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CANINE AND FELINE GASTRIC HELICOBACTERS: DIAGNOSIS AND SIGNIFICANCE IN CHRONIC GASTRITIS

by
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ACADEMIC DISSERTATION

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To the memory of American Cocker Spaniel
Fin Ch von Mein Heim Missisippie “Minttu” (27.10.1974-19.8.1985),
my loving, loyal, attentive, unforgettable tail-wagger
who led me into the challenging field of veterinary medicine.

CONTENTS

1. ABSTRACT

To elucidate the significance of canine and feline gastric helicobacters in the etiology of gastritis, five studies were performed. Diagnostic methods for detecting helicobacters: brush cytology, urease test, histologic examination, transmission electron microscopy (TEM), and bacterial culture, were compared in 10 apparently healthy dogs and cats. A detailed topographical mapping of helicobacters in the stomach was performed on these same 10 dogs and cats to find the most representative regions for sample-collection. The prevalence and colonization density of canine gastric helicobacters as well as their association with gastric inflammation were then investigated in 25 clinically healthy dogs and in 21 dogs with upper gastrointestinal signs. *Helicobacter* species were identified by TEM and by culture, with evaluation of the association between number of *Helicobacter* species and degree of gastric inflammation. The efficacy of human triple therapy on the eradication of gastric helicobacters, on clinical signs, and on gastric histology was assessed in nine dogs with upper gastrointestinal signs; the recurrence of the bacteria after successful eradication was evaluated in four of these dogs. Finally, the transmission of gastric helicobacters from two dams to their eight puppies and between the puppies was studied.

For demonstrating gastric helicobacters, brush cytology was the most sensitive method. The urease test and histologic examination revealed helicobacters less often only in the antrum, and therefore were also of value. TEM revealed helicobacters in 90 to100% of all cases, and culture in 40% and 17% of the positive cases in dogs and cats, respectively. TEM and culture, although laborious and more expensive, are needed for accurate identification of *Helicobacter* species.

The prevalence of gastric helicobacters was 60% in healthy cats, and 100% in healthy and 95% in affected dogs. No significant differences were detected in colonization density of helicobacters between healthy and affected dogs. Gastric *Helicobacter* species identified in dogs included *H. bizzozeronii*, *H. felis*, and *H. salomonis*; mixed infections were common. No significant association appeared between number of colonizing *Helicobacter* species and inflammation. *H. bizzozeronii*-like organisms and *H. felis* were identified from cats.

Gastric helicobacters were detected less frequently ($P<0.01$) and more superficially ($P<0.05$) in the antrum than in the cardia, fundus, and corpus in dogs but not in cats. The colonization density of helicobacters was also lowest in the antrum of dogs ($P<0.001$). One sample from the cardia, fundus, or corpus should thus be sufficient to demonstrate the organisms.

Mononuclear cells, neutrophils, eosinophils, and lymphocyte aggregates appeared both in clinically healthy and in dogs with upper gastrointestinal signs; mononuclear cells and neutrophils were relatively evenly distributed throughout the stomach. Degree of gastric inflammation did not differ significantly between healthy and affected dogs.

Colonization density of helicobacters was not associated with degree of gastric inflammation. In cats, lymphocyte aggregates were found only in helicobacter-positive cats, which also had more lymphocytes in the fundus and corpus than did helicobacter-negative cats ($P<0.05$).

Dogs commonly showed histologic changes comparable to those of mild chronic gastritis or of mild active chronic gastritis with the histologic criteria of human gastritis. Mild chronic gastritis was found in the antrum of all healthy cats, and more often ($P<0.01$) in the antrum than in other regions. Cats also showed a significant ($P<0.05$) association between helicobacters and chronic gastritis in the fundus and corpus. With the newly developed visual analogue scale for grading canine and feline gastritis, gastritis was also a common diagnosis, although it appeared more rarely in healthy dogs than in affected dogs ($P<0.05$). No macroscopic or microscopic evidence of gastric or duodenal ulcers was detected in any dog or cat. Atrophy and dysplastic epithelial changes were diagnosed in only a few clinically healthy and affected dogs. In cats, helicobacters may cause histologic changes comparable to those of chronic gastritis, but in dogs this association remained unclear. It still remains to be determined whether certain strains of *Helicobacter* species may induce canine gastritis.

Human triple therapy (amoxicillin, metronidazole, and bismuth subcitrate), eradicated gastric helicobacters in 8/9 positive dogs. One dog was successfully treated with tetracycline and omeprazole after unsuccessful eradication with triple therapy. Eradication of helicobacters alleviated clinical signs ($P<0.05$) but did not resolve them totally in any dog; additional therapies after triple therapy alleviated clinical signs further ($P<0.01$). Neither triple therapy nor additional therapies had a significant effect on gastric histologic changes. Gastric helicobacters recurred within three years of eradication treatment. Because gastric helicobacters alone do not appear to be responsible for upper gastrointestinal signs in dogs, routine eradication treatment seems not to be warranted.

During a challenge study with *H. bizzozeronii*, puppies of the non-challenged group that originated from two dams having an eradicated *H. salomonis* infection before delivery were found to be infected with *H. salomonis* despite strict isolation and hygienic procedures. *H. salomonis* isolates of dams and puppies studied by ribotyping (*Hae*III, *Cla*I or *Pst*I) and pulsed-field gel electrophoresis (PFGE) of *Not*I digests, showed that those in dam B and in non-challenged puppies were identical. Because *H. salomonis* isolates were metronidazole-resistant, the eradication therapy of the dams before delivery had merely suppressed their helicobacter infection. Hence, puppies may acquire gastric helicobacters from their dams during the lactation period, and puppies can infect each other during their early life.

2. LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers which are referred to in the text by their Roman numerals (**I-V**):

I Happonen I., Saari S., Castren L., Tyni O., Hänninen M.-L., Westermarck E. Comparison of diagnostic methods for detecting gastric Helicobacter-like organisms in dogs and cats. Journal of Comparative Pathology 1996; 115: 117-127.

II Happonen I., Saari S., Castren L., Tyni O., Hänninen M.-L., Westermarck E. Occurrence and topographical mapping of gastric Helicobacter-like organisms and their association with histological changes in apparently healthy dogs and cats. Journal of Veterinary Medicine, Series A 1996; 43: 305-315.

III Happonen I., Linden J., Saari S., Karjalainen M., Hänninen M.-L., Jalava K., Westermarck E. Detection and effects of helicobacters in healthy dogs and dogs with signs of gastritis. Journal of the American Veterinary Medical Association 1998; 213: 1767-1774.

IV Happonen I., Linden J., Westermarck E. The effect of human triple therapy on eradication of canine gastric helicobacters, upper gastrointestinal signs and gastric histology - a follow-up study. Journal of Small Animal Practice 1999; in press.

V Hänninen M.-L., Happonen I., Jalava K. Transmission of canine gastric *Helicobacter salomonis* infection from dam to offspring and between puppies. Veterinary Microbiology 1998; 62: 47-58.

3. TERMINOLOGY, DEFINITIONS, AND ABBREVIATIONS

In this thesis, terms used for describing anatomic regions of the stomach are cardia, fundus, corpus, antrum, and pylorus, according to the *Illustrated Veterinary Anatomical Nomenclature* (Schaller 1992). In the literature, the terms "body" and "fundus" are often used instead of "corpus". Because this thesis focuses on gastric helicobacters only, the term "helicobacters", although not preceded by the term "gastric", always refers to those of the stomach. The terms "positive" and "negative" refer to helicobacter-positivity or -negativity. The term "gastritis" is a histologic diagnosis, not a clinical diagnosis. The expression "upper gastrointestinal signs" refers to clinical signs the patient is showing, such as nausea, vomiting, and abdominal pain. Of the gastric helicobacter species, *Helicobacter pylori* refers always to that of human beings if not mentioned otherwise.

The following abbreviations appear in the text:

ALP, alkaline phosphatase
ALT, alanine aminotransferase
BabA, blood group antigen-binding adhesin
BHI, brain-heart infusion
BP, biochemistry panel
CBC, complete blood count
CCUG, culture collection of the University of Gothenburg
CFU, colony-forming units
DNA, deoxyribonucleic acid
EIA, enzyme immunoassay
ELISA, enzyme-linked immunosorbent assay
cagA, cytotoxin-associated gene A
HE, hematoxylin and eosin
HPF, high-power field (400x magnification)
HpSA, *H. pylori* antigen in stools
IL-8, interleukin-8
ISH, in situ hybridization
i.m., intramuscular
i.v., intravenous
MALT, mucosa-associated lymphoid tissue
MGG, May-Grünwald-Giemsa
MRU, modified rapid urease
NSAID, non-steroidal anti-inflammatory drug
PAF, platelet-activating factor
PPI, proton-pump inhibitor
PCR, polymerase chain reaction
PFGE, pulsed-field gel electrophoresis
REA, restriction enzyme analysis
RNA, ribonucleic acid
SEM, scanning electron microscopy
TEM, transmission electron microscopy
UBT, urea breath test
VacA, vacuolating cytotoxin A

4. INTRODUCTION

Chronic vomiting is a common gastrointestinal complaint in dogs and cats, and is often linked to chronic gastritis; spiral bacteria have been suggested as one etiologic factor for chronic gastritis (Guilford and Strombeck 1996). Spiral-shaped microorganisms were observed originally as early as the end of the 19th century within the stomachs of animals (Rappin 1881). Rappin (1881) found helicobacters in the gastric mucosa of a dog, and his work was confirmed by Bizzozero (1893) and Salomon (1896) in dogs, cats, and rats. These bacteria were first named *Spirillum* (Rappin 1881) or *Spirochete* (Lockard and Boler 1970), then they were classified *Campylobacter*, and now they belong to the genus *Helicobacter* (Owen 1998). At least four *Helicobacter* species may colonize the canine and feline stomach: *Helicobacter felis* (Lee et al 1988, Paster et al 1991), *Helicobacter bizzozeronii* (Hänninen et al 1996), *Helicobacter salomonis* (Jalava et al 1997), and *"Flexispira rappini"* (also called *"Helicobacter rappini"*) (Lockard and Boler 1970, Eaton et al 1996, Jalava et al 1998). Mixed infections are common in dogs and cats (Lockard and Boler 1970, Lee et al 1988). What has not been clarified is whether any one *Helicobacter* species is more pathogenic than another, or whether a mixed infection differs from that of one species. Dogs are not found to be naturally colonized with human *Helicobacter pylori* (formerly *Campylobacter pylori*). However, *H. pylori* has been shown to colonize gnotobiotic dogs in an experimental infection model (Radin et al 1990). One study reports *H. pylori* in commercial vendor cats (Handt et al 1994). *"Helicobacter heilmannii"* (formerly *"Gastrospirillum hominis"*), the second species detected in humans in a minority of cases (McNulty et al 1989a, Solnick et al 1993, Andersen et al 1996 & 1999), resembles by morphology *H. bizzozeronii*, and it has been speculated whether these are one and the same species. A recent detailed comparative analysis of a human isolate of other helicobacters revealed that the cultured human *"H. heilmannii"* strain examined (Andersen et al 1999) was in all characteristics identical to *H. bizzozeronii* (Jalava et al 1999a). An interesting suggestion has also been made that some reports of *"H. heilmannii"* may represent in vivo growth of *H. pylori* based on different morphology of the single strain of bacteria when it was grown on blood agar plates or in broth cultures (Fawcett et al 1999).

The genus of *Helicobacter* includes at least 18 formally described species and several novel species that have not been validly named (Fox and Lee 1997, Owen 1998, Wadström and Hänninen 1999). Several *Heliocbacter* species have been described from gastric mucosa also of other animals than the dog and cat, including *Helicobacter mustelae* in ferrets, *"Gastrospirillum suis"* in pigs, *Helicobacter nemestrinae* in monkeys, and *Helicobacter acinonyx (acinonychis)* in cheetahs (Fox and Lee 1997). In addition to the gastric mucosa, helicobacters have been isolated from the intestine and liver, such as *Helicobacter canis* from the intestine and liver of dogs (Stanley et al 1993, Fox et al 1996a), *Helicobacter cinaedi* from the intestine of humans and hamsters (Totten et al 1985, Gebhard et al 1989), *Helicobacter muridarum* and *Helicobacter rodentium* from the

intestine of mice (Lee et al 1992a, Shen et al 1997), *Helicobacter trogontum* from the intestine of rats (Mendes et al 1996), *Helicobacter pametensis* from the intestine of birds and swine (Dewhirst et al 1994), *Helicobacter pullorum* from the intestine and liver of poultry and from the intestine of humans (Stanley et al 1994), *Helicobacter bilis* from the liver and intestine of mice (Fox et al 1995), *Helicobacter hepaticus* from the liver of mice (Fox et al 1994), and *Helicobacter cholecystus* from the liver of hamsters (Franklin et al 1996).

Human *H. pylori* has been studied keenly for the past 15 years since its culture from man in 1982 and the discovery that it may cause gastritis (Warren 1983, Marshall 1983). Later, it was demonstrated that *H. pylori* plays an important role in the etiology of human dyspepsia, gastritis, and gastroduodenal ulceration (Marshall and Warren 1984, Goodwin et al 1986, Graham 1989, Sipponen et al 1993), and is a risk factor for gastric carcinoma and lymphoma (Parsonnet et al 1991, Sipponen et al 1992). Interest in canine and feline gastric helicobacters began to increase a few years ago when the strong association of *H. pylori* in man with gastric diseases became evident and universally accepted, and at present, these bacteria are under intensive study.

Colonization of the gastric mucosa of dogs and cats with large spiral helicobacters is common (Weber et al 1958, Henry et al 1987, Geyer et al 1993, Hermanns et al 1995, Eaton et al 1996, Yamasaki et al 1998). In cats, helicobacters have been found more often in adult than in juvenile cats (Weber et al 1958, Otto et al 1994), but in dogs this association is unknown. The true prevalence of gastric helicobacters among pet dogs and cats has remained unclear because previous studies either have been performed on laboratory animals (Weber et al 1958, Henry et al 1987), or the clinical background of the pet dogs has been obscure (Geyer et al 1993, Hermanns et al 1995, Yamasaki et al 1998). The route of transmission of gastric helicobacter in animals is also unknown. Human *H. pylori* is suspected to be transmitted in early life by oral-oral contact (Taylor and Blaser 1991), although the fecal-oral route is also suggested (Kelly et al 1994). Close personal contact seems to be an important factor in transmission of *H. pylori*. Dogs usually live in close social contact with each other; dams and puppies are especially in close contact during the lactation period. Dogs can therefore serve as an animal model for studying the transmission of gastric helicobacters during the early weeks of life.

Diagnosis of *H. pylori* infection in man can established by brush cytology, the urease test, histologic examination, electron microscopy, culture, serology, the urea breath test, and molecular methods (de Boer 1997). Of these methods, histologic examination, the urease test, and electron microscopy have been used most commonly for detecting helicobacters in animals. Comparisons between diagnostic methods for human *H. pylori* have been performed and reviewed from various angles (Chodos et al 1988, Brown and Peura 1993, Cutler et al 1995, de Boer et al 1997, Cutler 1997, Onders 1997), but the superiority of any one method has not been evaluated in animals.

It has been assumed that canine and feline helicobacters may be responsible for histologic changes seen in the stomach in conjunction with these organisms. However, such changes have been detected both in dogs and cats with upper gastrointestinal signs and in clinically healthy dogs and cats (Weber et al 1958, Henry et al 1987, Lee et al 1992b, Geyer et al 1993, Otto et al 1994, Hermanns et al 1995, Yamasaki et al 1998). Some of these conclusions have been drawn from studies on experimentally induced helicobacter infections (Lee et al 1992b). That the histologic definition of gastritis of dogs and cats has varied from study to study has made comparison of results very difficult. Thus far, no study has given an unambiguous answer to the question whether canine and feline helicobacters are of clinical importance. Gastric helicobacters differing from canine and feline ones have been associated with variable degrees of histologically verified gastritis in their hosts, e.g., *H. mustelae* in ferrets (Fox et al 1986 & 1990), *"G. suis"* in pigs (Mendes et al 1990), *H. nemestrinae* in monkeys (Bronsdon et al 1991), and *H. acinonyx (acinonychis)* in cheetahs (Eaton et al 1993).

In humans, eradication of *H. pylori* is more effective in preventing recurrence of gastroduodenal ulcers than is traditional treatment with acid blockers, and reinfection with *H. pylori* is rare after successful eradication (Seppälä et al 1992, Labenz and Börsc 1994, Uemura et al 1995). The present recommendation for *H. pylori* eradication therapy consists of amoxicillin or tetracycline with metronidazole, plus omeprazole or bismuth subcitrate (European Helicobacter Pylori Study Group 1997). Only a few preliminary reports have been published on eradication therapy for canine and feline gastric helicobacters (Lecoindre et al 1998) and none on its effect on gastrointestinal signs or gastric histology.

5. REVIEW OF THE LITERATURE

5.1. Canine and feline chronic gastritis

Histologically verified chronic gastritis is a poorly documented entity in dogs and cats, although it is considered a common cause of chronic vomiting in these species. The prevalence of canine and feline chronic gastritis and its association with upper gastrointestinal signs is, however, unknown.

Canine and feline gastritis may be classified as acute or chronic, based on the inflammatory cell type; and as superficial or diffuse, according to distribution of the cells (Guilford and Strombeck 1996). Various classifications of chronic gastritis have been used - including etiologic and histologic classification - which have overlapping clinicopathologic features. Etiologic classification is possible if the primary cause is identified, such as food allergy, nonsteroidal anti-inflammatory drugs (NSAID), or uremia. However, in most cases of chronic gastritis, the cause remains unknown (Guilford and Strombeck 1996). The fact that objective criteria for histologic assessment of canine and feline gastric biopsy specimens do not exist causes confusion when results of different studies are interpreted and compared.

Histologic features evaluated include the type and amount of cellular infiltrate, the area of mucosa affected and its topography, mucosal thickness, and amount of gastric glands. In acute gastritis, the number of neutrophils is increased. According to dominant cell-type, chronic gastritis may be divided into nonspecific (mononuclear cells), eosinophilic (eosinophils), and granulomatous/ histiocytic (macrophages) gastritis, the first being the most common. Both atrophic gastritis, characterized by thinning of gastric mucosa and reduced number of gastric glands along with increased numbers of lymphocytes and plasma cells, and hypertrophic gastritis, characterized by mucosal proliferation due to hypertrophy and hyperplasia of the foveolar and glandular epithelium accompanied by variable amounts of fibrous tissue and inflammatory cells, are not as common as nonspecific gastritis.

Chronic gastritis is often patchy, and therefore small gastric biopsy specimens are not representative of the whole stomach (van der Gaag and Happé 1989). Superficial gastritis often heals whereas, severe diffuse gastritis, atrophic, and hypertrophic gastritis may persist for months, even years (van der Gaag and Happé 1989).

5.2. Epidemiology and transmission of gastric helicobacters

Prevalence of canine and feline helicobacters is high. Prevalence rates of 86 to100% in healthy dogs, 61 to 82% in dogs with upper gastrointestinal signs, 41 to100% in healthy cats, and 56 to76% in affected cats have been reported (Weber et al 1958, Henry et al 1987,

Geyer et al 1993, Otto et al 1994, Hermanns et al 1995, Eaton et al 1996, Papasouliotis et al 1997, Yamasaki et al 1998, Neiger et al 1998, De Majo et al 1998). Most dogs studied have been adults of various ages, and the prevalence rate related to the age of the dogs is unknown. In cats, helicobacters have been found more often in adults than in juveniles (Weber et al 1958, Otto et al 1994).

Human *H. pylori* has a world-wide distribution, and its prevalence in healthy asymptomatic persons is 15 to70% depending on age, race, and social status (Kosunen et al 1989, Graham et al 1991, Mégraud 1993, Lambert et al 1995). The infection rate seems to be very low in younger children in many developed countries (Mégraud et al 1989, Kontiainen et al 1994). Epidemiological studies have demonstrated a correlation between colonization and age, low socio-economic status, and overcrowding, particularly during childhood (Webb et al 1994). The occurrence of *"H. heilmannii"* is rare, its prevalence being <1% (McNulty et al 1989a, Heilmann and Borchard 1991, Holck et al 1997).

