Prevalence of gastrospirillum-like organisms in pigs, cattle, and dogs: a comparison of diagnostic methods between species

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ABSTRACT: A survey was conducted to determine the prevalence of spiral-shaped bacteria in animals as a possible source of pathogens causing chronic changes in the human and animal stomach as well as in other parts of the digestive tract. This study was carried out in three different groups of animals, pigs, cattle and dogs. Swabs from the oral cavity of dogs (n = 198) were stained using Gram's method to evaluate gastrospirillum-like organisms (GLOs) and revealed two different types of GLOs with an incidence of 23.2% (46/198). The stomachs of the pigs (n = 104), cattle (n = 102), and dogs (n = 7) were collected for the urease test, brush cytology, light and electron microscopy, and PCR. A positive urease test was observed in 31.7% (33/104) of pigs, 90.2% (92/102) of cattle, and 85.7% (6/7) of dog samples. GLOs were detected in 37.5% (39/104) of pigs, 62.7% (64/102) of cattle, and 85.7% (6/7) of dog samples by brush cytology. Furthermore, positive PCR results were obtained in the stomach samples of dogs that had tested positive by both the urease test and brush cytology. The morphological study using brush cytology and scanning electron microscopy of a pig stomach revealed bacteria with the typical morphology of GLOs, which appeared to be similar to *Helicobacter heilmanii*. This study indicates that the urease test and brush cytology are useful tools for diagnosing GLOs in different animals. Moreover, the location of specimen collection can influence the diagnostic sensitivity of the examination.

Keywords: pigs; cattle; dogs; stomach; urease test; brush cytology

Many different gastrospirillum-like organisms (GLOs) colonize the stomach of humans and animals including domestic pets. The colonization of the human stomach with *Helicobacter pylori* (*H. pylori*) is accompanied by persistent chronic gastritis, which can lead to peptic ulceration and gastritis (Blaser, 1990), atrophic gastritis, and gastric adenocarcinoma (Talley et al., 1991). There does not appear to be any substantial reservoir of *H. pylori* aside from the human stomach. Although *H. pylori* is present in the stomach of approximately half of the world's population, its transmission route is not completely understood (Dunn et al., 1997). The presence of *H. pylori* in cats appears to be a case of human-to-animal transmission. The ecology of other species within the gastric mucosa is largely unknown (Jalava et al., 1998) and many other sources have been suggested. Dimola and Caruso (1999) reported that horses, cattle and pigs returned positive results when tested for *H. pylori*. Another *Helicobacter* was also found to be associated with a small subset of human gastritis cases (1%) (Heilmann and Borchard, 1991), and was later designated *H. heilmanii* (Debongnie et al., 1995). Currently, other *Helicobacter* species do not appear to be pathogenic to humans.

Gastric ulceration in the *pars oesophagea* is a well-known problem in swine that can lead to growth retardation, bleeding, and death (Guise et al., 1997). Spiral-shaped GLOs are present in the stomachs of pigs (Queiroz et al., 1990; Roosendaal et al., 2000). Recent phylogenetic analysis has clas-

sified this organism as belonging to the genus *Helicobacter*, and it has been renamed "*Candidatus* Helicobacter suis" (De Groote et al., 1999). Several studies have examined and confirmed the association between GLOs and ulcerative lesions in the *pars oesophagea* of swine stomachs (Barbosa et al., 1995). This uncultured porcine *Helicobacter* sp. has a similar morphology to *H. heilmanii* based on its ultrastructural features (Mendes et al., 1990), and a comparison of its 16S rDNA gene sequence with that of *H. heilmanii* revealed that they share 99.5% identity at the nucleotide level (De Groote et al., 1999).

H. pylori* can also be found within the oral cavity of human patients with recurrent aphthous stomatitis (Riggio et al., 2000), gastritis and peptic ulcers (Okuda et al., 2000), and duodenal ulcers (Dore-Davin et al., 1999). Some authors indicate that the oral mucosa does not appear to be the preferred site of colonization of *H. pylori* (Mravak-Stipetic et al., 1998). On the other hand, others have assumed that *H. pylori* is transmitted through the oral-oral route with the oral cavity possibly being their permanent reservoir (Song et al., 2000a).

