Detection of Helicobacter spp. DNA in the colonic biopsies of stray dogs: molecular and histopathological investigations

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Abstract

Background: In dogs, the gastric Helicobacter spp. have been well studied, but there is little information regarding the other parts of the alimentary system. The incidence of Helicobacter spp. infection in dogs is largely unknown and to our knowledge there are no data about their potential pathogenic role. In light of these considerations, the aims of this study were (i) to assess the prevalence of Helicobacter spp. in colonic biopsies of healthy and symptomatic stray dogs also (ii) we isolate and characterize helicobacters in canine colonic biopsies to compare the commonly used tests for the identification of Helicobacter spp. and to determine the occurrence of these species in dogs.

Methods: Tissues from fifteen stray dogs (8 males and 7 females, age 6 months – 10 years) were used in this study. From each stray dog, multiple colonic biopsies were taken for PCR. Biopsies for PCR of cecum and colon were immediately frozen and stored at −20°C until DNA extraction. Samples for histological examination were fixed in 10% neutral buffered formalin and embedded in paraffin wax.

Results: In the cecum and colon, Helicobacter spp. DNA was detected in all dogs. H.canis, H.bizzozeronii, H. bilis, H.felis, H.salomonis and H.pylori identified by specific polymerase chain reaction. Histopathology demonstrated that Helicobacter organisms were localized within the surface mucus and the intestinal crypts. Dogs with heavy Helicobacter spp. colonization were significantly in younger as well as had a higher level of mucosal fibrosis/atrophy than dogs with uncolonized or poorly colonized biopsies (p < 0.05).

Conclusions: We have indicated that the crypts of the cecum and colon of healthy and symptomatic dogs are heavily colonized by Helicobacter spp.. Combined molecular and histological approaches demonstrated that enterohepatic Helicobacter spp. infection is rather common in colonic biopsies of healthy and symptomatic stray dogs, with Helicobacter spp. specialy H. canis, H.bizzozeroni, H.bilis, H.felis and H. salomonis identified as the most common species.

Virtual Slides: The virtual slide(s) for this article can be found here: http://www.diagnosticpathology.diagnomx.eu/vs/1957989294118782.

Keywords: Dog, Inflammatory lesions, Helicobacter, Single PCR, Colonic biopsies

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**Background**

Based on their preferential site of colonization and phylogenetic analysis of 16S and 23S rRNA genes, members of the genus *Helicobacter* are divided into gastric and enterohepatic *Helicobacter* spp. [1,2]. *Helicobacter* spp. can be either part of the normal flora or associated with inflammatory and proliferative disorders of the alimentary tract in humans and animals [1]. A pathogenic role of the gastric and enterohepatic *Helicobacter* spp. in dogs has not yet been well elucidated. However, canine gastric and enterohepatic *Helicobacter* spp. have been identified in humans with gastrointestinal and systemic disorders [1], suggesting a potential zoonotic role, and warranting further investigations.

Since the first isolation of *Helicobacter pylori* from the stomach of humans by Warren and Marshall [3] several additional *Helicobacter* species have been identified in the gastrointestinal tract of various mammalian species and birds [1,4]. Similarly to humans, gastric infection with *Helicobacter* spp. has been associated with variable degrees of gastritis in various animals, including dogs, cats, ferrets, pigs, monkeys and cheetahs [5], gastric adenocarcinoma in ferrets naturally infected by *H. mustelae* [5] and in Mongolian gerbils experimentally infected by *H. pylori* [6].

The discovery of *H. pylori* in humans [7] and its relationship to gastritis, peptic ulcer, and gastric neoplasia has increased interest in gastric bacteria. Research has focused on four specific areas: (i) prevalence of gastric bacteria in dogs and cats; (ii) investigation of these domestic animals as a source of zoonotic *Helicobacter* infection of humans [8,9]; (iii) clinical significance of gastric Helicobacters in dogs and cats; and (iv) development of animal models of human disease.