The transmission of *H. pylori* is likely to occur by multiple routes affected by local conditions and behavior (Cave 1997, Mendall 1997). In humans, close personal contact seems to enhance the transmission of *H. pylori*, which is suspected to be transmitted by oral-oral or fecal-oral routes, which are evidenced by isolation of *H. pylori* from saliva and feces (Taylor and Blaser 1991, Thomas et al 1992, Kelly et al 1994, Fox et al 1996b). In animals, higher prevalence rates are suspected in those living in colonies in which transmission is enhanced (Weber et al 1958, Henry et al 1987, Eaton et al 1996), supporting the role of intimate contact in this transmission. Dogs usually have close contact with each other, such as dams and puppies during the lactation period. Family members have shown identical subtypes of the organisms studied by molecular methods (Wang et al 1993, Vincent et al 1994), suggesting transmission between family members. Otherwise, *H. pylori* isolates from different subjects show large genetic divergence; almost all isolates represent unique types (Akopyanz et al 1992, Owen et al 1992). Iatrogenic transmission via biopsy forceps or endoscope is also possible when proper cleaning and disinfection has not been performed (Langenberg et al 1990, Fantry et al 1995, Akamatsu et al 1996, Cronmiller et al 1999). Although water and vegetables have also been linked to *H. pylori* transmission, more comprehensive studies are needed to evaluate the importance of these vehicles as reservoirs for *H. pylori* (Fox 1995).

A study reporting *H. pylori* in commercial vendor cats led to a suggestion that *H. pylori* may be a zoonotic pathogen with transmission occurring from cats to humans (Handt et al 1994). However, *H. pylori* has not been isolated from the stray cats studied by El-Zataari et al (1997). Neither has there been any difference in *H. pylori* seropositivity between subjects who have owned a cat and those with other pets; this fails to support the hypothesis that *H. pylori* may be transmitted from cats to humans or that cat owners may have a higher risk of *H. pylori* infection than does the general population (Ansorg et al 1995, Webb et al 1996).

An assumption for zoonotic transmission of *"H. heilmannii"*-like helicobacters from dogs and cats to humans has been suggested by similar morphologic features of the organisms found in humans suffering from upper gastrointestinal symptoms and in animals handled by these persons (Lavelle et el 1994, Thomson et al 1994, Dieterich et al 1998). The symptoms were resolved after clearance of these organisms (Lavelle et el 1994), or in one case, only when helicobacter eradication therapy was instituted in both humans and the animals (Thomson et al 1994). However, this evidence has been based solely on similar morphologic features; firm evidence of their identity should be based on genetic criteria (Curry 1994, Kusters and Kuipers 1998). It has also been suggested that animals may be reservoirs in the transmission of *"H. heilmannii"*, because *"H. heilmannii"* infection has been found associated with contact with dogs, cats, or domestic animals (Stolte et al 1994, Meining et al 1998). Recently, it has been established that the isolated strain of *"H. heilmannii"* (Andersen et al 1999) and *H. bizzozeronii* represent the same species according to their phenotypic characteristics, whole-cell protein profile, 16S rRNA gene sequence, and DNA-DNA homology, and hence, this species can be transmitted from dogs to humans (Jalava et al 1999a). The possible risk of transmission of canine and feline large helicobacters may, however, be relatively slight because the prevalence of *"H. heilmannii"*-like bacteria (i.e., *H. bizzozeronii*) is high in pet dogs and cats, whereas the prevalence of *"H. heilmannii"* in humans is low (Strauss-Ayali and Simpson 1999).

5.3. Pathogenicity of helicobacters

Canine and feline gastric helicobacters have been suspected of inducing histologically verifiable gastritis. Histologic changes such as inflammation, lymphocyte follicles, and degeneration of the gastric glands and parietal cells in the presence of gastric helicobacters have been assumed to indicate the pathogenicity of these organisms (Weber et al 1958, Henry et al 1987, Lee et al 1992, Geyer et al 1993, Otto et al 1994, Hermanns et al 1995). However, such changes have been detected not only in dogs and cats suffering from gastrointestinal signs, but also in clinically healthy dogs and cats. It has been speculated that *H. felis* and *H. bizzozeronii* may differ in their pathogenicity, *H. felis* being more pathogenic than *H. bizzozeronii*, based on their cytopathogenic effects (Peyrol et al 1998, Norris et al 1999). In experimental animal models, *H. felis* has been demonstrated to induce gastritis in rats and mice (Lee et al 1990, Fox et al 1991a & 1993) and similarly *H. pylori*-induced atrophic gastritis in long-term infected mice (Lee et al 1993a). However, although hard evidence of the pathogenicity of canine and feline gastric helicobacters is still lacking, some features of human *H. pylori* may apply also to helicobacters of animals.

Evidence that *H. pylori* plays a role in the pathogenesis of human gastritis comes from volunteer studies, animal-model experiments, and therapeutic trials. Acute gastritis developed in two volunteers who ingested *H. pylori* (Marshall et al 1985, Morris and

Nicholson 1987). An experimental oral challenge of gnotobiotic piglets with *H. pylori* was associated with persistent colonization of the stomach and transient infiltrates of the gastric mucosa with neutrophils, followed by mucosal and submucosal infiltration with diffuse and follicular infiltrations of mononuclear cells, as in human *H. pylori* infection (Krakowka et al 1987). Disappearance of gastritis after eradication of *H. pylori* supports the view that this organism plays a causal role in pathogenesis of chronic gastritis (Valle et al 1991). There is also indirect or secondary evidence that supports the pathogenic role of *H. pylori* such as association of the organisms with only gastric epithelial cells, serologic response to infection, and lowering of antibody levels after eradication of the bacteria (Blaser 1990). Combining the pathogenetic data of *H. pylori* has clearly pointed out the fact that *H. pylori* is a major human gastrointestinal pathogen (Blaser 1990, Marshall 1991, Blaser et al 1992, Misiewicz 1992, Lee et al 1993b, Lambert et al 1995).

In addition to gastritis, human *H. pylori* has been recognized to be an important etiologic factor also in gastroduodenal ulceration (Marshall and Warren 1984, Goodwin et al 1986, Graham 1989, Sipponen and Hyvärinen 1993), and in gastric carcinoma and primary gastric low-grade lymphoma of mucosa-associated lymphoid tissue type (MALT) (Parsonnet et al 1991, Sipponen et al 1992, Sipponen 1994). MALT lymphoma has also been detected in association with *"H. heilmannii"* (Morgner et al 1997).

Gastric ulcers and tumors are rare in dogs and cats. In dogs, NSAID are the most common cause for gastric ulcers, which tend to be most frequent in the antrum (Guilford and Strombeck 1996). A variety of stressful events may also induce gastric erosions and ulcers, such as trauma, shock, sepsis, psychologic stress, and head injury. Carcinoma and lymphoma are the two most common types of gastric tumors, and are encountered most often in older animals (Gualtieri et al 1999). Of the *Helicobacter* species in animals, *H. mustelae* in ferrets has been suggested to be associated with gastric adenocarcinoma and gastric lymphoma (Fox et al 1997, Erdman et al 1997).

5.3.1. Gastric histopathology

H. pylori-induced gastritis is characterized by accumulation of neutrophils, lymphocytes, and plasma cells. Human gastritis is classified as acute, chronic, or special, and is graded according to morphological variables - *H. pylori* density, inflammation, glandular atrophy, and intestinal metaplasia - each as normal, mild, moderate, or marked (Price 1991, Dixon et al 1996). In acute gastritis, neutrophils infiltrate the gastric mucosa, whereas in chronic gastritis, the inflammatory cells consist of lymphocytes and plasma cells. Typically, *H. pylori* induces chronic active gastritis, and may also induce atrophy and intestinal metaplasia (Blaser 1992, Rugge et al 1993, Sipponen and Hyvärinen 1993, Kuipers et al 1995). Lymphoid follicles are associated with *H. pylori*-induced gastritis; they are found more frequently in the antrum than in the corpus, and on the lesser rather than greater curvature (Genta et al 1993a, Zaitoun 1995). The long-term course and consequences of canine and feline helicobacters is unknown, whereas in humans it has

been demonstrated that chronic *H. pylori* infection may heal spontaneously and inflammatory changes in the gastric mucosa may resolve partially or totally, and new *H. pylori* infection may occur in adulthood, although these events are rare (Niemelä et al 1995, Villako et al 1995, Valle et al 1996). On the other hand, duodenal ulcer disease is associated with persistent *H. pylori* infection, gastritis usually persists, and atrophic gastritis as well as intestinal metaplasia may appear, and the risk for development of gastric cancer may increase (Kuipers et al 1995, Niemelä et al 1995, Villako et al 1995, Valle et al 1996).

"H. heilmannii" has also been associated with gastritis which, however, is usually less severe than *H. pylori*-induced gastritis (Heilmann and Borchard 1991, Holck et al 1997, Stolte et al 1997). In addition, one study has reported *"H. heilmannii"* to be associated with gastric ulcers (Debongnie et al 1998).

Canine and feline helicobacters have been found throughout the stomach, most often in the corpus, and are located superficially on the mucosal surface including gastric pits, and deeper in gastric glands and in parietal cells (Henry et al 1987, Geyer et al 1993, Yamasaki et al 1998). *H. pylori* usually colonizes both the antrum and the body, but the number of bacteria is usually higher in the antrum (Genta and Graham 1994, Stolte et al 1997), and the organisms are located within the mucus and in close proximity to epithelial cells only (Hazell et al 1986, Thomsen et al 1990). However, one study reports *H. pylori* found also in parietal cells (Chen et al 1986). *"H. heilmannii"* colonizes predominantly the antrum, and is located primarily on the mucosal surface but may also penetrate deeper into gastric glands and even into parietal cells (Heilmann and Borchard 1991, Stolte et al 1997).

5.3.2. Mechanisms of tissue injury

Thus far, very little is known about the pathogenic properties of canine and feline helicobacters. The putative pathogenic determinants of human *H. pylori* can be divided into maintenance factors and virulence factors (Dunn et al 1997). Maintenance factors allow the organisms to colonize and remain within the host, and include for instance motility and adhesion to the gastric mucosa, and the production of urease enzyme. Virulence factors contribute to the pathogenetic effects of the bacterium, and the main effects are gastric inflammation, disruption of the mucosal barrier, and alteration of gastric physiology.

Their corkscrew-like **motility**, the spiral shape, and flagella of helicobacters allow free movement in viscous gastric mucus, and therefore, motility is assumed to ease the colonization of the organisms (Hazell et al 1986, Eaton et al 1992). In an experimental study with gnotobiotic piglets, the motile strains of *H. pylori* showed a 100% infection rate, whereas aflagellated, nonmotile strains were unable to colonize (Eaton et al 1989). **Adherence** to the gastric mucosa may play an important role in the colonization and pathogenicity of *H. pylori* and *H. mustelae* but not *H. felis* (Taylor et al 1992). Adhesion

occurs by adhesins such as hemagglutinins and blood group antigen-binding adhesin, BabA (Evans et al 1988, Taylor et al 1992, Kobayashi et al 1993, Ringner et al 1993, Clyne and Drumm 1993 & 1997, Ilver et al 1998). However, adhesion appears not to be essential for colonization for all gastric helicobacters because for instance *"H. heilmannii"* and *H. felis* do not attach but maintain a close proximity to the mucosa, presumably by their active motility (Lee et al 1993b).

Gastric helicobacters of both humans and animals produce the **urease enzyme** which hydrolyzes urea to ammonia, which raises gastric pH adjacent to the mucosa up to a level where bacteria may survive (Marshall et al 1990), although this does not appear to be the primary function of urease (Eaton et al 1994). Hypochlorhydria may occur via parietal cell failure, possibly due to direct or indirect toxic effects of the ammonia or due to the organisms themselves, or via obstruction of the necks of the gastric glands by sloughed epithelium, mucus, and inflammatory cells (Chen et al 1986, Graham et al 1988). Parietal cell acid secretion may also be inhibited by interleukin-1 (IL-1) which has been shown to increase in *H. pylori* infection (Peek et al 1995, Beales and Calam 1998). Additionally, ammonium itself may be directly toxic to epithelial cells (Smoot et al 1990). In an experimental study, a urease-negative *H. pylori* strain did not colonize gnotobiotic piglets, whereas a urease-positive strain did, indicating that urease activity appears to be essential for colonization of *H. pylori* (Eaton et al 1991). Acute *H. pylori* infection appears to cause usually transient hypochlorhydria, promoting bacterial colonization before acid secretion returns to normal (Morris and Nicholson 1987, Barthel et al 1988, Cave and Vargas 1989, Graham et al 1988, Kelly et al 1993). The period of hypoacidity (from weeks to months) is probably of sufficient duration for the bacteria to colonize (Blaser 1990). In ferrets, *H. mustelae* also produces a transient elevation in gastric pH (Fox et al 1991b).

Mechanisms by which *H. pylori* may induce **gastric inflammation** consist of several factors including interleukin-8 (IL-8) secretion, neutrophil adherence, platelet-activating factor (PAF), and urease (Dunn et al 1997). The inflammation caused by *H. pylori* is suspected to be due to chemotactic factors, such as IL-8, induced by the bacterium, which stimulate leukocyte accumulation (Craig et al 1992, Mai et al 1992, Nielsen and Andersen et al 1992, Hatz et al 1996). It has also been demonstrated that *H. pylori* strains possess 150-kDA protein, which increases neutrophil adherence to endothelial cells and metabolizes the nonulcerogenic lyso-PAF into PAF, a phospholipid mediator which is a potent ulcerogenic agent, and urease which stimulates mononuclear phagocyte activation and inflammatory cytokine production (Dunn et al 1997).

Disruption of the protective gastric mucosal barrier may be due to phospholipases, mucinase, and vacuolating cytotoxin produced by *H. pylori* as well as be due to induction of the apoptosis (Dunn et al 1997). Extracellular protease of *H. pylori* may alter the quality the of the mucus layer by reducing viscosity and making the layer thinner (Slomiany et al 1987 & 1988, Sarosiek et al 1988, Hills et al 1993), although it seems contradictory that

helicobacters would destroy the mucus which is the natural habitat of these organisms (Lee et al 1993b).

H. pylori strains may possess **cytotoxin-associated gene A (*cagA*)** which codes production of CagA, an immunogenic protein (Dunn et al 1997). There is evidence that *cagA*-positive *H. pylori* strains may be more infective and cause more inflammation than *cagA*-negative strains (Gunn et al 1998). An *H. pylori* gene, ***iceA*** (**induced by contact with epithelium**), has recently been discovered, and of the two main variants, the *iceA1* has also been associated with peptic ulceration and increased mucosal concentration of IL-8, an inflammatory mediator (van Doorn et al 1998, Peek et al 1998).

All *H. pylori* strains possess the cytotoxin-coding *vacA* gene and approximately half the strains produce a **vacuolating cytotoxin A (VacA)** which induces cytoplasmic vacuoles in epithelial cells (Leunk et al 1988, Cover and Blaser 1992). Neutralizing antibodies against VacA cytotoxin have been detected in the serum of patients infected with cytotoxin-producing strains (Cover et al 1992, Leunk et al 1990). Most of the *H. pylori* strains isolated from patients having gastric ulcers generate cytotoxin, but also a significant number are negative for cytotoxin (Figura et al 1989). The role of cytotoxin in gastroduodenal ulcerogenesis is not fully known. However, *H. pylori* strains of *vacA* s1a type have been shown to produce the highest level of cytotoxin, and have been associated with enhanced gastric inflammation and duodenal ulceration (Atherton et al 1995 & 1997), whereas *H. mustelae* in ferrets has not been shown to produce cytotoxin in vitro (Morgan et al 1991), although it causes gastritis in its host (Fox et al 1990). In vivo vacuolization is a nonspecific phenomenon, and thus may be attributable to normal epithelial turnover or to inflammatory cell mediators because vacuoles may be present in both infected and uninfected individuals, as shown in an experimental infection model (Eaton et al 1989).

It has been demonstrated that *H. pylori* induces alterations in the gastric epithelial cells, including enhanced proliferation and increased programmed cell death, **apoptosis** (Moss et al 1996). *H. pylori* infection has also been shown to induce **autoimmunity** in susceptible individuals, which may lead to corpus atrophy, loss of parietal cells and consequent decrease in acid secretion, and increased gastrin levels as a reaction to increased pH (Appelmelk et al 1998).

Increased gastrin release, diminished responsiveness of parietal cells to gastrin, and decreased somatostatin release lead to altered gastric physiology. Colonization of human *H. pylori* is associated with **hypergastrinemia** (Levi et al 1989a, Smith et al 1990, Prewett et al 1991), parallel to *H. mustelae* in ferrets (Perkins et al 1996a). The suggested causes for hypergastrinemia include suppressed antral somatostatin content (Kaneko et al 1992, Moss et al 1992, Haruma et al 1995), stimulation of antral G cells by mediators of inflammation (Mullholand et al 1993), especially in *H. pylori*-induced pangastritis (Valle et al 1992, Götz et al 1995), and alkalinization of the gastric mucus layer by bacterial urease (Chittajallu et al 1991). Increased acid secretion reduces somatostatin secretion, which in turn stimulates the secretion of gastrin (Blaser 1992, Lamers 1992). It has also

been speculated that a high gastrin level increases secretion of hydrochloric acid which may promote gastric metaplasia in the duodenum, offering a favorable environment for helicobacters to colonize, and therefore a predisposition to duodenal ulcers (Misiewicz 1992). The suggestion that increase in acid secretion occurs at the very beginning of the infection and lasts for only a short period of time before development of hypochlorhydria causes confusion in this matter (Graham et al 1988).

In experimental dogs infected with *H. felis* and in uninfected control dogs, the gastric secretory axis, i.e., fasting, meal-stimulated, and pentagastrin-stimulated gastric acid secretion, mucosal gastrin and somatostatin immunoreactivity, and fasting gastric pH, has been reported to be similar, suggesting that *H. felis* may not be a gastric pathogen in dogs (Simpson et al 1999).

Pepsinogen I and II concentrations have also been reported to be higher in *H. pylori*-positive compared to *H. pylori*-negative subjects (Mossi et al 1993). Along with the eradication of *H. pylori*, gastrin and pepsinogen concentrations normalize, indicating that both hypergastrinemia (Levi et al 1989b, Graham et al 1990, Chittajallu et al 1992, Verhulst et al 1995) and hyperpepsinogenemia (Fraser et al 1992, Di Mario et al 1996, Gisbert et al 1996) are induced by *H. pylori* infection.

5.4. Diagnostic methods for detecting gastric helicobacters

Diagnostic methods to detect gastric helicobacters can be classified as invasive or non-invasive. Invasive methods require endoscopically obtained gastric biopsy specimens or mucus and include brush cytology, the urease test, histologic examination, electron microscopy, culture, the polymerase chain reaction techniques (PCR), and in situ hybridization (ISH). Noninvasive methods consist of serologic methods, the urea breath test (UBT), and detection of bacteria, as well as bacterial DNA and antigens in stool. Helicobacters can be visualized directly in a brush cytology sample, in histologic and TEM specimens, and in cultured samples. The presence of the organisms can demonstrated indirectly by the urease test, serology, and UBT. Serology and UBT are used in humans for epidemiologic studies and in treatment monitoring of *H. pylori* infection. In animals, PCR, serologic methods, and UBT are not in routine use thus far.

5.4.1. Quick tests

Brush cytology (Gad 1989, Mendoza et al 1993, Carmona et al 1995) is performed by spreading gastric mucus on a slide which is then air dried and stained with Romanovsky-type stain, if a rapid result is required, or with May-Grünwald-Giemsa (MGG). The spiral bacteria are readily seen under a microscope at 400x magnification. Similarly, rapid diagnosis of helicobacters can be made by stained touch preparations of mucosal biopsies (Montgomery et al 1988). It has been suggested that *"H. heilmannii"* in

humans may be better diagnosed by touch cytology than by biopsy specimens (Debongnie et al 1995).

A typical feature of gastric helicobacters is that they are potent urease producers, and the **urease test** reveals their enzyme production (Hazell et al 1987). It is an easy, quick, and inexpensive test to perform. Gastric mucosal biopsy specimens are placed into a test reagent containing unbuffered urea in distilled water and an indicator color for pH change. Ammonia released by the urease enzyme causes a pH change, and subsequently, a color change in the test reagent. The time for a positive result is usually proportional to the number of helicobacters, i.e., when a positive result is obtained rapidly it is likely that the helicobacter count is high (Hazell et al 1987). Several reports describe and compare urease tests for the detection of human *H. pylori* (Borromeo et al 1987, Marshall et al 1987, Goldie et al 1989, McNulty et al 1989b, Katelaris et al 1992).