This study examined the prevalence of *Helicobacter* microorganisms in the stomachs of slaughtered pigs and cattle in Korea, as well as in necropsied dogs using various diagnostic tools, in order to explain the gross and histological changes in the stomachs of the animals investigated. The mouth cavities of pet dogs were also observed. The results of this study will provide a valuable clue to help identify useful methods for the diagnosis of *Helicobacter* infections in animals. Furthermore, this report aims to contribute to explaining the possible animal to human transmission route of *Helicobacter* spp.

MATERIAL AND METHODS

Samples

Gastric mucosal samples were taken from the antral, fundal, and pyloric regions of the stomachs of 104 pigs and 102 cattle immediately after slaughter in the Jeonju slaughter house during March of 2001, and from seven dogs in a pathology necropsy laboratory, College of Veterinary Medicine, Chonbuk National University, Jeonju city, South Korea. The mucus for the brush cytology and biopsy specimens for the urease test and histopathological examina-

tions was obtained from the pylorus, fundus, and cardia of the stomachs. The samples for electron microscopy were recovered from the fundus of eight cattle, as well as from the pylorus of eight pigs and two dogs. Swabs for brush cytology were taken from the mucosal surface of the mouth cavity from 198 dogs examined in private veterinary hospitals in Jeonju and Busan city.

Urease test

A modified rapid urease test was used on the small mucosal specimens cut with scissors and placed in test tubes with two drops of a physiological solution (distilled water) for all collection times in the slaughterhouse. The tubes were brought to the laboratory, the solution was removed and 1 ml of reagent was added to every sample (10% unbuffered urea in distilled water, pH 6.8, to which 1% phenol red suspension was added). The results were recorded at 30 min and 60 min after each specimen had been placed in the reagents. A change of color from pale yellow to bright pink was considered a positive reaction (Figure 1).
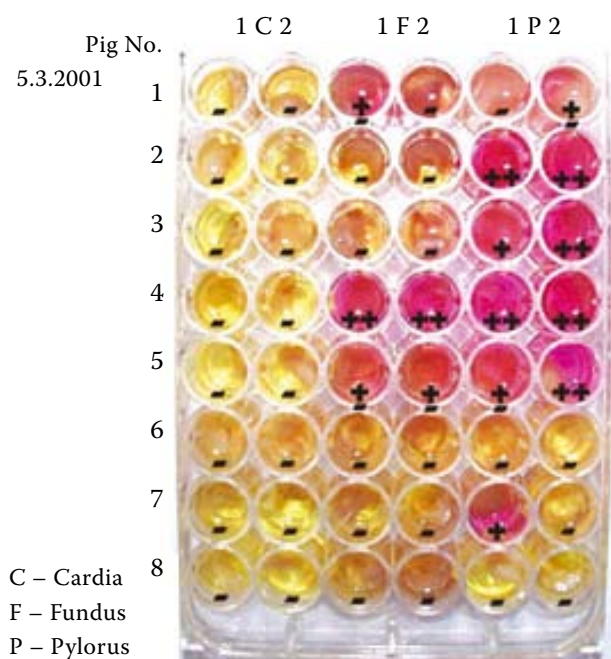

Figure 1. Urease activity test showing a positive and negative reaction. Small mucosal specimens taken from various parts of the stomach were placed in each well. 1 ml of urea reagent was then added to every sample. A positive reaction was defined as a color change in the urea reagent from yellow to pink within one hour at room temperature or 37°C

Brush cytology

Mucus collection was carried out with small brushes prepared earlier in the laboratory. The brush was rolled over the mucosa at the sample site and subsequently rolled onto a clean slide. The preparations were then air dried, Gram stained and examined using an oil immersion lens at ×100 magnification. The number of gram negative GLOs was recorded in 25 fields with the highest density of collected mucus.

