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RESEARCH ARTICLE

Gastric Helicobacter-like Organisms in Stray Cats: Identification, Prevalence, and Pathologic Association

Serpil Dağ[1]*, Mahmut Sözmen[2], Mete Cihan[3], Recai Tunca[4], Başak Kurt[3], Alparslan Kadir Devrim[5] and Hasan Özen[1]

[1]Department of Pathology, Faculty of Veterinary Medicine, Kafkas University, Kars, Turkey; [2]Department of Pathology, Faculty of Veterinary Medicine, Ondokuzmayıs University, Samsun, Turkey; [3]Department of Surgery, Faculty of Veterinary Medicine, Kafkas University, Kars, Turkey; [4]Department of Pathology, Faculty of Veterinary Medicine, Adnan Menderes University, Aydın, Turkey; [5]Department of Biochemistry, Faculty of Veterinary Medicine, Kırıkkale University, Kırıkkale, Turkey
*Corresponding author: serpildag06@hotmail.com

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Total of 30 domestic stray cats (*Felis cattus*) were investigated for the presence of different species of gastric *Helicobacter*-like organisms (GHLO) by immunohistochemistry and polymerase chain reaction (PCR) analysis. The severity and distribution of pathologic changes in different regions of stomach were assessed microscopically. GHLO were present in all areas of the stomach in 93.3% cats investigated. Morphologically two different types of spiral bacteria were recognized. In 53.3% cats *H. felis* like organisms and in 76.7% cats *H. heilmannii* like organisms were determined. Mixed presence of both bacteria was seen in 43.3% cases. *H. pylori* was not detected in any of the cats. Mild to severe gastritis were observed in 90.0% cats. GHLO were present in fundus, corpus and pyloric antrum regions in similar densities. The most striking histopathological changes were lymphocyte and neutrophil infiltrations, fibrosis in the lamina propria, and lymphoid follicle formation. There was no significant relationship between the degree of bacterial density and the extent of histopathological changes. GHLOs were present on the mucosal surface, in the lumen of gastric glands, and in the cytoplasm of parietal cells. In conclusion, PCR and immunohistochemistry can be successfully used in detection of GHLOs. The results of the study show also that *H. heilmannii* and *H. felis* are frequent agents in stray cats, and hence suggest that these animals might be common reservoirs for these microorganisms. However, the bacteria do not seem to be solely responsible for gastritis observed in some stray cats.

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INTRODUCTION

Spiral microorganisms are long known to be present in the stomach of various animals as well as human beings. After the first identification of *H. pylori* in human stomach and determination of its relevance to gastritis, studies on Helicobacters have increased enormously. Helicobacters were reported to be located in the mucosal layer of stomach, gastric pits, gastric adrenal lumens and cytoplasm of parietal cells (Erginsoy and Sozmen, 2006; Bridgeford *et al*., 2008; Takemura *et al*., 2009). These bacteria were recorded to cause chronic gastritis, peptic ulceration, and neoplastic changes in animals and human (Morgner *et al*., 1995; Ménard *et al*., 2014; Pozdeev *et al*., 2015; Jankowski *et al*., 2015).

In cats, *H. felis* is the first helicobacter isolated. Thereafter, other helicobacters such as *H. heilmannii*, *H. bizzozeronii*, *H. salomonis*, *H. rappini*, *H. canis* (in Bengal cats), *H. cynogastricus*, and *H. marmotae* were reported (Stacey and Sutton, 2008). *H. heilmannii* was also identified in the human stomach and suggested to cause zoonozis (Stolte *et al*., 1994). Similarly, in various studies performed on house and stray cats, *H. pylori* was shown to be present in saliva, gastric content, and feces and hence suggested to be zoonotic (Handt *et al*., 1994; Fox *et al*., 1995). However, not be able to showing *H.*

pylori in cats in other studies suggested that the agent is transferred by anthroponotic way.

Prevalence of gastric Helicobacter caused infections in cats is quite high (41-100%) (Priestnall *et al*., 2004; Van den Bulck *et al*., 2005; Araujo *et al*., 2010; Macêdo *et al*., 2012). In spite of the high incidence rate in cats, an association between the Helicobacter species and gastritis is still suspicious. It was reported that Helicobacters cause gastritis and glandular degeneration in some cats, however clinical symptoms are not seen in most of them (Happonen *et al*., 1996; Yamasaki *et al*., 1998).

