การศึกษาอุบัติการณ์การติดเชื้อแบคทีเรีย *Helicobacter pylori* ด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส และความสัมพันธ์ของอาการทางคลินิกกับการติดเชื้อแบคทีเรีย *H. pylori* ในกระเพาะอาหารสุนัข

นายวรพัฒน์ ประชาศิลป์ชัย

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STUDIES ON THE INCIDENCE OF HELICOBACTER PYLORI INFECTIONS BY POLYMERASE CHAIN REACTIONS AND THE RELATIONSHIP OF CLINICAL SIGNS OF H. PYLORI INFECTIONS IN CANINE STOMACHS

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วรพัฒน์ ประชาศิลป์ชัย: การศึกษาอุบัติการณ์การติดเชื้อแบคทีเรีย *Helicobacter pylori* ด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส และความสัมพันธ์ของอาการทางคลินิกกับการติดเชื้อแบคทีเรีย *H. pylori* ในกระเพาะอาหารสุนัข อ. ที่ปรึกษา: อัจฉริยา ไศละสูต อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศุภชัย เนื้อนวลสุวรรณ จำนวนหน้า 70 หน้า. ISBN 974-17-6151-1

ศึกษาการติดเชื้อ *Helicobacter pylori* ในกระเพาะอาหารสุนัขจำนวน 75 ตัวอย่าง สุ่มเก็บตัวอย่างกระเพาะอาหารที่ตำแหน่ง cardia fundus body และ pyloric antrum ของสุนัขที่ส่งชันสูตรซาก ณ ภาควิชาพยาธิวิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ระหว่างเดือนเมษายน 2546 ถึง มิถุนายน 2547 จุดประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาอุบัติการณ์การติดเชื้อแบคทีเรีย *H. pylori* ในกระเพาะอาหารสุนัขด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส เปรียบเทียบกับการศึกษาทางพยาธิวิทยาด้วยวิธีย้อมสีพิเศษและอิมมูโนฮีสโตเคมี และศึกษาหาความสัมพันธ์ของอาการทางคลินิกในสุนัขที่ติดเชื้อแบคทีเรีย *H. pylori* ในกระเพาะอาหารสุนัข ผลการศึกษาการเปลี่ยนแปลงทางจุลพยาธิวิทยาของเยื่อบุกระเพาะอาหาร 60.0 % (45/75) ประกอบด้วยเยื่อบุกระเพาะอักเสบระดับอ่อน 64.44 % (29/45) ระดับปานกลาง 11.11 % (5/45) และ ระดับรุนแรง 24.44 % (11/45) ไม่พบการเปลี่ยนแปลงทางจุลพยาธิวิทยาของเยื่อบุกระเพาะอาหาร 40.0 % (30/75) ศึกษาอุบัติการณ์การตรวจพบ *Helicobacter spp.* ด้วยการย้อม H&E พบ 17.33 % (13/75) ย้อมสีพิเศษ Warthin Starry stain พบ 46.67 % (35/75) และตำแหน่งการกระจายของเชื้อ พบที่ luminal crypt 18.67 % (14/75) gastric pit 22.67 % (17/75) gatric gland 21.33 % (16/75) และ gastric epithelium 8 % (6/75) ตามลำดับ ย้อมด้วยเทคนิคอิมมูนโนฮิสโตเคมีด้วย rabbit polyclonal anti-*H. pylori* แอนติบอดีพบ 30.67 % (23/75) และปฏิกิริยาลูกโซ่โพลีเมอเรส พบ 10.67 % (8/75) จากการศึกษาครั้งนี้ไม่พบการติดเชื้อแบคทีเรีย *H. pylori* ในเนื้อเยื่อกระเพาะอาหารสุนัข ไม่พบความแตกต่างของการเปลี่ยนแปลงทางจุลพยาธิวิทยาของกระเพาะอาหารในแต่ละบริเวณ ($p>0.05$) ทำการเปรียบเทียบการติดเชื้อแบคทีเรีย *Helicobacter spp.* ในกระเพาะอาหารสุนัขด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรสกับการศึกษาทางพยาธิวิทยาด้วยวิธีย้อมสีพิเศษ WSS และอิมมูโนฮีสโตเคมีพบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p>0.05$) ศึกษาหาความสัมพันธ์ของการติดเชื้อแบคทีเรีย *Helicobacter spp.* ในกระเพาะอาหารสุนัขด้วยวิธีทางพยาธิวิทยาด้วยการย้อมสีพิเศษ WSS อิมมูโนฮีสโตเคมี และปฏิกิริยาลูกโซ่โพลีเมอเรส กับอาการทางคลินิกในสุนัขที่ติดเชื้อแบคทีเรีย *Helicobacter spp.* ในกระเพาะอาหารสุนัขพบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p>0.05$) ทำการเปรียบเทียบการตรวจพบ *Helicobacter spp.* ด้วยวิธี H&E และย้อมสีพิเศษ WSS พบว่ามีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p<0.001$)

ภาควิชา.....พยาธิวิทยา.....ลายมือชื่อนิสิต.....

สาขาวิชา.....พยาธิวิทยาทางสัตวแพทย์.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2547.....ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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WORAPAT PRACHASILCHAI : THESIS TITLE. (STUDIES ON THE INCIDENCE OF HELICOBACTER PYLORI INFECTIONS BY POLYMERASE CHAIN REACTIONS AND THE RELATIONSHIP OF CLINICAL SIGNS OF H. PYLORI INFECTIONS IN CANINE STOMACHS) THESIS ADVISOR : ACHARIYA SAILASUTA, THESIS COADVISOR : SUPHACHAI NUANUALSUWAN, PAGES 70 pp. ISBN 974-17-6151-1

A study on *Helicobacter pylori* infection in canine stomach was performed. A total of 75 biopsied samples of cardia, fundus, body and pyloric antrum from necropsied dogs, submitted to the Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, during April, 2003 to June, 2004, were investigated. The objective of this study was focused on the prevalence of *H. pylori* in canine stomach by polymerase chain reaction (PCR) in comparison to histochemistry and immunohistochemistry (IHC) as well as relationship between clinical significance of and gastric *H. pylori* infection. The histopathological results revealed 60.0 % (45/75) as mild gastritis 64.44 % (29/45), moderated gastritis 11.11 % (5/45) and severe gastric 24.44 % (11/45) and no histopathological lesions as 40.0 % (30/75). The presence of *Helicobacter spp*. using Hematoxylin & Eosin (H&E) staining, showed positive results at 17.33 % (13/75), Warthin Starry staining (WSS), 46.67 % (35/75) and the *Helicobacter spp*. was localized in the luminal crypt 18.67 % (14/75), gastric pit 22.67 % (17/75), the gastric gland 21.33 % (16/75), and the gastric epithelium 8 % (6/75), by IHC with rabbit polyclonal anti-*H. pylori* antibody 30.67 % (23/75) and by PCR 10.67 % (8/75), but none of *H. pylori* could be detected in all samples. There was no significantly different in histopathological changes in any part of the stomach ($p>0.05$). The *H. pylori* diagnosis by polymerase chain reaction (PCR) in comparison to WSS and IHC was not significantly different ($p>0.05$). There were no relationship between pathological study by H&E, WSS and IHC, especially PCR and clinical signs of *H. pylori* infections in canine stomachs ($p>0.05$). The present study revealed significantly different correlations for *Helicobacter spp.* detection between H&E and WSS ($p<0.001$).

Department……Pathology…….Student signature…….

Field of study……Veterinary Pathology……..Advisor's signature……..

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Abbreviations

A	ampare
Bp	base pairs
^{o}C	degree celsius (centrigate)
Cag	cytotoxin associated gene
CFU	colony forming unit
CU-VDL	Veterinary Diagnostic Laboratory, Chulalongkorn University
DF	degree of freedom
DG-DGGE	double gradient-denaturating gradient gel electrophoresis
DNA	deoxyribonucleic acid
DW	distilled water
ELISA	enzyme-linked immunosorbant assay
et al.	et alii, and others
FRET	fluorescence resonance energy transfer
g	gram (s)
GI	gastrointestine

h	hour (s)
H&E	hematoxylin & eosin
HLO	*Helicobacter*-like organisms
Ig	Immunoglobulin
IHC	Immunohistochemistry
kb	kilobase pair
kd	kilodalton
Mab	Monoclonal antibody
min	minute (s)
ml	milliliter (s)
mm^3	millimetre (s)
n	number of sample (s)
µl	microlitre
µm	micrometer (s)
OD	optical density

PAI	pathogenicity island
PCR	polymerase chain reaction
pH	the negative logarithm of hydrogen ion concentration
RFLP	restriction fragment length polymorphism
rpm	round per minutes
RT-PCR	reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid
SD	standard deviation
s	second (s)
V	Volt
Vac	vaculating cytotoxin protein
WSS	Warthin-Starry stain

CHAPTER 1
INTRODUCTION

The discovery of the association of *Helicobacter pylori* with chronic gastritis, peptic ulcers and gastric neoplasia, mucosa-associated lymphoid tissue-type lymphoma and carcinoma (Solte et al., 2002; Uemura et al., 2001; Fox and Wang, 2001; Eaton, 1999), has led to fundamental changes in the understanding of gastric disease in humans (Lee et al., 1993; Smoot and Hamilton, 1995; Tompkins and Falkow; 1995; Simpson et al., 1999(a)). Some humans with *H. pylori* infection develop only mild, asymptomatic gastritis. Whether more severe disease develops thought to be influenced by individual host factors and pathogenicity of the bacteria involved (Eaton, 1999). The odd of developing symptomatic *H. pylori* infection varies by geographic location and age (Nakajima et al., 2000). Different strains of *H. pylori* have recently been identified. Therefore, *H. pylori* should be considered a population of closely related but genetically heterogenous bacteria of different genotypes and virulence (Herbrink and van Doorn, 2000).

A gastric (spiral) bacterium of genus *Helicobacter spp.* is gram-negative, spiral-shaped bacteria. At least 13 species have been reported, and most are suspected or proven gastric or hepatic pathogens (Hall, 2000). Investigation of the relationship of gastric disease with *Helicobacter spp.* infection in other species has resulted in the discovery of *H. mustelae* in ferrets with gastritis and peptic ulcers, *H. acinonyx* in cheetahs with severe gastritis, *H. heilmannii* in pigs with gastric ulcers (Eaton et al., 1993; Fox et al., 1990; Queiroz et al., 1996).

In humans, the reservoir for *H. pylori* is stomach. Risk factors for *H. pylori* infection in humans include age and socioeconomic status, with children and those with low socioeconomic status at greater risk (Deltenre and Koster, 2000). The role of *Helicobacter spp.* in gastrointestinal disease in dogs and cats is uncertain. It has been known for years that gastric *Helicobacter*-like organisms (HLO) are commonly present in stomach of dogs but the relationship of these organisms and gastric disease is unresolved. (Eaton et al., 1996;Geyer et al., 1993; Happonen et al., 1996; Henry et al., 1987; Hermanns et al., 1995; Yamasaki et al., 1998). The study of canine gastric

Helicobacter spp. infection was performed in Thailand. This study revealed significantly different correlations for bacterial detection between Hematoxylin & Eosin and Warthin Starry stains (Pirarat et al., 2003). Infection with HLO is highly prevalent in dogs. It is seen in 61-80% of dogs presented for the clinical sign of vomiting (Gayer et al., 1993; Hermanns et al., 1995; Yamasaki et al., 1998) and in 67-86% of clinically healthy pet dogs (Eaton et al., 1996; Yamasaki et al., 1998).

H. pylori transmission is proposed to be fecal-oral, oral-oral, and gastro-oral (via vomited fluids) (Deltenre and Koster, 2000). The exact details of transmission are still unclear (Blanchard and Czinn, 2001). A higher incidence of *H. pylori* infection has been reported by gastroenterologists, suggesting that transmission from patient to physician is possible (Lin et al., 1994). It has been suggested that *Helicobacter spp.* infection might be zoonotic by contact with dogs and cats and has been correlated with human *H. heilmannii* infection. There is no correlation between pet ownership and human *H. pylori* infection (Deltenre and Koster, 2000; Meining et al., 1998; Fox, 1998).

Helicobacter spp. produce urease, which breaks down urea into ammonia and bicarbonate ions. In stomach, ammonia has a buffering effect that may help *Helicobacter spp*. colonize on mucosa in the acidic gastric environment. In addition, ammonia is directly toxic to gastric epithelial cells (Hall, 2000). *H. pylori* infection is associated with increased gastric acid secretion (hyperacidity), which causes inflammation of the gastric antrum (antral gastritis) and duodenal ulceration. It has been proposed that hyperacidity is caused by hypergastrinemia resulting from the inhibition of somatostatin-secreting cells (somatostatin inhibits gastrin release) (Moss et al., 1992). Hypergastrinemia also increases parietal cell mass through a trophic effect on gastric mucosa. *H. pylori* infection can also be associated with lack of gastric acid (achlorhydria). This is thought to occur when *H. pylori* causes mucosal atrophy in the gastric fundus and body or inhibits functioning of the parietal cells (El-Omar et al., 1997; Simpson et al., 1999(b)). Chronic gastric inflammation may progress to chronic atrophic gastritis and intestinal metaplasia, which are precancerous conditions (Nakajima et al., 2000). It appears that *Helicobacter spp.* infection significantly alter gastric acid secretion in dogs (Hall, 2000).

Diagnostic tests for *Helicobacter spp.* can be divided into invasive (those requiring a gastric mucosal biopsy specimen) and noninvasive methods. *H. pylori* may exhibit a patchy distribution in gastric mucosa and may cause atrophic gastritis (with resultant of low bacterial numbers in the gastric mucosa). Both factors may limit the sensitivity of biopsy-based diagnostic test (Herbrink et al., 2000).

Direct observation of *Helicobacter spp.* organisms in biopsy specimens usually requires special stains (e.g. Giemsa's, Warthin-Starry, Genta's, alcian yellow-toluidine blue). *H. pylori* is typically not visualized by hematoxylin and eosin stain (Vaira et al., 2000). Current standards in human medicine dictate that an estimate of *H. pylori* density, activity and grade of gastritis, and comments on the presence of atropic gastritis or intestinal metaplasia should be provided when histopathological examination is made (Leodolter et al., 2001). The specificity of histopathology is 100%, and sensitivity is of greater than 90% (Vaira et al.,2000; Montiero et al., 2001). Immunohistochemical staining (Herbrink and van Doorn, 2000; Vaira et al., 2000) and electron microscopy may also be used to evaluate gastric biopsy specimens.