Electron microscopy

Samples for scanning electron microscopy were trimmed into 1 mm^3 pieces, fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer at pH 7.3 and stored in a refrigerator. The samples were postfixed in 1% osmium tetraoxide, dehydrated in a graded series of alcohol and isoamylacetate, dried in a critical point drier (PCD), coated with gold particles and examined by scanning electron microscopy (SEM, JSM-5310LV, Jeol; Tokyo, Japan).

Polymerase chain reaction (PCR)

Small pieces of a formalin fixed pylorus from the pigs and cattle were cut and washed in PBS for the PCR. Fresh frozen samples of the dog stomach were used. The DNA was isolated from the urease positive or brush cytology positive samples using a DNeasy Tissue Kit (Qiagen, Germany). The extracted DNA was used as a template for PCR amplification. *Helicobacter* genus specific primers were used to amplify a 409 bp fragment of the 16S rRNA gene (Cosmo Genetech, Korea). The primer sequences were: 5'-GCCCTAAACGATGGATGCTA-3' (sense) and 5'-AGGGCCATGATGACTTGACGT-3' (antisense).

RESULTS

Pigs

Keratinization of different grades of ulceration in the cardial region (29.8%, 31/104, Figure 2A) and hemorrhages (Figure 2B) of the fundal mucosa (7.7%, 8/104) were the most commonly observed macroscopic lesions. A positive reaction to testing for Gram-negative GLOs was observed in 31.7% (33/104) of the pigs investigated by the urease test (Table 1). Among stomach parts examined, the pylorus testing positive most commonly in the urease test (29.8%, 31/104). In 37.5% (39/104) of pigs, GLOs were observed by brush cytology from the mucosal surface (Figure 3A) and were detected mainly in the fundus (20.2%, 21/104) or pylorus (28.9%, 30/104) of the stomach. Positive findings were obtained in 46 pigs (44.2%, 46/104) by either the urease test or brush cytology. Moreover, 24 pigs (23.1%, 24/104) tested positive to both the urease test and brush cytology. There was no correlation
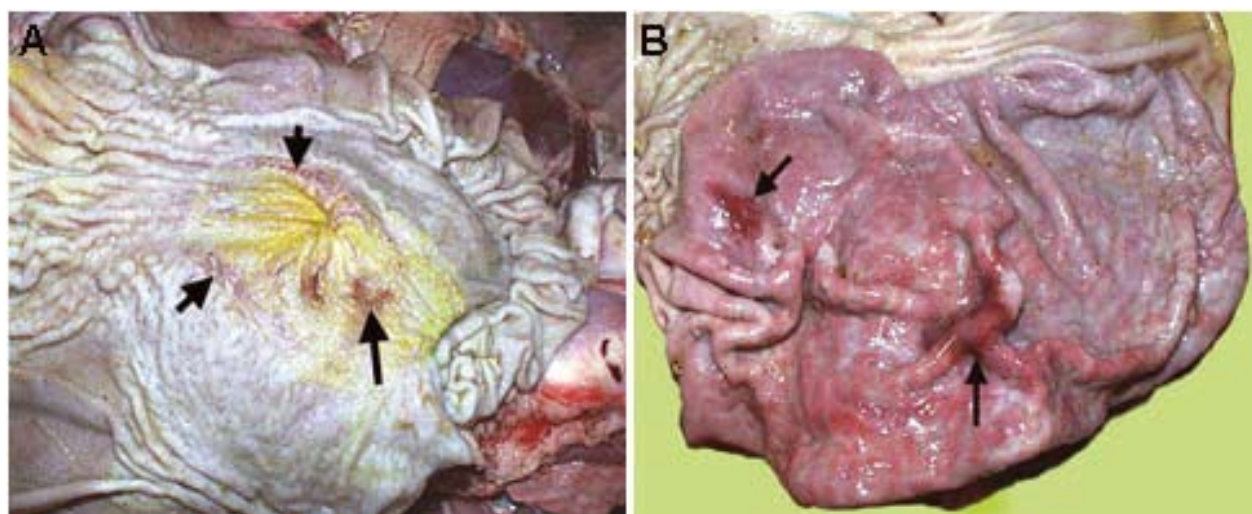