It has been stated that doubling the amount of tissue (size or number) in the urease test hastens the positive result by approximately 1.5 to 2 hours (Laine et al 1996a). Similarly, incubation of the urease test at 37°C has been shown to hasten the time to a positive result in comparison to incubation at room temperature (22-24°C), and therefore warming of the urease test is recommended when the final result of the test is desired within 1 to 2 hours of biopsy, within which period this difference appears to be significant (Laine et al 1996b). Accidental blood in the biopsy specimen does not seem to alter urease test results (Laine 1998). The urease test has been reported to be less accurate in human patients receiving H_2-receptor antagonists (Lerang et al 1998).

5.4.2. Histology

Gastric biopsy specimens for **histologic examination** are usually fixed in buffered formalin, embedded in paraffin wax, and sectioned. Helicobacters can be visualized with routine hematoxylin and eosin (HE) staining, and with special stains such as Warthin-Starry silver staining (Stevens 1990), Giemsa staining (Madan et al 1988), Genta staining (Laine et al 1997), and alcian yellow/toludine blue staining (Leung et al 1996). When HE, Giemsa, and Genta stains were compared, it was found that Giemsa stain appeared to be the preferred stain for *H. pylori* diagnosis, although the HE stain was also accurate when bacterial density was high (Laine et al 1997). The Warthin-Starry staining method is sensitive for demonstrating spiral organisms, but it is more expensive and difficult to perform and does not have any advantage over Giemsa staining (Barthel et al 1988, Madan et al 1988). Genta stain is also good for helicobacter evaluation and allows visualization of histologic features simultaneously, but it is a laborious and expensive method (El-Zimaity et al 1998). It has been shown that the Sayeed staining method also allows simultaneous identification of helicobacters and visualization of tissue morphologic features, and is inexpensive, quick, and easy to perform and interpret (Cohen et al 1997). It may be difficult to detect helicobacters in a gastric biopsy specimen when the density of the organisms is low (El-Zimaity et al 1998). Histologically, in addition to demonstrating

the presence and location of gastric helicobacters, lesions such as inflammation can be evaluated. Immunohistochemical staining techniques have also been shown to be sensitive in detecting *H. pylori* in gastric biopsy specimens (Ashton-Key et al 1996).

5.4.3. Electron microscopy

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) reveal not only the presence of gastric helicobacters but also their typical morphology (Weber and Schmittdiel 1962, Lockard and Boler 1970, Henry et al 1987, Lee et al 1988, Geyer et al 1993, Handt et al 1994, Utriainen et al 1997). The size of the bacterium, number of coils, location, and presence of polar flagellae and periplasmic fibrils can be evaluated by TEM and SEM. Canine and feline gastric helicobacters have a tightly or loosely coiled spiral morphology. They possess bipolar multiple flagellae at their terminal ends. They may have periplasmic fibrils around the cell body, or the cell surface may be smooth. The size of the bacteria ranges from 5 to10 μm in length and from 0.3 to1.2 μm in width.

At least four morphologically different *Helicobacter* species occur in biopsy samples. The first spiral organism is *H. felis*, which has one, two, or three periplasmic fibrils around the cell wall (Lee et al 1988, Paster et al 1991). The second, which does not have fibrils, is *H. bizzozeronii* (Hänninen et al 1996). The third, the loosely coiled or wavy and rather thick *H. salomonis*, has been described recently (Jalava et al 1997). The fourth is *"F. rappini"*, a more cylindrical organism with numerous net-like filaments around the cell body (Lockard and Boler 1970, Utriainen et al 1997). Human *"H. heilmannii"* and the newly described non-fibrillated *H. felis* (Eaton et al 1996) are highly similar to *H. bizzozeronii*, based on their morphological features in TEM and SEM. Therefore, electron microscopy is not a sensitive enough method to draw conclusion as to similarity of these species (Curry 1994).

5.4.4. Culture

Culture allows a detailed characterization of isolates and antimicrobial susceptibility testing. Several reports on culture techniques and growth media for *H. pylori* have been published (Goodwin et al 1985, Veenendahl et al 1993, Hachem et al 1995). Culture of canine and feline gastric helicobacters is, however, difficult and laborious. An adequate transport medium should be used, and rapid transportation of the biopsies to a microbiological laboratory is essential for the viability of the bacteria (Veenendaal et al 1993, Xia et al 1993). A freshly made brain-heart infusion (BHI) agar with horse blood has been described as the first-choice recommendation for primary isolation of *H. pylori* (Hachem et al 1995), and similar media with selective antibiotics have appeared most suitable also for isolation of canine and feline helicobacters (Hänninen et al 1996). The plates are incubated microaerobically at 37°C up to 10 days because helicobacters grow slowly. The bacterial growth is seen as a thin, spreading non-hemolytic film.

Contamination of the plates is often encountered and can be minimized by fasting the animals before sampling biopsies (Jalava et al 1998).

5.4.5. Molecular methods

PCR techniques can demonstrate helicobacter DNA from gastric biopsies even when their number is too low to be detected by other methods. This technique amplifies a fragment of genomic DNA from a given bacterium. *H. pylori* has been detected with this technique but is usually not cultured from dental plaque, saliva, or feces (Banatvala et al 1994, Li et al 1996). Several primers have been designed for *H. pylori* chromosomal DNA amplification (Ho et al 1991, Valentine et al 1991, Clayton et al 1992, Hammar et al 1992, Kong et al 1996). The gene sequence 16S rRNA is highly similar between *H. felis*, *H. bizzozeronii*, *H. salomonis*, and *"H. heilmannii"* and therefore does not seem suitable as a target for species-specific PCR for discriminating among gastric helicobacters in animals (Jalava et al 1997 & 1998, Norris et al 1999). One study reports the use of PCR with primers specific for the 26-kDa product in detection of helicobacters in cats after eradication therapy (Perkins et al 1996b). Currently, PCR is not in routine use in veterinary medicine for detection of gastric helicobacters.

An **in situ hybridization** technique has been used successfully for identifying *H. pylori* from formalin-fixed, paraffin-embedded tissue sections (Bashir et al 1994, Karttunen et al 1996, Barret et al 1997, Park and Kim 1999). In ISH methods, nucleic acid probes are produced by selecting, cloning, and synthesizing genomic sequences specific for a particular group of infectious agents; these probes in turn hybridize with DNA or RNA targets in the clinical specimen (Tang and Persing 1999).

5.4.6. Serological tests

Serological tests measure circulating antibodies (IgG, IgA), formed by response to gastric helicobacters, and these tests are accurate, relatively fast and simple to perform, and fairly cheap. Several methods are used to measure *H. pylori* antibodies, including bacterial agglutination and complement fixation, latex agglutination, passive hemagglutination, and immunoblotting tests (von Wulffen 1992). However, the enzyme-linked immunosorbent assay (ELISA) is the most commonly used method because it is sensitive and easy to perform, and therefore suitable for screening large populations in epidemiologic studies (von Wulffen 1992). Selection of an antigen is crucial in diagnostic serology, and in general, three types of antigens have been used: crude antigens such as whole cells and whole cell sonicates, cell fractions such as glycine extracts and heat-stable antigens, and enriched antigens such as urease and a 120-kDa antigen (Dunn et al 1997). Sensitive and specific results have been gained by ultracentrifuged sonicate, acid glycine extracts, urease preparations, and purified protein preparations as antigens (Hirschl et al 1988, von Wulffen and Grote 1988, Czinn et al 1989, Evans Jr. et al 1989, Newell and Stacey 1989). Serology may also be used to monitor *H. pylori* eradication treatment, and a 20 to 50% reduction in

IgG concentrations by six months after eradication therapy has been associated with successful treatment (Kosunen et al 1992, Cutler et al 1993). Commercial kits are available for *H. pylori* serology (Talley et al 1992), but not for animals. Recently, a serologic method has been described for diagnosing gastric helicobacters in dogs (Strauss-Ayali et al 1999).

5.4.7. Urea breath tests

The urea breath test (UBT) diagnoses helicobacter infection by demonstrating the urease activity of the bacteria. After an overnight fast, baseline breath samples are obtained, and then radiolabeled urea is administered orally as a substrate. Urea can be labeled with ^{13}C, a nonradioactive isotope (Graham et al 1987, Logan et al 1991), or with radioactive ^{14}C (Marshall and Surveyor 1988, Veldhuyzen van Zanten et al 1990). Exhaled air is sampled usually 30 minutes after ingestion, and the level of radioactivity is measured with a mass spectrometer (^{13}C) or a scintillation counter (^{14}C). Safe and simple microdose ^{14}C- and ^{13}C-urea breath tests have also been validated in humans. In these methods, a low dose of urea is given orally in a capsule, and a single breath sample is collected after 10 minutes; the result can be obtained within 15 minutes if a counting instrument is nearby (Bielański et al 1996, Bielański and Konturek 1996, Peura et al 1996). UBT is useful in for treatment monitoring, because after antimicrobial treatment and eradication of *H. pylori*, serologically detected antibody titers show a diagnostic decrease after 5-6 months (Kosunen et al 1992), whereas UBT becomes negative in only about a month (Marshall and Surveyor 1988). The disadvantages of the UBT are higher cost because of special equipment and the requirement of a radioactive isotope if ^{14}C is used.

The ^{13}C-urea breath test has been evaluated in dogs and seems to provide a noninvasive procedure to detect canine helicobacters (Cornetta et al 1998). The ^{14}C-urea breath test has been used successfully to diagnose *H. mustelae* infection in ferrets and *H. felis* infection in mice (McColm et al 1993, Glauser et al 1996).

5.4.8. Helicobacter detection in stool

Culture and PCR techniques have been used to detect *H. pylori* and its DNA in feces (Kelly et al 1994, Gibson et al 1995, Makristathis et al 1999). In addition, enzyme immunoassay (EIA) for detection of ***H. pylori* antigens in stool (HpSA)** has recently been described (Vaira et al 1999). This noninvasive EIA method is commercially available (*HpSA* Premier Platinum HpSA, Meridian Diagnostics, Cincinnati, OH, USA), and has been shown to be a reliable tool and easy to use for diagnosing *H. pylori* infection. One report describes a PCR method for diagnosing helicobacters in feces as well as in dental plaque of *H. pylori*-infected cats (Fox et al 1996b).

5.5. Identification and molecular typing of helicobacter isolates

Preliminary identification of *Helicobacter* species is made on the basis of morphologic characteristics and motility determined in vivo by light microscope, and of urease positivity. Helicobacters are helical, gram-negative, actively motile, urease-, oxidase- and catalase-positive organisms. Accurate species identification requires morphologic examination by electron microscopy, biochemical, tolerance, and phenotypic tests, as well as molecular methods on cultured helicobacters (Paster et al 1991, Hänninen et al 1996, Jalava et al 1997).

Genotyping, a DNA-based typing of bacteria, uses microbial DNA as a target in analyzing sequence variation and restriction sites at the whole genome level or at certain loci. Comparison of genotypes within species provides information on the genetic relationship of strains. This information can be used to trace the source of infection in epidemics or, for example, to follow transmission of an infectious agent in a population of animals or humans (Maslow et al 1993). Methods used for genotyping include ribotyping and pulsed-field gel electrophoresis (PFGE) (Tenover et al 1995). In ribotyping, digested DNA is hybridized with 16S rDNA, 23S rDNA or 16S + 23S rDNA probes. In PFGE, with the use of rare-cutting enzymes, a restricted number of fragments are produced which are electrophoresed (Maslow et al 1993).

Molecular typing methods have been applied to study the diversity of *H. pylori* isolates from various geographical areas (Akyopyanz et al 1992, Owen et al 1992). Similar methods have been used for typing of *H. mustelae* (Taylor et al 1994), *H. bizzozeronii*, and *H. salomonis* (Hänninen and Hirvi 1999) as well as *H. felis* (Jalava et al 1999b).

5.6. Eradication therapy for gastric helicobacters

Studies concerning eradication therapy against canine or feline gastric helicobacters are few but the preliminary studies, in which combinations of amoxicillin, metronidazole, and famotidine or omeprazole were evaluated, indicate that human therapy effective for *H. pylori* eradication may also apply to cats and dogs, although recurrent infections after these therapies was also reported (DeNovo and Magne 1995, Perkins et al 1996, Cornetta et al 1998, Lecoindre et al 1998). Multi-drug regimens have been shown to yield the best eradication results in humans (Ciociola et al 1996, Unge 1996). The regimes studied have consisted of a bismuth preparation, an H_2-receptor antagonist, or a proton-pump inhibitor (PPI), in combination with one, two, or three antimicrobial agents (Rokkas et al 1988, Unge et al 1989, Louw et al 1992, Hentschel et al 1993, Bell et al 1993, Yousfi et al 1995, 1996). In the standard triple therapy, two antimicrobial agents: amoxicillin or tetracycline in cases of penicillin allergy, and metronidazole, were combined with bismuth subcitrate (Borody et al 1989). In humans, the side-effects of this regimen, such as diarrhea, nausea,

vomiting, or abdominal pain, may cause cessation of the therapy; therefore other regimens aiming to fewer side-effects have been instituted (Bell et al 1993).

A recent recommendation for *H. pylori* eradication therapy involves a seven-day treatment consisting of amoxicillin or tetracycline or clarithromycin with metronidazole, plus omeprazole (PPI) or bismuth subcitrate (European Helicobacter Pylori Study Group 1997). Combining omeprazole with two antimicrobials, amoxicillin, clarithromycin, or metronidazole, and giving this twice daily for one week has been shown to produce a high eradication rate of *H. pylori*, and to be well tolerated by the patients (Lind et al 1996). The European Society for Primary Care Gastroenterology (ESPCG) has recommended PPI, clarithromycin, and amoxicillin for eradication therapy of *H. pylori* infection in primary care; however, awareness of the local resistance rates for metronidazole and clarithromycin is essential for effective treatment (Rubin et al 1999). Metronidazole resistance has been reported as causing failures in eradication therapies containing this antimicrobial component (Glupczynski et al 1990, Bell et al 1991, Noach et al 1994). Primary resistance may, however, be non-stable, explaining the discrepancy observed between results of in vitro susceptibility tests and the eradication obtained in vivo (van Zwet et al 1995). One study reports that addition of omeprazole may reduce the impact of primary resistance of *H. pylori* to metronidazole as well as to clarithromycin (Lind et al 1999).

It has been shown that eradicating *H. pylori* is more effective in preventing recurrence of gastroduodenal ulcers than is traditional treatment with acid blockers and that reinfection is rare after successful eradication (George et al 1990, Seppälä et al 1992, Cutler and Scubert 1993, Labenz and Börsc 1994, Tytgat 1994, Uemura et al 1995). This has also been verified in two recent studies which demonstrated that omeprazole-amoxicillin or metronidazole-clarithromycin one-week therapies were safe and effective for eradication of *H. pylori*, healing and preventing relapse of gastric and duodenal ulcers after successful eradication (Malfertheiner et al 1999, Veldhuyzen van Zanten et al 1999).

Indications for *H. pylori* eradication therapy have been given, and therapy is strongly recommended, for instance, for peptic or bleeding ulcer disease; recurrent dyspepsia, and gastritis with severe abnormalities (European Helicobacter Pylori Study Group 1997, Rubin et al 1999). Interestingly, MALT lymphomas associated with *H. pylori* or *"H. heilmannii"* infections may resolve after cure of helicobacter infection (Chiang et al 1996, Morgner et al 1997).

To confirm that the therapy has been successful, three large gastric mucosal samples for biopsy are recommended to study the histology (El-Zimaity et al 1995). To ensure the total eradication of the bacteria, a check-up should be performed at the earliest 4 weeks after therapy, and the use of more than one method for helicobacter detection is recommended (van der Ende et al 1997). Endoscopy and biopsy or ^{13}C-UBTt have been recommended as diagnostic tests for *H. pylori* infection, and ^{13}C-UBT as the most useful test for monitoring eradication (Rubin et al 1999). The decrease in the number of inflammatory cells occurs rather slowly: one to two years after *H. pylori* eradication, some

increase in mononuclear cells can still be detected (Valle et al 1991, Genta et al 1993a). A slow decrease in lymphoid follicles also occurs after eradication therapy (Genta et al 1993b).

6. AIMS OF THE STUDY

The purpose of this thesis was to establish the significance of canine and feline gastric helicobacters in the etiology of gastric disorders. The specific objectives were:

1. to compare brush cytology, the urease test, histologic examination, TEM, and culture as diagnostic methods for detecting gastric helicobacters (I);

2. to examine the prevalence and colonization density of gastric helicobacters in clinically healthy pet dogs and cats and in dogs with upper gastrointestinal signs (II, III);

3. to perform a detailed topographic mapping of gastric helicobacters (II) in order to discover the most representative regions for sample-collection for their detection (III);

4. to study the association between colonization densities of gastric helicobacters and degree of gastric inflammation in clinically healthy pet dogs and cats and in dogs with upper gastrointestinal signs in various gastric regions (II, III);

5. to identify gastric helicobacters by TEM as well as by culture (I - III) and to evaluate the association between number of *Helicobacter* species and degree of gastric inflammation (III);

6. to evaluate the efficiency of human triple therapy for the eradication of gastric helicobacters, and its effect on clinical signs and on gastric histology, as well as to estimate the recurrence of gastric helicobacters after eradication (IV);

7. to study the transmission of gastric helicobacters from dams to offspring and between puppies (V).

7. MATERIALS AND METHODS

7.1. Animals

To evaluate diagnostic methods and to perform topographic mapping, 10 dogs and 10 cats, recently euthanized, were acquired (Studies I-II). All were apparently healthy pets and had been euthanized at their owners' request (Table 1). The history of these pets revealed no gastrointestinal signs, and they were not examined further.

In order to determine the prevalence and density of gastric helicobacters as well as gastric histology (Study III), 25 clinically healthy dogs, the health of which was verified by history and further examinations, and 21 dogs with upper gastrointestinal signs were obtained (Table 1). One of these healthy dogs came from a pet shelter; the other healthy dogs as well as all affected dogs were obtained from breeders. Owners allowed the use of their pets in this study. Some of the dogs had undergone routine surgical procedures, e.g., ovariohysterectomy or castration. All owners filled out a questionnaire to ensure that the dogs had shown no gastrointestinal signs and that none had received medication during the preceding month. Dogs with only a few instances of pica (grass), borborygmus, and flatulence were considered clinically normal and were included in the study. For the dogs with upper gastrointestinal signs, client-owned dogs were selected from among those that had undergone an endoscopy procedure due to signs the cause of which had not been obvious before endoscopy. These 46 dogs had been given no nonsteroidal anti-inflammatory agents, antibiotics, sucralphate, acid suppressants (e.g., cimetidine, ranitidine, omeprazole), or any other medications for at least the 2 weeks prior to the study. Clinical examination and the hematologic and biochemical analyses (Table 1) were performed on all healthy and affected dogs, and abdominal radiography on affected dogs. Dogs with metabolic diseases, gastrointestinal foreign bodies, and tumors were excluded.

Chronic intermittent vomiting, most often consisting of bile-stained fluid or mucus and foam, was reported in 18 of 21 (86%) dogs with upper gastrointestinal signs. Two of the three dogs that did not vomit had signs of abdominal pain, borborygmus, flatulence, and marked pica (grass), which were also reported commonly for the dogs which vomited; the third dog had, in addition, bouts of abdominal bloating. Duration of clinical signs ranged from 1 month to 3 years (mean 8.5 months).

Nine dogs with upper gastrointestinal signs participated in the eradication therapy follow-up study (IV) (Table 1). Prior to the study, the pet owners had filled in a questionnaire in which the nature and duration of the gastrointestinal signs, feeding habits, environment, contacts with other animals, and previous medications were determined. These dogs had suffered from upper gastrointestinal signs for 4 months to 4 years and had been on medications such as antibiotics, sucralphate, cimetidine, or metoclopramide. Obvious reasons for the gastrointestinal signs were ruled out, as in Study III, and also by use of tests specific for intestinal disorders (Table 1); the criteria for patient exclusion were

also the same. Three dogs with mildly to moderately elevated alanine aminotransferase (ALT) and alkaline phosphatase (ALP) concentrations were included in the study because the suspected causes for their elevated liver enzymes was reactive hepatopathy due to the gastrointestinal disorder and administration of glucocorticoids (Twedt 1998). Complete blood counts and biochemical analyses were repeated in follow-up controls. ALP and ALP levels normalized during the study when clinical signs had resolved, and when corticosteroids were discontinued.

