigs, cats, and dogs, the frequent identification of *H. salomonis* in human gastric biopsy samples is in contrast to its rare identification in pet carnivore samples, urging us to suspect other sources of infection.

Spiral organisms have been described in the gastric mucosae of cats and dogs since the 19th century and are considered common inhabitants of the gastric mucosal niche (18). Three different morphological types were identified after ultrastructural analyses of the bacteria in situ; these types originally were presumed to represent various stages in the movement of one organism (25). Recent polyphasic taxonomy studies involving various isolates, however, revealed at least three different species belonging to the genus *Helicobacter*, namely, *Helicobacter felis* (24), *Helicobacter bizzozeronii* (17), and *Helicobacter salomonis* (21); these species are both phenotypically and phylogenetically highly related (21). These tightly coiled organisms were largely ignored by the scientific community until the isolation of *Helicobacter pylori* from the human gastric mucosa renewed interest in gastric bacteria (27).

H. pylori, colonizing the stomachs of half of the world's human population, was proved to be the primary cause of gastritis and peptic ulceration (13) and is considered a major risk factor in the development of gastric adenocarcinoma (32) and mucosa-associated lymphoid tissue lymphoma (36). A limited number of human gastric biopsy samples, however, were shown to harbor bacteria which were morphologically different from *H. pylori* (6, 19). These tightly coiled bacteria were mostly associated with chronic active gastritis characterized by an inflammatory process less intense than that of *H. pylori*-related gastritis (6, 19, 37), and sporadic cases of gastric ulcers or erosions (7, 10, 38, 43) and cancer (29, 42) were also reported. Genetically and morphologically based studies directed the classification of the helical organisms into the genus *Helicobacter*, and the provisional name "*Helicobacter heilmannii*" was proposed (34). However, this name fell into disuse as two kinds of spiral organisms could be distinguished in the human stomach. The first type is closely related and probably identical to "*Candidatus* Helicobacter suis," a hitherto unculturable porcine gastrospirillum (9). The second type most likely represents a mixture of species, including the *Helicobacter* species naturally occurring in the gastric mucosae of cats and dogs (9) as well as the recently described "*Candidatus* Helicobacter heilmannii," a *Helicobacter* species detected in the gastric mucosae of a human being and wild carnivores (31). The marked morphological resemblance and the genetic relatedness between the spiral organisms identified in both animal and human gastric tissues led to the hypothesis that spiral gastric bacteria are zoonotic agents. Moreover, contact with dogs,

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cats, and pigs has been determined to be a risk factor for human non-*H. pylori Helicobacter* infection, indicating that animals are a source for this infection (28). This hypothesis is supported by a unique report on the in vitro culturing of a tightly coiled bacterium, subsequently identified as *H. bizzozeronii*, from a human stomach (1, 22).

The exploration of the actual occurrence of each individual non-*H. pylori Helicobacter* species in human, canine, and feline stomachs, however, has been hampered by the very high similarity within the group of canine and feline gastric helicobacters, combined with the marked fastidious nature of these microorganisms. Nevertheless, this knowledge is essential to better understanding the implications of these organisms for both animal health and human health. In the present study, a recently developed multiplex PCR, which allows the identification of the three carnivorous *Helicobacter* species and "*Candidatus* Helicobacter suis" (3), was used to identify tightly coiled bacteria in human, feline, and canine gastric biopsy specimens.

MATERIALS AND METHODS

Human patients. A total of 126 paraffin-embedded gastric biopsy specimens were selected from 126 patients on the basis of the histological presence of tightly coiled spiral bacteria. Seventy-two patients were male, and 39 were female, with ages ranging from 18 to 89 years. The age and gender were unknown for the remaining 15 patients. The samples were taken in four different hospitals, three in Belgium ($n = 46$) and one in Germany ($n = 80$), between January 1991 and May 2004. The study protocol was approved by the institutional ethics committee of Ghent University Hospital.

Animals. Samples from the antral, cardiac, and body regions were collected from the stomachs of 110 dogs (65 male and 45 female, ranging in age from 1 day to 18 years) and 43 cats (25 male and 18 female, ranging in age from 7 weeks to 18 years) of various breeds, with various pathologies, and presented for autopsy at the Department of Pathology, Faculty of Veterinary Medicine, Ghent University, between November 2001 and September 2003. The samples were immediately frozen at −20°C until DNA extraction was performed.

DNA extraction. From each paraffin-embedded gastric biopsy specimen from human patients, five sections 10 μm thick were cut and collected in a vial. Care was taken during section preparation to avoid contamination from the microtome blade, both by using disposable blades and by washing with 95% ethanol followed by 0.1 N HCl. To each vial, 250 μl of digestion buffer (50 mM Tris-HCl [pH 8.0], 0.5% Triton X-100, 10% proteinase K solution [10 mg/ml]) was added; the samples were incubated at 56°C for 18 h and then for 10 min at 95°C. DNA extracts were heated for 10 min at 56°C prior to use in PCR mixtures.