Figure 2. Hyperkeratosis in the cardial part of the stomach showing chronic ulcerative changes (A, arrows) and hemorrhage (arrows) in the stomach mucosa (B)

Table 1. Presence of GLOs in pig and cattle stomachs (in %/number)

Part of stomach	Pig		Cattle	
	urease test	brush cytology	urease test	bBrush cytology
Cardia	0 (0/104)	4.8 (5/104)	63.7 (65/102)	30.4 (31/102)
Fundus	4.8 (5/104)	20.2 (21/104)	62.7 (64/102)	38.2 (39/102)
Pylorus	29.8 (31/104)	28.9 (30/104)	70.6 (72/102)	26.5 (27/102)
Total positive results in each stomach	31.7 (33/104)	37.5 (39/104)	90.2 (92/102)	62.7 (64/102)

between macroscopic changes and a positive result in the urease test and/or brush cytology. Spiral-shaped microorganisms were observed in the SEM images of the pylorus of a urease positive stomach (Figure 3B).

Cattle

Chronic mucosal changes in the pylorus were sometimes observed after ulcer formation (Figure 4).

The urease test was positive in 63.7% (65/102) of samples from the cardia, 62.7% (64/102) from the fundus, and 70.6% (72/102) from the pylorus of cattle (Table 1). Positive results were returned in 90.2% (92/102) of the abomasa. The microscopic examination by brush cytology revealed Gram-negative GLOs in 30.4% (31/102) of the cardia, 38.2% (39/102) of the fundus, and 26.5% (27/102) of the pylorus samples examined. According to the morphological features no typical *Helicobacter*-like microorganism was observed by brush cytology.

Table 2. Presence of GLOs in the mouth cavities dog according to whether it was kept inside or outside

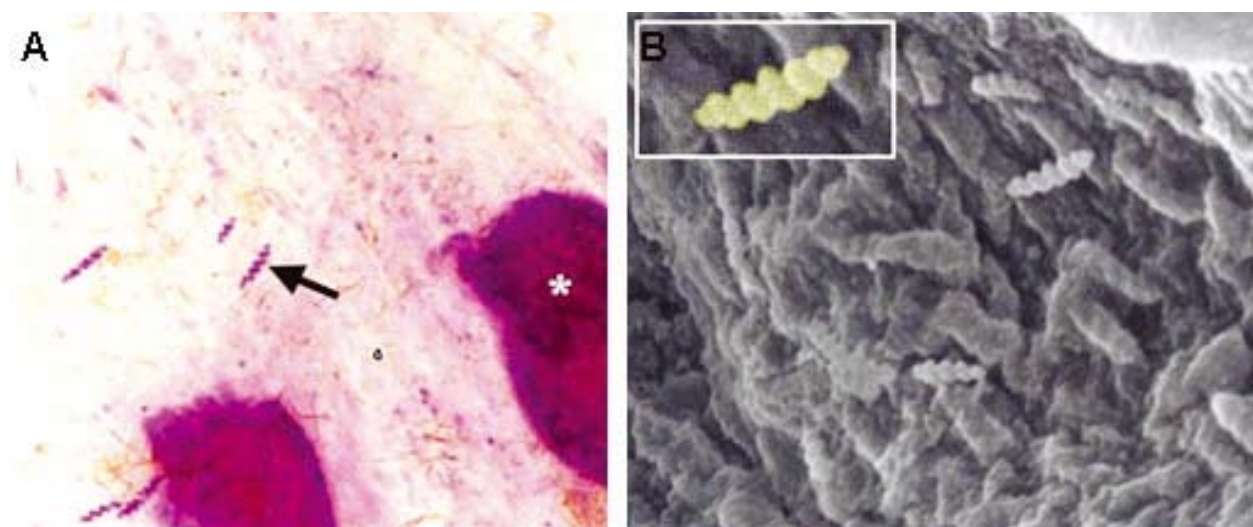| Type of keeping (number of animals investigated)	Negative for GLOs (%/number)	Positive for GLOs (%/number)	
		thinner type	thicker type
Outside (31)	74.2 (23/31)	12.9 (4/31)	12.9 (4/31)
Inside (167)	77.2 (129/167)	22.2 (37/167)	0.6 (1/167)
Total (198)	76.7 (152/198)	20.7 (41/198)	2.5 (5/198)