Recent reports indicate that in the canine stomachs three different culturable helicobacters can be present and they are regarded as different species: *H. felis*, *H. bizzozeronii* and *H. salomonis* [10,11].

Enterohepatic *Helicobacter* spp., including *H. bilis*, *H. canis*, *H. cinaedi* and other unclassified *Helicobacter* spp. have been recovered from the faeces of healthy and diarrhoeic dogs [1,12-14]. In healthy dogs, enterohepatic *Helicobacter* spp. were described to preferentially colonize the crypts of the cecum and colon [15]. However, despite the extensive literature on enterohepatic *Helicobacter* spp. infection associated with IBD in laboratory animals, the association between *Helicobacter* spp. infection and gastrointestinal inflammatory and proliferative disorders has not been established in dogs so far.

The goals of this study were to investigate the prevalence of *Helicobacter* spp. in colonic biopsies of pet dogs and its six different species in colonic biopsies of stray dogs, and to evaluate the effect of the *Helicobacter* spp. colonization status (heavily colonized, poorly colonized and uncolonized biopsies) on the presence of histological lesions and DNA sequencing.

**Methods**

**Animal and samplings**

Tissues from fifteen stray dogs (8 males and 7 females, age 6 month –10 years) were used in this study. Stray dogs were housed in the same research facility (Table 1). None of the animals had been treated with antibiotics within 4 weeks before examination. Endoscopy and biopsy sampling were performed as part of clinical investigation with the owners’ agreement. The study was approved by the Ethical Committee of of the Science and Research Branch, Islamic Azad University of Tehran. Tissue samples from the gastrointestinal tract of each dog were collected in a sterile manner, with care taken to avoid cross-contamination between different sites. From each stray dog multiple colonic biopsies were taken for PCR. After each sampling, the endoscope and the biopsy forceps were thoroughly cleaned, then sterilized using an activated aldehyde solution. Biopsies were immediately frozen and stored at −20°C until DNA extraction. Samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax for histological examination.

**Polymerase chain reaction (PCR) amplification**

DNA was extracted from biopsies by using a DNA easy tissue KIT® (Qiagen, Hilden, Germany) according to the manufacturer’s Instructions. The 16S rRNA gene of members of the family Helico-Bacteraceae was amplified by primers H276f/H676r [16], by using a single PCR. Primers P17f/P17r [17] were used to amplify specifict protein of Helicobacter bilis. Primers Fe11F/Fe3R [18] were used to amplify urease gene of *H. bizzozeronii*. Furthermore, primers HSALF/ HSALR [19] were used to amplify HP60 of *H. salomonis* and primers HP-for/HP-REV [20] were used to amplify the urease C gene of *H.pylori*. Primers canis3F/canis4R (EU233451) were used to amplify the HSp60 of *H.canis*. All PCR reactions were performed in an ABI Applied Biosystems Thermocycler (2720 thermal cycler, M/PCR/23), all the amplifications were performed in a final volume of 25 μl containing 5 μl of DNA extracted, 10.3 μl ultra-pure distilled water, 2.5 μl of 10×Tag polymerase buffer (Qiagen), 0.5 μl MgCl2 (Qiagen), 0.2 μl of Tag polymerase enzyme and 0.5 μl of each primer (MWG), 0.5 μl DNTP (Qiagen), 0.5 μl DNA Nucleotide (Qiagen), 5 μl Q Solution (Qiagen). Samples were heated at 94°C for 15 min and 30 s, denaturation phase, followed by 30 s at primer annealing temperatures that were different for the species and the family Helicobacteraceae. Primer annealing temperature for the family Helicobacteraceae was 52°C, for *H.bilis* was 55°C, for *H.felis* was 52°C, for *H. bizzozeronii* was 54°C, for *H. salomonis* was 51°C, for *H. pylori* was 49°C and for *H.canis* was 52°C, followed by 30 s...
in 72°C (extension phase) this cycle repeated 35 times, followed by 7 min in 72°C, final extension step and repair (Table 2). Ten microliters of each amplification product were analyzed by gel electrophoresis in 1.5% agarose gels in Tris-boric acid EDTA buffer (TBE). The gel was stained with ethidium bromide (0.5 mg/L) and examined under UV transilluminator for the presence of amplified DNA; the size of the expected fragments was compared to a 100 bp reference marker (Fermentas-e), (Table 3).