Table 1. The number, signalment and status of the animals in the studies.

Study	Number and status of animals	Sex, age and breed	Selection criteria
I, II	10 dogs Apparently healthy	8 ♀; 2 ♂ 1-13 yrs (mean 7.1 yrs) 6 different breeds	No GI signs.
	10 cats Apparently healthy	6 ♀; 4 ♂ 1-17 yrs (mean 7.4 yrs) Domestic shorthair	No GI signs.
III	25 dogs Clinically healthy	14 ♀, 1 (♀); 10 ♂ 1.5-8 yrs (mean 4.5 yrs) 11 different breeds	No GI signs. No medication for 1 month. No abnormalities in CBC, BP.
	21 dogs Affected	9 ♀, 3 (♀); 9 ♂ 1-11 yrs (mean 4.5 yrs) 16 different breeds	uGI signs without obvious reason. No medication for 2 weeks. No abnormalities in CBC, BP, radiography.
IV	9 dogs Affected	2 ♀, 1 (♀); 6 ♂ 1-5.5 yrs (mean 2.8 yrs) 9 different breeds	uGI signs without obvious reason. Dietary hypersensitivity ruled out. No abnormalities in CBC, BP, radiography, serum folate and cobalamine, fecal flotation for parasites.
V	2 dogs Clinically healthy	2 ♀ (dams) 1.5 and 2 yrs Beagles	No GI signs. Helicobacter-negative after eradication therapy.
	8 dogs Clinically healthy	2 ♀; 6 ♂ (puppies) 7 weeks Beagles	No GI signs. Helicobacter-negative before experimental challenge.

(♀) = neutered female
GI signs = gastrointestinal signs, i.e., nausea, vomiting, abdominal pain or discomfort, abdominal bloating, pica, borborygmus, flatulence, constipation, diarrhea.
uGI signs = upper gastrointestinal signs, i.e., nausea, vomiting, abdominal pain or discomfort, abdominal bloating, pica.
CBC = complete blood count.
BP = biochemistry panel (sodium, potassium, calcium, alanine aminotransferase [ALT], alkaline phosphatase [ALP], urea, creatinine, total proteins, albumin, cholesterol, glucose).

The animals in the transmission study (V) were two conventional Beagle dams acquired from a breeding unit producing experimental dogs, and their eight puppies, four from each dam (Table 1). Pregnant dams (A and B) were acquired 3 weeks before the expected delivery from an experimental dog-breeding unit. The dams were placed in a separate unit for experimental animals of The Faculty of Veterinary Medicine where their puppies were also kept. The dams were found to be helicobacter-positive before delivery and were therefore treated with eradication therapy, and checked 7 weeks later when they were negative. The negative helicobacter status of the puppies was verified before the experimental challenge test.

7.2. Diagnostic methods

7.2.1. Comparison of diagnostic methods and topographic mapping (I-II)

The stomach was detached immediately after euthanasia by ligating it at the distal esophagus and proximal duodenum; it was cut open along the greater curvature and rinsed gently with tap water (37°C) to remove the contents. The samples were collected from three regions of the stomach: the fundus (including the cardia), the corpus, and the antrum (including the pylorus). A total of 17 samples were obtained from each dog, and 14 from each cat. In dogs, there were four sample sites in the fundus, eight in the corpus, and five in the antrum, and in cats four, six, and four, respectively (Figure 1). The sample sites were marked with sterile injection needles before sample collection, and instruments sterilized with 2% glutaraldehyde were used to obtain each sample. Mucus for brush cytology and biopsy specimens for the urease test and histologic examination were obtained from each sample site (Tables 2 and 3). To identify *Helicobacter* species, samples were also taken for TEM from three dogs and six cats, and for culture from eight dogs and six cats from the fundus and corpus provided, that these regions had produced positive result in the urease test.

7.2.2. Endoscopy and biopsy procedures (III-V)

Before endoscopy, food was withheld from the dogs for 12 hours. Endoscopy was performed under general anesthesia (III-V). Anesthesia was induced with intramuscular (i.m.) or intravenous (i.v.) medetomidine 30-40 µg/kg followed by propofol 1-2 mg/kg i.v., and was maintained with halothane, or parenterally with propofol i.v. (0.5-1 mg/kg as needed). Puppies from 7 weeks to 3 months of age were anesthetized with fentanyl 0.002 mg/kg i.m. and medetomidine 20 µg/kg i.v. followed by propofol 1-2 mg/kg, from 3 to 6 months of age with medetomidine 40-50 µg/kg and ketamine 4 mg/kg i.m., and from 6 months of age forward as for the adult dogs.

For endoscopy, the dogs were placed in left lateral recumbency. A videoendoscope with an outer diameter of 9.8 mm and biopsy channel diameter of 2.8 mm (Olympus EVIS

GIF-100, Tokyo, Japan) was used for medium- and large-sized dogs, and a fiberoptic endoscope with an outer diameter of 5 mm and biopsy channel diameter of 2 mm (Olympus
GIF-N30, Tokyo, Japan) for small dogs and puppies. Equipment and instruments were cleaned thoroughly and sterilized in 2% glutaraldehyde for at least 20 minutes before use (Henkel-Ecolab Disinfectant® or Henkel Hygiene GmbH, Düsseldorf, Germany; Cidex®, Johnson & Johnson, Sollentuna, Sweden). During endoscopy, the gastric mucosa was evaluated for mucosal abnormalities.

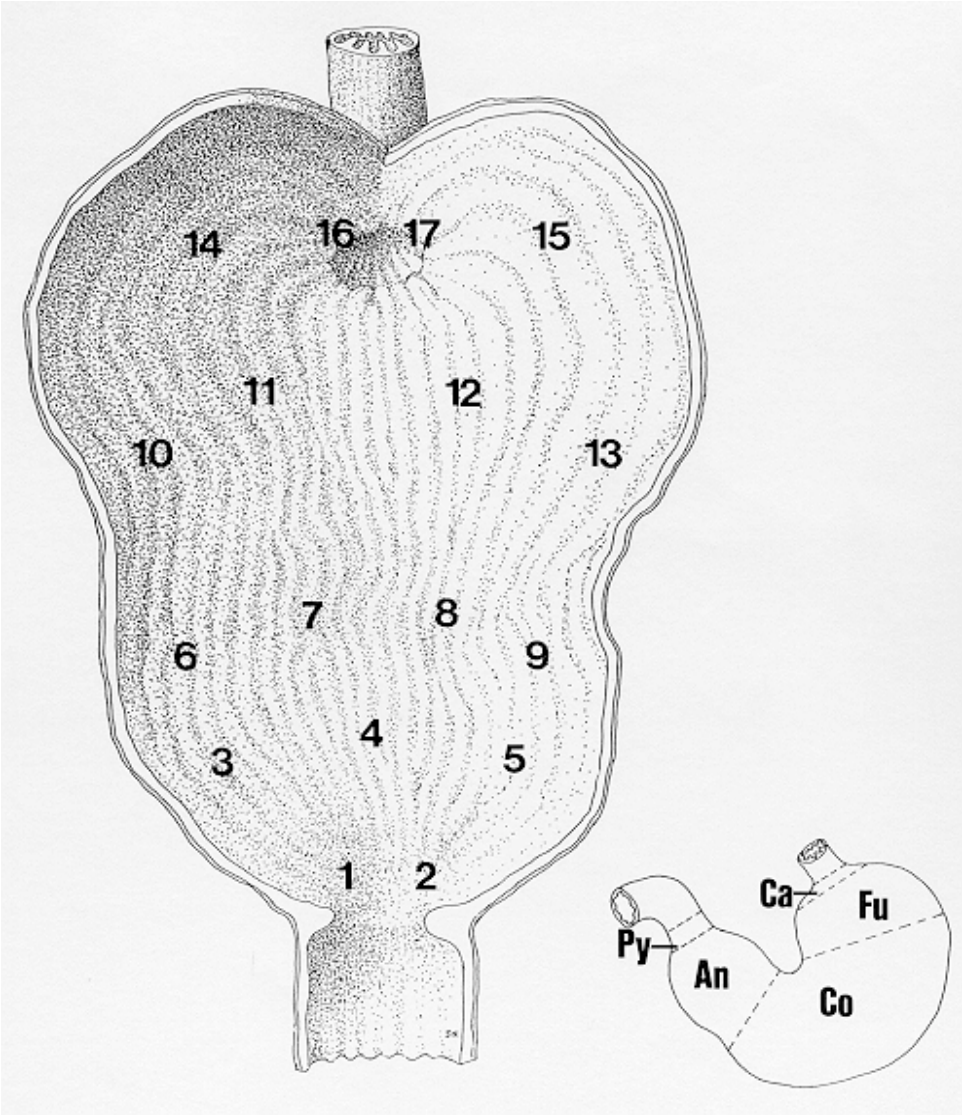

Figure 1. *Schematic drawings of anatomy of the canine and feline stomach, indicating gastric regions and sample sites (numbers): Py = Pylorus (1-2), An = Antrum (3-5), Co = Corpus (6-13), Fu = Fundus (14-15), and Ca = Cardia (16-17). In cats, samples were not obtained from sites number 5, 9, and 13 due to the smaller size of the stomach. The stomach is opened along the greater curvature.*

Mucosal biopsy specimens were obtained with biopsy forceps (Olympus FB-24Q or FB-19K, Tokyo, Japan) for the urease test, histologic examination, TEM, and culture. After taking mucus for brush cytology, biopsy specimens were collected mainly from the corpus for the urease test, and then for culture and TEM. Culture and TEM were performed if the urease test gave a positive result. For histologic examination, one or more biopsies were always from the corpus and the others from the cardia, fundus, or antrum; in most cases, all gastric regions were sampled. The sampled gastric regions and number of samples in various studies are detailed in Tables 2 and 3.

7.2.3. Brush cytology (I-V)

For brush cytology, mucus was collected from the gastric mucosa with small, round brushes (Interdental No. 3 Mini®; Jordan, Norway), a clean, disposable brush being used on each sample site (I), or with an endoscopic cytology brush which was washed thoroughly and disinfected after each use (hand wash: Cidex®, Johnson & Johnson, Sollentuna, Sweden; machine wash: Henkel-Ecolab Disinfectant®, Henkel Hygiene GmbH, Düsseldorf, Germany) (III-V). The mucus was spread on a slide which was then air dried and stained with May-Grünwald-Giemsa (MGG). Helicobacters were sought in each gastric mucus smear under a microscope at 400x (III-V) or 1000x (I-II) magnification (Figure 2). The sampled regions and criteria for recording results are detailed in Table 2. In Studies I-II, the criteria for quantification were modified from McNulty et al (1989a).

7.2.4. Urease test (I-V)

A modified rapid urease (MRU) test (Katelaris 1992) was used to demonstrate the urease positivity of gastric biopsies. Small mucosal specimens were cut with scissors from the sample sites (I, II), or they were obtained with biopsy forceps (III-V). Mucosal specimens were placed immediately into the test reagent containing 10% unbuffered urea in distilled water (pH 6.8) and 1% phenol red. The Eppendorf tubes with 0.5 ml of test reagent in each tube were stored in the freezer (−20°C) and warmed up to room temperature before testing. A color change from pale yellow to bright pink was considered positive. The test was incubated at room temperature for up to 24 hours, and the time of a positive result was recorded (Table 2).

7.2.5. Histologic examination (I-IV)

The biopsy specimens for histologic examination were fixed immediately in 10% buffered formalin, embedded in paraffin wax, sectioned (3-4 μm), and stained with hematoxylin and eosin (HE). Biopsy specimens negative for helicobacters with HE staining were re-evaluated with Warthin-Starry staining (I, II), or with Giemsa staining (IV). Full- thickness specimens, which included all layers of the stomach wall, were obtained with punch biopsy instruments (diameter 5-6 mm) from all the sample sites (I, II). Endoscopic biopsy specimens were taken with biopsy forceps (for instruments, see section

7.2.2.) (III-V). Mucosal biopsies for histologic examination were collected from the gastric regions as listed in Table 3 (I-V) and shown in Figure 1 (I, II). The biopsy specimens were collected together and then evaluated by one pathologist who was not blinded to clinical or microbiological information.

Of the endoscopically obtained samples, only good-quality specimens were accepted for histologic evaluation. Each acceptable biopsy specimen was sufficiently large, contained epithelium including glands and lamina propria, and showed only minimal damage from crushing and stretching. Histologic evaluation of each specimen included the detection, colonization density, and location (mucosal surface including gastric pits, gastric glands, and parietal cells) of helicobacters as well as the detection, number, and distribution of inflammatory cells, i.e., mononuclear cells (lymphocytes and plasma cells), neutrophils, and eosinophils. The number of lymphocyte aggregates, i.e., accumulation of lymphocytes regardless of size of accumulation or germinal center, in a specimen was also counted. Tissue changes, e.g., atrophy, dysplasia and metaplasia were evaluated. Atrophy was recorded when the gastric glands were atrophic and reduced in number and the mucosa thinned, without evidence of active tissue destruction or regeneration. Dysplasia was recorded when epithelial lesions characterized by proliferating glandular elements irregular in shape and size were evident. Metaplasia was recorded when gastric epithelium appeared to have an intestinal pattern or goblet cells were detected. Ulcers as well as intraepithelial inflammatory cells were also observed.

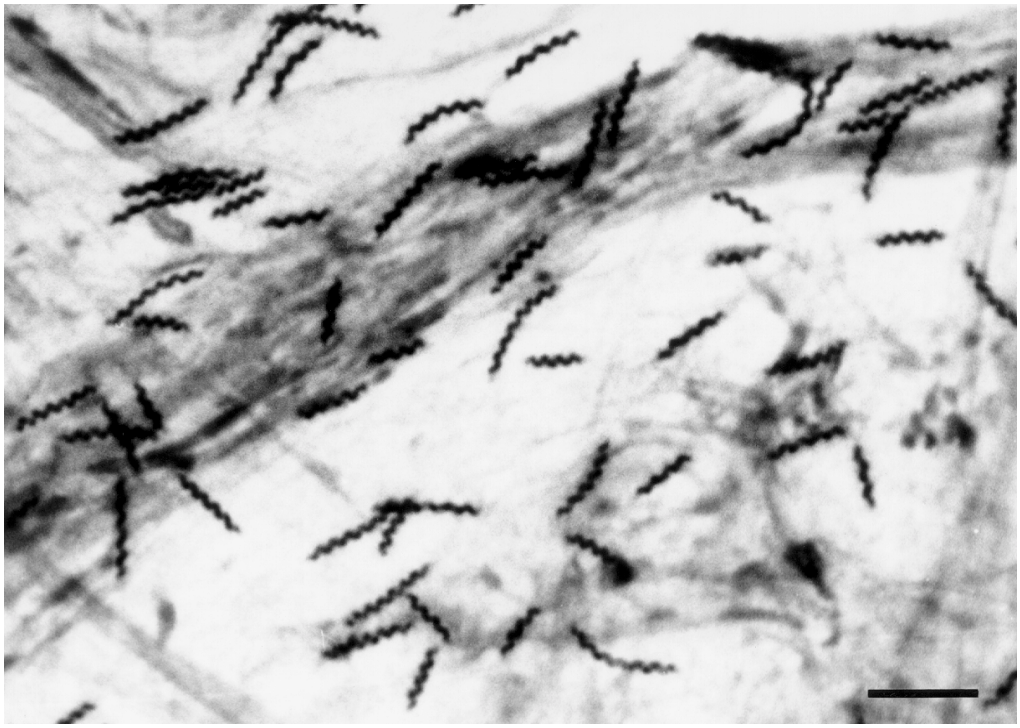

Figure 2. *Photomicrograph of brush cytology specimen from corpus of a canine stomach, demonstrating numerous helicobacters with distinctive spiral morphology. May-Grünwald-Giemsa stain; bar = 10 µm.*

Table 2. Diagnostic methods for detecting gastric helicobacters, grading systems for the bacteria, sampled gastric regions, and number of dogs tested (Studies I-V).

Method	Study	Sampled gastric region (number of samples dog/cat)	Number of animals tested	Grading / recording system
Brush cytology	I, II	All regions [1] (Ca 2/2, Fu 2/2, Co 8/6, An 3/2, Py 2/2)	10/10 of each: apparently healthy dogs & cats	Scale: 0, 1, 2 [2]
	III	Corpus (1)	24/25 clinically healthy dogs 11/21 dogs with upper GI signs	Scale: none, few, moderate, many, numerous [3]
	IV	Corpus (1)	3/9 dogs at 1st visit 3/9 dogs at check-ups 4/4 dogs at long-term check-up	Result positive or negative
	V	Corpus (1)	2/2 dams, 8/8 puppies	Result positive or negative
Urease test	I, II	All regions (Ca 2/2, Fu 2/2, Co 8/6, An 3/2, Py 2/2)	10/10 of each: apparently healthy dogs & cats	Positive results recorded at 30 & 60 min
	III	Corpus (1)	25/25 clinically healthy dogs 21/21 dogs with upper GI signs	Followed up to 24 h, times for positive results recorded
	IV	Corpus (1)	9/9 dogs at 1st visit 9/9 dogs at check-ups 4/4 dogs at long-term check-up	Followed up to 24 h, times for positive results recorded
	V	Corpus (1)	2/2 dams, 8/8 puppies	Followed up to 24 h, times for positive results recorded
TEM	I, II	Fu (1), Co (1) if urease-positive	3/10 app. healthy dogs 6/10 app. healthy cats	Morphologic features [4]
	III	Corpus (1) if urease-positive	22/25 clinically healthy dogs 5/21 dogs with upper GI signs	Morphologic features [4]
Culture	I, II	Fu (1), Co (1) if urease-positive	8/10 app. healthy dogs 6/10 app. healthy cats	See text for details
	III	Corpus (1) if urease-positive	25/25 clinically healthy dogs 6/21 dogs with upper GI signs	
	V	Corpus (1) if urease-positive	2/2 dams & 8/8 puppies	

1) Gastric regions: Ca = cardia, Fu = fundus, Co = corpus, An = antrum, Py = pylorus.
2) Predominant score of bacterial number based on evaluation of three representative fields after checking the whole specimen (1000x): 0 = no helicobacters; 1 = occasional (1–10/field), not in all fields; 2 = numerous (>10/field).
3) Whole specimen evaluated (400x): none (0), few (1–10), moderate (10–30), many (30–50), numerous (>50).
4) Large spiral bacteria without periplasmic fibrils having morphology resembling either that of *H. bizzozeronii*, *H. salomonis*, or non-fibrillated *H. felis*, or *H. felis* with periplasmic fibrils.
GI = gastrointestinal.

Table 3. Histologic evaluation of gastric biopsy specimens.

Study	Gastric region sampled [1] and number and status of animals	Grading system
I, II	All gastric regions [2] 10/10 of each: apparently healthy dogs and cats	Number of lymphocyte aggregates per sample (200x): 0, 1-2, 3 Number of neutrophils, eosinophils, lymphocytes & plasma cells as the mean of three fields (400x): 0 = none, 1 = <10, 2 = 10-50, 3 = >50 Helicobacter numbers (400x): 0 = none, 1 = 1-50 (few), 2 = >50 (numerous) Helicobacter location (400x): 0 = none, 1 = on mucosal surface and in gastric pits, 2 = 1 + in gastric glands, 3 = 1 + 2 + in parietal cells
III	Corpus + 1-3 other regions [3] 25/25 clinically healthy dogs 21/21 dogs with upper GI signs	VISUAL ANALOGUE SCALE: **Colonization density of helicobacters** (400x): Mild = a few organisms on the mucosal surface ± single in gastric glands or in parietal cells . Moderate = several helicobacters on the mucosal surface ± some in gastric glands and/or in parietal cells, **or** a few helicobacters on mucosal surface + some in gastric glands and/or in parietal cells. Marked = several or numerous organisms on the mucosal surface and several in gastric glands and/or parietal cells. **Number of mononuclear cells** (/HPF): Normal = none or a few cells Mild increase = several cells Moderate increase = many cells Marked increase = numerous cells **Number of neutrophils and eosinophils** (/HPF): Normal = none or only single sporadic cells in a specimen Mild = a few cells Moderate = several cells Marked = numerous cells
IV	Corpus + 1-3 other regions [3] Dogs with upper GI signs: 9/9 dogs at 1st visit 9/9 dogs at check-ups 4/4 dogs at long-term check-up	VISUAL ANALOGUE SCALE (same as in Study III)

1) Gastric regions: cardia, fundus, corpus, antrum, pylorus.
2) Number of samples: cardia 2; fundus 2; corpus 8 (dogs) / 6 (cats); antrum 3 (dogs) / 2 (cats); pylorus 2.
3) Number of samples: ≥ 1/ region.
HPF = high-power field, 400x magnification.
GI = gastrointestinal.