Figure 3. Observation of *Helicobacter*-like microorganisms in a pig stomach. (A) *Helicobacter*-like microorganisms (arrow) in the brush cytology of a pig stomach (white asterisk – epithelial cell, ×150, Gram's staining). (B) Scanning electron micrograph of *Helicobacter*-like microorganisms in the pylorus of a pig (bar – 5 μm, ×5 000)
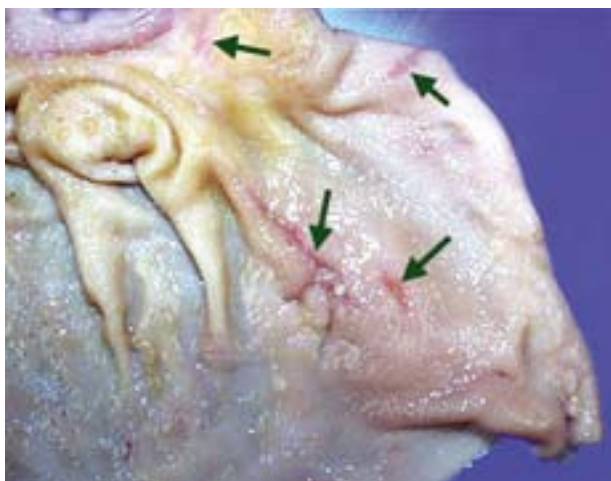
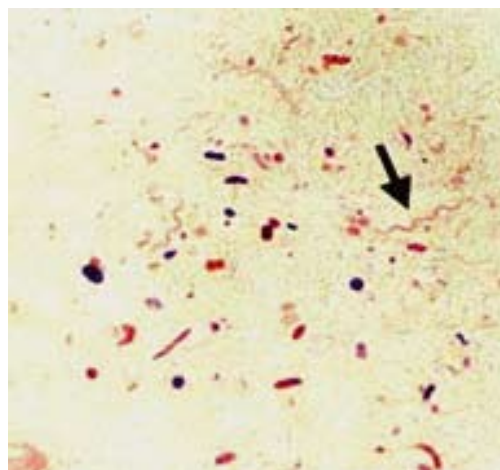Figure 4. Numerous traumatic changes (arrows) in the mucosal surface of the pylorus of cattle

Figure 5. Gastrospirillum-like organisms (arrow) in the cattle abomasums (×150, Gram's staining)