**DNA purification and sequencing**

PCR products (there were 53 PCR products) were purified by Sanger method and then sequenced on the ABI 3730XL DNA Analyzer which provides high quality of

| Dog no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|
| Helicobacteraceae (H276f/H676r) | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| H.bilis (P17f/P17r) | - | - | + | - | + | + | + | - | + | - | - | - | - | - |
| H.bizzozeronii (Bi1F/Bi2R) | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| H.canis (canis3F/canis4R) | - | + | - | - | - | - | - | - | - | - | - | - | - | - |
| H.felis (Fe1F/Fe3R) | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| H.pylori (HP-for/HP-REV) | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| H.salomonis (HSALS/HSALR) | + | + | + | - | - | + | - | + | + | + | + | + | + | + |

- Negative; +, positive.
sequence analysis data (Sequencing Service from Bioneer, Republic of Korea). Sequencing results were extracted and corrected in FASTA format by FinchTV as a trace file viewer. The identities of the gene sequences (16S rRNA gene, specific protein of H. bilis, urease gene of H. felis, HSP60 of H. salomonis, urease C gene of H. pylori, HSP60 of H. canis) were verified by comparison of the sequences to the GenBank database through the algorithm BLASTn. Finally, the verified sequences were aligned in the MEGA5 software [4] and the related phylogenetic tree was constructed for each group of sequences.

Histological evaluation
Formalin-fixed paraffin-embedded 4 μm sections of colonic biopsies were stained with hematoxylin and eosin (H&E) and Periodic Acid-Silver Methenamine (PEM). The biopsies after staining were scored by a single pathologist. These criteria included the evaluation of inflammatory (infiltrates of lymphocytes and plasma cells, eosinophils, neutrophils and macrophages in the lamina propria) and morphological changes (surface epithelial injury, crypt hyperplasia, crypt dilation, distortion, mucosal fibrosis and atrophy). The degree of severity of both inflammatory and morphological changes was scored as follows: normal (0), mild (+), moderate (++) and marked (+++).

Statistical analysis
The software, SPSS Version 16.0 for Windows (SPSS Inc., Chicago, IL), was used for analysis. To compare the data chi-square tests were used. Differences were considered significant when \( P < 0.05 \).

Mean spirochete colonization status in 10 microscopic fields (PEM,×1000) evaluated also scored for the presence of Helicobacter spirochetes in the superficial mucus and in the crypts as follows: 0 = uncolonized; +1 = colonization <10 organismes; +2 =10 organismes ≤ colonization <50 organismes; +3 = colonization ≥50 organismes.

Results
PCR
Results of the overall PCR amplification of Helicobacter spp. DNA, and the group-specific canine colonic Helicobacter spp. are summarized in Table 3. Helicobacteraceae DNA was detected with single PCR in all the colonic biopsies (100%), furthermore, H. canis and H. bizzozeronii DNA in 100%, H. bilis DNA in 44%, H. felis DNA in 40%, H. salomonis DNA in 80% and H. pylori DNA in 1 of the 15 dogs examined (7%) were detected.

DNA sequence analysis
Evolutionary relationships of taxa for H. canis, H. bilis and H. Salomonis were inferred using the UPGMA method [1]. The optimal tree with the sum of branch
length = 1.04374900, 0.04752769, 0.74197122 are shown, respectively. The evolutionary distances were computed using the Kimura 2-parameter method [2] and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. Evolutionary analyses were conducted in MEGA5 [4] (Figures 1, 2, and 3).