Most studies on natural (Yamasaki *et al.*, 1998; Erginsoy and Sozmen, 2006) or experimental (Simpson *et al*., 2000) gastric Helicobacter infections are focused on the histomorphological characterization and identification of pathological lesions observed. However, identification of Gastric helicobacter species under light microscope is sometimes very difficult and specific techniques, such as bacteriological culture, immunohistochemistry, 16S rRNA analysis, DNA hybridization and electron microscopy may be required (Jalava *et al*., 1998; Neiger *et al*., 1998; Norris *et al*., 1999; Sjodin *et al*., 2011; Smet *et al*., 2012).

The aim of this field study is to determine the prevalence of Helicobacter species in stomach of stray cats using polymerase chain reaction analysis and immunohistochemical staining methods, and to correlate the results with histopathological lesions to make an estimate for significance.

MATERIALS AND METHODS

Materials: Total of 30 adult stray cats (*Felis cattus*) (15 female and 15 male) were used in this study. Under general anesthesia gastric samples from cardia, fundus, and pyloric antrum were taken applying gastroscopy. Halves of the biopsy samples were fixed in 10% Phosphate buffered formaldehyde for histopathological and immuno-histochemical investigations and the other halves were saved for Polymerase chain reaction (PCR) analysis.

Histopathology: Fixed tissue samples were routinely paraffin embedded, and tissue sections were cut and then stained routinely with hematoxylin and eosin for light microscopic evaluation. Semi-quantitative evaluation of histopathological changes was performed based on lymphoid follicle formation, bacterial density and presence of gastritis. The scales used in microscopic evaluations were based on the study of Erginsoy and Sozmen (2006). Simply, mean values for each parameter (lymphoid follicle formation, bacterial density and presence of gastritis) recorded for each animal were used for statistical comparison.

Immunohistochemistry: Tissue sections of cardia, fundus and pyloric antrum obtained from each animal were stained for Rabbit anti-*H pylori* polyclonal antibody (Dako® Diagnostica GmbH, Cat No: B0471), which is known to detect helicobacters in general, by Avidin Biotin Peroxidase immunohistochemistry (IHC) technique. The primer antibody was diluted 1:150 in phosphate buffered saline and antigen retrieval was performed by microwave treatment in citric acid solution (pH 6.0). Immunohisto-chemical procedure was routinely followed with DAB/ H_2O_2 reaction and hematoxylin background staining.

PCR-Gel Electrophoresis Analysis: Paraffin embedded cardia, corpus and pyloric antrum tissue blocks were used in PCR analysis. Total DNA was extracted from the bocks by Ex-Wax™ DNA extraction kit (Chemicon; Cat No: S4530). Urease B gene of *H. pylori, H. felis* and *H. heilmanni* was amplified using the primer pair shown in Table 1. PCR mixture was composed of 50µl 1X PCR buffer, 200 µM dNTPs, 100 pmol each of primer pair (Microsynth GmbH, Balgach, Isvicre) and 2.5 U Taq DNA polymerase. Negative controls were done by replacing extracted DNA samples with distilled water. PCR time and temperature cycles were as follow; Initial denaturation of 2 minutes at 94°C, followed by 31 cycles of 0.5 minute at 94°C denaturation, 0.5 minute at 57°C annealing and 1 minute at 72°C extension, with a final extension step of 5 min at 72°C. The amplicons were visualized on 1% agarose gels that contained 0.5 µg ethidium bromide. The bands were calculated as reference to the DNA ladder marker.

Statistical Analysis: Statistical differences in histopathological observations and presence of GHLO in three different stomach regions were tested by Chi square analysis at SPSS version 20.0 for Windows. $P<0.05$ was accepted significant.