Polymerase Chain Reaction (PCR) allows identification of the particular *Helicobacter spp*. or strain and can be the sample of PCR gastric biopsies, gastric juice, dental plaque, or feces. Sensitivity of detection method was varied by the primer used but sensitivity is considerable high (Herbrink and van Doorn, 2000; Vaira et al., 2000; Montiero et al., 2001). In addition, PCR allows identification of specific *H. pylori* genes (so-called virulence factors) associated with an increased incidence of peptic ulcer or cancer. (Herbrink and van Doorn, 2000).

Antibodies (IgG) against *H. pylori* can also be detected in saliva, but this method had lower sensitivity and specificity (81% and 73%, respectively) in a recent multicenter study of 213 dyspeptic patients (Luzza et al., 2000). IgG against *H. pylori* has also been detected in urine, and a urine-based ELISA test recently validated in Japan had an accuracy comparable to that of serum ELISA test (Leodolter et al., 2001).

Noninvasive test methods for detecting *Helicobacter spp.* (e.g. urea breath testing, antibody testing, stool antigen testing) are not routinely available for dogs. Urease testing (breath and blood) has been investigated in dogs but is not widely available (Cornetta et al., 1998). Antibody testing in dogs is potentially more difficult than

that in humans because of the variety of *Helicobacter spp.* infecting dogs. Nonetheless, antibody testing is being investigated, and infected animals are known to develop antibodies (Straus et al., 2002). Theoretically, stool antigen testing could be useful in *H. pylori*-infected in animals infected with *Helicobacter spp.* that have antigenic homology and thus cross-reactivity with *H. pylori*. This has not been investigated.

The only way to confirm the presence of *Helicobacter spp.* in dogs with the invasive methods is already discussed. Endoscopically obtained gastric mucosal biopsies are commonly used. Direct observation of organisms (via histological examination of biopsies or cytological examination of brush cytology specimens) and rapid urease testing are common method of identifying *Helicobacter spp.*. Because *Helicobacter spp.* distribution in the stomach may be patchy, the evaluation of multiple biopsies and anatomic locations (i.e. cardia, fundus, antrum) are recommended (Neiger and Simpson, 2000). Unfortunately, the lack of noninvasive diagnosis testing for *Helicobacter spp.* in veterinary medicine makes response to treatment difficult to assess. Repeat endoscopy or biopsy is required, which is expensive and unappealing for many pet owners. However, an advantage of follow-up endoscopy over noninvasive testing is the opportunity to reassess gastric morphologic changes.

The hypothesis of this study was focused on the implement of polymerase chain reaction for diagnosis *H. pylori* infection in dogs in Thailand.

The objectives of this study were focused on two specific areas: (i) possibility of *H. pylori* diagnosed by polymerase chain reaction in comparison to routine histopathology, histochemistry and also immunohistochemistry (ii) clinically significances and evidence gastric *H. pylori* infection in dogs.

H. pylori became one of the subject for the intensive investigations in the history of medicine as new knowledge revealed that half or more of all people and their pet dogs worldwide are infected by this organism and their life long infection is a proven risk-factor in development of gastric cancer, one of the most common and frequently lethal of all cancers in humans. The results from this experiment could be useful of the epidemiology and diagnosis of *H. pylori* in dogs in Thailand.

CHAPTER 2
REVIEW LITERATURES

The latter quarter of the 20th century discovered the emergence of many new pathogens or disease syndromes, including Legionnaires' disease, toxic shock syndrome, Lyme disease , and AIDS. Perphaps the most suprising finding was that a microbe (*Helicobacter pylori*) was associated with duodenal and gastric ulcers. Prior to the findings of Warren and Marshall in 1983 (Warren and Marshall,1983) and until general acceptance of the findings by the 90's, most believed that gastric ulcers were due to the life style, behavior type, or diet. Interestingly, the spiral-shaped microaerophilic bacteria associated with gastric biopsy material had been previously observed at the beginning of the century, but the association with gastric disease was never established. The pharmaceutical industry, once the benefactors of billion dollar ulcer treatment therapeutics was invested, now focused on the development of new antimicrobials for the potentially worldwide market. While others focused on development of diagnostic tools from endoscopic devices to rapid diagnostic kits for singling out those bacteria for eradicative therapy.

A gastric spiral bacterium of superkingdom bacteria, phylum proteobacteria, subphylum delta/epsilon subdivisions, class epsilonproteobacteria, order campylobacter, family helicobacteraceae, genus *Helicobacter spp.* (Tomb et al., 1997) is gram-negative, spiral-shaped bacteria. At least 13 species have been reported, and most are suspected or proven gastric or hepatic pathogens (Hall, 2000). *Helicobacter spp*. have been reported in humans: mainly *H. pylori*, nonhuman primates: *H. nemestrinae*, cats and dogs various species, including *H. pylori* (Buczolits et al., 2003), *H. felis, H. salomonis, H. rappini, H. heilmannii* (Stoffel et al., 2000), and *H. bizzozeronii*, pigs: *H. heilmannii* , ferrets: *H. mustelae* , and cheetahs: *H. acinonys* (Simpson et al.,1999 (b)). More recently we have learned that nearly all mammmals harbor their own species of *Helicobacter* infection (Dewhirst et al., 2000). Some are suggesting now that infection might be benign or even beneficial by protecting againts development of esophageal reflux and cancer of the esophagus (Loffeld et al., 2000).

1. *H. pylori* in human

H. pylori is one of the major causes of chronic gastritis and plays an important role in the pathogenesis of peptic ulcer, gastric carcinoma, gastric adenocarcinoma (Huang et al., 1998; Nomura et al., 1991; Parsonnet et al., 1991), and primary B-cell gastric lymphoma (Graham, 1997; Graham and Yamaoka, 1998; Issacson and Spencer, 1993; Nomura et al., 1991; Parsonnet et al., 1995). *H. pylori* is the second most common cause of cancer morbidity and mortality worldwide, and the development of gastric non-Hodgkin's lymphoma (Parsonnet et al., 1994; Zucca et al., 1998). Histological gastritis is essentially universal among *H. pylori*-infected individuals, but only a few develop a clinically significant outcome, such as peptic ulcer disease or gastric cancer. The clinical significance of this bacterium has recently been emphasized by a National Institutes of Health consensus panel and thus recommending antibiotic therapy for the large majority of peptic ulcer patients who are infected with *H. pylori* (Anonymous, 1994 (a)) and by classification of *H. pylori* as a classification of *H. pylori* as a class I (definite) carcinogen by the World Health Organization (Anonymous, 1994 (b)). The bacteria were often seen in malignant or ulcerated gastric tissue (Freedberg and Barron, 1940), and the possibility of an infectious cause of peptic ulcer was considered (Barber and Frankin, 1946).

2. Pathogenesis of *H. pylori* in human

H. pylori often estabishes life-long infections of the gastric mucosa. These bacteria produce a powerful urease that is regulated in response to acid (Scott et al., 2000). So the ammonia and carbonate produced by this enzyme most likely create an alkaline microenvironment. This mechanism is unprecedented. Further study shows that the high density of colonization by *H. pylori* occurs in the antrum (lower portion of the stomach) where conditions are less acidic. As the infection becomes more pronounced or under conditions where the antrum becomes more alkaline, the motile bacteria migrate up into the cardia (body) of the stomach. Infection with *H. pylori* bacteria is basically located in three dimensions, as these bacteria not only can move north and south in the mucosa in response to acid levels, but they are able to move freely up and down in the mucus layer that coats the gastric mucosa and provides a protective barrier

against the diffusion of strong acid onto the epithelium. The notion of being "off shore" and therefore out of reach of the macrophages and cells of host immune defense may also play an important role in survival of these bacteria. Finally, the mounting evidence suggests that *H. pylori* may control the immune response through mimicry (LPS displaying Lewis antigens) and selective release of inflammatory factors. The balance between increase of inflammation and immune suppression is a key to the persistence and an area where novel therapeutics, perhaps in combination with vaccine strategies, could be directed.

The discovery of *Helicobacter spp.,* a relative of *Campylobacter spp*. (bacterial pathogens of the lower GI tract), fortunately coincided with the beginning of the genomics era, and is the beneficiary of two completely sequenced genomes of *H. pylori* (Tomb et al., 1997; Alm and Trust, 1999). The results reveal a small genome (1.67 megabases) containing some 1553 genes encoding around 1,300 proteins. Despite possessing a limited number of genes, *H. pylori* displays auxotropy for only a few amino acids and appears to possess most catabolic and anabolic pathways found in bacteria with larger genomes. Recent studies examining essentiality testing on a genome scale suggested that there are few redundancies and backups in metabolic pathways and thus the percentage of *H. pylori* genes found essential may be greater than expected for organisms with larger genomes perhaps opening a door for development of *Helicobacter* selective therapeutics (Chalker et al., 2001). Other caveats from genome gazing is that there are few regulatory genes particularly two component signal transduction systems, that are so typical of enteric bacteria and many pathogens. (Berg et al.,1997; Marais et al., 1999; Doig et al., 1999).

3. CagA

Major interest has focused on genes clustered in a pathogenicity island (PAI) that is associated with strains causing pathology and gastric manifestations. CagA (Cytotoxin associated gene A) positive stains posses a 40 kilobase-region encoding genes with orthologs in the type IV secretion and pathogenesis system of *Agrobacterium*, *Bordetella pertussis*, and etc.(Censini et al., 1996). CagA gene is located at one end of the PAI and serves as a marker for cag status. Strains lacking the

cag PAI are characterized as less virulent as indicated by colonization at lower microbial densities and producing less inflammation than Cag^+ strains. However, there is still debate over relative association with ulcers and more severe disease. Genes within the Cag PAI promote secretion of a vacuolating cytotoxin protein (VacA) whose gene is located outside of the Cag locus. Recently, studies show that the Cag PAI is also responsible for delivery of the CagA protein into mammalian cells and that this protein exhibits tyrosine phosphatase activity (Odenbreit et al, 2000). Thus, these toxins are considered important in the pathogenesis and severity of disease. New applications of bioinformatics and proteomics have also contributed to expanded knowledge of protein interactions (Ryan et al., 2001) and assignment of function to many of the *H. pylori* genes whose function is unknown. Experience with other bacterial pathogens suggests that *H. pylori* strain-specific factors may influence the pathogenicity of different *H. pylori* isolates. *H. pylori* studies have primarily focused on two groups of putative bacterrial virulence factors, the Cag PAI for which CagA is a marker and the vacuolating cytotoxin VacA (Xiang et al., 1995; Yamaoka et al., 1999). The presence of an intact Cag PAI is associated with increased interleukin-8 production and mucosal inflammation (Yamaoka et al., 1999). Overall, the data support the notion that infection with a CagA-positive isolate increases the risk but does not predict the presence of a clinically significant outcome (Graham and Yamaoka, 1998; Yamaoka et al., 1997; Yamaoka et al., 1998).

Advancements in diagnostics, both serologic, stool antigen test, and invasive endoscopy have led to sorting out infected people with upper GI ailments from those who are uninfected. Studies now show that nearly 35% of the world's population suffers from dyspepsia, but only 5 to 20% of these individuals are also infected with *H. pylori* that colonizes the gastric mucosa of some 50% of the world's population, and is one of the most common of human infectious agents (Dunn et al., 1997). In western societies, even fewer are infected with more severe CagA strains and in either case, studies have shown that eradication of *H. pylori* infection may not necessarily resolve dyspeptic symptoms (Talley, 1999). Thus, general practitioners tend not to investigate the basis for dyspepsia and often simply treat the symptoms with anti-acids and or acid secretion blockers. The assumption that *H. pylori* infection is rather benign-particularly in western societies where gastric cancer is rare in the first place seems to justify the practice.

Where alarm symptoms are noted, referral to a specialist and further investigation by endoscopy is generally pursued. As mentioned earlier, the worry over cure of *H. pylori* and the subsequent development of gastric esophageal reflux disease (GERD) has raised concerns among physicians and the notion that the only good *Helicobacter* is a dead one, has been challenged. It is not clear what the future will bring, but given that 75 to 80% of North Americans are or never were infected with *H. pylori*, needs to be considered in this argument. Of the remaining 20-23% that are infected in North America, are they more likely upon cure to have a different outcome than those who have never been infected. Otherwise, the study of prevalence of virulence factors producing strains of *Helicobacter pylori* and their association to peptic ulcer in Thai patients was concluded no association of CagA nor VacA might not be used as the markers for peptic ulcer disease in Thai patients. However, CagA protein might be considered an antigen for serodiagnosis and a candidate vaccine against *H. pylori* infection (Suthienkul et al., 2000).

4. Treatment of *H. pylori* infection

Much of the concern associated with whether to treat or not stems from the fact that treatment regimes have variable outcomes. While *H. pylori* is susceptible to a large variety of antimicrobials in vitro, the therapeutic activity in the gastric milieu is often nil and monotherapies have generally demonstrsted poor efficacy. The most common practice is to use combinations of drugs, the so called triple or quadruple therapies containing amoxicillin, macrolides like clarithromycin, and or nitroimidazoles like metronidazole in combination with proton pump inhibitors (omeprazole) that reduce acidity and improving therapeutic action of the antimicrobials (Boer and Tytgat, 2000). Treatments can also contain bismuth salts and tetracycline. The fact that all the current therapeutic agents are old drugs and generally of broad spectrum has alarmed many who do not understand why the pharmaceutical industry has not developed novel therapeutics specifically for treating *Helicobacter* infections. Unfortunately, despite the obvious need, the drug industry market analyses show a rather small market and one that is disappearing-reinfection rates is so low in developed countries that patients would only be treated once and the market would eventually disappear. The need for

new therapeutics is still very real for developing countries where resistance to metronidazole and macrolides renders many cerrent therapeutics nearly useless. Unfortunately, the cost of drug development could never be recouped through sales in these markets where need is greatest. In western societies, the educational initiatives associated with efforts to reduce the wide spread practice of prescribing antibiotics have succeeded in that many physicians are reluctant to prescribe first line broad spectrum antibiotics for treatment of non-life threatening infections as *H. pylori* out of fear of contributing to drug resistance.

