

Investigation with Real-Time PCR and Histopathology on the presence of *H. felis*, *H. heilmannii* and *H. pylori* in dogs

Investigación con PCR en Tiempo Real e Histopatología sobre la presencia de *H. felis*, *H. heilmannii* y *H. pylori* en Perros

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ABSTRACT

Helicobacter species such as *H. heilmannii*, *H. pylori*, *H. felis*, *H. bizzozeronii* and *H. salomonis* have been identified in cats and dogs, and research suggesting that these species may be zoonotic agents and has been studied intensified in recent years. The aim of this study was to reveal the presence, comparing the histopathological findings and Real-time PCR results of *H. felis*, *H. heilmannii*, and *H. pylori* in the stomach and liver tissues taken during the necropsies of owned, stray or shelter dogs. The material of the study consisted of stomach and liver tissues taken from 35 dogs that died for different reasons and were brought for necropsy. DNA copies of *H. heilmannii* were detected by Real-time PCR in the liver samples of 30 dogs using *H. heilmannii*-specific primers. In the case of gastric samples, Real-time PCR detected *H. heilmannii* in 13 cases, *H. pylori* in 3 cases, both *H. heilmannii* and *H. pylori* in 13 cases, and *H. felis*, *H. heilmannii* and *H. pylori* in 3 cases. Microscopically, neutrophil leukocyte infiltration, epithelial degeneration, fibrosis and oedema in the lamina propria, and lymphoplasmacytic cell infiltration were determined in the stomachs. In the Hematoxylin Eosin staining of the sections, 5 cases and 14 cases in the Warthin-Starry staining were found positive for *Helicobacter*-like microorganisms. Microscopically, dissociation of the remark cords and hydropic degeneration in hepatocytes, and focal mononuclear cell infiltrations in some sections were determined in the livers. In conclusion, with this study, it was understood that Real-time PCR analyzes are very useful in the diagnosis of *H. felis*, *H. heilmannii*, and *H. pylori*. However, histopathological examinations are necessary to associate the presence of bacteria with the development of the disease.

Key words: Canine; histopathology, real-time PCR; *H. felis*; *H. heilmannii*; *H. pylori*

RESUMEN

Especies de *Helicobacter* como *H. heilmannii*, *H. pylori*, *H. felis*, *H. bizzozeronii* y *H. salomonis* han sido identificadas en gatos y perros, y en los últimos años se han intensificado las investigaciones que sugieren que estas especies pueden ser agentes zoonóticos. El objetivo de este estudio fue por medio de la comparación de los hallazgos histopatológicos y los resultados de PCR en tiempo real, revelar la presencia de *H. felis*, *H. heilmannii* y *H. pylori* en los tejidos del estómago e hígado tomados durante las necropsias de perros con dueño, callejeros o procedentes del refugio. El material del estudio consistió en tejidos de estómago e hígado tomados de 35 perros fallecidos por diferentes causas y que fueron traídos para la realización de la necropsia. Se detectó ADN de *H. heilmannii* mediante PCR en tiempo real en las muestras de hígado de 30 perros utilizando primers específicos de *H. heilmannii*. En el caso de las muestras gástricas, la PCR en tiempo real detectó *H. heilmannii* en 13 casos, *H. pylori* en 3 casos, tanto *H. heilmannii* como *H. pylori* en 13 casos, y *H. felis*, *H. heilmannii* y *H. pylori* en 3 casos. Microscópicamente en el tejido estomacal se observó infiltración de leucocitos neutrófilos, degeneración epitelial, fibrosis y edema en la lámina propia e infiltración de células linfoplasmocitarias. En la tinción de Hematoxilina y Eosina de los cortes, 5 casos resultaron positivos para microorganismos tipo *Helicobacter* y 14 casos en la tinción Warthin-Starry. Microscópicamente, en el tejido hepático se observó disociación de las columnas de Remark y degeneración hidrópica en los hepatocitos, e infiltraciones de células mononucleares focales en algunas secciones. En conclusión, con este estudio se comprendió que los análisis de PCR en tiempo real son muy útiles en el diagnóstico de *H. felis*, *H. heilmannii* y *H. pylori*. Sin embargo, los exámenes histopatológicos son necesarios para asociar la presencia de dichas bacterias con el desarrollo de la enfermedad.

