TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2016) 40: 81-88
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doi:10.3906/vet-1502-82

Detection of *Helicobacter heilmannii* type II and *Helicobacter pylori* in dogs and their role in the development of gastritis

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Received: 25.02.2015 • **Accepted/Published Online:** 21.10.2015 • **Final Version:** 05.01.2016

Abstract: The aim of this study was to detect the presence of *Helicobacter pylori* and *Helicobacter heilmannii* type II in dogs and evaluate the role of *Helicobacter* spp. in chronic gastritis. Gastroscopy was performed in a total of 40 dogs (22 symptomatic and 18 asymptomatic) admitted to the small animal clinic of İstanbul University. In this study, the density of spiral bacteria and the degree and type of gastritis by histopathology were determined. Rapid urease tests were performed on gastric biopsy samples, and the species identity was determined using nested polymerase chain reaction to assess for the presence of *H. heilmannii* type II and *H. pylori.* The relationship between symptoms and gastroscopic findings was significant ($P < 0.001$). Gastritis was diagnosed in 38 of the 40 dogs histopathologically, of which 71% were atrophic. Thirty-four dogs were found to be positive for *H. heilmannii* type II. Although general opinion indicates that dogs do not harbor *H. pylori*, 3 dogs with gastric symptoms and 1 asymptomatic dog were found positive for *H. pylori* in this study. No significant correlation was found between *Helicobacter* spp. density and gastritis severity. However, *H. pylori* had a high pathogenic effect as higher infiltration rates and increased intensity of mononuclear inflammatory cells and lymphoid follicles were observed in cases of *H. pylori*-related gastritis.

Key words: Dog, gastritis, gastroscopy, *Helicobacter pylori*, *Helicobacter heilmannii* type II

1. Introduction

Over the last decade, at least 23 *Helicobacter* species have been identified in humans and animals (1), and there is a high prevalence of *Helicobacter* spp. in dogs (2). Both '*Gastrospirillum hominis*' and '*Helicobacter heilmannii*' have no official standing in the bacterial nomenclature. Analysis of the 16S rRNA gene sequences showed that they were not identical (96.6% similarity) and consequently they became known as '*H. heilmannii*' type I and type II. Morphologically similar organisms have also been described in cats (*Helicobacter felis*), dogs (*Helicobacter bizzozeronii*, *Helicobacter salomonis*), and pigs (*Candidatus* Helicobacter suis). Although the 16S rRNA gene sequence analysis is a very useful phylogenetic tool, it is not sufficiently sensitive to discriminate among these species (3). *Helicobacter heilmannii* type I and *Candidatus* Helicobacter suis are considered to be identical (4). However, *Candidatus* H. heilmannii (type II) is also associated with *Helicobacter felis*, *Helicobacter salomonis*, and *Helicobacter bizzozeronii* affecting dogs and cats, as they share >98% of the 16S rRNA gene sequence similarity (3,4). Moreover, *Helicobacter pylori* is known to be the cause of gastritis in humans. Although molecular studies of *H. pylori* in dogs are rare, according to certain reports, the general opinion is that dogs do not harbor *H. pylori* (5–10). Helicobacteriosis is generally seen as a subclinical infection because no significant correlation between gastritis and infection severity has been found (6,7,11). Due to differences in pathogenicity of *Helicobacter* spp., it is difficult to evaluate the relationship between helicobacteriosis and gastritis (12,13). Therefore, further studies are necessary to clarify the connection between *Helicobacter* spp. and gastritis, and to determine the factors affecting pathogenicity (2,14). The aim of the present study was to determine the presence and histopathological effect of *H. pylori* and *H. heilmannii* type II in the development of gastritis in dogs.