The gastric samples from cats and dogs were thawed at room temperature, and DNA was extracted by using a DNeasy tissue kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer.

β-Globin PCR assay. To evaluate the quality of the DNA isolated from paraffin-embedded gastric biopsy specimens, an initial PCR was performed with each DNA extract from human gastric biopsy specimens. Fragments of 110 bp were amplified from the β-globin coding gene portion of the human genome as described elsewhere (26). Positive controls consisted of DNA extracted from nonfixed, nonembedded gastric biopsy specimens.

***Helicobacter pylori*-specific PCR.** Human gastric biopsy specimens were subjected to a PCR to detect the presence of *H. pylori*. Primers HP-FOR and HP-REV, based on the *ureC* gene of *H. pylori*, were used as previously described (33). A DNA extract from a pure culture of *H. pylori* SS1 was used as a positive control.

Pet carnivore *Helicobacter*-specific PCR. Human and animal gastric biopsy specimens were screened for the presence of *H. bizzozeronii*, *H. salomonis*, and *H. felis* by a PCR amplifying a unique 78-bp-long DNA fragment of the 16S rRNA coding gene, which these three species have in common. PCRs were performed as described before (8). DNA extracts from pure cultures of *H. bizzozeronii* (CCUG 35545^T), *H. salomonis* (CCUG 37845^T), and *H. felis* (CCUG 28539^T) and a DNA extract from a swine stomach sample histologically found positive for "*Candidatus* Helicobacter suis" served as positive controls.

The PCR products of these three PCRs were analyzed by gel electrophoresis as described elsewhere (3).

Multiplex PCR. All samples of human and animal origins were subjected to a multiplex PCR, enabling the identification of *H. felis*, *H. bizzozeronii*, *H. salomonis*, and "*Candidatus* Helicobacter suis" on the basis of the tRNA intergenic spacers of *Helicobacter* species and on the urease gene of *H. felis* as previously described (3). Fluorescently labeled PCR products, obtained by using fluorescently labeled primers (TET, HEX, and NED), were analyzed by capillary electrophoresis. Samples were identified as *H. felis* when a TET-labeled fragment of 137 bp and a NED-labeled fragment of 434 bp were obtained. The identification of *H. bizzozeronii* resulted when a TET-labeled amplicon of 136 bp and a HEX-labeled fragment of 373 bp were obtained. The presence of merely a TET-labeled amplicon of 134 bp resulted in *H. salomonis* identification. "*Candidatus* Helicobacter suis" was identified when a TET-labeled amplicon of 136.5 bp and a NED-labeled fragment of 447 bp were retrieved.

Sequencing of the 16S rRNA gene. Biopsy samples which produced a 135-bp TET-labeled fragment in the multiplex PCR were subjected to 16S rRNA gene amplification with primers complementary to the conserved edges. Consensus primers αβ-NOT (5′-TCA AAC TAG GAC CGA GTC) and ωMB (5′-TAC CTT GTT ACT TCA CCC CA) were used as previously described (2).

A 1,500-bp amplicon amplified in this PCR was sequenced after cloning into *Escherichia coli* cells as described elsewhere (5). Sequence analysis was performed by using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, Calif.), and sequences were aligned with GenBank sequences by using BLAST.

RESULTS

β-Globin PCR. Of the 126 human gastric biopsy specimens, 123 showed a positive signal in the β-globin PCR, indicating that the DNA extraction from paraffin-embedded samples was successful. The remaining three samples with a negative result were excluded from further study.

***H. pylori*-specific PCR.** Seventeen human gastric biopsy samples showed a positive signal in the PCR specific for detecting urease of *H. pylori*; 11 of these represented a single infection of *H. pylori*, without evidence of other *Helicobacter* species being present.

Pet carnivore *Helicobacter*-specific PCR. The positive control DNA from *H. felis*, *H. bizzozeronii*, and *H. salomonis* produced an amplicon of 78 bp, while the DNA from the porcine gastric mucosa tested negative in the *Helicobacter*-specific PCR. Fifty-eight human gastric biopsy specimens (47.2%) selected on the basis of the histological presence of tightly coiled organisms yielded a positive signal in this group-specific PCR. For the canine and feline stomach specimens, positive signals were obtained in 71.8% and 79.1%, respectively.

Multiplex PCR. The results of the multiplex PCR are summarized in Table 1. The positive control DNA from *H. felis*, *H. bizzozeronii*, and *H. salomonis* produced the expected PCR fragments, as did the DNA from the swine stomach specimen that was positive for spiral organisms.