Palabras clave: Canino, histopatología, Real-Time PCR, *H. felis*, *H. heilmannii*; *H. pylori*

INTRODUCTION

After the detection of *Helicobacter pylori* in humans and its association with gastritis, peptic ulcer and gastric neoplasia, research on spiral-shaped bacteria in both humans and cats (*Felis catus*) and dogs (*Canis lupus familiaris*) has increased [2, 16, 23]. *Helicobacters* are known as gram-negative, comma-shaped, 'S' or spiral-shaped, flagellate, sporeless, non-encapsulated, microaerobic and non-acid-fast microorganisms [12, 18]. *Helicobacter* species such as *H. heilmannii*, *H. pylori*, *H. felis*, *H. bizzozeronii*, *H. salomonis* were detected in cats and dogs [4]. In addition, studies have been published stating that these agents are found in 0.2-6% of human gastric biopsies and that they may be zoonotic agents [10, 33].

H. pylori is known as a pathogen that infects almost half of the population of the World [7, 34]. It has been reported that *H. heilmannii* is found in the gastric mucosa of many animal species, especially dogs, cats, pigs (*Sus scrofa domestica*) and primates (*Macaca* spp.), and its pathogenicity is lower than that of *H. pylori* [5]. Numerous researchers have reported that they have identified *Helicobacter* species in both healthy dogs and suffering from gastritis [28, 31, 32]. It was stated that although *Helicobacter* species were observed in the histopathology of the stomach of cats and dogs, they were not related to the severity of the inflammation [21]. Using scraping cytology and histopathological examination methods for diagnosing *Helicobacter* spp., it was reported that gastric *Helicobacter* spp. was detected in dogs at a rate of 84.4% in scraping cytology and 65.6% in histopathological examinations [6].

Although basic gastrointestinal findings such as vomiting and diarrhoea have been reported clinically in *Helicobacter* infections in cats and dogs [14, 35] some researchers reported that most cats and dogs with *Helicobacter* were asymptomatic [8, 27, 30]. Mild gastritis and mononuclear cell infiltration are commonly observed in dogs with histopathologically determined *Helicobacter* spp. in the gastric mucosa [8, 13, 26]. In a previous study, it was reported that glandular degeneration was more common in the *fundus* region in *Helicobacter* spp.-infected cats and dogs compared to non-infected ones. Additionally, pycnotic parietal cells and enlarged canaliculi have also been reported [35].

Diagnosis of *Helicobacter* species is made with tests like rapid urease test, histopathology, culture, Polymerase Chain Reaction (PCR) from tissue samples or non-invasive tests such as urea breath test, blood tests, and serological tests [3, 13, 15, 26]. The aim of this current study was to reveal the presence of *H. felis*, *H. heilmannii*, and *H. pylori* in the stomach and liver tissues taken during necropsy from owned, stray dogs and dogs kept in shelters, which died from different causes in the first stage, by Real-time PCR and histopathological examination. Then, compare the histopathological findings with Real-time PCR results to determine the incidence of *H. felis*, *H. heilmannii* and *H. pylori*.

MATERIALS AND METHODS

Animal material

The material of the study consisted in the stomach and liver tissues of 35 dogs that died between 2019 and 2022 due to different reasons and were brought to Selcuk University Veterinary Faculty Pathology Department for necropsy. Of the dogs studied in the study, thirteen were stray dogs staying in the shelter, and thirteen were brought in by city officials and animal lovers. The remaining nine were owned dogs. The study was approved by SÜVDAMEK (Decision number: 2022/76).

Histopathological examination

Stomach and liver samples taken for histopathological examinations were fixed in 10% buffered formalin solution and underwent routine tissue follow-up procedures. Afterwards, the tissues were embedded in paraffin, and paraffin blocks were obtained. 4-5 micron (μm) sections were taken from paraffin blocks to slides and stained with Hematoxylin Eosin (HE) and Warthin Starry (WS) methods [22].