2. Materials and methods

2.1. Data collection

Forty dogs between the ages of 2 and 11 years, brought to the İstanbul University Faculty of Veterinary Medicine

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Animal Hospital, were included in this study in accordance with the ethical standards approved by the Ethical Committee of İstanbul University. The dogs were divided into 2 groups, I and II. Group I included 22 dogs exhibiting at least one gastric symptom, such as nausea, vomiting, grass eating, abdominal pain, slouch position, coprophagy, allotriophagy, anorexia, ructus, or meteorism. Dogs with these symptoms caused by secondary disorders such as metabolic disorders, organ failure, infectious diseases, or gastritis owing to the presence of a foreign body were excluded from the study. Group II (the control) included 18 dogs that were found to be healthy by physical examination and laboratory findings and showed none of these gastric complaints. The animals were placed on a fast overnight and were anesthetized with xylazine hydrochloride (3.5 mg/kg, intramuscular) and ketamine hydrochloride (10 mg/kg, intramuscular). Endoscopic examinations were performed with subjects on the left lateral recumbent position; 10–12 biopsy samples were taken from the fundus, corpus, and pylorus of the stomach.

Rapid urease tests (Helident; RTA, Kocaeli, Turkey) were performed on the samples collected from the dogs. A positive result was recorded when the color of the urea solution changed from yellow to magenta.

For histopathological examination, biopsy samples were fixed with 10% buffered formalin and embedded in paraffin blocks. Hematoxylin–eosin, Giemsa, and Warthin–Starry stained sections were examined by two pathologists under a light microscope (Olympus bx50, Olympus, Tokyo, Japan) to determine the presence of histopathological alterations and the intensity of spiral bacteria. Samples were scored based on the number of bacteria observed per visual field ((–) for no bacteria present; (+) for 1–10; (++) for 11–25; or (+++) for >25) and on the intensity of spiral bacteria as previously described (15). Gastritis grading was performed following a method described by Erginsoy and Sozmen (15) with modifications. The severity of gastritis was defined as follows: "no gastritis" meant no lymphoid aggregates, <5 leukocytes per high power field, and normal mucosal epithelium; "mild gastritis" meant fewer than two lymphoid aggregates per low-power field and/or 5 to 25 leukocytes per high-power field, and normal mucosal epithelium; "severe gastritis" meant more than two lymphoid aggregates per low-power field, >26 leukocytes per high-power field, and mild or marked gastric epithelial changes.

2.2. DNA extraction and primer design

For DNA isolation, gastric biopsies taken by endoscopy were extracted using a commercial DNA extraction kit (High Pure PCR Template Preparation Kit; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. DNA samples were stored at –80 °C until use. Primers (Table 1) were selected by the authors from publically available (GenBank) urease gene sequences and adapted for nested polymerase chain reaction (PCR) (http://www.ncbi.nlm.nih.gov/genbank/). Primers HHCF1 (Promega, Madison, WI, USA) and HHCR1 (Promega) (Table 1) were used for the detection of *Helicobacter* spp. Species specific primers (HHCF2 and HHCR2) (Promega) were designed from the urease gene sequences of *H. pylori* (Table 1). Species specific primers (HHCF2 and HHCR-heil) (Promega) were designed from the region urease gene sequences of *Helicobacter heilmannii* type II (Table 1).

2.3. PCR analyses

The thermal cycling conditions for the PCR were as follows: 94 °C for 3 min, followed by 45 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2.30 min and a final extension for 10 min at 72 °C. For the second round of amplification, 2 µL of the first-round PCR product were added to 48 µL of a fresh PCR mixture. Primers HHCF2 (Table 1) and HHCR2 (Table 1) were used to amplify *H. pylori.* Optimal thermal cycling conditions for nested PCR (MJ Research, PTC-200, GMI, Ramsey, MN, USA) were 94 °C for 3 min, followed by 45 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2.30 min and a final extension for 10 min at 72 °C. Primers HHCF2 and HHCR-heil (Table 1) were selected to amplify *H. heilmannii* type II with the thermal cycling conditions set at 94 °C for 3 min, followed by 45 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.30 min and for a final extension of 10 min at 72 °C. The samples were separated by gel electrophoresis and

Table 1. Positions, time of melting, and primers for *H. pylori* and *H. heilmannii* type II group.