Table 3. Presence of GLOs in dog stomachs

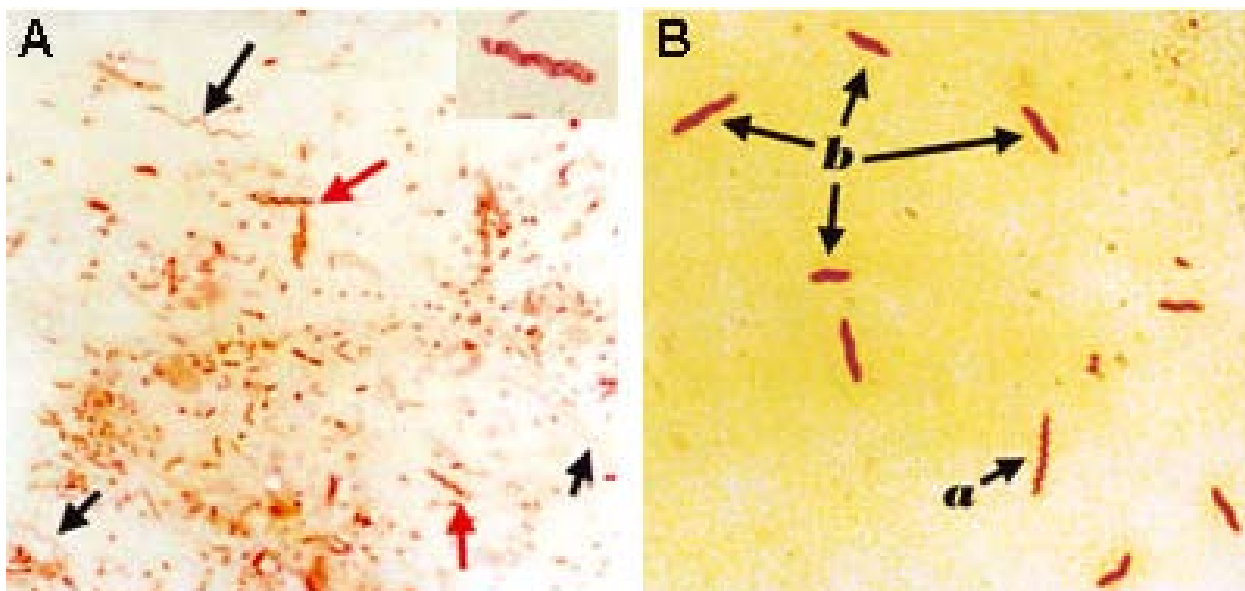| Part of stomach	Number of positive animals (%/number)		
	urease test	brush cytology	PCR
Cardia	71.4 (5/7)	71.4 (5/7)	71.4 (5/7)
Fundus	71.4 (5/7)	71.4 (5/7)	71.4 (5/7)
Pylorus	71.4 (5/7)	71.4 (5/7)	71.4 (5/7)
Total positive results in each stomach	85.7 (6/7)	85.7 (6/7)	85.7 (6/7)

Figure 6. *Gastrospirillum*-like organisms in the mouth cavity and stomach in dogs. (A) GLOs in the mouth cavity in dogs (black arrows – thinner type, red arrows – thicker type, ×100) and thicker *Helicobacter*-like type of GLOs in the mouth cavity of dogs (right upper box, ×150). (B) Two different types (a and b arrow) of *Helicobacter*-like microorganisms in the stomachs (×150, Gram's staining)

All microorganisms were evidently thinner with a number of irregular spiral turns of the microbial body ranging from two to five (Figure 5). There was no correlation between the mucosal changes and a positive result for GLOs by brush cytology and/or the urease test.

Dogs

Brush cytology of the mouth cavity revealed the presence of GLOs in 23.2% (46/198) of samples (Table 2). Two different types were observed using optical microscopy (Figure 6A). The thinner type of spiral-shaped bacteria with no regularly formed turns and of varying length were observed with greater frequency. The thicker type of GLO was structurally similar to *Helicobacter*-like microorganisms. The bacterial cells had three to five spiral turns and were approximately four microns long and 0.5 microns wide. They were evidently (five to six times) thicker than the spiral-shaped bacteria (thinner type) and were present at higher levels in dogs kept outside (12.9%, 4/31) than in those kept inside (0.6%, 1/167). The type of food they ate, gender or clinical status of the animals investigated had no correlation with the results. A higher prevalence of the helical-shaped thinner bacteria was observed only in dogs of more than one year old (25.1%, 40/159) compared with dogs less than one year of age (2.6%, 1/39).

Brush cytology found typical *Helicobacter*-like microorganisms in the samples from all parts of the dog stomachs investigated (Table 3). These samples also tested positive in the urease test and PCR, which suggests a close correlation between detection methods. Furthermore, more types of *Helicobacter*-like microorganisms (the thicker GLOs) were identified by light microscopy according to their shape and number of spiral turns (Figure 6B).