5. *H. pylori* infection in animals

Helicobacter-like bacteria have been identified in the stomachs of all mammalian species examined to date. Many epidemiological studies have shown a strong association between chronic *H. pylori* infection and subsequent development of gastric carcinoma in humans. Studies of *H. pylori* and gastric carcinoma from the view point of animal model showed that persistent *H. pylori* infection has recently been achieved in the Japanese monkeys and Mongolian gerbil models, with results demonstrating that the sequential histopathological changes in the gastric mucosa are closely mimic the gastric mucosal changes caused by *H. pylori* infection in humans. Gastric mucosa infected with *H. pylori* exhibited significantly higher gastritis score, reduction in glandular height, increase in the number of Ki-67 positive cells and over expression of p53 protein and p53 gene mutation in the Japanese monkey model. In the Mongolian gerbil model, *H. pylori* infection enhances gastric carcinogenesis in combination with known carcinogens such as MNU and MNNG, and also demonstrated that *H. pylori* infection alone can result in the development of gastric carcinoma (Fujioka et al., 2002).

6. *H. pylori* infection in dogs

Gnotobiotic dogs were used as experimental hosts for *H. pylori* infection in one early study (Radin et al., 1990). All dogs tested were successfully colonized with *H. pylori*. In addition, two inoculated dogs co-housed with experimental dogs also became colonized, indicating transmission of infection. The subsequent use of dogs as *H. pylori* models has been limited. One recent study showed that conventionally housed dogs are

also susceptible to experimental infection (Rossi et al., 1999). In addition to experimental studies, efforts have been made to determine the presence of natural gastric *Helicobacter* infections in dogs. Surveys of pet dogs have repeatedly failed to show natural infections in dogs. Surveys of pet dogs have repeatedly failed to show natural infection with *H. pylori* (Happonen et al., 1998; Eaton et al., 1996; Marini et al., 2000). However, natural infection with other gastric *Helicobacters* commonly occurs. *H. felis, H. bilis, H. bizzozeronii, H. salomonis, H. heilmannii* and *Flexispira rappini* have all been identified in surveys of gastric infections in dogs (Happonen et al., 1998; Eaton et al., 1996; Cattoli et al., 1999; Peyrol et al., 1998). The pathological significance of these organisms in the dog is currently unknown. A significant association between their presence and the occurrence of gastritis has never been demonstrated.

7. Diagnosis of *H. pylori* infection

The cultivation of *H. pylori* and the recognition of its clinical significance served to renew interest in bacteria associated with the gastrointestinal and hepatobiliary tracts of humans and other animals, many of which have now been identified as novel species of *Helicobacter*. These organisms are of interest both because of their pathogenic role in humans and animals and because of their value as models of human disease. Other bacteria have also been newly identified, or in some cases reclassified, as novel *Helicobacter* species that in fact humans (Solnick and Schauer, 2001). Culture is the most specific diagnostic method for *H. pylori* infection but its sensitivity is usually substantially lower than that of the other methods. Even if culture rarely gives additional positive results, it is an important method as isolates for the traditional susceptibility testing obtained. PCR was found to be as sensitive as culture in detecting *H. pylori* in gastric biopsies. Indeed it has the advantage of allowing the detection of virulence factors such as CagA, VacA alleles, IceA, etc. A novel multiplex PCR has been proposed by Chisholm et al. to detect VacA alleles in one step. They found a perfect correlation with the genotypes found on the corresponding strains.The VacA s1m1 genotype represented 52% of the strains in England (Chisholm et al., 2002). Genotyping can be performed on paraffin-embedded gastric biopsy specimens. Scholte et al. found corpus atrophy associated with CagA and VacA ss1m1 status as determined by the line

probe assay. Multiple genotypes were detected in 14% of their cases (Scholte et al., 2002). Direct genotyping of virulence factors using the Line probe assay was also used in the gastric carcinnoma (Figueiredo et al., 2002). A CagA-positive status was associated with a higher bacterial load and activity but also with elevated serum TNF-γ and gastrin levels as well as intestinal metaplasia in a large series of 345 adult patients in Turkey (Saruc et al., 2002). In New York city, a study of 200 patients showed a high prevalence of CagA positive strains (81%) but no association with a specific disease and little allelic heterogeneity with respect to VacA and IceA subtypes (Straus et al., 2002). *H. pylori* fingerprinting can be performed directly from biopsy specimens by Polymerase Chain Reaction Restriction Fragment Length Polymorphism. Thus, Luman et al. (2002) showed that strains present in 13 couples were different from one another using a glmM (UreC) PCR and *Mbo*I or *Hba*I as restriction enzymes (Luman et al., 2002). Real-time PCR is now becoming more popular. The TaqMan technology was applied to determine the bacterial load in gastric mucosa. Another important application is the direct detection of resistance to clarithromycin. A fluorescence resonance energy transfer (FRET) assay was developed by Oleastro et al. It is based on amplification of the 23S rRNA gene region where the three main mutations concerned are located, hybridization with labelled probes, and detection of the signal in a Light-Cycler apparatus. This test was validated on a large batch of biopsies after failure of a first line therapy. It allows detection of clarithromycin resistance within 2 h and therefore has the potential to be used in the management of *H. pylori*-positive patients (Oleastro et al., 2003).

Another approach was the use of double gradient-denaturating gradient gel electrophoresis (DG-DGGE) to detect the mutations in the amplicons. This method revealed altered homoduplex molecules and/or formation of heteroduplex molecules (Scarpellini et al., 2002). Pena et al. looked for *Helicobacter* species by PCR in *H.pylori*-negative gastric biopsy specimens. Interestingly, out of 126 cases they found two positive with their 16S rRNA genus specific probe and *glm*M-negative. After cloning and sequencing, they were identified as *Helicobacter cinaedi*, a urease negative enterohepatic helicobacter (Pena et al., 2002). The availability of a PCR to be used on stools with excellent sensitivity and specificity is a future goal. A study using a semi-

nested UreA PCR reported results with high specificity but low sensitivity (26%) (Wisniewska et al., 2002), inferior to previously published results (Monteiro et al., 2001). PCR has also been used to detect *H. pylori* in various sites. The bacterium was detected in 2 out of 46 appendix specimens from patients with appendicitis (4%) (Pavlidis et al., 2002), 4 out of 12 ethmoid specimens from patients with chronic rhinosinusitis (33%) probably following reflux (Ozdek et al., 2003), but in none of the 22 atherosclerotic plaque specimens (Dore et al., 2003).

Noninvasive diagnostic methods include urea breath testing, a stool antigen test, and serum antibody testing. Although consensus exists among gastroenterologists that noninvasive testing should be conducted first in patients suspected of *H. pylori* infection, agreement is lacking regarding which noninvasive test is the best (Chey and Fendrick, 2001). Urea breath testing (which detects urease activity) and the stool antigen test (which measures *H. pylori* antigen) are direct tests that detect active infection. Serum antibody tests are indirect and cannot distinguish between actively infected patients and those that were previously infected. Direct testing is thus preferred. However, serum antibody testing is currently the most commonly used screening method because of convenience, availability, and low cost (Chey and Fendrick, 2001).

Like the rapid urease test, urea breath testing finds *Helicobacter spp*. by identifying urease activity. The patient swallows labeled (with nonradioactive carbon 13 or radioactive carbon 14) urea. If *Helicobacter spp*. is present, urease hydrolyzes the labeled urea and the marker (carbon-labeled bicarbonate) is detected in the breath after 30 to 60 minutes (Neiger and Simpson, 2000; Cornetta et al., 1998). A test meal is given to delay gastric emptying (Vaira et al., 2000). Antisecretory drugs increase gastric pH and decrease urease activity (Chey et al., 1997; Cornetta et al., 1998); thus test results may be false-negative immediately after treatment and must be interpreted in relation to treatment. The current recommendation in human medicine is to wait 4 weeks following cessation of therapy before conducting a follow-up urea breath test (Metz, 2000), although research has demonstrated that the effect of antisecretory drugs on the urea breath test can resolve as early as 5 days after cessation of treatment (Chey et al., 1997). Sensitivity and specificity of urea breath testing are high (greater than 95%)

(Vaira et al., 2000), and the urea breath test is considered a reliable, noninvasive way of documenting eradication after treatment (Metz, 2000). Carbon-labeled bicarbonate can also be detected in serum, but FDA approval of this as a diagnostic test for *Helicobacter spp.* is pending (Metz, 2000).

The stool antigen test, an enzyme immunoassay test for detecting *H. pylori* antigen, is the newest noninvasive technique for diagnosing *Helicobacter spp.* infection in humans. Initial test kits employed polyclonal antibodies to *H. pylor*i, but a newer test using monoclonal antibodies has been developed. Based on a review of 44 studies encompassing 4769 untreated patients, Gisbert and Pajares (Gisbert and Pajares, 2001 (a)) found that sensitivity and specificity for *H. pylori* stool antigen testing were high (greater than 92%). The optimal time for using stool antigen testing after treatment to check for eradication is uncertain. The accuracy of this test in patients with GI bleeding is uncertain (Gisbert and Pajares, 2001 (b)). It is also uncertain whether cross-reactivity with other *Helicobacter spp.* exists (Montiero et al., 2001).

Serology identifies antibodies against *H. pylori*, which can be detactable for more than 1 year following infection. Three types of antibody tests are available: quantitative serum ELISA, Western blot test using serum, and qualitative rapid whole blood tests (in-office, rapid latex agglutination or flow-through, membrane-based enzyme immunoassays). Serum ELISA testing is the most commonly used method and can detect IgG and IgA. The serum IgG ELISA is considered the most sensitive, although sensitivity depends on characteristics of the population tested and particular *H. pylori* antigen preparation used to make the test kit Herbrink and van Doorn (2000) reported that sensitivity and specificity of commercial ELISA assay vary between 60% and 100%, and most had values over 85%. Western blot testing of serum permits more detailed analysis of the patient antibody profile but has limited usefulness because of labor-intensive methodology. Variable sensitivity has been reported for in –office, rapid, whole blood tests designed for use in primary care clinic (Herbrink and van Doorn, 2000).

CHAPTER 3
MATERIALS AND METHODS

Tissue samples

Gastric tissues were randomly collected from 75 necropsy dog cases, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. Their signalments were recorded as in routine necropsy cases such as breed, sex, age, clinical signs, cause of illness or died.

The stomach of each dog was then opened along the greater curvature and inspected for gross lesions. The two pieces of gastric mucosa tissue were then divided into 4 different site of stomach from cardia, fundus, body and pyloric antrum (Fig 1). One piece of each part was then placed in steriled Eppendorf tube containing a 0.9% sterile NaCl solution and frozen in -70^{O}C for DNA extraction and polymerase chain reaction (PCR) analysis. The others four remaining pieces were placed in 10% buffered formalin pH 7 and conducted for histophathology (Pirarat et al., 2003). The full-thickness tissue of gastric mucosa approximately 1.5 cm in length from the cardia, fundus, body and pyloric antrum of each dog were embedded in paraffin, cut in to 4-6 µm sections, and stained with Hematoxylin & Eosin (H&E) and Warthin-Starry (WSS) stain (see appendix A).

Histopathological examinations (Microscopic findings)

The formalin-fixed tissues were trimmed and processed in the tissue processor. Briefly, tissues were dehydrated by 70, 80, 95 and 100% ethanol and xylene, respectively. The tissues were embedded in a paraffin block and cut into 4-6 µm thickness by microtome, then sections were placed on slide (for H&E staining and WSS staining) or poly-L-lysine coat slide (for Immunohistochemistry; IHC).

The paraffin-embedded sections were deparaffinized and rehydrated by xylene, 100, 95, 85, 70% ethanol and distilled water, respectively. The slides were stained with Harris's hematoxylin and eosin and then were dehydrated by 70, 80, 95, 100% ethanol and xylene, respectively.

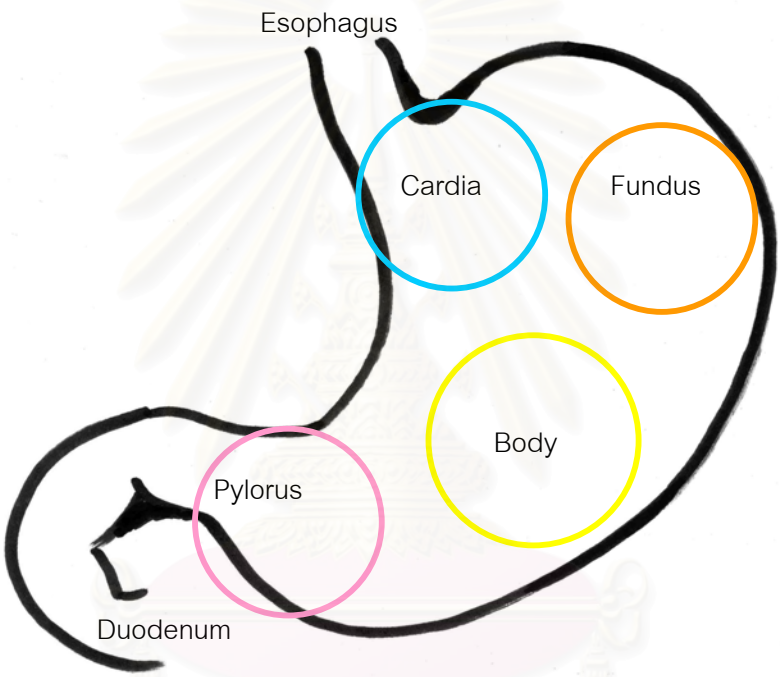


Fig 1 Diagram for collected samples of cardia, fundus, body and pyloric antrum after the stomach was cut on greater curvature.

Histopathology evaluation

H&E stained slides, evaluated on a blind-coded basis, were assigned as a gastritis score according to the following criterias: 0, normal; 0 to 10, inflammatory cells (not including those within lymphoid aggregates) per high-power field, with no lymphoid follicular aggregates and normal mucosal epithelium; 1, mild gastritis, 10 to 50 inflammatory cells per high-power field, fewer than two follicles per low-power field, and normal mucosal epithelium; 2, moderated gastritis, 10 to 50 or more inflammatory cells per high-power field, with greater than two follicles per low-power field and mild gastric epithelial changes; 3, severe gastritis, greater than 50 inflammatory cells per high-power field and marked epithelial changes. Epithelial changes included individual cell necrosis, cytoplasmic basophilia and glandular dilation. The high-power field had a total magnification of X400, and the low-power field had a total magnification of X20. The type and location of inflammatory cells and the number of lymphoid follicles per low-power field were noted, as were spiral organisms. The *Helicobacter spp.* was basophilic color spiral shape, 2.5 - 5.0 μm long, 0.5 – 1.0 μm wide(Handt et al.,1995; Pirarat et al., 2003).