Real-time PCR examination

Deoxyribonucleic acid (DNA) copies of *H. felis*, *H. heilmannii* and *H. pylori* were investigated by Light Cycler 2.0 Real-time PCR (Roche, Life Sciences, Germany) device using primer probes prepared by a private company. Deionized water was used as negative control. Primer sequences used in Real-time PCR analysis are given in TABLE I. DNA isolation from stomach samples taken for PCR examinations was performed in accordance with the commercial DNA isolation Kit (Roche, MagNA Pure LC DNA, Cat No; 03264785001) procedure. The obtained DNAs were stored at -20°C (Frigidaire 20.0 Cu. Ft. Upright Freezer, Turkey). Reaction mixture; 10 microliters (μL) of enzyme and dNTP mixture, 0,5 μL of forward primer, 0,5 μL of reverse primer, 0,2 μL of TaqMan probe (20 μM) and 3.8 μL of nuclease-free water, in total, 15 μL were prepared. The mixture was spun, and then 15 μL of the reaction mixture was transferred to the capillaries for each reaction, and 5 μL of sample or control sample was added to each capillary. Thermal cycle; It was 10 minutes (min) for denaturation at 95°C , 30 seconds (seg) for annealing at 55°C , and 1 seg for elongation at 72°C . The Light Cycler 2.0 software was used for the presence-absence data analysis.

TABLE I
Primary probe sequences used in the study

Genes and Species	Primer sequences	Reference
üreA, üreB Genes (<i>H. felis</i>)	F: 5'-GTGAAGCGACTAAAGATAAACAAT-3' R: 5'-GCACCAAATCTAATTCATAAGAGC-3'	[9]
üreB Gen (<i>H. heilmannii</i>)	F: 5'-GGGCGATAAAGTGCCTTG-3' R: 5'-CTGGTCAATGAGAGCAGG-3'	[24]
glmM (<i>H. pylori</i>)	F: 5'-GGATAAGCTTTAGGGGTGTTAGGGG-3' R: 5'-GCTTACTTTCTAACACTAACCGCC-3'	[20]

"F, forward; R, reverse"

RESULTS AND DISCUSSION

Macroscopic findings

It was determined that the pathological diagnoses of the necropsied dogs were bronchopneumonia in four, hepatitis in four, hydronephrosis in one, meningoencephalitis in one, myocarditis in one, nephritis in one, trauma in ten and gastroenteritis in thirteen.

Microscopic findings

Neutrophil leukocyte infiltration, epithelial degeneration, fibrosis and oedema in the lamina propria, and lymphoplasmacytic cell infiltration were determined in the microscopic examination of HE stained preparations prepared from samples taken from the antrum regions of the stomachs during necropsy (FIGS. 1, A-B-C). In addition,

5 cases in HE staining and 14 cases in WS staining were positive for *Helicobacter*-like microorganisms (FIGS. 1, F). The microscopic examination of HE stained preparations from liver samples revealed dissociation in the remark cords and hydropic degeneration in hepatocytes, and focal mononuclear cell infiltrations in some sections

(FIGS. 1, D-E). *Helicobacter*-like organisms could not be detected in HE stains and WS stains. Histopathological findings determined in the microscopic examination of stomach and liver tissues are given in TABLE II.