The animals, sample data, and histologic evaluation are summarized in Table 3. In Studies I-II, the criteria for quantity of helicobacters and inflammatory variables were modified from Handt et al (1994), and the results were also recorded according to Sydney system nomenclature for human gastritis (Price 1991). In Studies III-IV, colonization density of helicobacters and number of inflammatory cells were evaluated by the visual analogue scale (Dixon et al 1996) modified for canine gastric biopsy specimens and expressed as the average of the whole specimen (Figure 3). In these two studies, criteria for normal gastric histology were: no cells to a few mononuclear cells per high-power field (HPF; 400x magnification) and none to occasional scattered neutrophils and eosinophils without tissue changes. Sparse lymphocyte aggregates without an increase in inflammatory cells were considered normal. Chronic gastritis was diagnosed if the number of mononuclear cells was increased and was graded as mild, moderate, or severe (Figure 4). Increased number of neutrophils indicated active gastritis which was also graded as mild, moderate, or severe (II). Gastritis was diagnosed as regional if it did not appear in all gastric regions, and as focal if it was not diagnosed in all biopsy specimens from one particular gastric region. The terms ”superficial” and ”diffuse” were used to describe the distribution of inflammatory cells in the gastric mucosa.

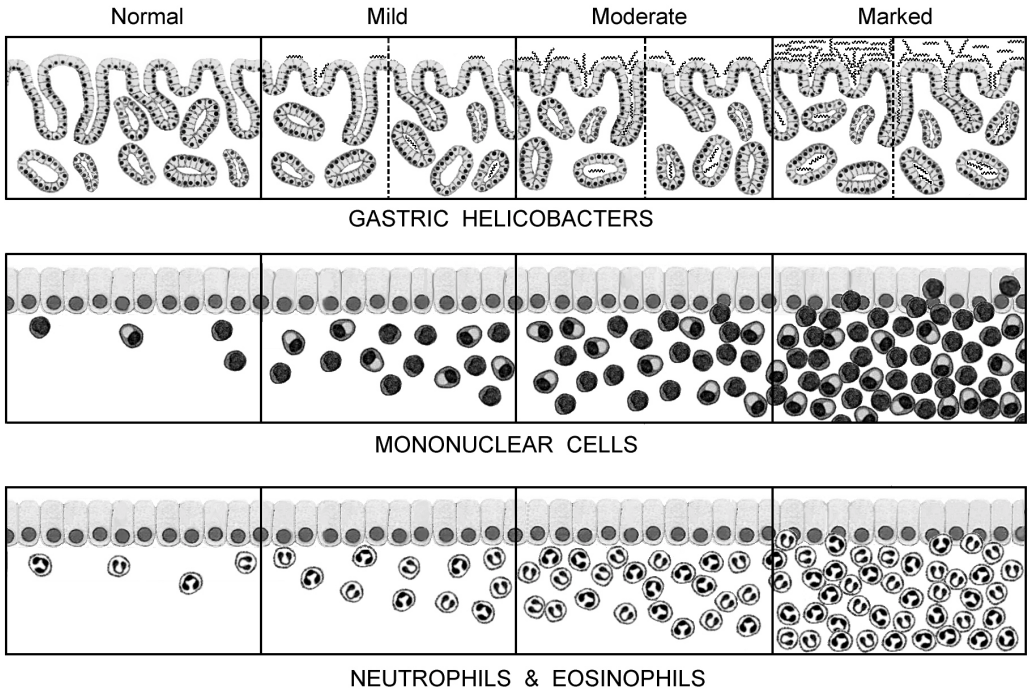

Figure 3. *Schematic visual analogue scale for evaluating and grading helicobacter organisms and inflammatory cells in canine gastric biopsy specimens. Each feature is observed and recorded by matching its appearance with the grading panel. When considerable variation in intensity is evident in the same biopsy specimen, the mean for several areas is determined and the specimen scored accordingly. Broken vertical lines in the top panel indicate two methods for grading mild, moderate, or marked colonization density.*

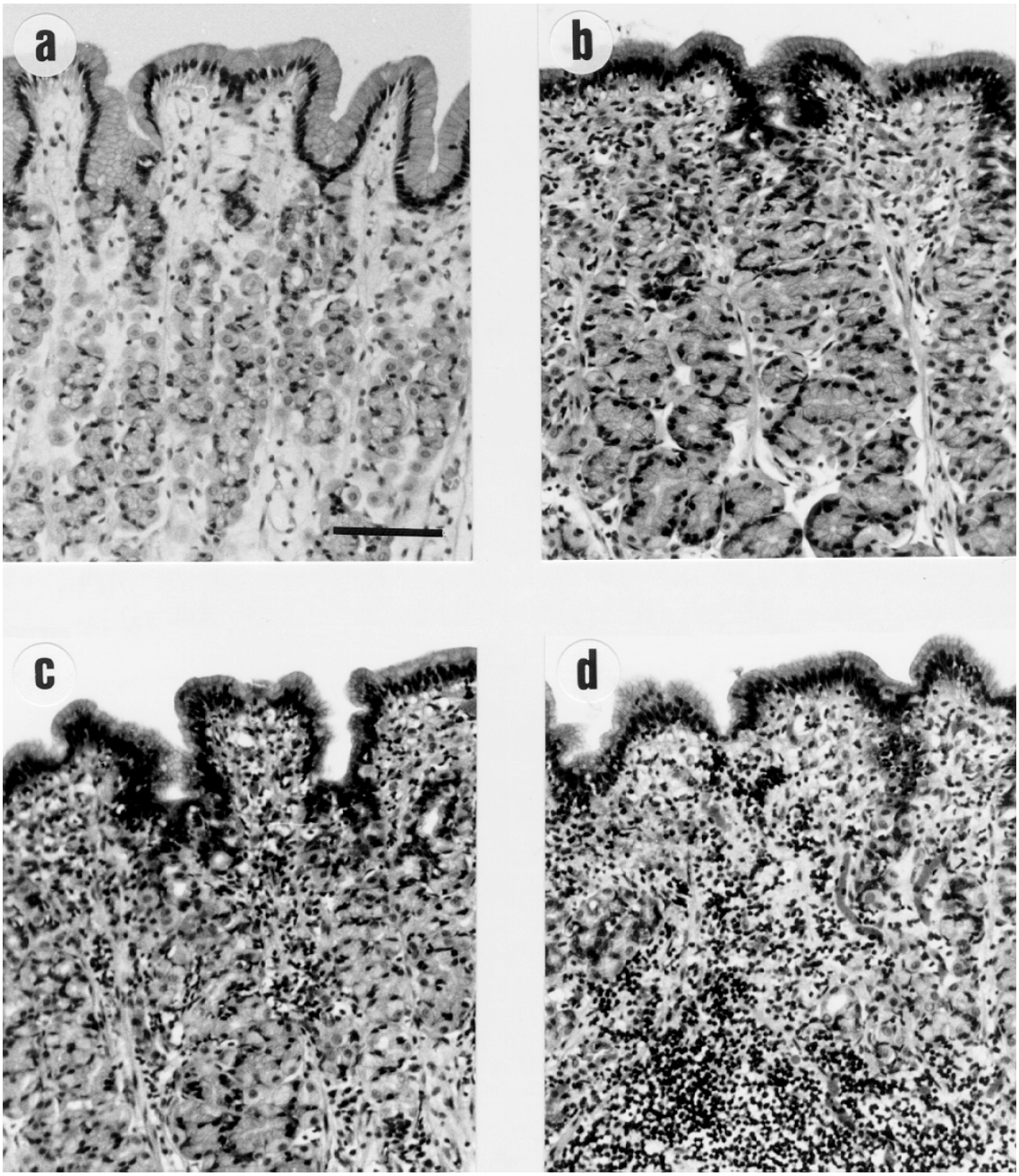

Figure 4. *Photomicrographs of canine biopsy specimens obtained from the corpus of the stomach showing (a) normal gastric histology, and (b) mild, (c) moderate, and (d) severe chronic gastritis. HE stain; bar = 100 µm.*

7.2.6. Transmission electron microscopy (I - III)

Samples for TEM were trimmed into about 1 mm^3 pieces, fixed for 2 h in 2.5% glutaraldehyde in 0.1M Sörensen phosphate buffer (pH 7.3), and stored in the buffer. The samples were post-fixed in 1% osmium tetroxide, dehydrated in acetone, embedded in epoxy resin (Epon LX 112; Ladd, USA), and polymerized. Ultrathin sections (0.06 µm) were placed on grids, stained with uranyl acetate and lead citrate, and examined with a JEM 100 S transmission electron microscope (Jeol, Tokyo, Japan). *Helicobacter* species were determined by TEM according to their morphological features: size of the bacterium, number of coils, and presence or absence of periplasmic fibrils (Weber and Schmittdiel 1962, Lockard and Boler 1970, Lee et al 1988, Solnick et al 1993). Evaluated samples and rough identification of *Helicobacter* species are presented in Table 2 and Figure 5.

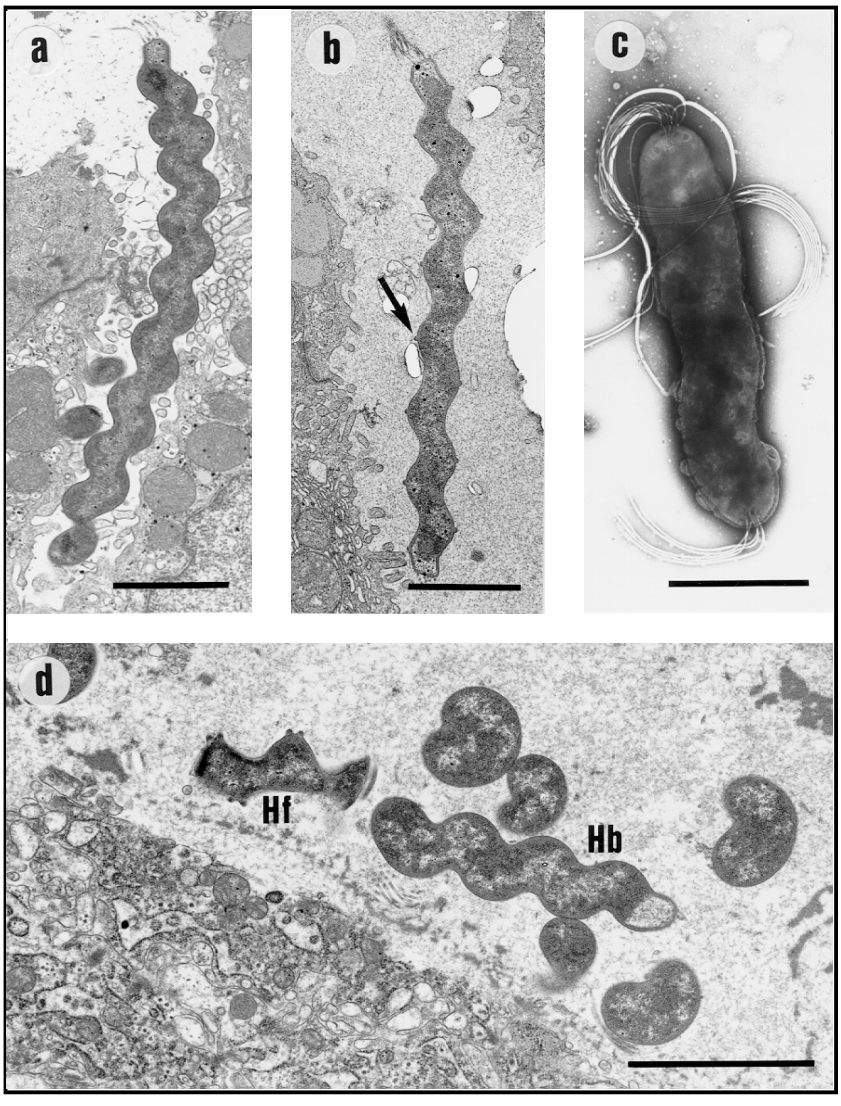

Figure 5. *Transmission electron micrographs of canine gastric mucosa revealing typical morphologic features of (a) Helicobacter bizzozeronii, (b) H. felis with periplasmic fibrils around the cell body (arrow), and (c) cultured H. salomonis, as well as (d) mixed infection with H. bizzozeronii (Hb) and H. felis (Hf). Uranyl acetate & lead citrate stain (a, b, d), negative stain (c); bar = 2 µm.*

7.2.7. Culture (I-III, V)

Biopsy specimens were obtained from the corpus and fundus (I, II) or only from the corpus (III, V) if a positive urease test result was obtained from these regions. The specimens were placed in a transport medium (Portagerm Pylori®, bioMérieux sa, Marcy l'Etoille, France) and cultured within 1 to 24 (I-II) or 2 to 8 (III, V) hours after sampling. Specimens were swabbed on fresh BHI blood agar or brucella blood agar containing selective antibiotics (trimethoprim 2.5-5 µg/ml, vancomycin 5-10 µg/ml, polymyxin-B 1.25 IU/ml, and cycloheximide 50 µg/ml or amphotericin B 2 µg/ml), or alternatively, specimens were crushed and placed in 300 to 500 µl of BHI broth containing 7% horse serum, and the preparation was spread on BHI blood agar plates containing the selective antibiotics. Plates
were incubated at 37°C microaerobically (5% O_2, 10% CO_2 and 85% N_2) up to 10 days. Growth was usually visible as a spreading film after 3 to 10 days. Preliminary identification of *Helicobacter* spp. was made on the basis of morphologic characteristics determined from examination of gram-stained slides and a positive result on the urease test. Additional identification was performed by use of TEM, phenotyping tests, and DNA-DNA hybridization analysis. Bacterial culture and characterization of cultures have been described in detail earlier (Paster et al 1991, Hänninen et al 1996, Jalava et al 1997). The isolates were stored in sterile skim milk with 15% glycerol at -70°C for later studies. Cultured biopsy specimens are listed in Table 2.

The fecal samples were studied for fecal excretion of helicobacters (V) by filtration of the fecal extract through a membrane filter with a pore size of 0.45-0.65 mm (Steele and McDermott 1984) or by inoculating fecal material on BHI blood agar with antibiotics. This fecal study was performed on the dams before the antimicrobial treatment and on the puppies after the last challenge with *H. bizzozeronii* and 3 months later.

7.2.8. Genotyping (V)

Restriction fragment analysis (REA) and **ribotyping** were used to study similarities of the isolates from dams and from puppies. Chromosomal DNA was isolated and purified by the guanidium thiocyanate method (Pitcher et al 1989). The concentration and purity of DNA was determined spectrophotometrically (Popovich et al 1993). The DNA (5 µg) was digested with the restriction enzymes *Hae*III, *Pst*I, and *Cla*I according to the recommendations of the manufacturer (New England Biolabs, Hertfordshire, UK). The digested DNA was electrophoresed at 25 V for 16 h in horizontal (0.8% w/vol) agarose in buffer (40 mM/l Tris-acetate and 2 mM/l EDTA). After electrophoresis, the gels were stained in ethidium bromide (1 µg/ml) and photographed. After photography, the gels were transferred by vacuum transfer (Vacu Gene XL, Pharmacia-LKB, Uppsala, Sweden) to MSI nylon membrane (MSI, Westboro, MA, USA). The probe, prepared from 16S +23S RNA of *Escherichia coli* by reverse transcriptase (Boehringer Mannheim, Germany), was labeled with digoxigenin. The samples were prehybridized at 58°C for 3 h and

hybridized at 58°C for 18 h (Popovich et al 1993). The hybridization products were visualized colorimetrically with antidigoxigenin-alkaline phosphatase (Boehringer Mannheim). Two unrelated *H. bizzozeronii* stains were included in the studies.

For further comparison of the isolates, **pulsed-field gel electrophoresis (PFGE) analysis** was performed. The isolates were grown on BHI blood-agar for 3 days at 37°C in a microaerobic atmosphere. The bacterial cells were harvested and treated with formaldehyde to inactivate endogeneous nucleases (Gibson et al 1994). Otherwise, DNA was prepared by the method of Maslow et al (1993). The bacteria were embedded in 1% low-melting agarose plugs (SeaPlaque GTG, FMC Bioproducts, Rockland, ME, USA). The plugs were washed and stored at 4°C in Tris-EDTA buffer, pH 7.5. Two-mm slices of the agar plugs were digested with *Not*I and *Spe*I (New England Biolabs). The DNA fragments were separated with Gene Navigator (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in 1% agarose gel in 0.5 X TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) at 200 V. *Not*I fragments were separated with a ramped pulse from 4 to 80 sec for 22 h and *Spe*I fragments were separated with a stepping program (0.5 sec/1 h, 0.7 sec/1 h, 0.9 sec/1 h, 2 sec/3 h, 4 sec/4 h, and 6 sec/4 h).

7.2.9. Metronidazole sensitivity testing (V)

Sensitivity to metronidazole was tested by adding 5, 10, or 15 mg/l of metronidazole (Sigma, St. Louis, MO, USA) into BHI blood-agar medium (Rautelin et al 1992) and spreading *H. bizzozeronii* culture on the plates. The medium without metronidazole served as the control medium. All plates were incubated microaerobically for up to 7 days. If no growth occurred, the isolate was regarded as sensitive.

7.3. Eradication therapy and follow-up study (IV)

Endoscopy was performed at entry and repeated one to three times during the follow-up period ranging from 1 to 12 months (mean 5.5). Control endoscopies were performed within 12 weeks (mean 3) after eradication therapies and within 3.5 months (mean 1.5) after additional therapies. In addition, four of the dogs that became helicobacter-negative after eradication therapies were reevaluated 1.5 to 3 years (mean 2.5) later.

At entry to the study, dogs with dietary hypersensitivity were excluded. Triple therapy, used for the eradication of human *H. pylori* (Tytgat 1994), consisted of orally administered amoxicillin 20 mg/kg and metronidazole 10 mg/kg twice a day for 10 to 14 days, as well as bismuth subcitrate 6 mg/kg twice a day for 2 to 4 weeks. If gastric helicobacters persisted after triple therapy, alternative eradication therapy with tetracycline 20 mg/kg twice daily and omeprazole 0.7 mg/kg once a day was administered orally for 10 and 14 days, respectively. If the clinical signs still persisted after eradication of helicobacters, additional therapies such as tylosin, sucralfate, cimetidine, prednisolone, and cisapride were prescribed individually. The owners maintained a detailed standardized

follow-up diary recording clinical signs daily during each treatment protocol for evaluation of gastrointestinal signs. In the reexaminations, hematological and biochemical analyses, abdominal radiographs, and endoscopy were repeated, and helicobacter-status and gastric histology were verified.

7.4. Transmission study (V)

At their arrival, the pregnant dams were verified as helicobacter-positive, and subsequently, were treated before delivery with triple therapy similarly to that of Study IV, but amoxicillin, metronidazole, and bismuth subcitrate were administered twice a day for 10 days. Seven weeks after cessation of the antimicrobial treatment, the biopsy samples of the dams were negative in the urease test, and no spiral organisms appeared in brush cytology of mucus specimens. The dams nursed the puppies for 7 weeks in separate isolated rooms, and after weaning the dams were transferred to another unit for experimental dogs of the Faculty of Veterinary Medicine. New biopsy samples were obtained from the dams 5 months after treatment and once again 2 years later.

The puppies were verified as helicobacter-negative after weaning, at 7 weeks of age. Eight puppies were divided into two groups, each of which consisted of two puppies from dam A and two from dam B. Three puppies in the first group were orally challenged with a suspension of 10^8 CFU (colony-forming units) of *H. bizzozeronii* CCUG 35045 in 2 ml of brucella broth daily for 2 consecutive days; the fourth puppy was left exposed to infection. The puppies of the second group served as non-challenged controls. Biopsy samples from the puppies were taken before the inoculation, and 2 weeks, 3 months, and 7 months after the challenge. The puppies were raised in separate rooms located on different floors with strict hygienic control to prevent transmission of infection.