DISCUSSION

Queiroz et al. (1990) first described *Helicobacter*-like organisms in the stomachs of pigs in 1990 based on a histology study. The former "*Gastrospirillum suis*" was newly named "*Candidatus* Helicobacter suis" (De Groote et al., 2000). Different nonspecific diagnostic methods have been used, and newly developed tools have been proposed for the detection of the organism (Mendes et al., 1990; Happonen

et al., 1996a; Utriainen and Hanninen, 1998; De Groote et al., 2000). This study used urease testing, scanning electron microscopy, a *Helicobacter*-specific PCR assay, and the Gram staining detection method. The results show that the efficiency of each diagnostic method for the detection of GLOs is influenced by various factors including the site of the stomach which is sampled, species of animal, and the condition of preserved samples. In this study, the percentage of positive results in the pylorus of the pig stomachs determined by the urease test (29.8%) and brush cytology (28.9%) was similar. However, there were more positive results in the cardia and fundus by brush cytology than with the urease test. This indicates that brush cytology is a more sensitive method for detecting GLOs in the cardia and fundus of pigs. In contrast to the pigs, there was much higher positive urease activity in the stomach of cattle than there were positive results by brush cytology, and the area sampled did not influence the results. Furthermore, similar positive results were obtained in all parts of the dog stomach by the urease test, brush cytology, and PCR. However, it is unclear why the PCR positive probes for samples of the dog stomachs were negative in the pig and cattle stomachs (data not shown). It is possible that after urease testing or fixation in formalin the biological material was unsuitable for the primers used.

Helicobacter heilmanii is another helicobacter-like organism that is sporadically associated with human gastric pathology and is morphologically similar to "*Candidatus* H. suis" (Debongnie et al., 1995). Research has revealed the existence of at least two different types of this organism in humans (Solnick et al., 1993). A 99.5% 16S rDNA sequence homology between *H. heilmanii* type 1 and "*Candidatus* H. suis" has recently been reported, which indicates that both sequences are derived from organisms belonging to the same species, suggesting a possible zoonotic role of "*Candidatus* H. suis" (De Groote et al., 1999). There are no reports describing other strains of GLOs structurally similar to *Helicobacter*-like organisms in the pig stomach mucosa. The association of the "*Candidatus* H. suis"-like organism with porcine gastric ulcer disease is still a subject of discussion (Grasso et al., 1996; Queiroz et al., 1996). "*Candidatus* H. suis"-like organisms were found in the fundal and pyloric part and in only 4.8% of cardia, and no gross changes observed. It is believed, as reported previously (Melnichouk et al., 1999), that cardial

ulcerative changes are not caused by "*Candidatus* H. suis"-like organisms.

The mucosa of the abomasums of cattle is strongly acidic and therefore has fewer microorganisms. However, it is not an entirely germfree environment. There are no published reports that can explain the high positive urease activity observed in this experiment. Braun et al. (1997) reported a relatively high positive urease activity in the pylorus of cattle (56.3%), which is still, however, significantly lower than the current finding (70.6%). These authors also observed *Helicobacter*-like microorganisms using light microscopy and cultivated them in a special culture broth. No typical *Helicobacter*-like bacteria were observed by brush cytology in the present study. However, they have been described in reports documenting microflora which cause no histopathological changes or in connection with the lymphocytic infiltration of the mucosal surface of abomasums (Braun et al., 1997) and even with the formation of the generalized form of T-cell lymphoma in calves (Kadota et al., 2001). Mostly thinner Gram-negative bacteria, which are similar to *Camplylobacter*-like microorganisms, were found. Similarly, some authors observed *Campylobacter*-like organisms mainly in the pyloric region (Gunther and Schulze, 1992). Several species of *Campylobacter*, particularly *C. jejuni*, are recognized as a major cause of human diarrheal disease. The prevalence of *Campylobacter* spp. in cattle varies according to their geographical location from very low to 100% (Busato et al., 1999). Important sources of infection for humans are the consumption of contaminated foods of animal origin and polluted water. Raw or undercooked beef, fresh poultry, and raw milk represent a particular hazard (Butzler and Oosterom, 1991). It should be noted that cooling and freezing can lead to the lengthy survival of *Campylobacter* spp. in foods.