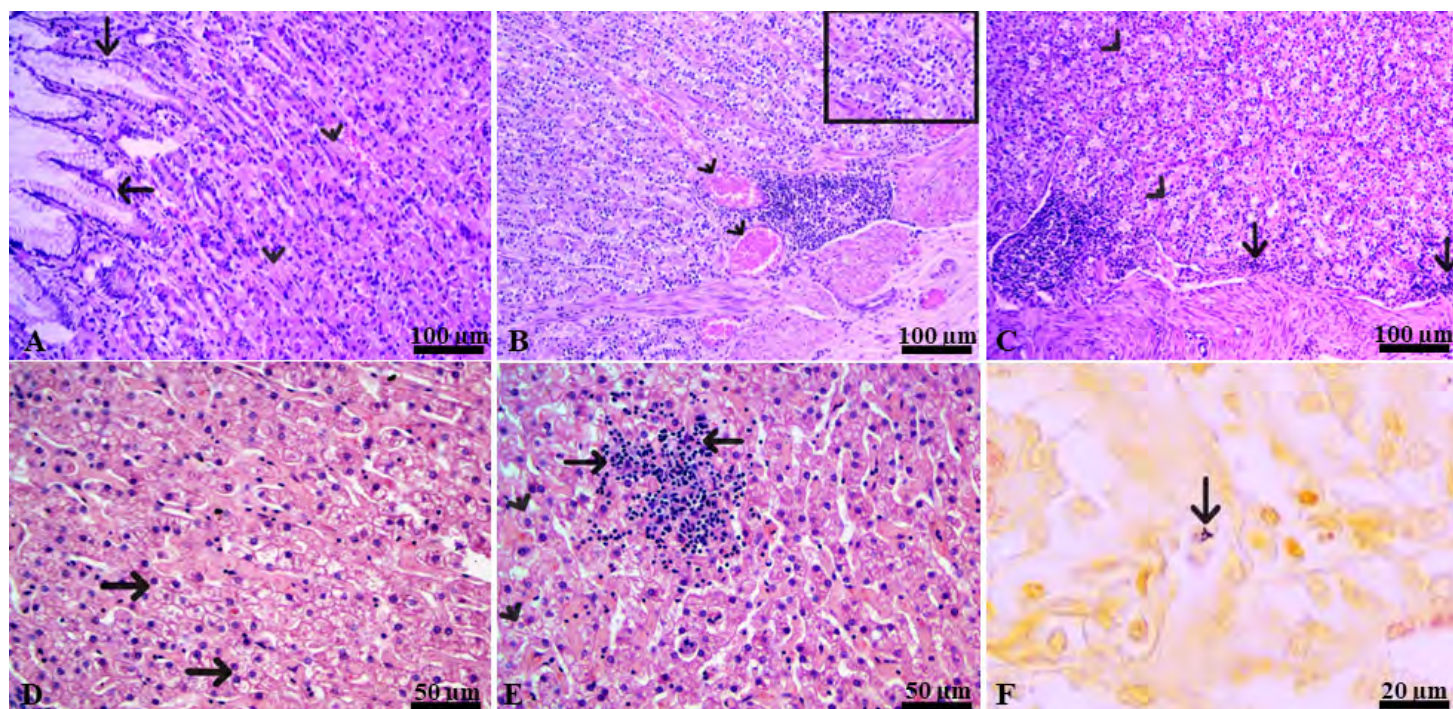


FIGURE 1. A. Degeneration of gastric mucosal epithelium (arrow) and fibrosis in the propria (arrowhead), x200, HE. B. Inflammatory cell infiltration (small picture) and congestion (arrowhead) in the case with *H.pylori*, x200, HE. C. Inflammatory cell infiltration (arrow) and degenerative changes in gland structures (arrowhead) in which all three agents are present (*H. heilmannii*, *H. pylori*, *H. felis*), x200, HE. D. Hydropic degeneration of the liver (arrow), x400, HE. E. Focal inflammatory cell infiltrates in the liver (arrow), x400, HE. F. *Helicobacter*-like microorganisms (arrow), WS Staining. (HE; Hematoxylin Eosin, WS; Warthin-Starry)

TABLE II
Histopathological findings determined in the histopathological examination of stomach and liver tissues

Microscopic Results	<i>H. heilmannii</i>	<i>H. pylori</i>	<i>H. felis</i> + <i>H. heilmannii</i> + <i>H. pylori</i>	<i>H. heilmannii</i> + <i>H. pylori</i>
Epithelial degeneration	4/13	1/3	1/3	4/13
Lymphoid cell infiltration in Lamina propria	6/13	1/3	1/3	3/13
Fibrosis in Lamina propria	4/13	1/3	1/3	4/13
Edema in Lamina propria	4/13	2/3	1/3	3/13
Degeneration of gastric gland epithelium	4/13	0/3	1/3	2/13
Degeneration in hepatocytes	8/13	1/3	1/3	3/13
Remark cords dissociation	8/13	1/3	1/3	3/13
Mnh cell infiltration in the liver	5/13	0/3	0/3	2/13
Agent in HE	2/13	0/3	1/3	2/13
Agent in WS	4/13	0/3	2/3	8/13

(Mnh: Mononuclear cell infiltration, HE: Hematoxylin Eosin, WS: Warthin-Starry)

Real-Time PCR (qPCR) findings

The detection rates of *H. felis*, *H. heilmannii* and *H. pylori* in stomach samples by Real-time PCR and the rates of detection of *H. heilmannii*, *H. pylori* and *H. felis* in gastric samples according to age and gender are given in TABLE III. The relevant amplification curves are given in FIG 2.

The presence in gastric samples of *H. heilmannii* in 13 cases, *H. pylori* in 3 cases, both *H. heilmannii* and *H. pylori* in 13 cases, and *H. felis*, *H. pylori* and *H. heilmannii* in 3 cases was determined by Real-time PCR.