In human gastric biopsy specimens, "*Candidatus* Helicobacter suis" was identified as the most prevalent *Helicobacter* species; it was detected in 45 specimens (36.6%). Twenty-eight of these biopsy samples were derived from the German clinical center. *H. felis*, *H. bizzozeronii*, and *H. salomonis* were identified as single infections or as part of mixed infections. A TET-labeled fragment of 135 bp, different from the TET-labeled fragments detected for *H. felis*, *H. salomonis*, and *H. bizzozeronii*, was demonstrated in nine samples (7.3%). The sequence of the 16S rRNA gene with the TET-labeled fragment of 135 bp (GenBank accession number AY756181) was found to have 99% similarity with a sequence representative of "*Candidatus* Helicobacter heilmannii" (AF506786) and ≥97% similarity with *H. felis*, *H. bizzozeronii*, and *H. salomonis* sequences. The

TABLE 1. Prevalence of non-*Helicobacter pylori* spiral organisms in gastric samples from humans (n = 123), dogs (n = 110), and cats (n = 43)

Helicobacter species[a]	No. (%) of multiplex PCR-positive samples from:		
	Humans	Dogs	Cats
Helicobacter felis only	11 (8.9)	4 (3.6)	4 (9.3)
Helicobacter bizzozeronii only	3 (2.4)	22 (20.0)	0
Helicobacter salomonis only	14 (11.4)	0	0
"*Candidatus* Helicobacter suis" only	38 (30.9)	0	1 (2.3)
HLO135 only	9 (7.3)	4 (3.6)	5 (11.6)
Helicobacter bizzozeronii + *Helicobacter felis*	1 (0.8)	23 (20.9)	3 (7.0)
Helicobacter bizzozeronii + HLO135	0	5 (4.5)	4 (9.3)
Helicobacter felis + HLO135	1 (0.8)	6 (5.4)	11 (25.6)
Helicobacter bizzozeronii + *Helicobacter salomonis*	1 (0.8)	2 (1.8)	0
Helicobacter felis + *Helicobacter salomonis*	4 (3.3)	0	0
"*Candidatus* Helicobacter suis" + *Helicobacter salomonis*	6 (4.9)	0	0
Helicobacter bizzozeronii + *Helicobacter felis* + HLO135	0	17 (15.5)	8 (18.6)
Helicobacter bizzozeronii + *Helicobacter felis* + *Helicobacter salomonis*	0	6 (5.4)	0
Helicobacter felis + *Helicobacter salomonis* + HLO135	0	0	1 (2.3)
Helicobacter felis + *Helicobacter salomonis* + "*Candidatus* Helicobacter suis"	1 (0.8)	0	0
Helicobacter bizzozeronii + *Helicobacter felis* + *Helicobacter salomonis* + HLO135	0	2 (1.8)	0
None (negative)	34 (27.6)	19 (17.3)	6 (14.0)

[a] HLO135 is a newly identified *Helicobacter*-like organism based on the length of the tRNA intergenic spacer.

organism was referred to as *Helicobacter*-like organism (HLO) 135 (HLO135). Mixed infections with different *Helicobacter* species, including *H. pylori*, were detected in 20 biopsy specimens (16.3%), while 34 samples (27.6%) tested negative in the multiplex PCR.

Mixed infections were detected in gastric samples from 41 dogs (37.3%), while in samples from 30 dogs (27.3%), only one *Helicobacter* species was demonstrated. *H. bizzozeronii* was the most prevalent *Helicobacter* species in samples from canine origin, as it was identified in 22 dogs (20.0%) as single infections and in 55 dogs (50.0%) as mixed infections. Single infections with *H. felis* or HLO135 were sporadically encountered, while single infections with *H. salomonis* were not identified in any sample. "*Candidatus* Helicobacter suis" was not identified in samples from canine origin.

In cats, *H. felis* and HLO135 were the most frequently detected *Helicobacter* species, either as single or as mixed infections. The other *Helicobacter* species were only occasionally demonstrated in the feline gastric samples.

DISCUSSION

The prevalence of spiral organisms in the gastric mucosae of cats and dogs has been determined by means of different techniques and ranges between 40 and 100% (11, 14, 18). The present study made use of a recently developed multiplex PCR (3) and revealed equivalent results, as pet carnivore helicobacters were detected in 82.7% of the canine stomachs and 86.0% of the feline stomachs. This PCR is a simple and effective method for discriminating the closely related species *H. felis*, *H. bizzozeronii*, *H. salomonis*, and "*Candidatus* Helicobacter suis" in one reaction mixture, in contrast to other PCRs, in which only one species (9, 16) or an assembly of species can be identified (8, 30).