7.5. Statistical analyses

Cochran's Q-test was used to compare differences between gastric regions in the occurrence of helicobacters and in active as well as in chronic gastritis (I), and when gastric regions were compared for differences between the results produced by brush cytology, the urease test, and histologic examination (II). **Friedman two-way analysis of variance** was used to analyze whether any differences existed between different gastric regions in the number of inflammatory cells and in the location of helicobacters (I). The **Kruskal-Wallis one-way analysis of variance** was used to test among groups with various numbers of helicobacters whether any differences existed in the number of inflammatory cells and the location of helicobacters (II). The groups were classified as cats with none, few, or numerous helicobacters and as dogs with few or numerous

helicobacters. **The Fisher exact test for 2 x 2 tables** served to determine the statistical association between occurrence of helicobacters and active or chronic gastritis (II).

Differences in prevalence and density of helicobacters and in gastric inflammatory parameters between clinically healthy dogs and dogs with signs of gastritis, as well as between dogs with a single and a mixed *Helicobacter* species infection, were tested by the **two sample proportion test** and **Mann-Whitney test** (III). **Cochran's Q-test**, **Friedman two-way analysis of variance**, and the **Wilcoxon signed rank test** were used to examine differences between gastric regions (III). The association between colonization density of helicobacters and gastric inflammatory parameters was studied by the **Spearman rank correlation coefficient** (r_S) (III). If several biopsies were obtained from one particular gastric region, that with the most pronounced finding was used for statistical analysis.

The sign test was used to analyze whether any changes existed in clinical signs and in gastric histology after triple therapy and after additional therapy (IV). **The Mann-Whitney test** was used to study the differences in upper gastrointestinal signs between helicobacter-positive and helicobacter-negative dogs.

P-values < 0.05 were considered significant.

8. RESULTS

8.1. Comparison of diagnostic methods for detecting gastric helicobacters (I, III)

Results of individual diagnostic methods are presented in Table 4. Brush cytology was the best method for detecting gastric helicobacters. A few to numerous helicobacters, most often many, were demonstrated by brush cytology. The urease test and histologic examination (HE staining) were also good methods, because only in the antrum did they produce more negative results than did brush cytology. In most cases, the results from brush cytology, the urease test, and histology were concordant. In 5/44 cases, brush cytology was the only method showing helicobacters (III, IV). The urease test was positive generally after 15-30 minutes; none of the positive results being recorded at 24 hours. Warthin-Starry staining revealed helicobacters in one of the canine samples in which HE staining had failed to demonstrate them (I). When the urease test and histologic examination were both negative, the number of helicobacters detected by brush cytology was usually low. However, in seven clinically healthy dogs and in one affected dog, only a few helicobacters were detected by brush cytology, although their urease tests rapidly became positive, and in histology several or numerous helicobacters were visible (III). TEM also revealed helicobacters in the majority of cases. Culture, however, was not as rewarding as these methods, although culture was more successful in dogs than in cats. Four of the ten cats were helicobacter-negative by brush cytology, the urease test, and histologic examination.

When gastric regions were compared in dogs, significant differences between the results produced by brush cytology, the urease test, and histologic examination were detected (I). These differences were most evident in the antrum, where the urease test gave a positive result in only 62% and histologic examination in 74% of the samples, whereas brush cytology results were positive in 100% of the samples (Table 4). In cats, no differences between the three diagnostic methods were found in any gastric region.

When the sensitivity and specificity of the methods were tested, the urease test and histologic examination were each compared with brush cytology, which was invariably capable of detecting the presence of gastric helicobacters (I). The sensitivity of the urease test at 30 and 60 min was 85.7% and 87.5% in dogs, and 94% and 100% in cats, respectively, and the specificity was 100% in both dogs and cats. The sensitivity of the histological examination was 92.3% in dogs and 97.6% in cats, and the specificity 100% in both species.

Table 4. Percentages of helicobacter-positive samples obtained by different diagnostic methods in positive animals, i.e., those positive by at least one method, in various gastric regions (number of animals in parentheses).

Method	Apparently healthy cats (I)	Apparently healthy dogs (I)	Clinically healthy dogs (III)	Dogs with upper GI signs (III)
Brush cytology	Co 100% Fu 100% An 100% *14 sites/animal* (n = 6)	Co 100% Fu 100% An 100% *17 sites/animal* (n = 10)	Co 100% *1 site/animal* (n = 25)	Co 100% *1 site/animal* (n = 20)
Urease test	Co 100% (30 min) Fu 88% (30 min) 100% (60 min) An 92% (30 min) 100 % (60 min) *14 sites/animal* (n = 6)	Co 96% (30 min) 100% (60 min) Fu 95% (30 & 60 min) An 62% (30 & 60 min)[a)] *17 sites/animal* (n = 10)	Co 96% (15-30 min) *1 site/animal* (n = 25)	Co 90% (15-30 min) 1 *site/animal* (n = 20)
Histologic examination (HE)	Co 100% Fu 100% An 92% *14 sites*/animal (n = 6)	Co 100% Fu 100% An 74% [b), c)] *17 sites/animal* (n = 10)	96% *All regions combined* (n = 25)	90% *All regions combined* (n = 20)
TEM	Fu+Co 100% *1 site/region/animal* (n = 6)	Fu+Co 100% *1 site/region*/animal (n = 3)	Co 91% *1 site/animal* (n = 22)	Co 100% *1 site/animal* (n = 5)
Culture	Fu+Co 17% *1 site/region/animal* (n = 6)	Fu+Co 37% *1 sample site/region* (n = 8)	Co 40% *1 site/animal* (n = 25)	Co 50% *1 site/animal* (n = 6)

GI = gastrointestinal.
Co = corpus, Fu = fundus (including cardia), An = antrum (including pylorus).
HE = hematoxylin and eosin staining.
TEM = transmission electron microscopy.

Significant differences between results produced by various diagnostic methods:
a) Urease test compared to to brush cytology ($P < 0.001$).
b) Histologic examination compared to urease test at 30 and 60 minutes ($P < 0.05$).
c) Histologic examination compared to brush cytology ($P < 0.001$).

8.2. Prevalence (II, III) and colonization density (III)

An animal was considered helicobacter-positive when any one test produced a positive result. All of the ten apparently healthy dogs (II) and 25 clinically healthy dogs (III) were helicobacter-positive (100%), and gastric helicobacters were also detected in 20 of 21 (95%) affected dogs (III). Of the ten apparently healthy cats, six were positive (60%) (II). Helicobacters were found both in young and in old animals and in both males and females. Gastric helicobacters were more common in clinically healthy dogs than in dogs with upper gastrointestinal signs in all gastric regions, but without statistically significant differences (III).

Nor were significant differences detected in the colonization density of helicobacters in any gastric region between clinically healthy and affected dogs (III). Age of dog was not associated with colonization density. When helicobacters were present, histologic examination most often revealed moderate to marked colonization density in both groups of dogs. Mild colonization density was detected in only one healthy dog and in two affected dogs, and their urease tests yielded positive results within 30 to 45 minutes. Brush cytology showed many helicobacters in the healthy dog but was not performed on the two affected dogs.

8.3. Topography of gastric helicobacters (II, III)

Gastric helicobacters were detected thoroughout the stomach. However, significant differences existed in helicobacter prevalence between gastric regions in dogs, and helicobacters were found less frequently in the antrum than in other regions (Study II $P<0.01$; Study III $P<0.05$). A significant difference ($P<0.001$) existed also in colonization density of helicobacters between different gastric regions in clinically healthy and affected dogs, with the lowest number of helicobacters detected in the antrum (III). In positive cats, helicobacters occurred in every gastric region without differences between regions.

Helicobacters were detected superficially on the mucosal surface and in gastric pits, and deeper in gastric glands and in parietal cells both in dogs and in cats. In Study II, the location of the organisms was significantly ($P<0.05$) more superficial in the antrum than in the fundus and corpus in dogs but not in cats. Helicobacters were located significantly ($P<0.01$) deeper in the corpus in cats with histologically verified chronic gastritis than in those without.

8.4. Association of helicobacters with gastric inflammation

A total of 309 gastric biopsy specimens were obtained endoscopically for histologic examination in Studies III and IV. Of these samples, 17% (52/309) were rejected because of non-diagnostic quality. From the antrum, 49% (33/67) were non-diagnostic, from the

cardia ,13% (9/69), from the fundus, 6% (4/64), and from the corpus, 6% (6/109). Therefore, the majority of non-diagnostic samples, 33/52 (63%), were from the antrum, and the best quality specimens were obtained from the corpus and fundus.

8.4.1. Gastric helicobacters and inflammatory parameters (II, III)

In apparently healthy dogs, significant associations did not exist between number of helicobacters (few versus numerous) and any inflammatory cell type (II). Colonization density of helicobacters was not positively associated with number of gastric inflammatory parameters in any gastric region either in clinically healthy dogs or in dogs with upper gastrointestinal signs (III). Among affected dogs, colonization density was negatively correlated with the number of mononuclear cells in the cardia and body and with the neutrophils in the cardia ($P<0.05$). Similar negative correlations were evident also among clinically healthy dogs, but these were not significant. When these two group of dogs were combined, negative correlations still remained ($P<0.05$).

Lymphocytes, plasma cells and lymphocyte aggregates were present in the stomachs of all dogs, with neutrophils and eosinophils seen less frequently. Study II showed no statistically significant difference in the number of neutrophils, eosinophils, lymphocytes, or lymphocyte aggregates between gastric regions, i.e., the fundus (including cardia), corpus, and antrum (including pylorus), but significantly more ($P<0.05$) plasma cells were found in the antrum than in the corpus. In Study III, mononuclear cells and neutrophils were relatively evenly distributed throughout the stomach in clinically healthy dogs and in dogs with upper gastrointestinal signs without any significant differences between gastric regions, whereas the number of lymphocyte aggregates was greatest in the fundus of healthy dogs and in the corpus of affected dogs ($P<0.05$). Eosinophils in affected dogs were fewest in the corpus ($P<0.05$).

The prevalence of lymphocyte aggregates, mononuclear cells, neutrophils, and eosinophils between clinically healthy dogs and dogs with upper gastrointestinal signs differed significantly ($P<0.05$) (III). In affected dogs, lymphocyte aggregates were detected more often in the corpus, mononuclear cells in the cardia and antrum, and eosinophils in the cardia. Minor differences were evident in the density of inflammatory parameters between healthy and affected dogs among gastric regions, but these were not significant.

Helicobacter-positive cats with numerous helicobacters demonstrated significantly more lymphocytes in the fundus and corpus than did helicobacter-negative cats ($P<0.05$) (II). Lymphocytes and plasma cells were present in the stomach of all ten apparently healthy cats. Plasma cells were significantly more ($P<0.05$) in the antrum than in the corpus of positive cats, but in helicobacter-negative cats they were seen only in the antrum. Lymphocyte aggregates were seen only in helicobacter-positive cats, and in at least one region. Neutrophils were found in five positive and four negative cats in at least one region, most commonly in the antrum. Three positive cats had eosinophils in the fundus.

8.4.2. Helicobacters and gastritis

Gastric histologic diagnoses in Studies II-IV are summarized in Table 5. For the first set of histologic criteria, mild chronic gastritis in the fundus, corpus, and antrum was recorded in all ten apparently healthy dogs, with no statistically significant difference in occurrence of chronic or active gastritis between gastric regions (II). In conjunction with chronic gastritis, mild active gastritis was seen in six dogs in one, two, or all three regions. No statistically significant association between active gastritis and the number of helicobacters (few or numerous) appeared.

All apparently healthy cats had mild chronic gastritis in the antrum whether or not helicobacters were present; chronicity was accompanied by activity in four positive cats and all negative cats (II). Both chronic and active gastritis were seen significantly ($P<0.01$) more often in the feline antrum than in other regions. A significant ($P<0.05$) association between the presence of helicobacters and chronic gastritis was found in the corpus and fundus. In contrast, no significant association existed in cats between helicobacters and active gastritis.

The criteria of the visual analogue scale revealed normal gastric histology in all gastric regions significantly ($P<0.05$) more often in clinically healthy dogs (13/25, 52%) than in dogs with upper gastrointestinal signs (4/21, 19%) (III). Two affected dogs had mild scattered eosinophilic infiltration alone, and the histologic diagnosis for the remaining dogs was most commonly mild to moderate gastritis, with or without scattered neutrophilic or eosinophilic infiltrates, or both. Chronic gastritis in these dogs was most often regional and superficial. Active gastritis was detected most often in association with moderate or severe chronic gastritis. Severe chronic gastritis was diagnosed in one healthy and in two affected dogs, and it was classified as regional and diffuse. Colonization density of helicobacters in these two affected dogs was only mild, whereas in the healthy dog it was moderate.

In Study IV, the most common initial histologic diagnosis was also mild chronic gastritis, detected initially in six of nine dogs. Mild eosinophilic activity in addition was found in three of these dogs, and eosinophilic activity alone was present in two. One dog had normal gastric histology. The degree of chronic gastritis in different regions among clinically healthy dogs and cats and among dogs with upper gastrointestinal signs is presented in Figure 6.

Gastric ulcers or erosions, macroscopic or microscopic, were detected in no dog or cat. Mild to moderate atrophy, dysplastic epithelial changes, or metaplasia were diagnosed in only a few clinically healthy and affected dogs (III). These changes were always associated with chronic gastritis and were most common in conjunction with moderate to severe gastritis. Similar changes were not found in apparently healthy dogs or cats (II).

Except for parietal cells, helicobacters were not seen in other glandular cells or in epithelial cells. In the presence of helicobacters, the appearance of parietal cells varied

from normal to vacuolated. When helicobacters were demonstrated in parietal cells by TEM, they appeared mainly in dilated intracellular canaliculi.

Table 5. Gastric histologic diagnoses in various groups of animals in Studies II-IV classified separately for the antrum and body of the stomach.

Histologic diagnosis	Apparently healthy cats (II): Hb-neg (n=4) / Hb-pos (n=6)	Apparently healthy dogs (II): All Hb-pos (n=10)	Clinically healthy dogs (III): All Hb-pos (n=25)	Dogs with signs of gastritis (III): All Hb-pos (n=21)	Dogs with upper gastrointestinal signs; Hb-pos (IV): Pre-ther (n=9) / Post-ther Hb-neg (n=8)[1)]
Any abnormality	4/6 (100/100%)	10 (100%)	12 (48%)	17 (81%)	8/8 (89/100%)
ANTRUM [2)]			*(n_1=13)*	*(n_1=14)*	*(n_1=4/7)*
Normal	0/0 (0/0%)	0 (0%)	9 (69%)	3 (21%)	2/2 (50/29%)
Chronic gastritis	4/6 (100/100%)	10 (100%)	4 (31%)	11 (79%)	1/1 (25/14%)
+ Active gastritis	4/4 (100/67%)	4 (40%)	2 (15%)	4 (29%)	0/1 (0/14%)
Eosinophils (± gastritis)	0/0 (0/0%)	4 (40%)	2 (15%)	5 (36%)	2/3 (50/43%)
BODY [3)]					
Normal	4/0 (100/0%)	0 (0%)	13 (52%)	6 (29%)	2/3 (22/38%)
Chronic gastritis	0/6 (0/100%)	10 (100%)	12 (48%)	13 (62%)	5/4 (56/50%)
+ Active gastritis	0/2 (0/33%)	5 (50%)	4 (16%)	4 (19%)	1/0 (11/0%)
Eosinophils (± gastritis)	0/0 (0/0%)	7 (70%)	4 (16%)	7 (33%)	4/2 (44/25%)

Hb-neg = helicobacter-negative; Hb-pos = helicobacter-positive.
Pre-ther = before eradication therapy; Post-ther= after eradication therapy; eradication therapy = amoxicillin, metronidazole, and bismuth subcitrate., or tetracycline and omeprazole after failed triple therapy in one case.
n_1= number of sampled animals if not the same as the total number of animals in various groups.
1) One dog remained helicobacter-positive after eradication therapy, and had a small number of eosinophils in the gastric body; the antrum sample was non-diagnostic.
2) Antrum includes pylorus.
3) Body includes corpus, fundus, and cardia.

8.5. Number of *Helicobacter* species and gastric inflammation (III)

Presumptive identification by use of TEM revealed one or two *Helicobacter* species. No *H. felis* occurred in any TEM specimen from cats but was cultured on one occasion. *Helicobacter* species identified by use of culture were *H. bizzozeronii*, *H. felis*, *H. salomonis*, and mixed-culture (Table 6). In the case of the mixed culture of *H. salomonis* and *H. felis*, TEM revealed only large spiral organisms. Culture usually identified only one of the two *Helicobacter* species seen during TEM of the specimen. When large spiral *Helicobacter* organisms were detected by TEM, alone or with *H. felis*, the most common species isolated by culture was *H. bizzozeronii*.

The number (one or several) of *Helicobacter* species had no statistically significant effect on number of lymphocyte aggregates, mononuclear cells, neutrophils, or eosinophils either in clinically healthy dogs or in dogs with signs of gastritis. Neither was there significant effect when these two group of dogs were combined.

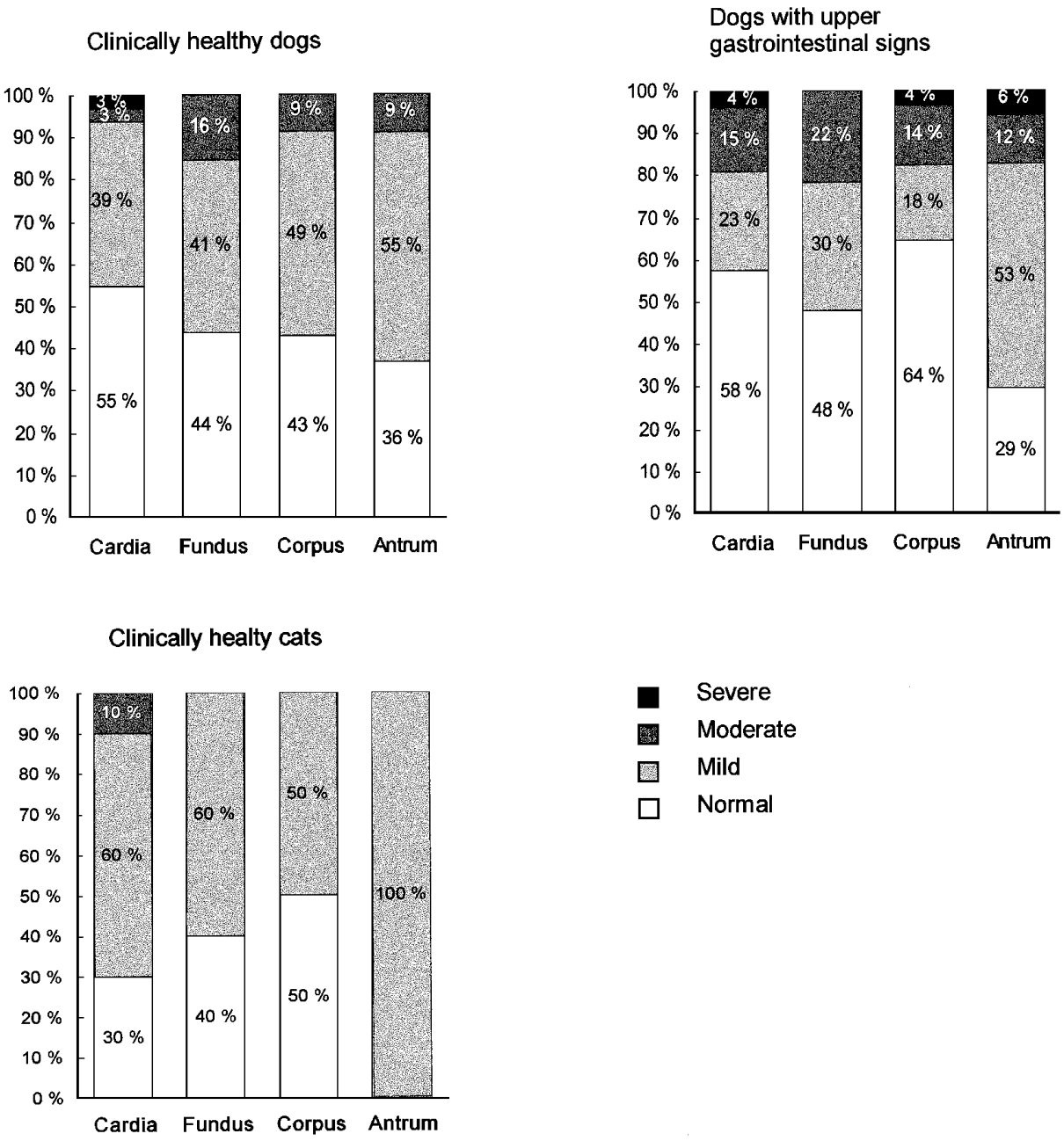

Figure 6. *Degree of chronic gastritis in various gastric regions in clinically healthy dogs, in dogs with upper gastrointestinal signs, and in clinically healthy cats, expressed as percentages. Both helicobacter-positive and helicobacter-negative animals are included.*

Table 6. Number of animals with different *Helicobacter* species identified by transmission electron microscopy (TEM) and culture in different animal groups in Studies II and III.