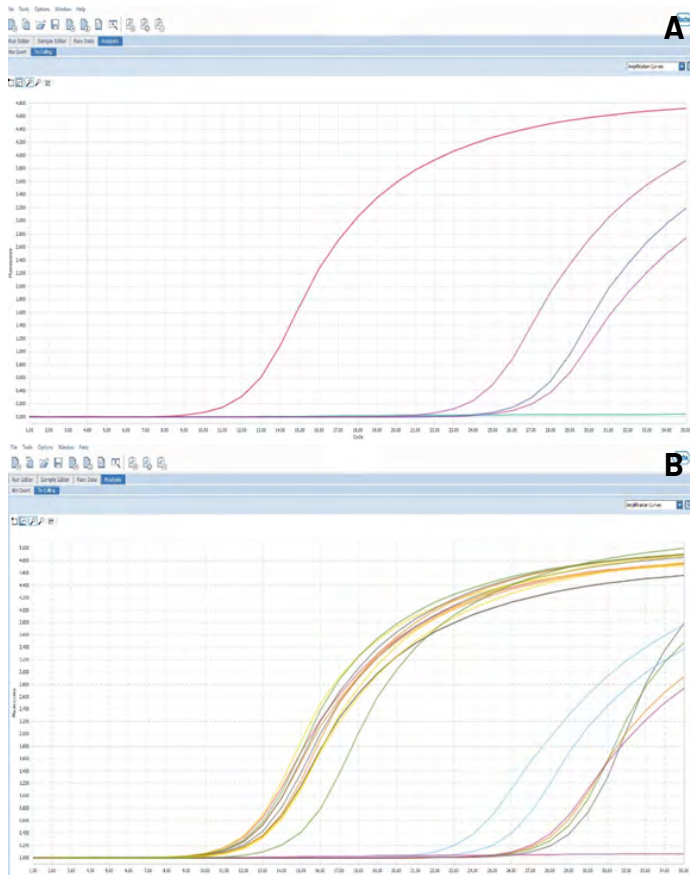


FIGURE 2. A. Real-time PCR, *H. felis* amplification curve. B. Real-time PCR, *H. heilmannii* amplification curve in male

In the study performed with Real-time PCR on liver samples using *H. heilmannii*-specific primers, the DNA of *H. heilmannii* was detected in 30 dogs, while DNA of *H. felis* and *H. pylori* could not be determined. The detection rates of *H. heilmannii* in liver samples by Real-Time PCR and the distribution of these rates according to age and gender are given in TABLE IV

**TABLE IV
Distribution of *H. heilmannii* by age and gender**

Agent	N	Male	Female	< 1 year old	1-3 years old	> 3 year old
<i>H. heilmannii</i>	30	16/35	14/35	10/35	9/35	11/35

It was observed that 16 of 30 cases of *H. heilmannii* determined by Real-time PCR from liver samples were male dogs, and 14 of them were female dogs. It was determined that 10 of the dogs with *H. heilmannii* were younger than 1 year old, 9 of them were between 1-3 years old, and 11 of them were older than 3 years old.

Determination of hepatitis and gastroenteritis in microscopic examination of stomach and liver samples, entity of *Helicobacter*-like organisms in HE and WS staining of samples taken from stomach and determination of *H. pylori*, *H. felis* and *H. heilmannii* DNA copies in these tissues by Real-time PCR are shown in TABLE V.

DNA copies of *H. felis*, *H. heilmannii* or *H. pylori* could not be determined in the liver of 5 dogs and in the stomach of 6 dogs in the examinations made with Real-time PCR. In 2 of these dogs, degeneration of gastric mucosal epithelium, degeneration of hepatocytes and dissociation of remark cords were detected in the liver. In one dog, in addition to the degeneration found in the gastric mucosa epithelium, oedema, fibrosis in the lamina propria and degeneration in the gland epithelium were observed.