WSS stain sections were evaluated, on a blind-coded basis, for the presence of organisms and the morphologies of the organisms. The quantity of organisms seen in each 1.5 cm. stained tissue section was graded by the following scale: 0, no organisms seen; 1, few organisms (<10 organisms per section); 2, moderate numbers of organisms (10 to 50 organisms per section); 3, large numbers of organisms (>50 organisms per section, usually too numerous to count) (Handt et al.,1995; Pirarat et al., 2003). Spiral-shape *Helicobacter spp.* was positive in dark brown, spiral shape, 2.5 - 5.0 μm long, 0.5 – 1.0 μm wide in yellow background. The locations of *Helicobacter spp.* in the stomach tissue were also demonstrated by WSS as luminal crypt, gastric pit, gastric gland and gastric epithelium (see in Appendix A).

Immunohistochemistry (IHC) the consequetive sections were also employed for immunohistochemical analyses by the ABC-peroxidase technique with a rabbit polyclonal anti-*Helicobacter pylori* antibody (DAKO®, Denmark), the evaluation was estimated as

followed: -ve, no *Helicobacter spp.;* +ve, The presence of *Helicobacter spp.* antigen was brown in color (Esteves et al., 2000; Pirarat et al., 2003) (see in Appendix B).

DNA extraction

From each gastric tissue sample, the mucosa was scraped from each stomach sample with a surgical blade. DNA was isolated from 25 mg of the scraping tissue, which was extracted by the QIAmp Tissue Kit (Qiagen Inc., CA., USA) according to the manufacturer's instructions. The approximately 25 mg of tissue was then cut into small pieces, placed in a 1.5 ml microcentrifuge tube, and added 180 µl of buffer ALT. 20 µl Proteinase K was added and mixed by vortexing used. The solution was incubated at 56°C was applied of samples until the tissues were completely digested in 3 h. The vortex was occasionally used during incubate to disperse the sample. The microcentrifuge tube was centrifuged to remove drops from the inside of the lid. The buffer AL to the sample was added mixed by pulse-vortexing for 15 s, and incubated at 70°C for 10 min. The microcentrifuge tube was centrifuged to remove drops from inside the lid. Ethanol (96-100%) 200 µl was added to the sample, and mixed by pulse-vortexing for 15 s. After mixed, briefly microcentrifuge tube was centrifuged to remove drops from inside the lid. Carefully the mixture was applied to the QIAamp spin column and centrifuged at 8,000 rpm for 1 min. The QIAamp spin column was placed in a clean 2 ml collection tube. Added 500 µl buffer AW1 and centrifuged at 8,000 rpm for 1 min. The QIAamp spin column was placed in a clean 2 ml collection tube, and the collection tube containing the filtrate was discarded. Buffer AW2 was added 500 µl and centrifuged at full speed 14,000 rpm for 3 min. The QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. Buffer AE was added. Tube was incubated at room temperature for 1 min, and then centrifuged at 8,000 rpm for 1 min. DNA was kept at –20 °C until needed.

Polymerase Chain Reaction (PCR)

Primers

Several primers were used for PCR, as shown in Table 1. Primers H 1, H 2 specific to the *Helicobacter* genus were prepared on the basis of the 16S rRNA gene sequence of *Helicobacter*-specific sequences. Primer pair H276f/H676r, was selected on the basis of alignments performed with EuGene software package (Baylor college of Medicine, Houston, Tex) (Riley et al., 1996) as described previously (Buczolits et al., 2001; Buczolits et al., 2003). Primers HP 1 and HP 2 specific to *H. pylori* were prepared on the basis of the 16S rRNA gene sequence of *H. pylori*, and were selected for PCR amplification based on published GenBank data (*H. pylori* ATCC 43504, GeneBank accession no. M88157; Choi et al., 2001). Primers 93089 and 93261 were selected from consensus regions of the available CagA gene sequences (GenBank accession no. L11714 and EMBL accession no. X70039; Covacci et al., 1993; Tummuru et al., 1993; Lage et al., 1995)

Table 1 Primers used for PCR and their sequences

Primer	Nucleotide sequence (5′→3′)	Position	Step of cycle	Temperature cycling		Cycle
				Temp (°C)	Time (min)	
Helicobacter genus specific						
H 1	CTA TGA CGG GTA TCC GGC	276-293	Initial denaturation	95	5	1
(H276f)			Denaturation	94	1	
H 2	ATT CCA CCT ACC TCT CCC A	676-658	Primer annealing	53	1	35
(H676r)			Extending	72	1	
			Final extending	72	5	1
H. pylori						
HP 1	CCT AAC CAA TTG AGC CAA GAA G	1176-1197	Initial denaturation	95	5	1
			Denaturation	94	1	
			Primer annealing	56	1	35
HP 2	CTT TCT AAC ACT AAC GCG CTC A	1579-1558	Extending	72	1	
			Final extending	72	5	1
H. pylori CagA$^+$						
93089	AAT ACA CCA ACG CCT CCA AG	2593-2612	Initial denaturation	95	5	1
			Denaturation	94	1	
			Primer annealing	56	1	35
93261	TTG TTG CCG CTT TTG CTC TC	2992-2973	Extending	72	1	
			Final extending	72	5	1

(Covacci et al., 1993; Tummuru et al., 1993; Lage et al., 1995; Riley et al., 1996; Buczolits et al., 2001; Choi et al., 2001; Buczolits et al., 2003)

PCR amplification

All reactions were performed in a 25 µl volume with an automated Thermo Hybaid HBPxE 0.2 thermocycler (Thermo Hybaid, United Kingdom) at Veterinary Diagnostic Laboratory (CU-VDL), Chulalongkorn University. Reaction mixtures contained each oligonucleotide primer at 5 µM, PCR buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM dNTP), 1 U of Hotstat Taq polymerase (MBI Fermentas, USA), 6 µl of template DNA and distilled water in a total volume of 50 µl.

Primers H 1, H 2: Samples were heated at 95°C for 5 min for initial dehydration step and then subjected to 35 cycles of denaturation step (94°C, 1 min), primer annealing step (53°C, 1 min), extending step (72°C, 1 min) and final extending step (72°C, 5 min).

The PCR products (10 µl) were subjected to electrophoretically separated in a 2% agarose gel (FisherChemical, USA) by electrophoresis at 100 V, 1.5 A for 1 h and were stained with ethidium bromide (Promega, USA) for 20 min then washed with distilled water. The DNA band was analyzed in the UV illuminator.

Primers HP 1, HP 2: The positive samples were used the first primer pair were heated at 95°C for 5 min for initial dehydration step and then subjected to 35 cycles of denaturation step (94°C, 1 min), primer annealing step (53°C, 1 min), extending step (72°C, 1 min) and final extending step (72°C, 5 min).

The PCR products (10 µl) were subjected to electrophoretically separated in a 2% agarose gel (FisherChemical, USA) by electrophoresis at 100 V, 1.5 A for 1 h and were stained with ethidium bromide (Promega, USA) for 20 min then washed with distilled water. The DNA band was analyzed in the UV illuminator.

The *Helicobacter pylori* from human used as positive control was kindly obtained from Department of Microbiology, Chulalongkorn Memorial Hospital.

Statistical analysis

The Chi-square analysis was used to examine relationship between locations of histopathological lesion and *Helicobacter spp.* infection.

Chi-square analysis was used to examine the relationship between pathological study by H&E, and WSS and IHC.

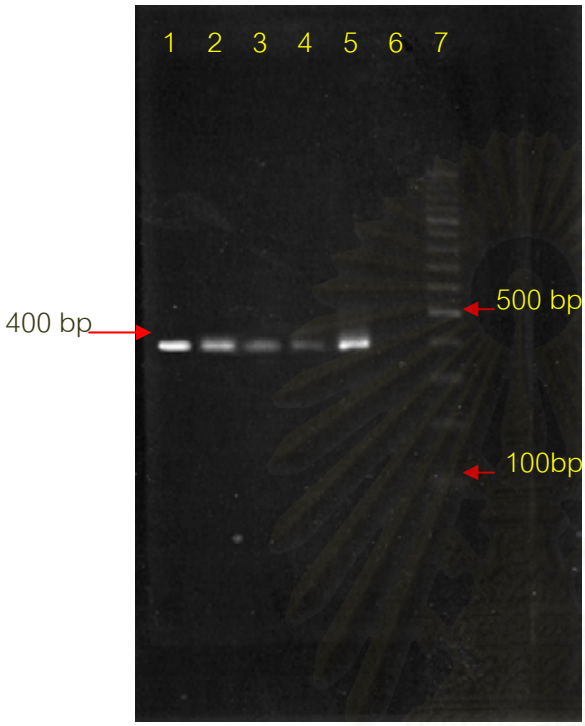
The Fisher's exact test was used to examine the relationship between pathological study by H&E, and PCR of *H. pylori* infections in canine stomachs.

The Fisher's exact test was used to examine the relationship between pathological study by H&E, WSS, IHC and PCR, and between clinical signs of *H. pylori* infections in canine stomachs.

The Fisher's exact test was used to examine the relationship between pathological study by H&E, WSS, IHC and PCR, and between kind of food intake in dogs.

Wilcoxon rank sums test was used to examine the relationship between pathological study by WSS and PCR.

Where significant effects ($p<0.05$) were detected. All statistical analyses were performed using software (SAS Institute, Cary, USA).



Lane 1 : *H. pylori* from human (10^5 CFU/µl)

Lane 2 : *H. pylori* from human (10^4 CFU/µl)

Lane 3 : *H. pylori* from human (10^3 CFU/µl)

Lane 4 : *H. pylori* from human (10^2 CFU/µl)

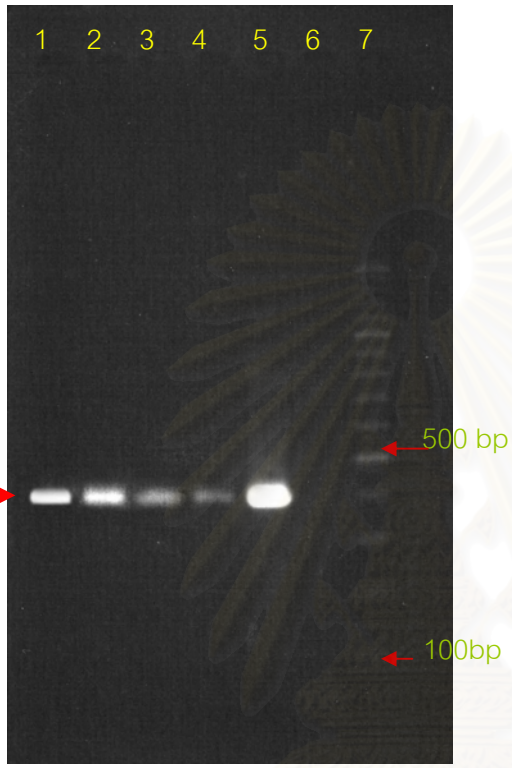
Lane 5 : Positive control, *H. pylori* from human (10^6 CFU/µl)

Lane 6 : Negative control

Lane 7 : 100 bp DNA ladder marker

Fig 2 Positive control: The bands 400 bp were seen by 2% agarose gel electrophoresis following PCR amplification of the *Helicobacter spp.* specific sequences.

(Riley et al., 1996; Buczolits et al., 2001; Buczolits et al., 2003).



Lane 1 : *H. pylori* from human (10^5 CFU/µl)

Lane 2 : *H. pylori* from human (10^4 CFU/µl)

Lane 3 : *H. pylori* from human (10^3 CFU/µl)

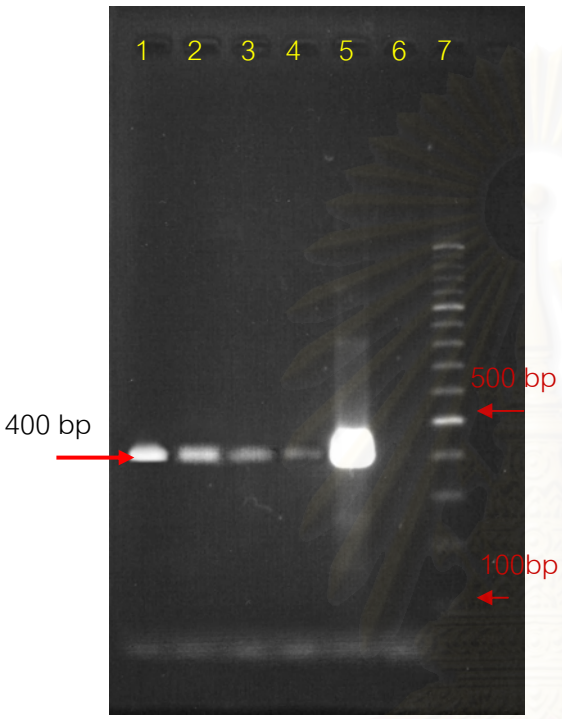
Lane 4 : *H. pylori* from human (10^2 CFU/µl)

Lane 5 : Positive control, *H. pylori* from human (10^6 CFU/µl)

Lane 6 : Negative control

Lane 7 : 100 bp DNA ladder marker

Fig 3 Positive control: The bands 403 bp were seen by 2% agarose gel electrophoresis following PCR amplification of *H. pylori*. (*H. pylori* ATCC 43504, GeneBank accesion no. M88157; Choi et al., 2001).



Lane 1 : *H. pylori* CagA^{+} from human (10^{5} CFU/µl)

Lane 2 : *H. pylori* CagA^{+} from human (10^{4} CFU/µl)

Lane 3 : *H. pylori* CagA^{+} from human (10^{3} CFU/µl)

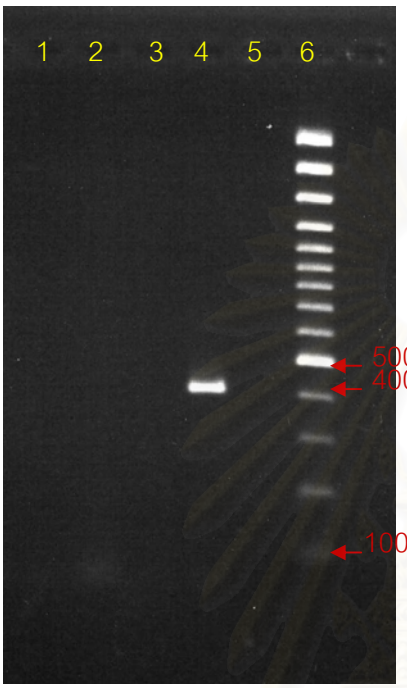
Lane 4 : *H. pylori* CagA^{+} from human (10^{2} CFU/µl)

Lane 5 : Positive control, *H. pylori* CagA^{+} from human (10^{6} CFU/µl)

Lane 6 : Negative control

Lane 7 : 100 bp DNA ladder marker

Fig 4 Positive control: The bands 400 bp were seen by 2% agarose gel electrophoresis following PCR amplification of *H. pylori* CagA^{+} (GenBank accession no. L11714 and EMBL accession no. X70039; Covacci et al., 1993; Tummuru et al., 1993; Lage et al., 1995)



Lane 1 : *E. coli* (10^6 CFU/µl)

Lane 2 : *Salmonella spp.* (10^6 CFU/µl)

Lane 3 : *Campylobacter spp.* (10^6 CFU/µl)

Lane 4 : Positive control (PC) *H. pylori* from human (10^6 CFU/µl)

Lane 5 : Negative control

Lane 6 : 100 bp DNA ladder marker

Fig 5 Positive control: The band 400 bp were seen by 2% agarose gel electrophoresis following PCR amplification of the *Helicobacter spp.* specific sequences (Riley et al., 1996; Buczolits et al., 2001; Buczolits et al., 2003).