RESULTS

Gastric helicobacter like organisms (GHLO) were detected in 28 out of 30 stray cats (93.3%) investigated by immunohistochemical staining. These bacteria were seen to be present either singly or in clusters in the stomach samples. They were observed to localize, focally or diffusely, in the mucous layer, gastric pits and gland lumens of the stomachs. Occasional intracellular localization was also noted. The most severe localization of the bacteria was recorded in the gastric pits and the gland lumens. In microscopic examination, two different types of bacteria were observed. One type was 5-9 µm long and has 6-8 spirals. This type of bacteria was detected in 16 cats and described as *H. felis* like organisms (HFLO) (Fig. 1). The other type was 7-9 µm long and has 7-9 spirals, therefore was described to have frequent spirals and named as *H. heilmannii* like organisms (HHLO) (Fig. 2).

Table 1: The primer pairs used in the amplification of Urease B genes of *H. pylori*, *H. felis* and *H. heilmannii*

Species	Primer pairs
H. pylori	F, 5'-GGAATTCCAGATCTATGAAAAAGATTAGCAGAAAAG-3' R, 5'-GGAATTCGTCGACCTAGAAAATGCTAAAGAGTTG-3'
H. felis	F, 5'-ATGAAACTAACGCCTAAAGAACTAG-3' R, 5'-GGAGAGATAAAGTGAATATGCGT-3'
H. heilmannii	F, 5'-GGGCGATAAAGTGCGCTTG-3' R, 5'-CTGGTCAATGAGAGCAGG-3'

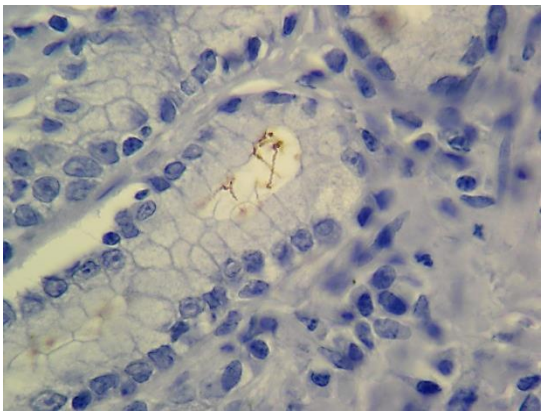

Fig. 1: *H. felis* like organisms in the gland lumen of a cat stomach. IHC, x40.

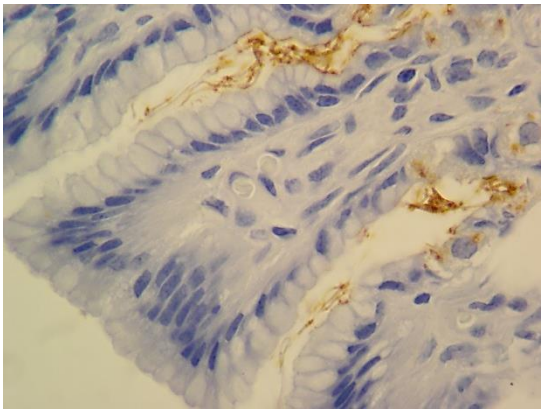

Fig. 2: *H. heilmannii* like organisms in the gland lumen of a cat stomach. IHC, x40.

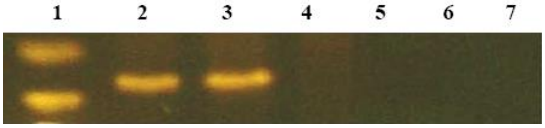

Fig. 3: PCR amplification and agarose gel electrophoresis of *H. felis* Urease B gene (1150 bp). 1, DNA ladder; 2, positive control; 3, cat no 6; 4, cat no 13; 5, cat no 20; 6, cat no 25; 7, negative control.

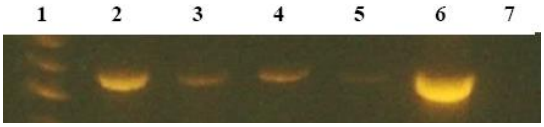

Fig. 4: PCR amplification and agarose gel electrophoresis of *H. heilmannii* Urease B gene (580 bp). 1, DNA ladder; 2, positive control; 3, cat no 4; 4, cat no 8; 5, cat no 11; 6, cat no 28; 7, negative control.