Method	*Helicobacter* species	Apparently healthy cats	Apparently healthy dogs	Clinically healthy dogs	Dogs with upper gastrointestinal signs
TEM	Large spiral non-fibrillated bacteria only [1)]	6	2	10	3
	Fibrillated *H. felis* only	0	0	0	0
	Mixed [2)]	0	1	10	2
	Not detected	0	0	2	0
		(n=6)	(n=3)	(n= 22)	(n=5)
Culture	*H. bizzozeronii*	0	2	6	2
	H. felis	1	1	1	1
	H. salomonis	0	0	2	0
	Mixed [3)]	0	0	1	0
	Not isolated	4	5	15	3
		(n=6)	(n=8)	(n=25)	(n=6)

1) Morphology resembling that of *H. bizzozeronii*, *H. salomonis*, or non-fibrillated *H. felis*.
2) Large spiral bacteria and fibrillated *H. felis*.
3) *H. salomonis* and *H. felis*.

8.6. Effect of eradication therapy on gastric helicobacters, clinical signs and gastric histology (IV)

At study entry, all nine dogs with upper gastrointestinal signs were positive for gastric helicobacters, and with triple therapy, seven became negative. One dog remained positive after triple therapy but became negative after treatment with tetracycline and omeprazole. One dog was initially considered to be helicobacter-negative after triple therapy according to HE-stained histologic specimens, but was subsequently found to be positive by Giemsa staining; therefore, the alternative therapy with tetracycline and omeprazole for eradicating helicobacters was not instituted, and instead additional therapy was prescribed.

As for upper gastrointestinal signs, with eradication therapy these were alleviated significantly ($P<0.05$); with additional therapies (tylosin, sucralfate, cimetidine, prednisolone, or cisapride), clinical signs were alleviated significantly further ($P<0.01$) (Table 7). With triple therapy, clinical signs were alleviated clearly or slightly in 7/9 dogs,

78% (SE 13.9%), and with additional therapy, the upper gastrointestinal signs resolved totally in 7/8 dogs, 88% (SE 11.7%). Changes in clinical signs after triple therapy between dogs remaining helicobacter-positive (n=2) and helicobacter-negative dogs(n=7) did not differ significantly.

In gastric histology, no significant change was evident after triple therapy (Table 7). Mild chronic gastritis persisted after triple therapy in 3/6 dogs. In the three other dogs, additional eosinophilic activity disappeared, whereas chronic gastritis persisted in one dog but resolved in the other two. In two dogs showing eosinophilic activity alone on initial examination, eosinophilic activity persisted in one and disappeared in the other. One dog had initially normal histology but developed mild eosinophilic activity during the follow-up period. No significant changes were detected in gastric histology between the first visit and after additional therapy or between the first visit and after long-term follow-up.

Table 7. Changes in clinical signs and gastric histology after triple therapy and additional therapies shown as number of dogs per number of examined dogs.

Change	After triple therapy		After additional therapies	
	Clinical signs [1]	Gastric histology	Clinical signs when compared to those after triple therapy [2]	Gastric histology
Improved [3]	7 / 9	2 / 9	8 / 8	3 / 5
Unchanged	2 / 9	6 / 9	0 / 8	1 / 5
Worsened	0 / 9	1 / 9	0 / 8	1 / 5

1)Significant alleviation after triple therapy ($P<0.05$).
2)Significant alleviation after additional therapy ($P<0.01$).
3)In gastric histology, improved refers to decrease in any inflammatory cell type.

8.7. Recurrence of gastric helicobacters after eradication (IV)

In the eight dogs that became helicobacter-negative after eradication therapies, gastric helicobacters remained absent at the end of the first follow-up period, i.e., up to 7.5 months. In four of these dogs, the helicobacter status was verified only once after therapy, within 0.5 to 3 months. In all four dogs at the long-term follow-up, helicobacters had recurred, but these dogs showed none or only occasional mild upper gastrointestinal signs that were controlled with diets or short-term medication. In two long-term follow-up dogs, helicobacter status was considered “likely positive”, as organisms were detected only in brush cytology and were few in number with morphology too indistinct to allow reliable identification.

8.8. Transmission of gastric helicobacters (V)

Large, spiral urease-positive organisms, later identified as *H. salomonis*, were isolated from the biopsy sample taken from dam B (isolate BI) before her eradication therapy. Although the biopsy sample from dam A was positive in the urease test, and large spiral organisms were visible in the brush cytology mucus specimen, bacterial culture was unsuccessful. Seven weeks after cessation of treatment, at the end of the lactation period, the biopsy samples from the dams were urease-negative, and helicobacters were no longer detected by brush cytology. Three and a half months after the weaning, *H. salomonis* was isolated from both dams (isolates AI and BII). Two years later, *H. salomonis* was isolated from dam B but not from dam A.

The urease tests and brush cytology specimens were positive in all four challenged-group puppies, and the inoculated strain of *H. bizzozeronii* (CCUG 35045) was isolated 2 weeks and 3 and 7 months after the challenge. The biopsy samples from three puppies of the non-challenged group were unexpectedly urease-positive 2 months after the beginning of the experiment. Spiral organisms were isolated from three non-challenged puppies after 3 months from the beginning of the challenge and then during the entire sampling period. No helicobacters were isolated from the fecal samples of the dams or the puppies at any stage.

REA of chromosomal DNA of the isolates, digested either with *Hae*III or *Pst*I, revealed that all isolates from dam B and from the non-challenged puppies were highly similar, but differed from the patterns of two non-associated *H. bizzozeronii* strains and from those of *H. bizzozeronii* CCUG 35045. The patterns of dam A had some banding differences from those of dam B and the puppies. Ribopatterns of *Helicobacter* isolates from both dams and from the non-challenged puppies were all identical. For one puppy, banding patterns of two isolates originating from different sampling periods were identical. Non-associated *H. bizzozeronii* strains produced different banding patterns. In PFGE analysis, *Spe*I patterns of the isolates of both the dam B and the non-challenged puppies were identical, but the pattern of dam A differed by two fragments. Furthermore, *Not*I pattern analysis revealed that the isolate of dam A clearly differed from the isolates of dam B and those of non-challenged puppies, indicating that helicobacters of the puppies originated from dam B.

H. bizzozeronii CCUG 35045 and the isolates from challenged puppies were sensitive to metronidazole. The isolates from the dams and non-challenged group were metronidazole-resistant (MIC >15 mg/l with a agar dilution method), which may explain the failure of triple therapy to eradicate helicobacters of dam B, and subsequently, the transmission of helicobacters to her puppies.

9. DISCUSSION

The pet animals of the studies were carefully selected to fulfill strict criteria such as the depth and completeness of the clinical background and medication data. Although the number of animals remained quite small, it enabled a thorough and detailed analysis of numerous variables. The pet owners were often difficult to reassure about the need for repeated invasive endoscopic procedures. Complete studies could not be performed on cats because of limited patient material.

The reliability of diagnostic methods for detecting gastric helicobacters is a strong determinant of whether or not bacteria are found. Therefore, application of different methods of assessing helicobacter status had to be evaluated in order to allow study of the association between helicobacters and unspecified signs of gastritis often seen in dogs and cats. Such comparison between diagnostic methods for the detection of helicobacters in animals had not been performed earlier. The methods used were adaptations of diagnostic techniques for human *H. pylori.* The sample collection in these methods requires endoscopy, which is an invasive procedure, making the diagnosis somewhat difficult. Serologic methods, UBT, PCR, and in situ hybridization assays are effective methods for demonstrating human *H. pylori* (Li et al 1996, de Boer 1997), but they were not available for the present studies. Although culturing of canine and feline helicobacters has been difficult and often unrewarding, our research team has been successful in isolating new helicobacter species, *H. bizzozernonii* (Hänninen et al 1995 & 1996) and *H. salomonis* (Jalava et al 1997), for the first time.

Histologic criteria were important in study of the association between helicobacters and gastritis. Objective standards to categorize canine and feline gastritis were lacking, and therefore a visual analogue scale was created for histologic evaluation of gastric biopsy specimens during the present studies.

Considering that the diagnostic methods used for verifying the success of the helicobacter eradication were valid for this purpose, human eradication therapies seemed to be effective also in dogs. An experimental infection study enhanced the understanding of the routes of transmission.

9.1. Diagnostic methods for detecting gastric helicobacters

Of the methods studied - brush cytology, the urease test, histologic examination, TEM, and culture (I) - only brush cytology revealed gastric helicobacters with high reliability regardless of sample site; it can therefore be considered the method of choice for demonstrating helicobacters. It is easy to perform by endoscopy, and a relatively large area can be sampled. The result can also be obtained almost immediately if a rapid staining reagent (Romanovsky type stain) is used. Helicobacters are recognized easily from a brush cytology specimen if they are moderate to numerous in number, whereas it is time-

consuming to detect a small number of bacteria or to conclude that they are absent. The characteristic spiral morphology of organisms can be discerned, although species identification is impossible. Brush cytology can be used as a semiquantitative method for evaluating the number of helicobacters. Although this method is able to detect helicobacters at a very low density, the results do not always correlate with histology or the urease test. In the same animal, histologic examination may demonstrate a high number of helicobacters, and the urease test may produce a positive result quickly, indicating high number of organisms, whereas brush cytology shows only a few bacteria. The small number of helicobacters in brush cytology when the correct number is actually high, may be attributable to inconsistency in the quality of the mucus specimen obtained or the site from which the specimen was collected. Gastric contents may also influence this method if debris reaches the slide covering the bacteria. If, however, brush cytology testing is not performed, but only histologic examination and urease testing, helicobacters may be missed, if low in number. For this reason, some failure to detect helicobacter-positive cases after eradication may have occurred (IV). In some cases, brush cytology detected helicobacters when histologic examination and the urease test gave negative results (III, IV). False-positive results with brush cytology might be possible if helicobacters originate from contaminated instruments or endoscopes. Even if the instruments and equipment are cleaned and disinfected properly, dead bacteria may cause a false-positive result in brush cytology. In addition, mucus strings which look like bacteria may cause misinterpretation.

Hazell et al (1987) showed that the number of positive results in the urease test increased with the time during which the test reagent was applied, especially when the helicobacter count was low. The same was noted in Study I, because more results were positive that were obtained when the test was read at 60 min than at 30 min. When a high number of helicobacters were present, the urease test a gave positive result usually in 15 minutes (III). However, the urease test may give some false negative results when a low number of helicobacters are present, as in samples from the canine antrum (I). False-positive results are thought to be rare, but can occur if the test produces a positive result after 12 to 18 hours; in such instances, a positive result may be due to other urease-producing gastric bacteria such as *Proteus mirabilis* or *Pseudomonas aeruginosa*, although late-occurring color change may also be possible if the number of helicobacters is low (Goldie et al 1989). In general, however, for the detection of gastric helicobacters, the urease test can be considered fairly reliable.

The advantage of histologic examination over brush cytology and the urease test is that the location of helicobacters in the gastric glands and parietal cells, and the presence of inflammatory cells as well as tissue changes, can also be evaluated. Its disadvantage is the length of time needed to obtain results. The mucus overlying the epithelium and consequently any superficial helicobacters may be easily wiped away if biopsy specimens are handled and processed carelessly. This was taken into account when the colonization density of helicobacters was graded in Study III by grading moderate colonization density

in two separate ways (Table 3). In the present studies, gastric helicobacters could be recognized histologically in HE-stained sections in most cases. The Warthin-Starry technique has been described as the most satisfactory staining method if one is searching only for spiral organisms (Stevens 1990). Giemsa staining may visualize helicobacters better than HE staining. However, when HE-stained specimens negative for helicobacters were stained with Warthin-Starry (I) or Giemsa (IV), both of these staining methods succeeded on only one occasion in revealing helicobacters where HE staining had failed. New sections were cut for other staining methods in these studies, and it may have been possible that gastric helicobacters were uncovered when more sections were cut from a paraffin block, since one section is approximately as thick as the cross-section of a helicobacter, a fact that needs to be considered in cases where HE-stained specimens fail to reveal bacteria. Therefore, to be assured that no helicobacters are present in the histologic gastric biopsy specimen, the whole paraffin block should be sectioned and evaluated, which is, however, time-consuming and impractical.

Spiral organisms were seen by TEM in all canine and feline specimens examined, suggesting that their number was high. Gastric helicobacters are, however, not always seen in a TEM specimen, since their distribution in the stomach may be patchy, and the sample is small and thin (McNulty et al 1989a). According to present findings, TEM is most rewarding to perform on urease-positive cases which indicate a high number of bacteria. The typical morphology of the bacteria, readily seen under the electron microscope, can be used for purposes of preliminary identification (Utriainen et al 1997). *H. felis* is easy to identify due to its periplasmic fibrils. However, although atypical non-fibrillated *H. felis* has recently been described (Eaton et al 1996), but it has not been isolated in Finland (Jalava et al 1998). Atypical *H. felis*, and *H. bizzozeronii*, *H. salomonis*, and human *"H. heilmannii"* are indistinguishable from each other by TEM because of similar morphologic features, but some conclusions may be made on the basis of the size of the bacterium. The most common helicobacter identified here in TEM specimens was a large spiral bacterium without periplasmic fibrils both in dogs and cats, and mixed infections with large spiral bacterium and *H. felis* in dogs.

The results of Studies I-III confirmed that culture of canine and feline gastric helicobacters is demanding and often unsuccessful. Fasting of the animal is crucial to decrease the number of contaminating organisms, and a fresh biopsy specimen, freshly prepared moist media, a long incubation period, and high atmospheric humidity are necessary to optimize bacterial growth (Jalava et al 1998). Despite that fact, culture and identification of a number of helicobacters was successful, in up to 50% of dogs and 17% of cats (I-III), in contrast to the meager results of other studies (Weber and Scmittdiel 1962, Henry et al 1987, Eaton et al 1996, Papasouliotis et al 1997, Neiger et al 1998). Culture is useful because isolates, if obtained, can be accurately characterized. *H. bizzozeronii* with or without *H. felis* was the most common species both in clinically healthy and affected dogs (III). It was surprising that *H. felis* was diagnosed in only one cat by culture (II)

because it has been reported to be a common finding in cats (Lee et al 1988). Mixed infections with *H. felis* and other *Helicobacter* species are also common in cats (Lee et al 1988) as well as in dogs (Lockard and Boler 1970), which in the case of dogs agrees with present results. The true number of mixed infections may have been even higher than detected, because TEM and culture may detect one species but fail to detect others. TEM and culture may also identify different species, indicating mixed infections, although only one species is detected by each of these methods.

9.2. Prevalence and colonization density

The high prevalence of gastric helicobacters, 95 to 100% in dogs and 60% in cats, confirmed the results from other studies (Weber et al 1958, Henry et al 1987, Eaton et al 1996, Yamasaki et al 1998). High prevalence rates are suspected to appear in animals living in colonies (Weber et al 1958, Henry et al 1987, Eaton et al 1996); for instance, all the beagle dogs that were kept in one laboratory environment had helicobacters (Henry et al 1987). However, lower prevalences have also been reported (Geyer et al 1993, Otto et al 1994, Hermanns et al 1995). The size of the biopsy specimen may have an influence on the prevalence rates as well. It may be more difficult to detect helicobacters in an endoscopically obtained pinch biopsy specimen than in a large, full-thickness biopsy, even though biopsy size did not affect the present results. Prevalence of the organisms may vary geographically. All dogs in the present studies were from urban areas of southern Finland, where the density of dogs is higher than in northern Finland. Consequently, this could have resulted in more contacts among these dogs, leading to enhanced transmission.

Limitations of the earlier studies may have included the method of selection of the dogs, such as obscure clinical history or experimental status of the animals, or the medications administered to them. All dogs in Study III were pets originally obtained from breeders, and the clinical and medical history of each dog was known. Therefore, the effects of drugs on the prevalence of helicobacters and on gastric histology could be better judged.

The similar prevalence rate and colonization density of gastric helicobacters both in healthy and affected dogs (III) suggests that these bacteria may be a part of the normal gastric flora. The prevalence rate was also equal in dogs of all ages and in both genders, and no association was detected between age and colonization density of helicobacters. Previous studies have mostly involved adult dogs of various ages. However, in cats, helicobacters have been found more often in adult cats than in kittens (Weber et al 1958, Otto et al 1994).

9.3. Topography

To verify the presence of gastric helicobacters, a biopsy sample from the corpus, fundus, or cardia proved to be reliable, because helicobacters appeared most frequently and were the highest in number in these three gastric regions. Thus, it was concluded that in clinical practice one sample from gastric regions other than the antrum (including the pylorus) may be sufficient to demonstrate the presence of gastric helicobacters, contrary to earlier reports of experimental infection studies. When gnotobiotic beagle dogs were infected with *H. pylori*, most of the bacteria were found in the fundus (Radin et al 1990), whereas when they were infected with *H. felis*, the heaviest colonization was detected in the fundus and antrum (Lee et al 1992b). Handt et al (1994) demonstrated *H. pylori* mostly in the corpus and antrum in cats. However, the terminology describing the anatomic regions of the canine and feline stomach may vary between studies, making accurate comparison of results difficult. Different results may also be attributable to the particular *Helicobacter* species used in the studies and to the origin of the dogs. The animals in the present studies were not gnotobiotic but had presumably normal gastric flora, a fact which may have affected the results. In this respect, the present results are likely to be clinically more relevant than those of the experimental studies. The distribution of helicobacters in dogs differs somewhat from that of human *H. pylori*, which predominantly colonizes the antrum (Genta and Graham 1994).

The location of gastric helicobacters on the mucosal surface and in gastric pits as well as in gastric glands and in parietal cells was consistent with earlier reports on dogs and cats (Weber et al 1958, Henry et al 1987, Geyer et al 1993, Hermanns et al 1995, Eaton et al 1996, Yamasaki et al 1998). This is in contrast to human *H. pylori*, which is located mainly on the mucosal surface and in gastric pits (Thomsen et al 1990), although one paper reports *H. pylori* also in parietal cells (Chen et al 1986). In a colony of cats, *H. pylori* was located only in the superficial mucus layer and in the gastric pits, whereas in other cats having different *Helicobacter* species, the organisms were seen also in parietal cells (Handt et al 1994). Thus, it seems that canine and feline helicobacters may have greater affinity to parietal cells than does *H. pylori* in man; this could be related to the predominance of the bacteria observed in gastric regions other than the antrum of dogs and cats.

9.4. Helicobacters and gastritis

In order to perform a reliable histologic assessment, high-quality biopsy specimens are required; they must be of adequate size and depth, properly oriented, and free of artifacts such as the crushing and stretching that distort gastric biopsy specimens. Artifacts can be avoided by the use of proper biopsy forceps and biopsy techniques. Overinflation of the stomach will flatten gastric folds and result in samples too small and superficial (Tams 1990). In contrast to other gastric regions, the antrum is difficult to

sample, and therefore, unless macroscopic changes are visible, some researchers do not routinely biopsy the antrum (Tams 1990). A great number of antral biopsy specimens had to be excluded also in the present studies because of poor quality.