Name	Position	Melting time (Tm)	Primer sequence
HHCF1	527–549	57	5'TAT GTT TCT ATG TAT GGD CCC AC 3'
HHCF2	605–626	54	5'AYT GCA CCA CTT ATG GYG AAG 3'
HHCR1	1257–1235	62.8	5'GAR TCG GTG TGG ATV GCY ACT TG 3'
HHCR2	1189–1169	60	5'GGT GTG CTD CCC CAG TCT TC 3'
HHCR-heil	803–783	60	5'GCY TTR CCA ATS CCR TGG 3'

compared with positive controls and negative controls. DNase and RNase free water was used as a negative control. Human clinical specimen confirmed positive for *H. pylori* was used as a positive control. The first detected sample at 198 bp was sequenced and confirmed as *H. heilmannii* type II; henceforth it was used as a positive control for *H. heilmannii* type II. The presence of a PCR product at 584 bp was positive for the presence of *H. pylori*, and the one at 198 bp was positive for *H. heilmannii* type II.

2.4. Statistical analysis

SPSS Version 13.0 (Chicago, IL, USA) was used for the evaluation of the collected data. Chi-square tests were performed within and between groups.

3. Results

3.1. Gastroscopy findings

The presence of any abnormal macroscopic findings detected by gastroscopy was evaluated as positive. These abnormal findings consisted of hyperemia, ulceration, multifocal bleeding, minor bleeding, erythema, stenotic sphincter images, thickening or reduction of the rugal folds, and rough mucosal surface image. Some of these abnormal findings are shown in Figure 1. Mostly multiple findings were detected. Positive gastroscopic findings were detected in 20 dogs of group I (n = 22) and no abnormality was found macroscopically in the remaining 2 animals (Table 2). The presence of gastroscopic findings was encountered only in 4 dogs of group II (n = 18) with no symptoms of gastritis (Table 2). The correlation between the presence of symptoms of gastritis and the presence of abnormal gastroscopic findings was significant ($P < 0.001$). However, no statistical correlation was found between the presence of abnormal gastroscopic findings and positive diagnosis of gastritis by histopathological examination. Nevertheless, antral hypertrophy was detected in 3 dogs from group I. Severe hypertrophic gastritis was also found histopathologically in these dogs. These dogs were found positive for *H. heilmannii* type II and 1–10 bacteria were detected histopathologically in the field. Ulceration was also detected in 2 dogs (positive for *H. heilmannii* type II) from group I. Severe atrophic gastritis was detected by histopathology. In the first dog 1–10 bacteria were found in the field, whereas there were 11–25 bacteria in the field in the histopathological examination of the other dog.

3.2. Histopathology findings

Biopsy samples positive for spiral bacteria, as detected by histopathologic examination, were evenly distributed between group I (16/22) and group II (14/18). Histopathologic alterations were observed in 38 dogs, distributed as follows: atrophic gastritis (27/38, 71%), hypertrophic gastritis (6/38, 16%), and lymphoplasmacytic gastritis (5/38, 13%). In one dog of each group (I and II), no histopathological alterations were found. The number of dogs with atrophic, hypertrophic, or lymphoplasmacytic gastritis in group I were 16, 3, and 2, respectively, as compared with 11, 3, and 3 dogs in group II. In tissues with atrophic gastritis, mononuclear cell infiltration and fibrosis were associated with increased bacterial load and