PCR amplification of DNA from bacterial strains was conducted by (NO.1-7) *H. bilis* P17F/P17r primer; (NO. 8-22) *H. canis* 3F/canis4r primer; (NO.23-34) *H. salomonis* HSALF/HSALR primer; (NO.35-37) *H. felis* Fe1F/Fe3r primer; (NO.38-52) *H. bizzozeronii* Bi1F/Bi2R primer; (NO.53) *H. pylori* HP-for/HP-REV Primer, and 100-bp molecular ladder (Figure 4).

**Histological examination**

Inflammatory changes in fifteen cases there were various numbers of lymphocytes and plasma cells in the lamina propria of the colonic biopsies, occasionally admixed with lower numbers of neutrophils and rare eosinophils, whereas detectable aggregates of macrophages were found. Mild to severe lymphoplasmacytic and neutrophilic infiltration was found in colonic biopsies (Figure 5A-D). Moreover, slides stained with Giemsa and Periodic Acid-Silver Methenamine (Figure 5A and B), the samples coinfected with less than 3 different species of Helicobacter (according to the PCR results) that had mild and unclear infection with helicobacter spirochetes. Although the mean value of neutrophilic infiltration was higher in heavily colonized biopsies compared to uncolonized and poorly colonized ones, this difference was not statistically significant.

**Morphological changes**

Morphological changes of the colonic mucosa were present in 12/15 examined stray dogs with different degrees of severity. A statistically significant difference was found between heavily colonized colonic biopsies and
both uncolonized and poorly colonized colonic biopsies for the parameter of mucosal fibrosis/atrophy (P < 0.05; Tables 1 and 2) as well as lieber kuhn’s glands secretion, crypt dilation, crypt hyperplasia and distortion were higher in samples with objective number of spirochetes than what there were in slides with poorly and non objective spirochetes, even though these differences were not statistically significant.

Discussion

Helicobacter spp. are usually found in large numbers in the stomach of dogs but few data are available on the presence of these bacteria in other parts of the alimentary system. To date, enterohepatic Helicobacter spp. have been identified by culture in the feces of dogs and there are no data about their spatial distribution within the canine intestinal tract [13]. The fastidious nature of most

Figure 3 Helicobacteraceae DNA was detected with single PCR in all the colonic biopsies: Evolutionary relationships of taxa for helicobacter salomonis the evolutionary history was inferred using the UPGMA, Kimura 2-parameter and MEGA5 methods.

Figure 4 The Polymerase Chain Reaction (PCR) on DNA isolated with standard protocol; PCR amplification of DNA from bacterial strains by (NO.1-7) H.bilis P17f/P17r primer; (NO. 8-22) H.canis canis3F/canis4r primer; (NO.23-34) H.salomonis HSALF/HSALR primer; (NO.35-37) H.felis Fe1f/Fe3r primer; (NO.38-52) H.bizzozeronii Bi1F/Bi2R primer; (NO.53) H.pylori HP-for/HP-REV Primer, and 100-bp molecular ladder.
Helicobacter spp. and the coexisting intestinal flora hinder their routine isolation from the intestine [23], therefore, we opted for a DNA sequence analysis approach to detect Helicobacter spp. in the canine alimentary tract. A combination of PCR, 16S rRNA gene cloning, sequencing and histopathology allowed us to identify specific regions of the alimentary tract that were colonized with Helicobacter spp. and to visualize morphologically intact microorganisms and their spatial distribution within the gastrointestinal tract of dogs, as previously reported in mice and children [24-26].