Gastric inflammation was a common finding in histologic specimens in dogs and cats, and mononuclear cells were more common than lymphocyte aggregates, neutrophils, or eosinophils. In the prevalence rates and densities of inflammatory parameters between gastric regions, some discrepancies occurred in dogs: in Study II, the antrum was more inflamed with plasma cells than was the corpus, whereas in Study III, mononuclear cells were distributed quite evenly throughout the stomach. These discrepancies may have been attributable to different criteria for histologic assessment. Neutrophils seem not to play as important a role in canine and feline gastritis as they do in *H. pylori* gastritis in man (Fox et al 1991a, Handt et al 1994). In dogs, there was no positive correlation between degree of gastric inflammation and colonization density of helicobacters, in agreement with some reports (Eaton et al 1996) but in conflict with others (Henry et al 1987, Yamasaki et al 1998). On the contrary, more lymphocytes were detected in helicobacter-positive cats having numerous helicobacters than in negative cats (II). In addition, lymphocyte aggregates were seen only in positive cats, indicating that in cats helicobacters and gastric inflammation may have some connection.

Criteria for histologic evaluation always affect the results. Since generally accepted objective criteria do not exist for dogs and cats, researchers have usually created their own criteria (Handt et al 1994, Eaton et al 1996), or they have used the Sydney system developed for categorizing human gastritis (Geyer et al 1993, Yamasaki 1998). In Study II, the modified Sydney system was applied for histologic diagnoses, and the results indicated that asymptomatic mild chronic gastritis is common in apparently healthy dogs and cats suggesting that the criteria used to diagnose human gastritis may not be suitable for these animals. For this reason and also because the biopsy sample was changed from full-thickness biopsy to endoscopic biopsy, the grading system in Studies III and IV was changed. A modified visual analogue scale from the updated Sydney system in humans (Dixon et al 1996) was developed because exact counting of each variable is laborious to perform routinely, and difficult in gastric biopsy specimens, most of which are heterogenous in appearance and often contain artefacts. More inflammatory cells were allowed in normal gastric histology, because seldom, if ever, is a gastric specimen without inflammatory cells. The importance of lymphocyte aggregates is debatable in dogs and cats; they have been speculated to be markers of gastric inflammation, but one report considers them a normal finding (Kolbjørnsen et al 1994). On the other hand, in humans, lymphoid follicles are linked with *H. pylori*-associated gastritis (Genta et al 1993a, Zaitoun 1995). Because no data were available on the significance of intraepithelial inflammatory cells, they were considered only when their number was clearly increased.

The new criteria, although modified to be less strict, still showed that mild chronic gastritis seems to be a common histologic diagnosis that is not associated with clinical

signs. It was surprising that many clinically healthy dogs had histologically verified mild to severe chronic gastritis, whereas many dogs with upper gastrointestinal signs had none or only mild gastritis. Most of the affected dogs had suffered from upper gastrointestinal signs over a long period before referral to endoscopy, and many had been treated with antibiotics and gastric protectants such as cimetidine and sucralphate more than two weeks prior to endoscopy. Therefore, in some affected dogs with mild chronic gastritis, previous medications may have played some role in histologic changes, although the effects of these drugs on gastric histology have not been reported in the literature. These findings raise concern about clinical interpretation of histologically verified chronic gastritis. Medication may also have reduced the number of gastric helicobacters, which could explain the mild colonization density of the bacteria in some affected dogs.

In addition to gastritis, degenerative lesions of parietal cells in conjunction with helicobacters have been found not only in affected but also in clinically healthy animals, as was demonstrated also in the present studies (II, III). These lesions have been supposed to indicate the pathogenicity of gastric helicobacters (Weber et al 1958, Henry et al 1987, Geyer et al 1993, Hermanns et al 1995), but it is unresolved whether helicobacters colonize degenerating parietal cells or induce their degeneration. Previous studies have shown an association between gastric helicobacters and chronic gastritis not only in cats but also in dogs (Lee et al 1992, Geyer et al 1993, Handt et al 1994, Hermanns et al 1995); however, this could not be established in the present studies. That the colonization density of gastric helicobacters showed no correlation with histologic changes suggests that helicobacters alone may not be responsible for those changes. Another explanation may be that different *Helicobacter* species or strains may differ in pathogenicity.

Only negative correlations were evident between the colonization density of helicobacters and the number of some inflammatory variables (mononuclear cells and neutrophils) in clinically healthy and affected dogs, but the mechanism and significance of this phenomenon remained unclear. Severe gastritis may decrease acid secretion, and consequently the number of bacteria; the small number of helicobacters may also be explained by destruction of bacteria due to an immune response induced by gastritis. Gastric pH measurement should be addressed in future studies to verify these hypotheses.

Because there were no differences between healthy and affected dogs in the prevalence rate or colonization density of gastric helicobacters or any positive association between colonization density and number of inflammatory variables, it would appear that helicobacters may be part of the normal gastric flora. However, the host response must also be considered, because specific dogs may react differently to bacterial flora. Large variation in severity of gastric inflammation has been reported in inbred and congenic mouse strains infected with *H. felis* (Mohammadi et al 1996), indicating that the genetic background of the host may play an important role in the clinical outcome of *Helicobacter* infections. In addition, because it was impossible to find a helicobacter-negative control group of dogs, conclusions could not be drawn regarding helicobacters' capability to

induce gastritis. Observed from the histologic aspect only, the clinically healthy status of an animal does not indicate whether or not an organism is pathogenic or part of the normal flora. Histologic criteria may need to be adjusted when the pathogenicity of these organisms has been resolved. Further studies are required to elucidate the role of the host as well as to clarify the significance of gastric helicobacters.

Mixed infections are common in dogs (Lockard and Boler 1970, Lee et al 1988), which agrees with results of Study III. *H. bizzozeronii* with or without *H. felis* was the most common species both in clinically healthy and in affected dogs; *H. salomonis* was identified only in a few cases. Successful isolation of a number of *Helicobacter* species made it possible to study the association between the number of species and degree of gastric inflammation, in order to resolve the possibility that one species might cause less inflammation than several species. The number of individual species isolations was too small to allow evaluation of any association between *Helicobacter* species and inflammation, an evaluation which could have provided more information on the possible pathogenicity of different species. Still, that no association could be demonstrated between number of *Helicobacter* species and degree of inflammation, may suggest that the *Helicobacter* species in the present studies seem to have been nonpathogenic. However, further studies are needed to establish the pathogenicity of various *Helicobacter* species and strains. For this purpose, successful laboratory culture of these organisms is needed, to enable the determination of biochemical, serological, molecular, and pathogenic features of the isolates.

9.5. Eradication

The follow-up study (IV) showed that the triple therapy used in human medicine to eradicate *H. pylori* seems also to be effective for eradicating canine gastric helicobacters. However, the result might have been less obvious if some uncontrolled variables, such as the time period between control endoscopies, could have been eliminated, or had more sensitive methods, such as the UBT or PCR assay (Li et al 1996, de Boer 1997), been used. These two methods are not used routinely in veterinary medicine at present, nor do they have 100% specificity or sensitivity. The use of more than one diagnostic method increases the sensitivity of helicobacter detection (van der Ende et al 1997). A ten-day duration of treatment appears to be long enough to eradicate gastric helicobacters. In humans, *H. pylori* status is usually evaluated four weeks after cessation of therapy, during which time recrudescence of the bacteria is likely to occur (van der Ende et al 1997). If this applies also to dogs, some of the control endoscopies may have been performed too soon after triple therapy (IV).

Metronidazole resistance may explain the eradication failure, a fact supported by the finding that one of the dogs that remained helicobacter-positive after triple therapy became negative with tetracycline and omeprazole (IV). Metronidazole resistance in *H. pylori* has

been reported (Glupczynski et al 1990, Bell et al 1991, Seppälä et al 1992, Logan et al 1993) and it seems to occur also in canine gastric helicobacters, as was shown in the transmission study (V). Only a limited number of *H. bizzozeronii* or *H. salomonis* isolates have been studied thus far, but they all have been sensitive to metronidazole (Hänninen et al 1996). In humans, about 20% of *H. pylori* isolates are resistant to metronidazole (Noach et al 1994). Molecular typing of metronidazole-resistant *H. pylori* strains in one study was identical in most cases before and after the unsuccessful therapy (Owen et al 1993). That metronidazole is used for treating gastrointestinal disorders and anaerobic infections in veterinary medicine may explain the resistance of the bacteria.

The general treatment protocol for *H. pylori* in humans has changed slightly since the beginning of the follow-up study (IV) five years ago. Instead of the bismuth subcitrate, amoxicillin, and metronidazole, the first-choice treatment today consists of a proton-pump inhibitor (e.g., omeprazole) together with amoxicillin and either nitroimidazole derivatives (metronidazole or tinidazole) or clarithromycin (European Helicobacter Pylori Study Group 1997). If triple therapy fails, the second-choice therapy is a proton-pump inhibitor and bismuth subcitrate with tetracycline or amoxicillin and metronidazole. In future, it may be worthwhile to test the eradication of canine gastric helicobacters according to these recommendations. Other drugs may also induce suppression of gastric helicobacters without eradicating them, drugs such as tylosin (personal experience) and sucralfate (Louw et al 1992).

Although triple therapy alleviated upper gastrointestinal signs to some extent, it failed to resolve the signs totally in any dog. Neither was there any difference in clinical response to triple therapy between negative dogs and dogs that remained positive. On the contrary, clinical signs subsided to a great extent with other treatments after gastric helicobacters already had been eradicated in most dogs. This is in contrast to humans, in which clinical signs caused by *H. pylori*-induced peptic gastric and duodenal ulcers resolve after eradication of *H. pylori* (Labenz and Börsc 1994), but it is still controversial what role *H. pylori* plays in non-ulcer dyspepsia (Talley and Hunt 1997). The results of triple therapy in the present study should, however, be interpreted cautiously because of the limited number of cases, i.e., seven dogs became negative and two remained positive with this therapy, and consequently, further studies are needed to draw final conclusions in this matter. The reason that the triple therapy had an alleviating effect on the clinical signs in dogs remained unclear. Although the primary causes of clinical signs may remain obscure, one or more of the three components in this therapy would be likely to alleviate clinical signs at least to some degree. The fact that the additional therapies prescribed, although not known to affect helicobacters, did control clinical signs in dogs better than did triple therapy did not support the view of gastric helicobacters being alone responsible for their upper gastrointestinal signs. A placebo control group might have been of value when the eradication therapy was assessed, but was not included in Study IV because of the pet owners' reluctance. Due to some diversity in clinical signs, problems originating from the

small intestine should also be considered as a possible cause for such clinical signs, although obvious intestinal problems were ruled out by performance of routine hematologic and biochemical analyses, and serum folate and cobalamine measurement; fecal flotation for parasites, and abdominal radiographs.

Histologically verified *H. pylori*-induced gastric inflammation in humans clearly decreases within one year of eradication of the bacteria (Valle et al 1991, Genta et al 1993a, Witteman et al 1995). Mild chronic gastritis, the most common histologic diagnosis in the dogs studied, persisted at almost the same level after eradication therapies as well as after other treatments, and also during the long-term follow-up period. The time-period between treatments and endoscopy may have affected the histologic diagnoses, because inflammatory changes might have persisted for a longer period than assumed after triple therapy. However, no published data are available on how quickly inflammatory changes disappear after eradication therapy in dogs or whether they resolve at all, and this matter remained unresolved here as well. Chronic gastritis may also have persisted because the eradication therapy had merely suppressed gastric helicobacters - assuming that the bacteria had been solely responsible for the inflammation in the first place. The reason for the eosinophilic activity in dogs with upper gastrointestinal signs remained obscure. In humans, eosinophilic infiltration is suggested to be associated with *H. pylori* gastritis (McGovern et al 1991), whereas any association between eosinophils and canine gastric helicobacters needs clarification. Dietary hypersensitivity and internal parasites may increase the number of eosinophils in the gastric mucosa, but since these conditions were ruled out at study entry, it is unlikely that they would have been responsible.

Gastric helicobacters appear to recur after eradication treatment. Reinfection in the case of successful eradication and recrudescence in the case of mere suppression are impossible to distinguish without identification of *Helicobacter* species pre- and post-treatment and similarity-testing of the isolates (van der Ende et al 1997). Culture of canine gastric helicobacters is difficult, and even if isolation succeeds, the high frequency of mixed infections in dogs may cause misinterpretation if only one species is identified. All the dogs that attended the long-term follow-up had become helicobacter-positive (IV), and had contacts with either another dog in the household or other dogs; this fact supports the transmission of helicobacters via the fecal-oral and/or oral-oral routes (Mendall 1997). Although the helicobacter status of the contact dogs was unknown, the likelihood for their being positive was strong because of the high prevalence of these organisms in dogs in general (Weber et al 1958, Henry et al 1987, Eaton et al 1996, Yamasaki et al 1998). Reinfection was most likely the cause of reappearance of gastric helicobacters in the dogs of the follow-up study because recrudescence of *H. pylori* occurs in most cases within four weeks after cessation of therapy (van der Ende et al 1997), but the bacteria in these dogs remained absent until the end of the first follow-up period, which ranged from one to twelve months (IV). However, triple therapy in some dogs may have merely suppressed gastric helicobacters to undetectable levels, in which case recurrence is more or less

inevitable. In humans, recurrence of *H. pylori* infection is low (3.4%) after successful eradication with triple therapy, and when it takes place, it appears to occur most commonly within the first year after treatment (Cutler and Schubert 1993).

9.6. Transmission

Early natural transmission of the gastric helicobacters in dogs either from the dams or among puppies received support in Study V. In humans, several studies support the acquisition of *H. pylori* infection early in life. A high level of positive sera (16.5-74%) is found among children under two years of age in developing countries (Mégraud et al 1989, Klein et al 1994, Oliveira et al 1994), whereas in many developed countries, children less than six years old often do not have antibodies to *H. pylori* (Kontiainen et al 1994). Correlations have been reported between colonization and age, low socioeconomic status, and overcrowding (Webb et al 1994). Although the infection route was not resolved, oral-oral transmission is likely because close nursing contact between dams and puppies during the lactation period will inevitably predispose puppies to the gastrointestinal infectious agents of their dams. Helicobacters could not be isolated from the fecal samples either of the dams nor of the puppies by the method used. Any transmission via personnel was prevented by strict hygiene, and via endoscopic equipment by thorough washing and disinfecting with glutaraldedyde, which has been shown to be effective in preventing cross-transmission of *H. pylori*; disinfection time should be at least 20 minutes in manual cleaning (Akamatsu et al 1996, Cronmiller et al 1999). In addition, the puppies had no contacts with other dogs.

Triple therapy seemed only to suppress rather than eradicate the infection of the dams. However, the number of helicobacters decreased to the level at which the urease test and brush cytology were negative after the treatment. The reason for unsuccessful treatment can be explained by the metronidazole-resistance of the isolates. Identical ribotyping and similar PFGE patterns of the isolates cultured from dam B before therapy, five months after therapy, and two years later (unpublished results) were suggestive for permanent infection of that dam with the same metronidazole-resistant strain of *Helicobacter* species. The infection level of dam A was considered lower than that of dam B because during the study, the urease test of the biopsy sample from dam A was either negative or weakly positive, and culture unsuccessful.

Although two of the puppies in the non-challenged group were born from dam A and two from dam B, the three *H. salomonis*-positive ones had identical *Helicobacter* genotypes confirmed by ribotyping and PFGE pattern analysis. At the beginning of the experiment, the animals in the challenged group were probably also infected with an *H. salomonis* strain originating from the dams, but a heavy experimental infection with *H. bizzozeronii* CCUG 35045 masked and suppressed the original infection. PFGE analysis revealed that the puppies of the non-challenged group were infected by the isolate from

dam B and not from dam A. Most probably, dam B had infected two of her puppies during the lactation period, and her puppies further infected the puppies of dam A in the group during the experiment.

Ribotyping of the present isolates revealed that the patterns of dams and puppies in the nonchallenged group were identical and differed from the patterns of unrelated strains. However, PFGE analysis was shown to be a better method for distinguishing between isolates than ribotyping, because it was able to distinguish the isolates of dam B from the isolate of dam A. Similarly, REA analysis suggested that the isolates of the dams were highly related but not identical. General genetic diversity of *H. bizzozeronii* and *H. salomonis* (Finnish strains) have been studied, showing that unrelated strains produce different ribo- and PFGE patterns (unpublished results), a finding supported by the present data.

9.7. Discussion summary

Despite these thorough studies, an unambiguous answer whether or not canine or feline gastric helicobacters are significant cannot be given, i.e., whether or not they may induce changes in gastric histology and signs of gastritis. However, several important factors were clarified: the high prevalence of the bacteria both in healthy and affected dogs as well as in healthy cats, the usefulness of routine diagnostic methods, and the efficacy of human triple therapy for eradicating helicobacters. The transmission of helicobacters was also elucidated. Further studies are needed to resolve the role of gastric helicobacters, for instance, placebo-controlled therapy trials and verification of the results by more sensitive methods. A detailed characterization of *Helicobacter* species and strains is also needed to establish their pathogenicity. The newly established zoonotic potential of *H. bizzozeronii* raises concerns in public health which should be dealt with.

10. CONCLUSIONS

1. Brush cytology was the method of choice for detecting gastric helicobacters. The urease test and histological examination were also of value. TEM produced positive results in the majority of cases; culture gave less positive results. Although the two latter methods are more demanding, they are needed to identify different *Helicobacter* species. Consequently, TEM and culture may assume greater importance if future studies show that different species or strains of canine and feline helicobacters differ in pathogenicity.

2. Gastric helicobacters were common inhabitants of the stomachs of dogs, with their prevalence varying from 95% in dogs with signs of gastritis to 100% in healthy dogs. Colonization density of helicobacters is also frequently high in both healthy dogs and affected ones. Age of dogs was not associated with colonization density. Helicobacters proved to be common also in cats but to a lesser extent (60%) than in dogs.

3. Gastric helicobacters can be found in all regions of the stomach, although less frequently and in the smallest quantity in the antrum of dogs. In cats, such regional differences seem not to be evident. Only one sample from the cardia, fundus, or corpus seems to be sufficient to demonstrate the presence of helicobacters in dogs and cats. Canine and feline helicobacters are located from the surface mucus down to the gastric pits and parietal cells.

4. Histologically verified chronic gastritis was very common in dogs, both in clinically healthy dogs (48%) and in dogs with upper gastrointestinal signs (81%). The colonization density of helicobacters could not be positively connected with inflammation of the gastric mucosa in either group. However, in cats, gastric helicobacters were associated with changes comparable to those of chronic gastritis. In dogs, a cause-and-effect relationship between gastric histology and clinical signs remained unclear, because all healthy dogs and the majority of affected dogs were helicobacter-positive, but more of the affected dogs had histologically verified chronic gastritis. It still remains to be demonstrated whether certain *Helicobacter* strains have specific virulence factors that may induce gastritis in dogs and in cats, and whether any host-associated factors play a role during development of gastritis. The histologic criteria for human gastritis as such seem not to be suitable for dogs and cats, whereas the modified visual analogue scale proved to be a satisfactory method.

5. Rough identification of *Helicobacter* species according to their morphologic features by TEM included *H. felis* with periplasmic fibrils, and large spiral non-fibrillated bacteria with a morphology resembling either that of *H. bizzozeronii*, *H. salomonis*, or non-fibrillated *H. felis*. Species accurately identified by culture comprised *H. bizzozeronii*, *H.*

felis, and *H. salomonis*. *H. pylori* was not detected in any dog or cat. The number of *Helicobacter* species seemed to have had no effect on degree of gastric inflammation.

6. Human triple therapy consisting of amoxicillin, metronidazole, and bismuth subcitrate appeared to be generally effective in eradicating canine gastric helicobacters. The alternative drugs tetracycline and omeprazole seemed also to be effective. Helicobacters do not appear to be a major etiologic factor in canine upper gastrointestinal disorders, since it was not demonstrated that these organisms alone were responsible for the clinical signs or gastric histologic changes. Helicobacters seemed also to recur after eradication, either because of reinfection or of recrudescence, without inducing clinical signs. Therefore, routine therapy for eradicating canine helicobacters appears not to be warranted or cost-effective until further evidence emerges of their pathogenicity.

7. Canine gastric helicobacters were acquired as early as during the lactation period, and the dam was the reservoir in the transmission. Puppies also infected each other. The infection route, however, whether oral-oral or fecal-oral, remains to be resolved. The transmission and persistence of the infecting strain could be demonstrated by molecular methods. Metronidazole-resistence may explain the persistence of helicobacters despite the eradication therapy. PFGE analysis seems to be better than ribotyping in distinguishing the similarity of the isolates.

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