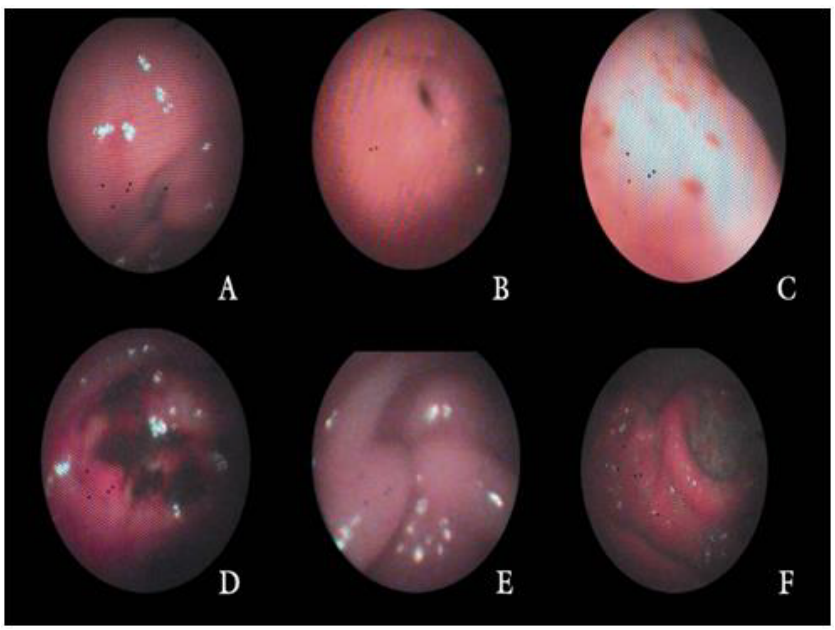

Figure 1. Abnormal gastroscopic findings (A. Erythema; B. Pyloric stenosis; C. Multifocal hemorrhage; D. Ulceration; E. Antral hypertrophy; F. Hyperemia and fundal fold surface roughening).

Table 2. Results of the different diagnostic methods and their distributions in the groups.

Diagnostic method	Group I (n = 22)	Group II (n = 18)
Endoscopic findings	20 (90.9%)	4 (22.2%)
Rapid urease test results	11 (50%)	9 (50%)
Histopathological examination		
Atrophy	16 (72.7%)	11 (61.2%)
Hypertrophy	3 (13.6%)	3 (16.6%)
Lymphoplasmacytic	2 (9.1%)	3 (16.6%)
Detection of spiral bacteria	16 (72.7%)	14 (77.7%)
PCR		
Helicobacter pylori	3 (16.6%)	1 (5.5%)
Helicobacter heilmannii type II	17 (77.2%)	17 (94.4%)

gastric gland degeneration (Figure 2). In tissues exhibiting signs of hypertrophic gastritis, mucosal proliferation was found; this was in contrast to cases involving severe diffuse lymphocyte and plasma cell infiltration, which occurred in dogs with lymphoplasmacytic gastritis. The distribution of bacteria was different among gastric mucus, glandular lumen, and parietal cells in 16 dogs of group I and 14 dogs of group II (Figure 2). No significant association could be found between bacterial overload and gastritis severity (Table 3). However, mononuclear inflammatory cell infiltration and increased lymphoid follicles were found in dogs positive for *H. pylori*.