CHAPTER 4
RESULTS

Clinical symptoms

The clinical symptoms in 75 necropsy dogs were reported as loss of appetite 32 % (24/75), vomiting 22.67 % (17/75) and diarrhea 6.67 % (5/75).

Gross findings

Macroscopic lesion was found at 34.67 % (26/75) such as focal ulcerative gastritis 10.67 % (8/75), catarrhal gastritis 8 % (6/75), hemorrhagic gastritis 13.33 % (10/75), erosive gastritis 1.33 % (1/75), and gastric polyps 1.33 % (1/75) as shown in Fig 6.

Histopathological findings

Microscopic lesions such as lymphoid follicular formation in submucosa, inflammatory cell infiltration in mucosa was presented most of neutrophils infiltration in ulcerative gastritis, crypt distortion and epithelial degeneration, in study location of stomach were found in cardia 34.67 % (26/75), fundus 30.67 % (23/75), body 30.67 % (23/75), and pylorus 21.33 % (16/75) as shown in Table 2.

Table 2 The microscopic lesions were found in each part of canine stomachs by H&E.

Part of canine stomach	Lesions were found (%) (n = 75)
Cardia	34.67
Fundus	30.67
Body	30.67
Pylorus	21.33

The histopathological results revealed lymphoid follicular formation 33.33 % (25/75); inflammatory cell infiltration 25.33 % (19/75); epithelial degeneration 17.33 % (13/75), and crypt distortion 13.33 % (10/75), respectively as shown in Fig 7. The histopathological lesions of the stomach were 60.0 % (45/75) which are Mild gastritis 64 .44 % (29/45), Moderate gastritis 11.11 % (5/45) and Severe gastritis 24.44 % (11/45) respectively (Handt et al., 1995). The presence of *Helicobacter spp*. using H&E staining, showed positive results at 17.33 % (13/75). The presence of *Helicobacter spp*. in different portion of stomach by H&E were cardia 10.67 % (8/75), fundus 6.67 % (5/75), body 12 % (9/75) and pylorus 8 % (6/75) as shown in Fig 8. *Helicobacter spp.* was observed in large numbers in the mucus covering the surface gastric epithelium, the gastric pits and the glandular lumina.

The histopathological evaluation of Warthin Starry stained (WSS) sections revealed the presence of spiral-shaped organisms measuring approximately 3 to 5 μm in length and 0.5 μm in width at 46.67 % (35/75). The *Helicobacter spp.* were localized in the luminal crypt 18.67 % (14/75), gastric pit 22.67 % (17/75), the gastric gland 21.33 % (16/75), and the gastric epithelium 8 % (6/75). The *Helicobacter spp.* was not seen in the cytoplasm of cells or the lamina propria. The presence of *Helicobacter spp*. in different portion of stomach by WSS was cardia 25.34 % (19/75), fundus 21.33 % (16/75), body 30.67 % (23/75), and pylorus 20 % (15/75) as shown in Fig 9.

The immunohistochemistry using rabbit polyclonal anti-*Helicobacter pylori* antibody (DAKO®, Denmark) revealed brown color in positive sites at 30.67 % (23/75). The presence of *Helicobacter spp*. antigen by IHC in cardia 16.00 % (12/75), fundus 13.33 % (10/75), body 10.67 % (8/75) and pylorus 12.00 % (9/75) as shown in Fig 10.

Table 3 Correlation between numbers of samples which cases presence of *Helicobacter spp*. in each part of canine stomachs by H&E, WSS and IHC.

Histopathologic methods	Location of canine stomach			
	Cardia	Fundus	Body	Pylorus
Helicobacter spp. by H&E	10.67[a] (8/75)	6.67[a] (5/75)	10.67[a] (8/75)	6.67[a] (5/75)
Helicobacter spp. by WSS	26.67[b] (20/75)	21.33[b] (16/75)	30.67[b] (23/75)	20.00[b] (15/75)
Helicobacter spp. by IHC	16.00[b] (12/75)	13.33[b] (10/75)	10.67[b] (8/75)	12.00[b] (9/75)

a, b significantly different effects ($p<0.05$) were detected

Polymerase chain reaction (PCR) results

1. Results from *H. pylori* from human

Positive control: The bands of approximately 400 bp were seen by 2% agarose gel electrophoresis following PCR amplification of the *Helicobacter spp.* specific sequences (Riley et al., 1996; Buczolits et al., 2001; Buczolits et al., 2003).

Positive control: The bands of approximately 403 bp were seen by 2% agarose gel electrophoresis following PCR amplification of *H. pylori* (*H. pylori* ATCC 43504, GeneBank accesion no. M88157; Choi et al., 2001).

Positive control: The bands of approximately 400 bp were seen by 2% agarose gel electrophoresis following PCR amplification of *H. pylori* CagA$^+$ (GenBank accession no. L11714 and EMBL accession no. X70039; Covacci et al., 1993; Tummuru et al., 1993; Lage et al., 1995)

2. Results from all the tissue specimens

Negative (-ve) for *H. pylori* from human

Negative (-ve) for *H. pylori* CagA$^+$ from human, cause of gastric neoplasia in human.

3. Results from *Helicobacter spp.*

The bands of approximately 400 bp were seen by 2% agarose gel electrophoresis following PCR amplification of the *Helicobacter spp.* specific sequences (Riley et al., 1996; Buczolits et al., 2001; Buczolits et al., 2003).

Positive results (+ve) were presented 10.67 % (8/75) as shown in Fig 11.

Table 4 Results of PCR detection

PCR detection	Positive control from human Band (bp)	PCR result (%) (n=75)
Helicobacter spp.	400	+ ve (10.67)
H. pylori	403	- ve
H. pylori CagA^{+}	400	- ve

Table 5 Results of number of samples were found *Helicobacter spp.* in canine stomach by H&E, WSS, IHC, and PCR.

Methods for detection of *Helicobacter spp.*	Number of sample (%) (n=75)
H&E	17.33 (13)
WSS	46.67 (35)
IHC	30.67 (23)
PCR	10.67 (8)

Table 6 The correlation of histopathology lesions in stomach, the presence of *Helicobacter spp.* by H&E, WSS and IHC and the clinical symptoms in *Helicobacter spp.* infected dogs and uninfected dogs by PCR method.

	Helicobacter + (%) (n = 8)	*Helicobacter* – (%) (n = 67)
1. Histopathology grading		
Mild gastritis	37.5 (3/8)	37.31 (25/67)
Moderate gastritis	0	7.46 (5/67)
Severe gastritis	12.5 (1/8)	14.92 (10/67)
2. H&E stain		
Presence of *Helicobacter spp.*	50 (4/8)	14.92 (10/67)
Absence of *Helicobacter spp.*	50 (4/8)	85.07 (57/67)
3. WSS stain		
Presence of *Helicobacter spp.*	62.5 (5/8)	40.29 (27/67)
Absence of *Helicobacter spp.*	37.5 (3/8)	59.70 (40/67)
4. IHC stain		
Presence of *Helicobacter spp.*	50 (4/8)	29.85 (20/67)
Absence of *Helicobacter spp.*	50 (4/8)	70.15 (47/67)
5. Clinical symptoms		
With clinical symptoms	25 (2/8)	53.73 (36/67)
Without clinical symptoms	75 (6/8)	43.28 (29/67)

Statistical analysis

The present study revealed statistically significantly different for *Helicobacter spp.* detection between H&E and WSS ($p<0.001$). And there was correlation between H&E and IHC ($p<0.05$), and PCR ($p<0.05$). The histopathological results were related with WSS in *Helicobacter spp.* infections ($p<0.05$). The pathological study by H&E, WSS and IHC, especially PCR and clinical signs of *Helicobacter spp.* infections in canine stomaches ($p>0.05$) were not statistically different. There was no statistically different significantly relationship with IHC and PCR. There was no significant correlation between locations of histopathological lesions and *Helicobacter spp.* infection ($p>0.05$). The number of *Helicobacter spp.*was not significantly different in each part of stomach ($p>0.05$).

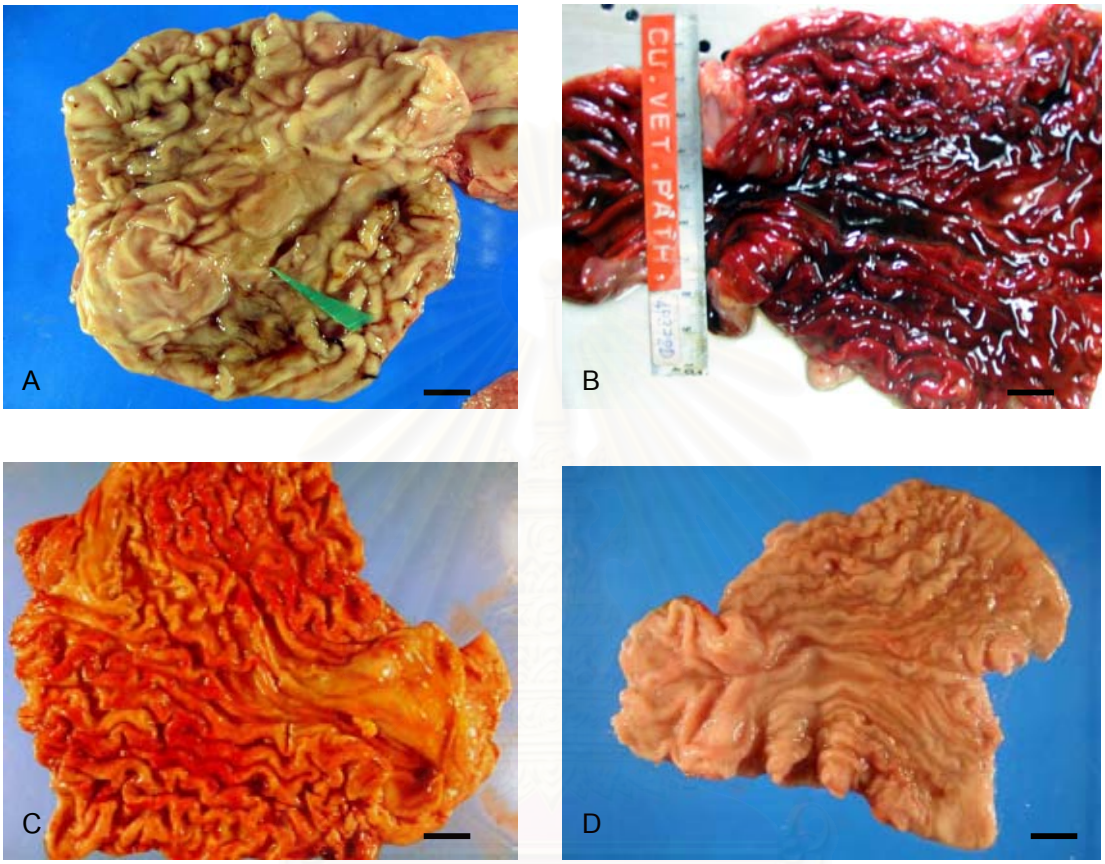


Fig 6 Macroscopic lesions were shown as Chronic mutifocal ulcerative gastritis; An ulcer with 0.5 x 1.5 cm was presented on the gastric mucosa at body part of stomach (green arrowhead) (A). Acute diffuse mucohemorrhagic gastritis (B).
Severe acute diffuse catarrhal gastritis (C) normal canine stomach (D). (Bar = 1 cm)