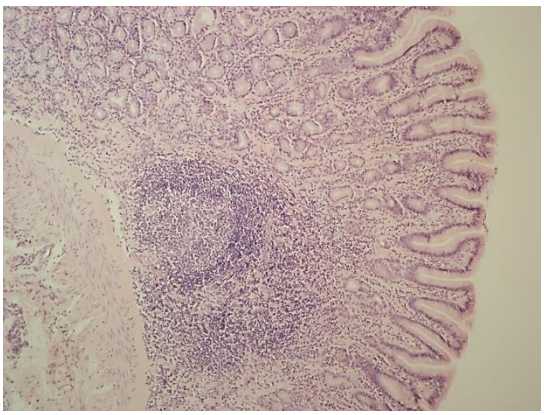

Fig. 5: Lymphoid follicle formation in pyloric antrum of a cat stomach. HE, x4.

Bacterial presence and localization of the helicobacters in three different stomach regions were summarized in Table 2. While HHLO was detected in total of 23 animals (76.7%), HFLO was seen in 16 animals (53.3%). HHLO were detected to be present only in one region of the stomach in 3.3% animals, in two different regions in 23.3% animals, and in all regions in 56.7% animals. HFLO were seen to localize in only one region in 23.3% animals, in two different regions in 16.7% animals and in all regions in 13.3% animals. These two types of bacteria were detected concomitantly in 43.3% cats, while HHLO alone were recorded in 40.0% animals and HFLO alone were determined in 6.7% cases. Results of immunohistochemical staining and PCR-Gel electrophoresis techniques used in order to determine the bacteria were generally parallel to each other. In gel electrophoresis an 1150 bp reaction band for *H. felis* (Fig. 3) and a 580 bp reaction band for *H. heilmannii* (Figure 4) urease B genes were observed. No reaction for *H. pylori* was detected in any of the animals investigated.

Light to moderate gastritis was detected in one or more regions of the stomach in 27 cats (90.0%) (Table 3). Gastritis was determined in 66.7%, 46.7% and 90.0% animals in fundus, corpus and antrum, respectively. Pyloric antrum was recognized to have more severe gastritis than the other regions of stomach. Mostly neutrophils and lymphocytes and occasionally plasma cells were noted in gastritis. In 3 cats, severe gastritis characterized by neutrophil and lymphocyte infiltration, basophilia in gland epithelia, fibrosis in mucosa and glandular dilatation was observed. In total of 10 cats, lymphoid follicle formation was determined. These lymphoid follicles were mostly seen in the pyloric antrum (Fig. 5). In 3 cats, no lesion was recorded in the stomach. None of the 30 cats showed any signs of erosion or ulceration in stomach.

Bacterial density was investigated and the results were given in Table 4. No significant difference was determined on the bacterial density among the three stomach regions ($P<0.05$). In 3 cats with light to moderate gastritis, no bacteria were detected, and conversely in another 3 cats with high bacterial presence, no gastritis was seen.

Results of lymphoid follicle formation in the stomach regions were shown in Table 5. Lymphoid follicle formation observed in 10 cats (9 infected and only 1 non-infected). Antrum was the most frequently formed place for lymphoid follicles ($P<0.05$). In light microscopic investigations, density and colonization regions of GHLO were similar to each other. No significant association was also determined between the gastritis and the bacterial colonization.

DISCUSSION

Discovery of helicobacters in human stomach and determination of its pathogenic effects has led to many investigations in animals as well to search for the presence of such an association over the past decades. In this study, helicobacters, their prevalence, pathological changes and probable relationship between the bacteria and the pathologies in stomach of stray cats were investigated by means of microscopy, immunohistochemistry and PCR-Gel electrophoresis analysis.

Table 2: Number of cats with presence of *Helicobacter felis* like organisms (HFLO) and *Helicobacter heilmanni* like organisms (HHLO) in different stomach regions

	Fundus	Corpus	Antrum
HFLO	7 (23.3)[a]	10 (33.3)[a]	12 (40.0)[a]
HHLO	22 (73.3)[a]	25 (83.3)[a]	19 (63.3)[a]

Values in parenthesis indicate percentage. Numbers with different superscripts in a row differ significantly (P<0.05).