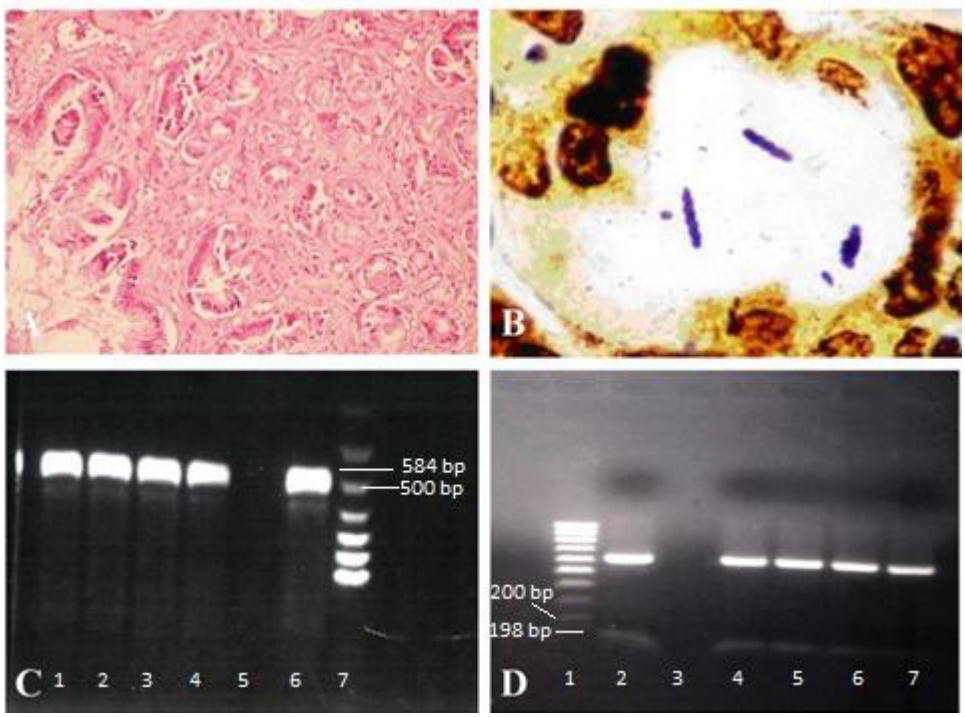

Figure 2. Histopathological view of atrophic gastritis and spiral bacteria. A. Atrophic gastritis (H&E 20×); B. Presence of spiral bacteria in glandular lumen (Warthin–Starry 100×); C. Gel electrophoresis of *H. pylori* positive results in 584 bp, lanes 1–4: some of the PCR (+) cases, lane 5: negative control, lane 6: positive control, lane 7: size marker; D. Gel electrophoresis of *H. heilmannii* type II positive results in 198 bp, lane 1: size marker, lane 2: positive control, lane 3: negative control, lanes 4–7: some of the PCR (+) cases.

Table 3. Distribution of gastritis types and severity depending on bacterial load in histopathological sections (A = no bacteria present; B = 1–10 bacteria; C = 11–25 bacteria; D = >25 bacteria).

		A (n = 10)	B (n = 19)	C (n = 8)	D (n = 3)
No gastritis (n = 2)		1	1	0	0
Mild gastritis (n = 13)		2	9	1	1
Severe gastritis (n = 25)		7	9	7	2
Atrophic gastritis	Mild	1	7	0	1
	Severe	3	7	6	2
	Total	4	14	6	3
Lymphoplasmacytic gastritis	Mild	1	1	1	0
	Severe	2	0	0	0
	Total	3	1	1	0
Hypertrophic gastritis	Mild	0	1	0	0
	Severe	2	2	1	0
	Total	2	3	1	0

3.3. PCR analyses

Helicobacter spp. was detected in 95% of the dogs (38/40) by nested PCR. Positive results for *H. heilmannii* type II (34/38) were as expected; however, 4 dogs were also positive for *H. pylori*. Dogs diagnosed with *H. heilmannii* type II might also be infected with one or more of the following: *H. felis*, *H. salomonis*, or *H. bizzozeronii*. As discrimination by sequence analysis was not performed, the positivity was ascribed to *H. heilmannii* type II. Of the dogs in group I (n = 22), 2 dogs were negative for all *Helicobacter* spp. tested by nested PCR, 3 dogs were positive for *H. pylori*, and 17 dogs were positive for *H. heilmannii* type II. Similarly to the dogs (n = 18) in group II, 17 samples contained DNA from *H. heilmannii* type II and 1 from *H. pylori*. Accordingly, the number of dogs infected with *H. heilmannii* type II (17/22) was greater than the number of dogs infected with *H. pylori* (3/22) (Figure 2). Findings from PCR, gastroscopic evaluation (abnormal findings described as positive), rapid urease test, and histopathology, and the presence and distribution of spiral bacteria, are presented in Table 2.