It is seen that the tendency towards adopting pets (cats and dogs) and meeting their needs such as care and nutrition is increasing day by day [1, 29]. *Helicobacter* species such as *H. heilmannii*, *H. pylori*, *H. felis*, *H. bizzozeronii*, *H. salomonis* were detected in cats and dogs [4]. In order to reveal the pathology and zoonotic importance of *Helicobacter* species, studies have been performed in the fields of both human and veterinary medicine [6, 9, 31]. In studies investigating the presence of *Helicobacter* spp. in dogs, gastric *Helicobacter* was determined at rates

**TABLE III
Distribution of relevant agents by gender and age in stomach samples**

	N	Stomach	Male	Female	< 1 year old	1-3 years old	> 3 year old
<i>H. felis</i>	-	-	-	-	-	-	-
<i>H. heilmannii</i>	13	13/35	7/35	6/35	4/35	5/35	4/35
<i>H. pylori</i>	3	3/35	2/35	1/35	1/35	1/35	1/35
<i>H. felis</i> + <i>H. pylori</i> + <i>H. heilmannii</i>	3	3/35	2/35	1/35	1/35	1/35	1/35
<i>H. pylori</i> + <i>H. heilmannii</i>	13	13/35	6/35	7/35	5/35	4/35	4/35

Table V
Determination of hepatitis and gastroenteritis in microscopic examination of stomach and liver samples, entity of *Helicobacter*-like organisms in H-E and WS staining of samples taken from stomach and determination of *H. heilmannii*, *H. felis* and *H. pylori* in these tissues by Real-time PCR

No	Histopathology		Agent		Real Time PCR					
	Hepatitis	Gastroenteritis	HE	WS	Stomach			Liver		
					<i>H. felis</i>	<i>H. heilmannii</i>	<i>H. pylori</i>	<i>H. felis</i>	<i>H. heilmannii</i>	<i>H. pylori</i>
1	-	+	-	+	-	+	+	-	+	-
2	-	-	-	-	-	+	-	-	+	-
3	-	+	-	-	-	+	+	-	+	-
4	-	-	-	-	-	+	-	-	+	-
5	+	+	+	+	-	+	-	-	+	-
6	-	-	-	-	-	-	-	-	-	-
7	-	+	-	+	-	+	+	-	+	-
8	-	+	-	+	+	+	+	-	+	-
9	-	-	-	-	-	+	-	-	+	-
10	-	-	-	-	-	-	+	-	-	-
11	-	+	-	+	-	+	-	-	+	-
12	-	-	-	-	-	-	+	-	-	-
13	+	-	-	-	-	+	-	-	+	-
14	+	-	-	-	-	+	+	-	+	-
15	-	-	-	+	-	+	+	-	+	-
16	-	+	-	-	+	+	+	-	+	-
17	-	-	-	-	-	+	-	-	+	-
18	-	+	+	+	+	+	+	-	+	-
19	-	+	-	+	-	+	+	-	+	-
20	-	-	-	-	-	+	+	-	+	-
21	+	-	-	-	-	+	-	-	+	-
22	-	-	-	+	-	+	+	-	+	-
23	-	+	+	+	-	+	+	-	+	-
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	+	-	-	+	-
26	-	-	-	-	-	-	+	-	+	-
27	-	-	-	+	-	+	+	-	+	-
28	-	+	+	+	-	+	+	-	+	-
29	-	-	-	-	-	+	-	-	+	-
30	-	-	-	-	-	+	-	-	+	-
31	-	-	-	-	-	+	-	-	+	-
32	-	+	-	+	-	+	+	-	+	-
33	-	-	-	-	-	-	-	-	-	-
34	-	+	+	+	-	+	-	-	+	-
35	-	-	-	-	-	+	+	-	+	-
	4	13	5	14	3	29	19	-	30	-

(HE: Hematoxylin Eosin, WS: Warthin-Starry)

ranging from 67 to 100%. For this reason, studies have focused on dogs with the suspicion that dogs with whom people share their habitats may be the source [3, 8, 18, 19]. In this study, *H. pylori* was determined in 54.28%, *H. heilmannii* in 82.85%, and *H. felis* in 8.57% by Real-time PCR in dogs, revealing the importance of studies on the prevalence and diagnosis of *Helicobacter*-like organisms in dogs.

Happonen et al. [12], in their study on the diagnosis of *Helicobacter* spp. in dogs and cats, reported that they determined *Helicobacter* spp. in 20 (95%) of 21 dogs with gastric clinical signs and in all (100%) of the 25 dogs without gastric clinical signs. Hwang et al. [17] reported the presence of *Helicobacter* in 77.5% of dogs with gastric symptoms and 67.5% of dogs with no gastric symptoms. The researchers interpreted that the prevalence of *Helicobacter* was determined at similar rates in dogs with and without gastric symptoms, as *Helicobacter* species may be a part of the normal gastric flora. In this study, DNA copies of *H. felis*, *H. pylori* and *H. heilmannii* were determined in 100% of the stomach samples of 13 dogs with gastroenteritis and in 20 (90.90%) of the stomach samples of 22 dogs without gastroenteritis, supporting the conviction that *Helicobacter*-like organisms may be a part of the stomach flora.