Table 3: Cats with gastritis in different stomach regions

	Fundus	Corpus	Antrum
None	10 (33.3)[a]	16 (53.3)[a]	3 (10.0)[b]
Light	12 (40.0)[a]	14 (46.7)[a]	18 (60.0)[a]
Moderate	7 (23.3)[a]	-	6 (20.0)[a]
Severe	1 (3.3)[a]	-	3 (10.0)[a]

Values in parenthesis indicate percentage. Numbers with different superscripts in a row differ significantly (P<0.05).

Table 4: Cats showing different bacterial densities in different stomach regions

Score	Fundus	Corpus	Antrum
None	5 (16.7)[a]	3 (10.0)[a]	3 (10.0)[a]
1-4	7 (23.3)[a]	9 (30.0)[a]	7 (23.3)[a]
5-10	12 (40.0)[a]	11 (36.7)[a]	14 (46.7)[a]
>10	6 (20.0)[a]	7 (23.3)[a]	6 (20.0)[a]

Values in parenthesis indicate percentage. Numbers with different superscripts in a row differ significantly (P<0.05).

Table 5: Cats with lymphoid follicle formation in different stomach regions

Score	Fundus	Corpus	Antrum
None	28 (93.3)[a]	27 (90.0)[a]	20 (66.7)[b]
1	1 (3.3)[a]	3 (10.0)[a]	7 (23.3)[a]
2-5	1 (3.3)[a]	-	1 (3.3)[a]
>5	-	-	2 (6.7)

Values in parenthesis indicate percentage. Numbers with different superscripts in a row differ significantly (P<0.05).

The prevalence of the spiral microorganisms in cat stomach was investigated by different detection techniques and reported to change between 41% and 100% (Priestnall *et al*., 2004; Van den Bulck *et al*., 2005, Erginsoy and Sozmen, 2006). In accordance with the previous reports, the prevalence of Helicobacters in stray cats was detected to be quite high (93%) in the current investigation, which applied both immunohistochemistry and PCR. In addition, *H. heilmannii*, *H. felis* and mix presence were detected in 25 (89.3%), 16 (57.1%) and 13 (46.4%) animals respectively among the total 28 cases in which Helicobacters were seen. In a similar study conducted by Strauss-Ayali *et al*. (2001), these prevalence rates were reported as 52.9%, 23.5% and 17.6%, respectively. Mix localization of Helicobacters in cats was also reported to occur in 25% (Priestnall *et al*., 2004) and 62.8% (Van den Bulck *et al*., 2005). In addition to presence of *H. felis* and *H. heilmannii* mix infection, *H.pylori*, *H. bizzozeronii* and *H. heilmannii* mix infection was also shown in cats (Canejo-Teixeira *et al*., 2014). This finding suggests that mix presence of the bacteria may also occur by other species of helicobacters, which may also affect the severity and variety of pathological changes that may require further investigations.

In the present study, immunohistochemical staining was shown to be a beneficial method in determining the micro-localization sites of *H. heilmannii* and *H. felis*. These microorganisms were determined to be present in the mucous layer, gland lumens and parietal cells of the stomach. No special localization tendency was seen among these sites. GHLO were observed as a single bacterium and also in groups. Similar presences were also reported in the literature (Erginsoy and Sozmen, 2006, Bridgeford *et al*., 2008; Takemura *et al*., 2009; Canejo-Teixeira *et al*., 2014; Sasani *et al*., 2014). Presence of mixed colony formations was previously suggested to cause genomic transmissions between different Helicobacter species (Priestnall *et al*., 2004). In this investigation, high incidence rate recorded for *H. heilmannii* complies with the previous investigations (Neiger *et al*., 1998; Norris *et al*., 1999). *H. pylori* infection was previously shown in laboratory and commercially sold cats, but not in house cats (Handt *et al*., 1994; Fox *et al*., 1995). Although *H. felis* was detected in this study, there are also studies not determined the agent both in domestic and feral cats (Ghil *et al*., 2009). On the other hand, *H. pylori* was not able to be detected as in the previous study conducted in South Korea either (Ghil *et al*., 2009). Therefore, regional differences may apply in the presence of Helicobacters species.