In present study a high prevalence of Helicobacteraceae (100%) with H. canis and H. bizzozeronii (100%) DNA was detected by PCR in examined colonic biopsies from healthy and symptomatic stray dogs. The incidence of other species including H. salomonis (80%), H. bilis (47%), H. felis (40%) and H. pylori (7%) were comparatively high to low, respectively. These results were in line with previous studies in which enterohepatic Helicobacter spp. were detected by PCR in 100% of the large intestines of a small group of healthy dogs [13]. Our study revealed higher outbreak of helicobacter spp. than some previous studies. Based on direct sequencing of the enterohepatic Helicobacter spp. PCR products, H. salomonis, H. bilis, H. felis and H. pylori were the most prevalent Helicobacter species present in the canine colonic biopsies that is in accordance with previous works [13,15].

The high incidence of enteric H. canis and H. bizzozeronii might be explained by anal–oral transmission due to typical canine social behaviour. The presence of H. canis and H. bizzozeronii in both cecum and colon is interesting from a zoonotic perspective since H. canis and H. bizzozeronii has caused infections in humans where contact with dogs was suspected [27]. H. bizzozeronii (100%) and H. salomonis (80%) were found in colon, supporting the idea that gastric Helicobacter spp. might transmit via oral contact [10,28]. The absence of gastric Helicobacter spp. in faecal samples is in agreement with previous studies [15].

Furthermore, Helicobacter spp. DNA was detected in the cecum and colon of all dogs. The degree of colonization assessed by histopathology showed variable amounts of positive bacteria in all the cecum and colon sections depending on the dog. In some of these samples, only a small amount of Helicobacter spp. DNA was detected by single PCR. The low level of colonization was reflected in

Figure 5 Colonic biopsies of Helicobacter spp. in the stray dogs. (A and B). Colonic biopsies: Moderate to severe amount of Helicobacter spp in the superficial mucus entering a colonic crypt (Wright Giemsa staining). C and D: Lymphoplasmacytic and neutrophilic infiltration (hematoxylin and eosin; 400x).
the weak band obtained by the *Helicobacter* spp. PCR. *Helicobacter* spp. detected by PCR also histopathology had a comparable distribution and were localized either in the intestinal lumen at the mucosal surface admixed to other bacteria or within the intestinal crypts extending from the superficial to the deep portions of the crypts. This finding together with the occasional amplification of gastric *Helicobacter* spp. DNA and the absence of gastric *Helicobacter* spp. sequences in clone libraries likely reflects their relative scarcity in the large intestine compared to colonic *Helicobacter* spp. that suggests passive intestinal carriage of gastric *Helicobacter* spp. DNA.

In the present study, a significantly higher level of mucosal fibrosis/atrophy was found in heavily colonized colonic biopsies compared to uncolonized or poorly colonized ones. Fibrosis is considered a common consequence of IBD that results from the reaction of intestinal tissue to the damage induced by chronic inflammation but its pathogenesis has not been fully elucidated [29]. Since *Helicobacter* spp. have been considered as likely candidates for involvement in IBD pathogenesis [30-33] that fibrosis is a common IBD-associated lesion. The association between heavy *Helicobacter* spp. colonization and mucosal fibrosis/atrophy found in this study support the role of enterohpetic *Helicobacter* spp. in the development of canine IBD [1,16,30,31,34]. Therefore, our data may well explain the discrepancy previous studies.

**Conclusion**

We have indicated that the crypts of the cecum and colon of healthy and symptomatic dogs are heavily colonized by *Helicobacter* spp.. Combined molecular and histological approaches demonstrated that *Helicobacter* spp. infection is rather common in colonic biopsies of healthy and symptomatic stray dogs, *Helicobacter* spp. Specialy *H. canis, H. bizzozeronii, H.bilis, H.felis and H. salomonis* identified as the most common species. Additional studies are necessary to investigate if *Helicobacter* spp. are involved in cecal and colonic inflammatory or proliferative disorders in dogs, as demonstrated in other species.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

FSA and FS participated in the histopathological evaluation, performed the literature review, acquired photomicrographs and drafted the manuscript and gave the final histopathological diagnosis. JJ performed sequencing alignment and manuscript writing. FSA, SHJ and FM edited the manuscript and made required changes. All authors have read and approved the final manuscript.

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