3.4. Rapid urease test results

Rapid urease test results were positive in half of the dogs with abnormal gastritis (12/24); these results were not significant. There was no correlation between the histopathologic severity of gastritis and the prevalence of bacteria. Abnormal gastroscopic findings were observed in 19 of the 34 dogs positive for *H. heilmannii* type II and in 3 of the 4 dogs positive for *H. pylori* by nested PCR. There was no correlation between the presence, or the type of gastritis, and positive rapid urease test results. However, a positive relation was found between the number of spiral bacteria observed and the positive rapid urease test results ($P < 0.05$). The classification of gastritis in dogs positive for *H. heilmannii* type II was as follows: 24 atrophic, 5 hypertrophic, and 3 lymphoplasmacytic. The classification of gastritis in dogs positive for *H. pylori* was 3 atrophic, 0 hypertrophic, and 1 lymphoplasmacytic. In the 2 symptomatic dogs with negative PCR findings, no spiral bacteria were observed histopathologically, although severe lymphoplasmacytic or hypertrophic gastritis was found.

4. Discussion

Vomiting in dogs is a very common symptom; however, dogs are mostly asymptomatic in cases of chronic gastritis, even with severe inflammation of the gastric mucosa (13). In the present study, significant relations were found between the presence of symptoms of gastritis and gastroscopic findings. Generally, the degree of agreement between clinical symptoms and abnormal gastroscopic findings has been reported between 40% and 78% (16–18). In the present study 91% of the symptomatic dogs (group I) had abnormal gastroscopic findings, indicating that gastroscopy was a reliable diagnostic method. No statistical correlation was found between abnormal gastroscopic findings and histopathological examination. However, specific findings like antral hypertrophy, stenosis of the pyloric sphincter, and rugal fold thickening were also supported by histopathology alongside the diagnosis

of severe hypertrophic gastritis. Some researchers have reported that histopathological evaluation after biopsy is necessary because of the risk of error in gastroscopic findings (18–20). However, the rate of gastritis diagnosed histopathologically in symptomatic and asymptomatic dogs with chronic vomiting is low: 35% and 26%–48%, respectively (2). High gastritis rates such as those found in our study (94%–95%) are not associated with the presence of symptoms. Similarly, chronic gastritis was detected histopathologically in 67% of asymptomatic cats (16), calling into question the significance and specificity of clinical symptoms.

The rapid urease test can be used to detect *H. heilmannii* type II; however, false-negative results may occur because of the variable dispersion of *H. heilmannii* type II as compared with *H. pylori* (21). In the present study, 50% of dogs with *H. heilmannii* type II were found to be positive in the rapid urease test results. Based on the significant correlation ($P < 0.05$) between rapid urease test results and the presence of bacteria, false-negative results could be identified based on the lower prevalence of bacteria.

In a previous study performed on dogs, spiral bacteria were found in 61% of symptomatic and 60% of asymptomatic dogs. There was no relationship between clinical symptoms and the presence of *Helicobacter* spp. in this study (17). Similarly, Yamasaki et al. (11) reported that no significant difference could be found between the presence of spiral bacteria and clinical symptoms. In the present study, spiral bacteria were detected in 73% of symptomatic and 78% of asymptomatic dogs. No relationship was found among bacterial load scores, clinical symptoms, and the presence of *Helicobacter* spp. Even in the dogs with ulceration ($n = 2$), a mild bacterial overload was detected in only one. Virulence factors in the bacteria contribute to changes in gastric physiology and mucosal barrier disruption (13). However, investigation of virulent strains of bacteria was not conducted in the current research. Although it has been reported that atrophic gastritis is rarely seen in dogs and the most common type of gastritis in dogs is lymphoplasmacytic (2), 71% of the dogs were diagnosed with atrophic gastritis in the present study. It must be kept in mind that no absolute criteria exist for the diagnosis of gastritis, and therefore there is no gold standard for research in this field. Happonen (12) reported that mild, chronic, asymptomatic gastritis was detected even in healthy dogs and cats by the Standard Sidney System.