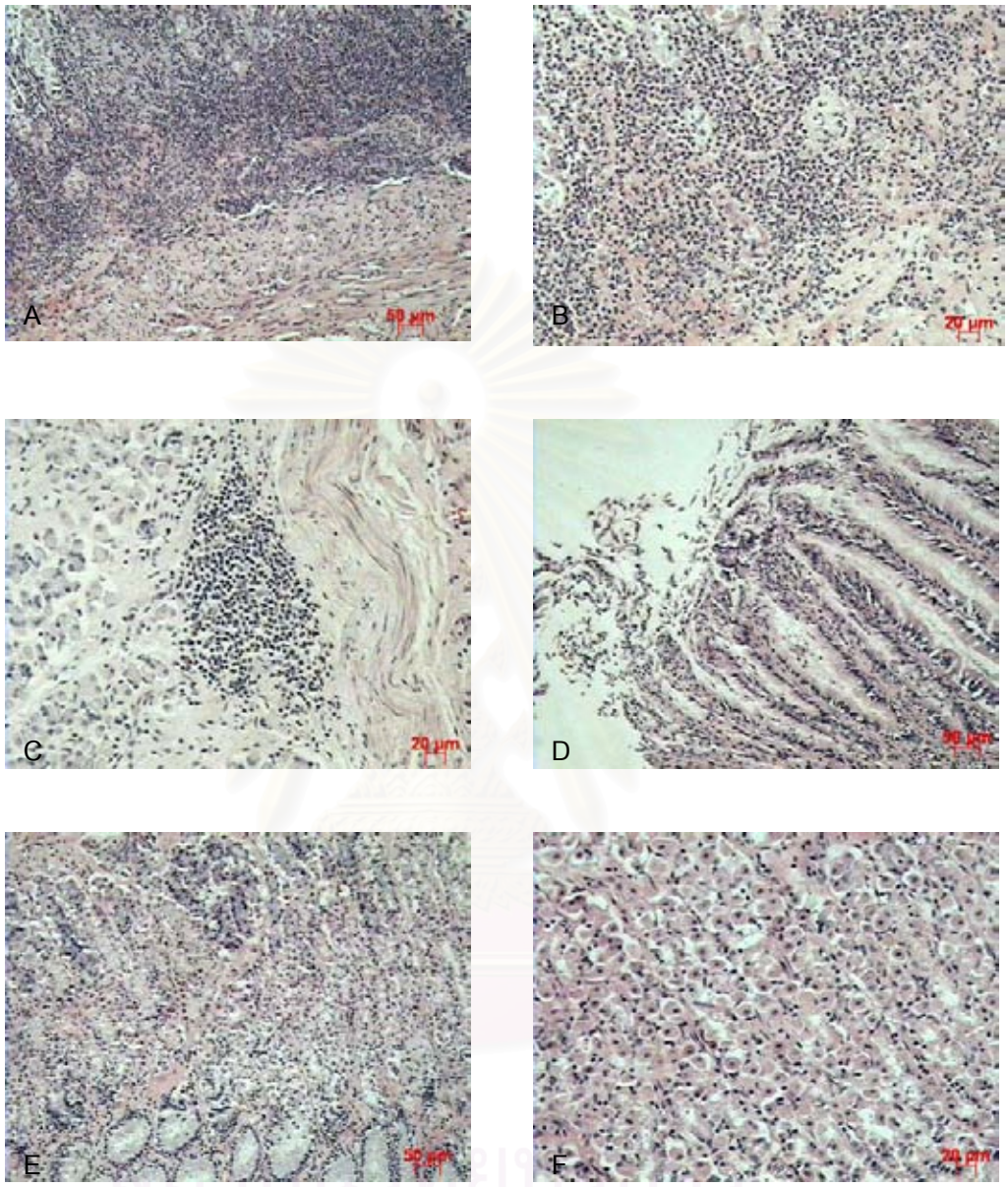


Fig 7 The histopathological results revealed inflammatory cell infiltration, numerous neutrophils infiltration in gastric mucosa as ulcerative gastritis (A and high magnification, B); lymphoid follicular formations (C); gastric epithelial sloughing (D) and crypt distortion (E and high magnification, F). (H&E, A, D, E Bar = 50 μm; B, C, F Bar = 20 μm)

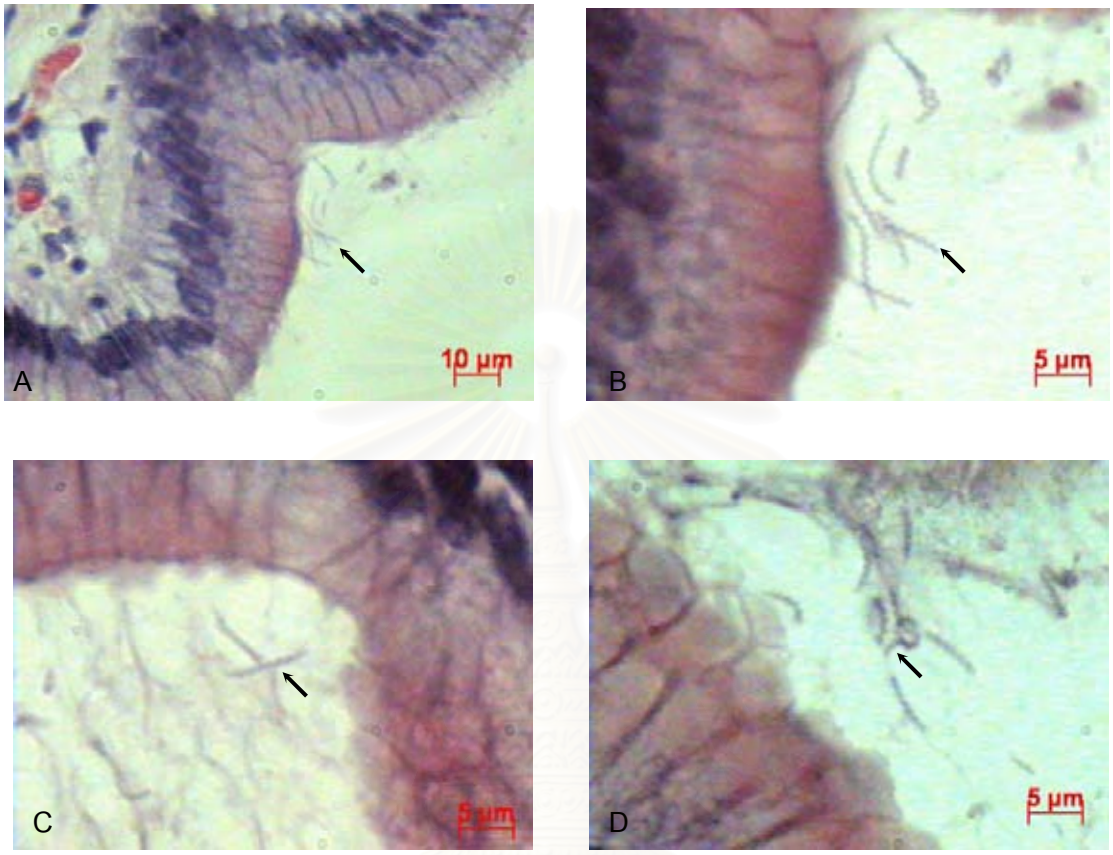


Fig 8 The numerous spiral shape organisms: *Helicobacter spp*. was presented in the surface of gastric mucosa in fundus part of stomach. (Arrow) (A) (H&E, Bar = 10 µm)

Helicobacter spp. was demonstrated on the gastric epithelium in higher magnification. (Arrow) (B) (H&E, Bar = 5 µm)

Helicobacter spp. was observed in the glandular lumina in fundus part of stomach. (Arrow) (C) (H&E, Bar = 5 µm) The *Helicobacter spp.* were found in large numbers in the mucus covering the surface epithelium of stomach. (Arrow) (D) (H&E, Bar = 5 µm)

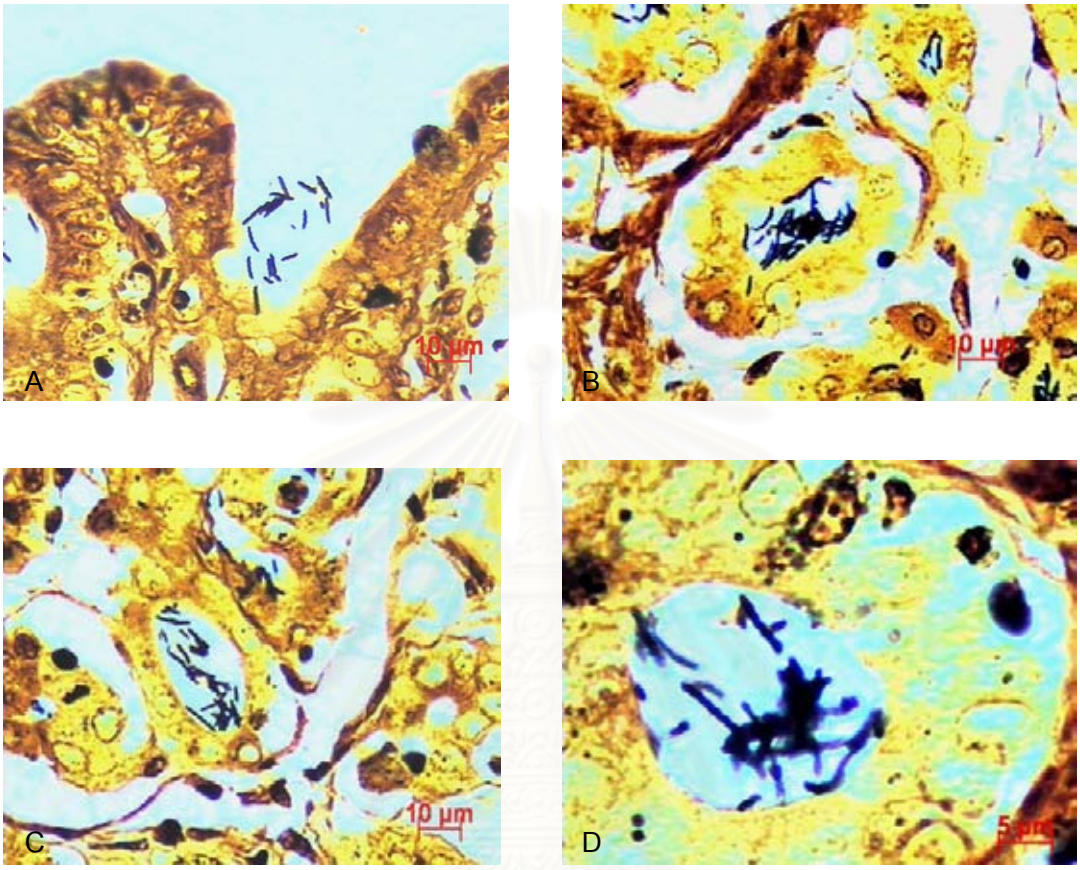


Fig 9 Spiral-shape *Helicobacter spp.* was positive in dark brown color in luminal crypts on the surface gastric mucosa in fundus part of stomach (A) (WSS, Bar = 10 µm) *Helicobacter spp*. was presented in the gastric pit in fundus (B, C) (WSS, Bar = 10 µm). In higher magnification of spiral-shape with dark brown color of *Helicobacter spp*. was shown 3.0 - 5.0 µm in length and 0.5 – 1.0 µm in width. (D) (WSS, Bar =5 µm)

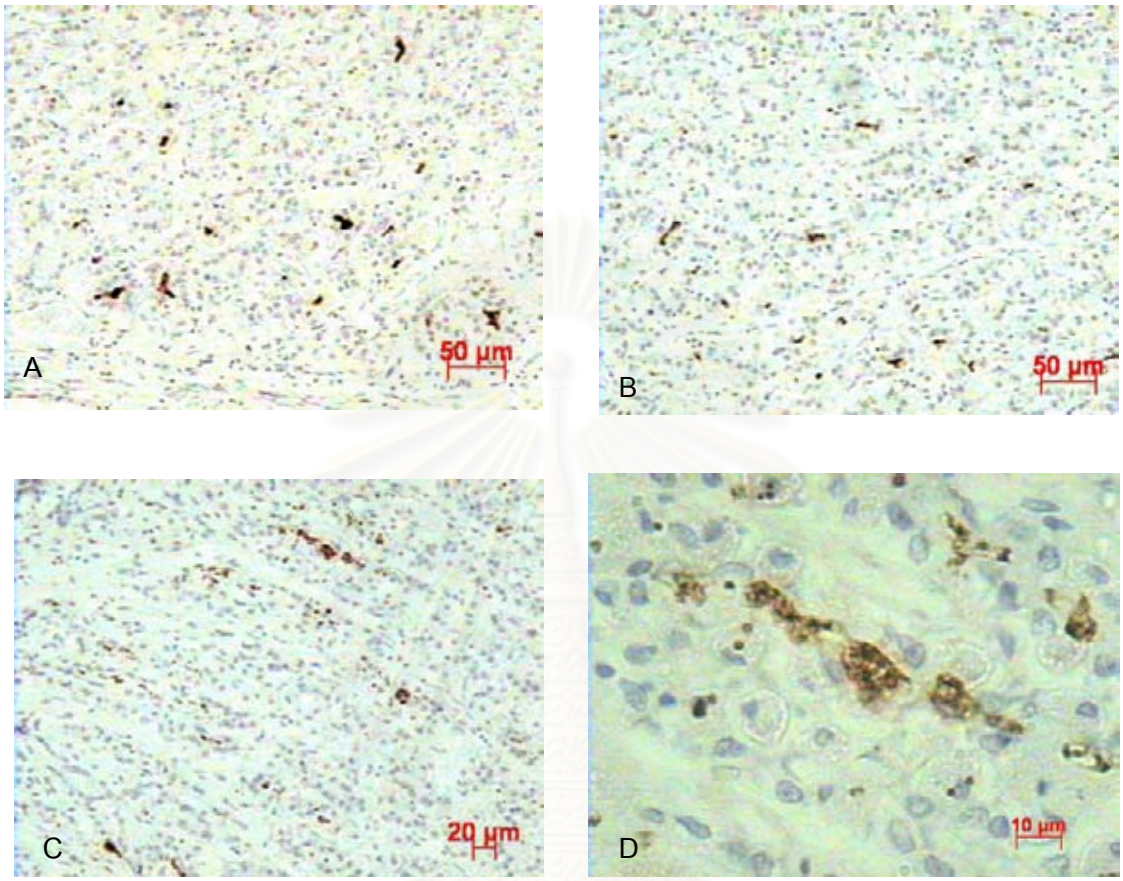


Fig 10 *Helicobacter spp.* demonstrated brown color in positive sites by Immunohistochemistry using rabbit polyclonal anti-*H. pylori* antibody in lumen of gastric gland in fundus part of stomach. Meyer's Hematoxylin counterstained, DAB (A,B Bar = 50 µm), (C Bar =20 µm) and (D Bar =10 µm)

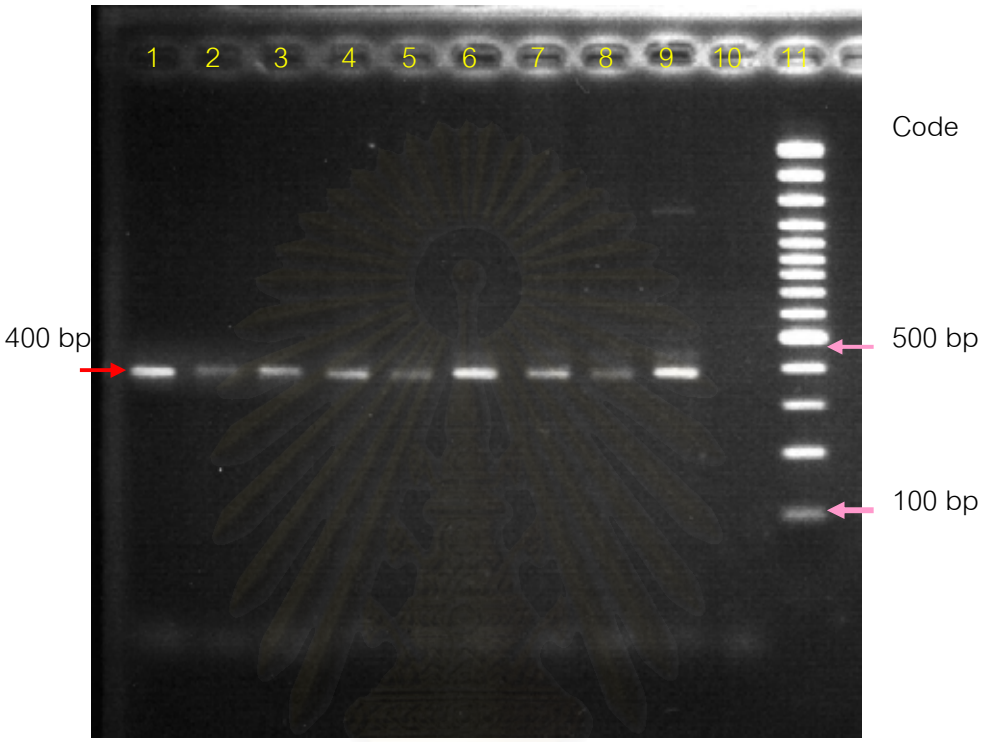


Fig 11 The bands of 400 bp were detected by 2% agarose gel electrophoresis following PCR amplification of the *Helicobacter spp.* specific sequences. Lane 1 – 8 Positive results from canine gastric tissues; Lane 9 Positive control *H. Pylori* from human; Lane 10 Negative control; Lane 11 100 bp DNA marker ladder.