Applying the multiplex PCR to animal and human gastric tissue samples led to the discovery of a 135-bp TET-labeled fragment in the stomachs of 63 animals, the vast majority of which were cats. This amplicon was clearly different from the TET-labeled PCR products obtained with DNA from *H. felis*, *H. bizzozeronii*, *H. salomonis*, and "*Candidatus* Helicobacter suis." 16S rRNA gene analysis of the organism with this 135-bp fragment justified classification into the genus *Helicobacter*, and the organism is referred to as HLO135. The sequence of the 16S rRNA gene showed high similarity to the sequence of "*Candidatus* Helicobacter heilmannii" (99%) and pet carnivore *Helicobacter* species (≥97.0%). Unfortunately, limited attempts to culture this organism remained unsuccessful (data not shown). This hitherto unculturable HLO could be a plausible explanation for the fairly low rate of successful isolation of spiral bacteria from canine and feline gastric mucosae (11, 23). Moreover, unculturable HLOs, morphologically similar to *H. bizzozeronii* but probably a different group of microorganisms, were found to represent a significant proportion of the spiral organisms visualized in canine and feline stomachs (4, 23). Clearly, more research is required to elucidate the actual identity, morphology, and pathogenicity of the newly identified member of the genus *Helicobacter*.

H. felis (51.2%) and the new genotype HLO135 (55.8%) were identified as the most prevalent *Helicobacter* species in cats, in both single and mixed infections. In dogs, *H. bizzozeronii* was found to be the main infecting HLO, as it was likewise identified in 70.0% of the stomachs. This result confirms former observations of *H. bizzozeronii* probably being the predominant organism in the canine stomach (23). Moreover, the present study corroborates the presence of mixed infections in the gastric mucosae of cats and dogs (23, 30, 35) and indicates the compositions as being variable and consisting of different combinations of gastric non-*H. pylori Helicobacter* species and/or the unknown HLO135. The high rates of mixed infections presently detected in feline (62.8%) and canine (55.5%) stomachs are in sharp contrast to their limited presence in human samples (11.4%).

Histological evidence of spiral organisms was present for 123 human gastric biopsy specimens. When these samples were tested with the multiplex PCR, 34 (27.6%) were found to be

negative. This phenomenon may be explained by the extremely patchy distribution of spiral organisms throughout the gastric mucosa (6, 20) or the presence of other species with a similar morphology (e.g., *Flexispira rappini*) (12). Strikingly, one-third ($n = 11$) of the negative samples were found positive for *H. pylori*. This observation illustrates the previously reported inconsistent morphology of *H. pylori* (15).

Variable rates of *H. felis*, *H. bizzozeronii*, *H. salomonis*, "*Candidatus* Helicobacter suis," and HLO135 were encountered in 89 human samples. "*Candidatus* Helicobacter suis" (36.6%) and *H. salomonis* (21.1%) were found to be the most prevalent *Helicobacter* species. Remarkably, "*Candidatus* Helicobacter suis" was detected in 47% of the samples derived from the German clinical center and in only 11.9% of the Belgian samples. A high rate of prevalence of the porcine gastrospirillum (78%) was also described previously for a group of exclusively German patients with histological evidence of HLO, although through a different technique (40). More research is required to determine sources of infection for humans—which might include pork consumption and, more likely, direct contact with pigs (28).

H. salomonis represented 51% ($n = 26$) of the human samples found positive for pet carnivore *Helicobacter* species, including the new genotype HLO135. The origin of this infection remains questionable, as *H. salomonis* was barely identified in cats and dogs. In this regard, it is interesting to speculate on other animal species acting as a host for these bacteria. The possibility that humans constitute the natural host for this organism also cannot be excluded.

H. felis and HLO135, the most prevalent *Helicobacter* species in cats, were identified in 14.6% and 8.1% of the 123 human samples, respectively. These data confirm earlier studies reporting that spiral bacteria in human patients probably originated from cats, as partial molecular characterization identified the same organisms (10, 41). Quite convincingly, the present results emphasize the role of cats as an important source of non-*H. pylori Helicobacter* infections in human beings.

In addition, contact with dogs has been considered an increased risk factor for acquiring a gastric infection with tightly coiled bacteria (28, 39). Compelling evidence came from the isolation of *H. bizzozeronii* from the human gastric mucosa (1, 22), indicating at least that dogs, designated earlier as the predominant host for *H. bizzozeronii* (23) and confirmed in the present study, may serve as a source of human non-*H. pylori* infections. However, *H. bizzozeronii* was currently identified in only five human samples, two of which were from mixed infections.

In conclusion, the present study is the first to identify gastric spiral bacteria up to the species level in a series of both pet animals and human patients. Our observations indicate that cats and swine are important in human gastric non-*H. pylori Helicobacter* infections but also urge us to suspect other sources of infection. In addition, the present study confirms the supposition that HLO is a mixture of *Helicobacter* species naturally occurring in the stomachs of animals.

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