Most experiments that have described *H. pylori* in the oral cavity of humans used the PCR to make a precise diagnosis. Using this method, they examined the dental plaque of the dens, and saliva (Song et al., 2000b), or patients with lesions located in the oral cavity (Riggio et al., 2000) and/or stomach (Okuda et al., 2000). This study confirmed the presence of *Helicobacter*-like microorganisms in the oral cavity of 2.5% of dogs kept outside without any age limitations. Morphologically, 20.7% of the positive samples in this experiment were *Campylobacter*-like organisms. *Campylobacter* spp. are another type of spiral-shaped or curved, motile, and microaer-

ophilic bacteria, which are often found in the oral cavity of humans (Okada et al., 2001) as well as in the fecal samples of animals (Steinhauserova et al., 2000). This Gram-negative spiral-shaped or curved microorganism represents a pathogen for humans and animals. Most commonly *C. jejuni*, *C. coli* and to a lesser extent *C. upsaliensis* are the causative agents of enterocolitis in animals and humans (Maden et al., 1996; Slutsker et al., 1998). The first evidence of a dog-to-human transmission of *C. jejuni* has also been reported (Wolfs et al., 2001). However, there are no reports dealing with the occurrence of GLOs in the oral cavity of dogs. Moreover, for a more precise examination of the oral microflora, which represent a possible source of pathogenic microorganisms such as *Campylobacter* spp. and *Helicobacter* spp. causing infections in humans, it is recommended that PCR be performed on samples prepared from the oral plaque and stones formed in the dog mouth cavity.

To date, the occurence of *H. felis*, *H. bizzozeronii*, *H. salomonis*, "*Flexispira rappini*", *H. bilis*, and *H. heilmanii* have been reported in the stomachs of dogs (Eaton et al., 1996; Jalava et al., 1998). Gastric *Helicobacter*-like organisms are quite prevalent in dogs, and have displayed a 100% detection rate in randomly-sourced dogs (Strauss-Ayali et al., 1999). These results are similar to the present observations (85.7%). The negative result in one dog from this experiment was possibly caused by the very young age of the animal; young animals may show less colonization than adults (Otto et al., 1994), but this is still controversial idea (Happonen et al., 1996b). A lower prevalence in sick animals might indicate the recent use of antibiotics in sick animals or perhaps a protective immune response (Yamasaki et al., 1998). More than one type of GLOs was identified in some of the dog stomachs examined by brush cytology.

Humans have very close interactions with working, food-producing, and companion animals. Several enteric pathogens, which were thought to be mainly restricted to animals, are a major cause of human diseases. GLOs also number among the zoonotic enteric pathogens. The prevalence of GLOs in the stomach of the animals used in this experiment appears quite high. In addition, the prevalence of spiral-shaped bacteria found by brush cytology of the mouth cavity in dogs was also high. Contact with dogs and sheep has also been identified as a powerful independent risk factor contributing to *H. pylori* infections (Dore et al., 1999; Mura et al., 1999).

The precise mode of *Helicobacter* transmission is unclear. Some studies have suggested a fecal-oral route because *H. pylori* can be cultured from human and cat feces, and suboptimal sanitary conditions in underdeveloped countries might favor such a mechanism of spreading (Dunn et al., 1997). Others have suggested an oral-oral route because *H. pylori* can be found in the saliva of infected humans, and the spouses of infected people have a higher prevalence of infection (Dunn et al., 1997). Similarly, gnotobiotic dogs infected with *H. pylori* experimentally transmitted the organism to uninfected dogs (Radin et al., 1990). Korea has a relatively high incidence of *H. pylori* infections and gastric cancer. The overall seroprevalence of *H. pylori* infections is 46.6% in asymptomatic people (Kim et al., 2001). However, it is also 22% in Korean children and appears to increase with age (Malaty et al., 1996). The nationwide epidemiological study of *H. pylori* infections in South Korea is very important because gastric cancer is the most common malignancy and leading cause of cancer death (Kim et al., 1995).

Campylobacter spp. and *Helicobacter* spp. have many common morphological, biochemical and physiological features. The relationships between these bacteria and their hosts are also comparable. They colonize the gastrointestinal tracts of both humans and animals, causing clinical disease in some individuals and acting as commensals in others (Newell, 2001). Therefore, further studies will be needed to determine the types of GLOs localized in the oral cavity of dogs using PCR, and to establish which of them are pathogenic to humans.

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