CHAPTER 5
DISCUSSION

The results of this study confirm other studies which suggested the presence of *Helicobacter spp*. is prevelently found in dogs at 46.67 % of study cases (Henry et al., 1987; Geyer et al., 1993; Hermanns et al., 1995; Pirarat et al., 2003). It has been reported that the gastric biopsies found 82 % of the dogs had an *Helicobacter*-like organism infection and dogs were postulated to be infected with several species of *Helicobacter spp. (*Hermanns et al., 1995). *Gastrospirillum hominis*, another *Helicobacter*-like organism, has so far not been cultivated in artificial media (Solnick et al., 1993). While,it is said to be the most commonly occurring of *Helicobacter*-like organisms in dogs and cats (Lee and O'Rourke, 1993). Most of the gastric tissues in this study were randomly collected from necropsy cases which was the first report in Thailand. The most frequently clinical symptoms were loss of appetite, vomiting, and diarrhea which could be noticed at 52 % (39/75) of the necropsy case in this study (Hermanns et al., 1995; Lage et al., 1995). But several recent reports have indicated that chronic gastritis, which is associated with intermittent vomiting, is also observed in dogs, and an etiology of this condition is rarely diagnosed (Flatland, 2002). Although, *Helicobacter spp*. produced the enzyme urease, which breaks down urea into ammonia and bicarbonate ions in the stomach rendering the toxicity to gastric epithelial cells, which could support the evidence of gastroenteritis by *Helicobacter spp*. infection in dogs. There was no correlation between *Helicobacter spp.* infection and clinical symptoms ($p>0.05$). Thus, role of gastric *Helicobacter* organisms in the development or potentiation of gastritis must be considered. Nevertheless, not all individuals infected with *H. pylori* develop gastric illness and this finding might be related to various factors such as the environment, host genetic factors, and bacterial virulent ability (Perng et al., 2003). In contrast, a clinical significant relationship between *Helicobacter spp.* infection and gastric pathology could not be established as upon the results the *Helicobacter spp*. infected dogs did not show any signs of gastritis (Eaton et al., 1996; Hermanns et al., 1995 and Yamasaki et al., 1998). In an experimental study of beagles

infected with *H. felis* and *H. bizzozeronii*-like organisms, Simpson et al., 2000 concluded that infected dogs showed no clinical signs. The mode the infection either in animals or in man has not yet been clarified, but oral transmission is assumed (Hermanns et al., 1995).

Gastric *Helicobacter spp*. was found in the cardia, fundus, body, and pylorus (Hermanns et al., 1995). In this study location of stomach were found in cardia 34.67 % (26/75), fundus 30.67 % (23/75), body 30.67 % (23/75) and pylorus 21.33 % (16/75) which was similar to Pirarat et al. (2003). *Helicobacter spp.* was demonstrated considerable affinity for parietal cells and is found in their intracellular canaliculi as well as in the cytoplasm. They had been observed in large numbers in the mucus covering the surface epithelium, the gastric pits and the glandular lumina (Hermanns et al., 1995). Even in the gastric body and cardia, *Helicobacter spp*. were presented in most necropsy dogs on the basis of an evaluation of histological sections in our study which is different from Dunn et al. (1997). It appears that *Helicobacter spp.* infection does not significantly alter gastric acid secretion in dogs. Study of naturally infected dogs has shown that Helicobacter predominantly colonizes the gastric cardia and fundus (Simpson et al., 2000; Flatland, 2002).

There was no statistically different significant correlation between locations of histopathological lesion and *Helicobacter spp.* infection using H&E ($p>0.05$). The presence of lymphoid follicles has traditionally been considered a common, nonspecific finding in the gastric mucosa of dogs. Most dogs had many bacteria and only mild gastritis. It is suggested that in dogs, the bacteria did not induce histological evident of certain diseases (Eaton et al., 1996; Simpson et al., 1999). In our study cases, the naturally infected with *Helicobacter spp.* the lymphoid follicles are frequently found in subglandular areas and sometimes extended between the glands as 33.33 % (25/75) (Henry et al., 1987; Lee et al., 1992). Moreover, it was reported that, gnotobiotic dogs which experimentally infected with *Helicobacter spp.* developed large numbers of lymphoid follicles throughout the gastric mucosa, while the two control animals had no follicles (Lee et al., 1992). It is possible to introduce that the occurrence of lymphoid follicles in this study cases were indicated the *Helicobacter spp*. infection from gastrointestinal specimens. And also the randomized

specimens from necropsies, lymphoid follicles are also diffusely located in another part of the stomach that could be missed by the collecting method. The *H. pylor*i infection in children frequently results in marked gastric lymphoid hyperplasia, have led to the hypothesis that lymphoid follicles in the human gastric mucosa can result from chronic *H. pylori* antigenic stimulation and, therefore, represent a specific immune response directed against the organisms. Also, by inducing lymphoid tissue formation in the gastric mucosa, *H. pylori* may be a necessary precursor for the development of primary gastric MALT lymphoma (Handt et al., 1995). It should be noted that the histopathological changes in the dogs was much less severe than those seen in *H. pylori* gastritis in human (Hermanns et al., 1995) which could be due to species variation. There was no correlation between *Helicobacter spp.* and histopathology lesion ($p>0.05$). It will be of interest to study dogs which have been subjected to different environmental conditions or dietary changes to ascertain if differences in the gastritis profile occur with manipulation of these variables.

Upon the study revealed significantly different correlations of *Helicobacter spp.* detection between H&E and WSS ($p<0.001$). *Helicobacter spp*. can be visualized at the high magnification with conventional H&E stained sections. Bacteria are located in the mucus adherent to the surface epithelium and are often found deep within the crypts. There were presented *Helicobacter spp.* by H&E in cardia 10.67 %, body 10.67 %, fundus 6.67 % and pylorus 6.67 %. However, H&E staining may be unclear when few bacteria are also presented (Dunn, 1997). In addition, luminal debris on the surface of the epithelium can be mistaken for *Helicobacter spp.* in H&E stained sections. Although these organisms with certain morphology 2.5-5.0 μm length, 0.5- 1.0 μm width and had five to nine helical turns could be notified. But the other organisms such as *Bacillus* organisms were difficult to histology evaluation. Because of the presented things were presented quite similar to *Helicobacter spp.* in color by H&E staining in histology evaluation (Handt et al., 1994). Using special stains such as WSS facilitates histological identification of bacteria. *Helicobacter spp.* was obviously presented in dark-brown color on yellow background. These bacteria were located mainly in the gastric pits and the upper portions of gastric glands, where they were often in intimate association with the epithelial cell surface. These

bacteria were located within the gland lumens, sometimes attached to the epithelial cell surface, and within the cytoplasm of parietal cells. WSS sections of stomach part revealed tightly coiled helical bacteria that were generally 2.5- 5.0 μm length. From the results, there were presented *Helicobacter spp.* in body 30.67 % (23/75), cardia 26.67 % (20/75), fundus 21.33 % (16/75) and pylorus 20.00 % (15/75). WSS is high occurence than H&E (Dunn et al., 1997). It is likely that the additional expense of WSS is offset by a reduction in time required for slide evaluation and improvement of identification of *Helicobacter spp.* infection. A sensitive staining technique consisting of a combination of H&E and WSS has been developed. WSS could be used for detecting *Helicobacter spp*. in gastric tissues short period of time, cheap and easy to use (Dunn et al., 1997). The method presented here enables examination of large numbers of sections in a relatively short period of time. However, the special staining procedure can be technically difficult, and require experiences (Pirarat et al., 2003). Its meaning acceptance among gastrointestinal pathologists has not been proved yet (Laine et al., 1997). Other factors that could influence gastric pathology include the number of organisms present, the host species and genotype, environmental factors such as diet, and concurrent infection with undetected organisms (Fox and Lee, 1997).

The present study revealed significantly different for *Helicobacter spp.* detection between H&E and IHC ($p<0.05$). IHC was much more sensitive for detecting infection than the routinely used H&E and WSS. IHC staining also have been developed to detect *Helicobacter spp*. antigen (Ashton-Key et al., 1996). In this study, the ABC-peroxidase technique with a rabbit polyclonal anti-*H. pylori* antibody (DAKO, Denmark) was used. IHC using monoclonal anti-*H. pylori* antibody is the best diagnostic tool for formalin-fixed samples. And IHC is very high specificity (Esteves et al., 2000). But IHC is rather expensive, long period of time and good experience. Such IHC are usually not necessary but may prove worthwhile in cases where stains are difficult to evaluate for confirmations (Dunn et al., 1997).

This study revealed significantly different correlations for *Helicobacter spp.* detection between H&E and PCR ($p<0.05$). PCR offers great promise as a highly sensitive

and specific technique for the detection of *Helicobacter spp.* and *H. pylori.* PCR technique for the detection of *H. pylori* in gastric biopsy specimens has been described by a number of laboratories although the accuracy of such technique varies widely (Ashton-Key et al., 1996). The identification of *Helicobacter spp.* in biopsies from dogs using PCR has not been reported yet and applied for detection of identification to species of *Helicobacter spp*. in dogs with naturally occurring gastric helicobacteriosis in Thailand. Several observations shown that PCR was sensitive and specific which is in agreement with studies in mice infected with *H. felis* and in humans and cats infected *H. pylori*, which showed that PCR was more sensitive than histology, bacterial culture, and urease mapping (Simpson et al., 1999). Factors affecting test accuracy of PCR include the choice of primers and target DNA, specimen preparation, bacterial density and technical issues. PCR demonstrated excellent accuracy for the detection of *H. pylori* infection in a limited number of samples. PCR technique for the detection of *H. pylori* is still in its infancy. It is unlikely that such technique will have widespread use in the initial detection of *H. pylori.* However, PCR method hold great promise in the detection of genetic differences between *H. pylori* strains for research and epidemiological studies (Dunn et al., 1997). Otherwise, in this study *Helicobacter spp.* were found at 10.67% (8/75) by PCR. There was no relationship between PCR and clinical symptoms of *H. pylori* infection in canine stomach ($p>0.05$).

The results of PCR technique were low-presented 10.67 % (8/75) in this study, because of factors affecting PCR detection technique include location of sampling, specimen preparation, the choice of primers and target DNA, bacterial density and technical issues (Dunn et al., 1997), the step of DNA extraction or inhibitor of the gastric juice and tissue should be concerned (Grant, 2003). In this study we randomly collected location of canine gastric tissues in each part of stomach with/without lesions. The choice of primers and target DNA were selected based on the earlier study. The bands of 10^6 CFU/µl positive control, *H. pylori* from human 400 bp were obviously seen by 2% agarose gel electrophoresis following PCR amplification of the *Helicobacter spp.* specific sequences. Which lesions could be confirmed the extraction method by the QIAmp Tissue Kit (Qiagen Inc., CA., USA) according to the manufacturer's instructions and the gold standard Phenol-

chloroform method. However, the DNA extraction from the tissue samples should be discussed, and possibly disturbed the amplification of the fragments of DNA because of some blocking factor (Ploskonosova et al., 1999). Otherwise, the use of gastric tissues without prior rinsed gastric juice could block PCR process for an detection for *Helicobacter spp.* in the gastric tissues (Grant, 2003).

From this study, *H. pylori* could be not detected by PCR in any of the 75 studied necropsy dogs. This is in agreement with the other studies which *H. pylori* were not described from dogs (Henry et al., 1987; Geyer et al., 1993; Hermanns et al., 1995). These findings are important, it is implied that pet dogs do not represent as a source of *H. pylori* for human population. In spite of, the frequent occurrence of *Helicobacter spp.* in dogs, the large number of pets, and their close contact with their human are not public health hazard. Recently, the certain *H. pylori* genes have been experimentally associate to with increased incidence of peptic ulcer and gastric neoplasia has increased interest in gastric bacteria also in dogs (Buczolits et al., 2003).

In conclusion, there is no single gold standard among the diagnostic tests for *H. pylori* infection but all of the tests have their pitfalls and limitations. The situation of the cases the specimen obtained as well as, the laboratory and technical facilities shold be considered, H&E is routine for histopathology evaluation but *Helicobacter spp.* could not seen clearly. Because of color of *Helicobacter spp.* in the tissue sections as same as background color. WSS is obviously detected, cheap and short time. IHC is confirmation to complete results. PCR technique use for small sample, follow-up treatment. The clinical symptoms showed no relation to *Helicobacter spp.* infection. No *H. pylori* could be demonstrated from studied cases. The *Helicobacter spp*. infection cases from PCR could possibly be other species such as *H. heilmannii*, *H. felis, & H. bizzozeronii* (Fox et al.,1995; Handt et al., 1995; Flatland, 2002).

The future study should be conducted in fresh canine gastric biopsy which might be a suitable specimens for study of *Helicobacter spp.* infections for bacterial isolation, PCR method as well as invasive method. The specific primers should be employed. Furthermore the PCR sequencing for correlation between *Helicobacter spp.* found in dogs and their

owner should be make a benefit for the Veterinary Public Health aspect. The obtained results will be useful in epidemiological, diagnosis of other species of *Helicobacter* infection in dogs in Thailand.

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Appendices

Appendix A

Preperation of Warthin – Starry staining

- Acidulated water

  Triple D.W.