Eaton et al. (6) reported that the prevalence of *Helicobacter* spp. in dogs was 67%–100% and the infection rate was not correlated with histopathological signs of gastritis. *Helicobacter* spp. were determined in 75% of positive dogs by histopathology, and 95% of positive dogs by PCR, including 91% of dogs displaying clinical symptoms and in asymptomatic dogs. No correlation was found between *Helicobacter* spp. presence and degree of gastritis.

Evidence of *H. heilmannii* type II obtained by PCR has been reported in 83%–90% of small animals tested (6,22,23). In contrast, PCR studies of *H. pylori* in dogs are rare. There is a general opinion that dogs do not harbor *H. pylori* (5–10). However, in two studies investigating the presence of *H. pylori*, no positive results were detected in gastric or oral samples of stray dogs and beagle dogs (5,7). Furthermore, in studies investigating the distribution of *Helicobacter* spp. in the stomach of dogs, *H. pylori* was absent in all samples (9,10). In one study, beagle dogs were experimentally infected with *H. pylori* and acute vomiting occurred (24). Similar to this result, of the 4 *H. pylori*-positive dogs in the present study, 3 exhibited severe gastrointestinal symptoms and gastroscopic findings were abnormal in all 4 dogs. From biopsy specimens of the 4 dogs, *H. pylori* or a very closely related species with moderate similarity to *H. pylori*, with 96.6%–98% genetic identity, was detected in 2 dogs. Moreover, previous reports have stated that the canine gastric mucosa may be naturally colonized by strains of *H. pylori* or a very closely related species (25). In research performed in the İstanbul region, the positivity rate of *H. pylori* infection was found to be 12.5% in feces of dogs by PCR (26). This high result is significant. Therefore, the risk of infection of dogs with *H. pylori* might be higher in countries where *H. pylori* is more prevalent in humans (82.5%) (27). Histopathological examination showed that the formation of lymphoid follicles was more remarkable in *H. pylori*-positive dogs than in those infected with *H. heilmannii* type II. These histopathological findings were in accordance with findings reported in a study on experimentally infected dogs (24). The higher infiltration rate and increased intensity of mononuclear inflammatory cells and lymphoid follicles in cases of *H. pylori*-related atrophic gastritis observed in the present study suggest that the pathogenic effect of *H. pylori* was greater than that of *H. heilmannii* type II.

Moreover, in the present study, a new primary sequence was selected using urease gene sequences of *H. pylori* and *H. heilmannii* type II available from GenBank. PCR was more reliable than other diagnostic procedures (rapid urease test and detection of the presence of bacteria by histopathological examination) for the diagnosis of gastric *Helicobacter* spp. infection.

The role of *Helicobacter* spp. in the development of chronic gastritis in dog is not known. Although high positivity for *H.heilmannii* type II was obtained in this study, no significant correlation was found between the density and presence of helicobacteriosis and gastritis

severity. Additional studies on virulence factors must be carried out to determine their relationship with upper gastrointestinal tract disorders. Contrary to general opinion, there may be a risk of infection with *H. pylori* for dogs. The gastroscopic findings, clinical signs, and severe gastritis observed during histopathological examinations indicated the high pathogenicity of *H. pylori* in dogs.

Acknowledgments

The present thesis work was supported by the Research Fund of İstanbul University, Project numbers T-794/27122005 and UDP-22051. The authors would like to thank Assoc Prof Dr Ömür Koçak, Prof Dr Ayşen Gargılı, Assoc Prof Dr Kenan Midilli, Prof Dr Tamer Dodurka, and Prof Dr Aydın Gürel for their help and guidance.

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