The results were shown that Helicobacters are present in corpus, fundus and pyloric antrum regions of stomach, and they equally localize in these regions. While this result is similar to that of Takemura *et al*. (2009), it contradicts the previous investigations reporting mostly fundus localization in cats and also dogs naturally or experimentally infected with Helicobacters (Happonen *et al*., 1996; Yamasaki *et al*., 1998).

In histopathological investigations, gastritis in varying degrees was observed both in Helicobacter present and not-present cats similar to the previous studies (Happonen *et al*., 1996; Neiger *et al*., 1998; Yamasaki *et al*., 1998; Strauss-Ayali *et al*., 2001; Macêdo *et al*., 2012; Sasani *et al*., 2014). In the present study, severity of the inflammation in pyloric antrum and fundus was seen to be significantly higher than that is observed in corpus (P<0.05). Pyloric antrum was the mostly infected stomach region. This finding was in accordance with the result of Sasani, *et al*. (2014), which also showed that the cardia was the least affected stomach region. In contrast, corpus was seen to be the least infected stomach region in the present study.

The finding of severe inflammatory infiltration in pyloris reported by Strauss-Ayali *et al*., (2001) partially complies with our results. The finding of lymphoid follicle hyperplasia in the antrum of stomach is also similar to the results reported in naturally infected cases (Hermanns *et al*., 1995; Happonen *et al*., 1996). On contrary to our results and others, Strauss-Ayali *et al*. (2001) reported few lymphoid follicle formations in pyloris. While some studies described an association between the infection and lymphoid follicle hyperplasia such an association was not seen in the current study (Neiger *et al*., 1998; Yamasaki *et al*., 1998; Norris *et al*., 1999). On the other hand, mononuclear cellular infiltration was observed as in the previous studies performed on naturally GHLO infected cats (Happonen *et al*., 1996; Neiger *et al*., 1998; Yamasaki *et al*., 1998; Norris *et al*., 1999). However, none or few non-infected cases in the studies makes difficult to evaluate the presence of an association between Helicobacters and the histopathological changes observed. But, such an association was described between the bacterial colonization severity in fundus and the degree of inflammation in some studies (Hermanns *et al*., 1995; Happonen *et al*., 1996; Yamasaki *et al*., 1998). In the

present study, degree of inflammatory cellular infiltration and lymphoid follicle formation in pyloric antrum were noted to be higher than those observed in the other regions of the stomach. Although we have not seen any difference on the colonization severity among the different stomach regions, less colonization has been previously described in pyloris by others (Happonen *et al*., 1996; Yamasaki *et al*., 1998).

PCR-Gel electrophoresis analysis have previously used in detection and differentiation of Helicobacter species (Neiger *et al*., 1998; Shojaee Tabrizi *et al*., 2010; Smet *et al*., 2012; Canejo-Teixeira *et al*., 2014). The results of the current investigation also show that PCR-Gel electrophoresis could be successfully used in determining gastric Helicobacter species in cats. Urease B genes of *H. pylori*, *H. felis*, and *H. heilmannii* were amplified by *Helicobacter* spp. specific primers. Absence of cross reactivity for the primers has been previously described (Li *et al*., 1996). Therefore, the results of the study can be said straightforward and reliable. Results of PCR-Gel electrophoresis analysis were also consistent with the results of immunohistochemistry, which visualized the presence of helicobacters. Therefore, it can be assumed that immunohistochemistry can be used in showing the presence of the bacteria. However, in this case species differentiation is still required PCR analysis.

In conclusion, presence and prevalence of *Helicobacter spp*. was investigated by PCR-Gel electrophoresis and immunohistochemistry as well as histopathological changes in the stomach of stray cats. The results show that cats are important reservoir for GHLO. Therefore, it can be assumed that cats may provide a source for non- *H. pylori* infections seen in human beings. The results also suggest that gastric Helicobacter infections might be associated with lympho-cytic hyperplasia in the pyloric antrum of cat stomach.

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Author's contribution: SD, MS, RT and HÖ designed the study and did histopathology, immunohistochemical staining and PCR, as well as evaluation of the findings. MC and BK did surgical manipulations for biopsy collection. AKD conducted PCR technique. All authors participated in manuscript writing and revision.

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