  Add enough 1% aq citric acid to bring water to pH 4 (3.6 – 3.8)

- 1% Silver nitrate

Silver nitrate	1	g
Acidulated water	100	ml

- 2% Silver nitrate

Silver nitrate	2	g
Acidulated water	100	ml

- 5% Gelatin Solution

Gelatin	5	g
Acidulated water	100	ml

- 0.15% Hydroquinone Solution

Hydroauinone	0.15	g
Acidulated water	100	ml

Warthin – Starry Methods

1. Deparaffinized and washing in clean and clear D.W.
2. 3 times D.W. and 1 time triple D.W.
3. Bath in acidulated water for 30 mins
4. Bath in 1% silver nitrate solution at 60°C for 1 hr (Impregnation)
5. Prepare developer
6. Keep all developer and D.W. in 60°C
7. After finished 4. Bath in developer and check control under microscope
8. Wash in D.W. 60°C quickly
9. Rince in D.W.
10. Dehydrate and mount

(Handt et al.,1995; Pirarat et al., 2003)

Appendix B

Immunohistochemical Staining For *H. pylori* (ABC kit)

1. Deparaffinization

   Xylene I (5 min) →Xylene II (5 min) →Xylene III (5 min) →Xylene+Alc (2 min) →Abs.alc I (2 min) → Abs.alc II (2 min) → 95%alc. (2min) →80%alc. (2min) → 70%alc. (2 min) →Running water (5 min) →D.W. (5 min) →PBS (5 min)
2. Pretreat slides by Trypsin For 30 min at 37°C
3. Wash in PBS 5 min x 3
4. Block endogenous peroxidase by 3%H_2O_2 30 min (Room Temperature)

   (Absolute Methanol 150 ml + 30% H_2O_2 1.5 ml)
5. Wash in D.W. 5 min x 1 , PBS 5 min x 2
6. Blocking skim milk 30 min (RT) (skim milk 1.5 g in D.W. 150 ml)
7. Wash in PBS 5 min x 3
8. Primary antibody *H.pylori* dilution 1:200 Overnight at 4°C
9. Wash in PBS 5 min x 3
10. Biotinylated IgG Goat Anti Rabbit dilution 1:400 30 – 60 min at 37°C

    Prepare ABC (A 45 µl + B 45 µl in PBS 5 ml) 30 min before use
11. Wash in PBS 5 min x 3
12. ABC kit 30 min at 37°
13. Wash in PBS 5 min x 3
14. DAB substrate (DAB 0.075 g + Tris buffer 150 ml + 30% H_2O_2 50 µl) 3 min
15. Stop and Wash in D.W.
16. Counterstain with hematoxylin 30 sec – 1 min
17. Wash in running water 5 min , Dehydrate and mount with mounting media

(Esteves et al., 2000; Pirarat et al., 2003)

Appendix C

Details of kinds of food in take, clinical sign, gross lesion, histopathological lesion score and detection of *H. pylori* by H&E, WSS, IHC and PCR

Case no.	Breed	Sex	Age	Food	Sign	Gross lesion	Histopatholagical lesion score	H&E	WSS	IHC	PCR
1	Chowchow	F	3 yr	Homemade	+	Diffuse erosive gastritis	-	-	-	-	-
2	Poodle	F	6 yr	-	+	NRL	-	-	-	+	-
3	Mongrel	F	5 mt	-	-	Mild acute catharrhal hemorrhagic gastritis	-	-	+1	-	+
4	Poodle	M	2 mt	Homemade	+	NRL	+1	-	-	-	-
5	Mongrel	F	8 yr	Homemade	-	NRL	+1	-	-	-	-
6	Pomeranian	F	1 yr	-	-	NRL	+1	-	+2	-	-
7	Rottweiler	M	10 yr	-	-	NRL	+1	+	+2	-	+
8	Doberman	F	4 yr	-	+	Marked massive hemorrhagic gastritis	+3	+	+3	-	-
9	Golden retriver	F	10 mt	-	+	NRL	+1	-	+3	+	-
10	Mongrel	F	-	Homemade	+	NRL	+1	-	-	+	-
11	Rottweiler	M	10 yr	Homemade	+	NRL	+2	-	-	-	-
12	Mongrel	M	5 yr	Homemade	+	NRL	+3	-	-	-	-

M: Male F: Female Food: - no detail Sign: - no clinical sign, + clinical sign NRL: no remarkable lesion

Histopatholagical lesion score -: no lesion, +1: mild, +2: moderate, +3: severe H&E: + positive, - negative

WSS: - no organism, +1 organism < 10, +2 organism 10-50, +3 organism > 50 IHC: + positive, - negative PCR: + positive, - negative

Case no.	Breed	Sex	Age	Food	Sign	Gross lesion	Histopatholagical lesion score	H&E	WSS	IHC	PCR
13	Mongrel	F	15 yr	-	+	Gastric congestion	+2	-	+1	-	-
14	Rottweiler	F	3 yr	Commercial	+	Large focal gastric polyps	+3	+	+2	-	-
15	Bulldog	M	2 mt	Commercial	-	NRL	-	-	-	-	+
16	Rottweiler	M	3 yr	Homemade	+	Hemorrhagic gastritis	-	+	+3	+	-
17	Poodle	M	6 yr	Homemade	-	NRL	-	+	+3	+	+
18	Poodle	F	7 mt	Homemade	+	NRL	+1	-	+3	-	-
19	Mongrel	F	-	-	+	NRL	+1	-	+2	+	-
20	Mongrel	F	10 yr	Homemade	+	NRL	+1	-	-	-	-
21	Bangkaew	F	7 yr	Homemade	+	NRL	+1	-	-	-	-
22	Poodle	M	3 yr	Homemade	+	Submucosal hemorrhagic gastritis	-	+	+1	+	-
23	Shizu	F	2 yr	-	+	Multifocal hemorrhagic gastritic	-	-	-	-	-
24	Mongrel	F	8 yr	-	+	NRL	-	-	-	-	-
25	Chowchow	M	10 yr	-	-	NRL	+1	-	-	+	-
26	Dalmatian	M	4 yr	-	-	Moderated ecchymotic hemorrhagic gastritis	+2	-	-	-	-
27	Shizu	F	2 yr	Commercial	-	NRL	+1	+	+3	+	-
28	Mongrel	F	6 yr	-	-	NRL	-	-	-	-	-

M: Male F: Female Food: - no detail Sign: - no clinical sign, + clinical sign NRL: no remarkable lesion

Histopatholagical lesion score -: no lesion, +1: mild, +2: moderate, +3: severe H&E: + positive, - negative

WSS: - no organism, +1 organism < 10, +2 organism 10-50, +3 organism > 50 IHC: + positive, - negative PCR: + positive, - negative

Case no.	Breed	Sex	Age	Food	Sign	Gross lesion	Histopathological lesion score	H&E	WSS	IHC	PCR
29	Doderman	M	7 yr	Commercial	-	NRL	-	-	-	+	-
30	Mongrel	F	-	Homemade	+	NRL	-	-	-	-	-
31	Mongrel	M	11 yr	Homemade	-	NRL	+1	-	+3	-	-
32	Bulldog	M	8 mt	Commercial	-	Large amount of blood clot	+1	-	+1	-	-
33	Mongrel	M	10 yr	Homemade	-	Catarrhal gastritis	-	-	-	-	-
34	Mongrel	M	3 mt	-	-	NRL	+1	-	+3	-	-
35	Mongrel	F	6 yr	-	-	NRL	-	-	-	+	-
36	Thai	F	4 yr	Homemade	+	Multiple ulcerative gastritis	-	-	-	-	-
37	English cocker spenial	F	3 yr	Homemade	+	NRL	+1	-	+1	-	-
38	Mongrel	F	3 yr	Homemade	-	NRL	+1	-	+2	+	-
39	Mongrel	F	1 yr 2 mt	-	+	Moderated acute multifocal mocohemorrhagic ulcerative gastritis	+3	-	-	-	-
40	Mongrel	F	15 yr	Homemade	+	NRL	-	-	+1	+	-
41	Dalmatian	M	9 mt	Homemade	+	Hemorrhagic gastritis	-	-	-	+	+
42	Eng. cocker spenial	M	2 yr	-	-	NRL	-	-	-	-	-

M: Male F: Female Food: - no detail Sign: - no clinical sign, + clinical sign NRL: no remarkable lesion

Histopathalogical lesion score -: no lesion, +1: mild, +2: moderate, +3: severe H&E: + positive, - negative

WSS: - no organism, +1 organism < 10, +2 organism 10-50, +3 organism > 50 IHC: + positive, - negative PCR: + positive, - negative

Case no.	Breed	Sex	Age	Food	Sign	Gross lesion	Histopathological lesion score	H&E	WSS	IHC	PCR
43	Doberman	F	10 yr	-	-	Ulcerative gastritis	+3	-	+1	-	-
44	Miniature Pincher	F	4 yr	-	-	NRL	+1	+	+3	+	-
45	Mongrel	M	6 mt	Homemade	+	NRL	+1	-	+1	+	-
46	Labrador retreiver	M	5 mt	-	-	Gastric congestion	+2	-	-	-	-
47	Labrador retreiver	M	3 yr	-	-	Hemorrhagic gastritis	-	-	+3	-	-
48	Mongrel	M	10 yr	Homemade	-	NRL	-	-	-	-	-
49	Mongrel	F	3 yr	-	-	NRL	-	-	-	-	-
50	Pug	M	2 yr	-	+	Severe diffuse catarrhal gastritis	-	-	-	+	-
51	Miniature Phincher	M	4 mt	-	-	NRL	+1	+	+3	+	+
52	Beagle	M	45 d	-	+	NRL	+1	-	-	-	-
53	Pomeranian	F	3 mt	-	-	NRL	+1	+	+3	+	-
54	Pomeranian	M	2 mt	Commercial	+	Catarrhal gastritis	-	-	-	-	-
55	Thai	F	18 yr	Homemade	+	Mild focal ulcerative gastritis	+2	-	-	-	-

M: Male F: Female Food: - no detail Sign: - no clinical sign, + clinical sign NRL: no remarkable lesion

Histopatholagical lesion score -: no lesion, +1: mild, +2: moderate, +3: severe H&E: + positive, - negative

WSS: - no organism, +1 organism < 10, +2 organism 10-50, +3 organism > 50 IHC: + positive, - negative PCR: + positive, - negative

Case no.	Breed	Sex	Age	Food	Sign	Gross lesion	Histopathological lesion score	H&E	WSS	IHC	PCR
56	German shepherd	F	5 yr	-	+	Large locally extensive hemorrhagic gastritis	+3	-	+1	+	-
57	Mongrel	F	8 yr	-	-	NRL	+1	-	+3	+	-
58	Mongrel	F	14 yr	-	+	NRL	+1	-	-	-	-
59	Pomeranian	M	2 mt	Commercial	+	Focal depression arreat the pyloric part	-	-	-	-	-
60	Mongrel	M	8 mt	-	-	NRL	+1	-	-	-	+
61	Mongrel	M	10 yr	-	+	Small focal ulcerative gastritis	+3	-	-	-	-
62	Thai	F	13 yr	Homemade	-	Hemorrhagic ulcerative gastritis	+3	-	+3	+	-
63	Mongrel	M	12 yr	Homemade	+	NRL	-	-	+1	-	-
64	Greatdane	M	8 mt	Homemade	-	Small diffuse ulcerative gastritis	+3	-	-	-	-
65	Mongrel	M	11 yr	Homemade	+	Small diffuse ulcerative hemorrhagic gastritis	+3	+	+3	+	+
66	Shizu	F	12 yr 3 mt	-	+	NRL	-	-	-	-	-
67	Dalmatian	M	6 yr	Homemade	-	NRL	+1	-	-	-	-

M: Male F: Female Food: - no detail Sign: - no clinical sign, + clinical sign NRL: no remarkable lesion

Histopatholagical lesion score -: no lesion, +1: mild, +2: moderate, +3: severe H&E: + positive, - negative

WSS: - no organism, +1 organism < 10, +2 organism 10-50, +3 organism > 50 IHC: + positive, - negative PCR: + positive, - negative

Case no.	Breed	Sex	Age	Food	Sign	Gross lesion	Histopathological lesion score	H&E	WSS	IHC	PCR
68	German Shepherd	M	7 yr	-	-	NRL	+1	+	+3	-	-
69	English cocker spenial	F	8 yr	Homemade	+	Catarrhal gastritis	-	-	-	-	-
70	Mongrel	F	9 yr	-	-	NRL	+1	-	-	-	-
71	Mongrel	F	13 yr	-	-	NRL	+1	-	-	-	-
72	Mongrel	M	3 mt	Homemade	+	Catarrhal gastritis	+3	+	+2	-	-
73	Border terrier	M	13 yr	-	-	NRL	-	-	-	-	-
74	Rottweiler	F	3 mt	-	+	NRL	-	-	-	-	-
75	Bulldog	M	8 mt	-	-	Severe acute hemorrhagic gastritis	-	-	-	-	-

M: Male F: Female Food: - no detail Sign: - no clinical sign, + clinical sign NRL: no remarkable lesion

Histopatholagical lesion score -: no lesion, +1: mild, +2: moderate, +3: severe H&E: + positive, - negative

WSS: - no organism, +1 organism < 10, +2 organism 10-50, +3 organism > 50 IHC: + positive, - negative PCR: + positive, - negative

Appendix D

Statistics of H&E by WSS in *Helicobacter spp.* infections using Chi-square analysis (n = 75)

H&E	WSS		
	Negative	Positive	Total
Negative	43	19	62
Positive	0	13	13
Total	43	32	75
DF = 1	Value = 21.132		$p<0.001$

Statistics of H&E by IHC in *Helicobacter spp.* infections using Chi-square analysis (n = 75)

H&E	IHC		
	Negative	Positive	Total
Negative	47	15	62
Positive	5	8	13
Total	52	23	75
DF = 1	Value = 7.049		p =0.008

Statistics of H&E by PCR in *Helicobacter spp.* infections using Fisher's exact test (n = 75)

H&E	PCR		
	Negative	Positive	Total
Negative	57	5	62
Positive	9	4	13
Total	66	9	75

p =0.043

Statistics of histopathological results (HO) by WSS in *Helicobacter spp.* infections using Wilcoxon rank sums test (n = 75)

WSS	Number of samples	Sum of scores	Expected under HO	SD under HO	Mean score
Negative	43	1424.5000	1634.0	87.3178310	33.1279070
Positive	32	1425.5000	1216.0	87.3178310	44.5468750

S = 1425.50 Z = 2.39355 Prob > $|Z|$ = 0.0167

VITA

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