Eaton et al. [8] detected *Helicobacter* spp. in all (100%) of 31 stray dogs and in 10 (67%) of 15 owned dogs. In this study, the detection by Real-time PCR of *H. felis*, *H. pylori* and *H. heilmannii* in 6 (66.66%) of 9 owned dogs was similar to the findings of Eaton et al. [8]. Again in this study, DNA copies of *H. felis*, *H. pylori* and *H. heilmannii* were determined in 10 (76.92%) of 13 shelter dogs and 10 (76.92%) of 13 stray dogs. These rates were found to be higher than the rates determined in owned dogs, which is consistent with the results of Happonen et al. [12] that the prevalence of *Helicobacter* spp in stray dogs is higher than in owned dogs.

Although Hänninen et al. [11] stated that the presence of *Helicobacter* spp. is higher in young dogs, Happonen et al. [12] stated that they found *Helicobacter* spp. at an equal rate in young and old animals in their studies on the prevalence of *Helicobacter*. In this study, of 32 cases identified as *H. felis*, *H. pylori* and *H. heilmannii* by Real-time PCR, 11 were under 1 year old, 11 were between 1-3 years old and 10 were over 3 years old. Similarly, in this study, out of 30 liver samples of *H. heilmannii*, 10 belonged to dogs under 1 year old, 9 to dogs between 1-3 years old and 11 to dogs older than 3 years. Our findings were consistent with those of [12].

Sagnak and Ozgur [32] found *Helicobacter* spp. DNA in 29 (58%) of the samples taken from 50 male dogs and 36 (78.3%) of the samples taken from 46 female dogs in the evaluation made by PCR in dog faeces; it was reported that the difference between female and male dogs was statistically significant ($P=0.034$). Considering the distribution of *Helicobacter* spp. DNA copies in both liver tissues and stomachs by gender in this study, it is understood that it was higher in males, unlike Sagnak and Ozgur [32]. However, a statistical comparison could not be made because the number of samples was insufficient.

Husnik et al. [16] reported in their studies using molecular, cytologic and histopathologic techniques to determine the prevalence of *Helicobacter* spp. and its correlation with gastric pathology, that they detected 71.4% *Helicobacter* spp. by PCR diagnostic method and *H. heilmannii* was the predominant species. Similar to the literature, in this study it was determined that *H. heilmannii* was the dominant species detected by Real-time PCR in both liver and stomach tissues. A positivity rate of 85.7% in liver tissues and 82.85% in stomach tissues was determined, which is slightly higher than the rate determined by Husnik et al. [16].

Hermanns et al. [14] reported that they detected *Helicobacter* in 82% of dogs in their study to determine the histopathological changes in cats and dogs carrying *Helicobacter* spp.. They reported that in these dogs, glandular degeneration with neutrophil granulocyte and lymphocyte infiltration, fibrosis and oedema in the lamina propria, and lymphoplasmacytic infiltrates were detected microscopically. Husnik et al. [16] reported the absence of a significant relationship between *Helicobacter* spp. infection status and the presence of inflammatory infiltration and gastritis, epithelial damage, and fibrosis. The histopathological findings determined in this study were consistent with the literature.

Diagnostic methods such as histopathology, cytology, culture, urea breath test and serological tests are constantly used in the diagnosis of *Helicobacter* infections in human medicine. However, the only way to identify agents at the species level is through PCR and culture tests [25]. When the findings of this study were evaluated, it strengthened the opinion that Real-time PCR was a rapid, specific, sensitive diagnostic test that can be used to detect *Helicobacter*-like organisms in dogs.

CONCLUSION

In conclusion, with this study, the presence of *H. felis*, *H. heilmannii* and *H. pylori* in stomach and liver samples taken during necropsy from dogs who died from different causes, living in shelters and on the street was revealed by Real-time PCR and histopathologically. It has been understood that Real-time PCR analyses are very useful in the diagnosis of *H. felis*, *H. heilmannii* and *H. pylori*, however histopathological examinations are necessary to correlate the presence of bacteria with the disease state.

Conflict of interest

The authors declare that they have no conflict of interest.

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