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ORIGINAL ARTICLE

Prevalence and diversity of enteric *Helicobacter* spp. in healthy and diarrheic cats

Reza Ahmadi[1], Farnoosh Arfaee[1*], Shahram Jamshidi[2], Pejman Mortazavi[3], Mahmoud Jamshidian[3]

[1]Department of Clinical Science, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]Department of Internal Medicine, School of Veterinary Medicine, University of Tehran, Tehran, Iran
[3]Department of Basic Science, Science and Research Branch, Islamic Azad University, Tehran, Iran

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ABSTRACT

Background and Objectives: Helicobacters are gastric and enterohepatic and live in the gut. The role of enterohepatic Helicobacters as intestinal pathogens is uncertain, while stomach Helicobacters are well-known. The prevalence of *Helicobacter* species in cat feces helps us understand their impact on cat health and human disease transmission. This study used PCR to identify *Helicobacter* spp. in feces samples from healthy and diarrhoeic cats, independent of the reason. The study also compared intestinal and stomach *Helicobacter* species.

Materials and Methods: PCR analysis was performed on fecal samples from 40 cats, with 20 cats having diarrhea and 20 cats showing no symptoms. The PCR analysis aimed to detect Helicobacter's presence using a method that identifies the bacteria through the 16S rRNA gene.

Results: The diarrhoeic group had a greater prevalence of infection (17:9 ratio), with an overall 65% infection rate detected. Cats that were older than 2 years showed a higher incidence of disease. *H. canis* had the highest occurrence rate (69.2%), followed by *H. bilis*, *H. bizzozeronii*, and *H. salomonis*. Significantly, *H. pylori*, *H. felis*, and *H. heilmannii* were not reported.

Conclusion: *H. canis* was the predominant species found in both healthy and diarrheic cats, indicating the need for more investigation. The detection of the gastric species *H. salomonis* and *H. bizzozeronii* further complicates the classification. This highlights the complex nature of *Helicobacter* infections in cats, emphasizing the need for further investigation to guide the development of preventative measures and treatment techniques for both veterinary and public health purposes.

Keywords: Cats; *Helicobacter* spp.; Feces; Diarrhea; Healthy

INTRODUCTION

The *Helicobacter* genus includes gram-negative, microaerophilic, and spiral creatures that rapidly evolve. These organisms continuously inhabit various mammal hosts and, in certain instances, can lead to significant clinical issues (1). Based on their preferred colonization site, the bacteria were classified into Gastric and enterohepatic groups. Gastric *Helicobacter* (GH) species account for one-third of all species in the genus. These bacteria can inhabit the stomach wall and produce large amounts of urease. The remaining two-thirds of *Helicobacter* species are enterohepatic *Helicobacter* (EHH) species. They can form colonies in either the intestinal mucosa or the hepatobiliary tract (2). The limited understanding of the pathogenicity of *Helicobacter* infections in dogs and cats causes challenges when selecting treatment

*Corresponding author: Farnoosh Arfaee, DVM, DVSc, Department of Clinical Science, Science and Research Branch, Islamic Azad University, Tehran, Iran. Tel: +98-912-2785724 Fax: +98-21-22110193 Email: f.arfaee@srbiau.ac.ir

options (3). Moreover, *Helicobacter* infections have proven challenging to eliminate across many veterinary trials (4). This bacterial genus has been linked to the emergence of multiple diseases in humans, including liver and gallbladder issues, gastroenteritis, and inflammatory bowel disease. However, the reservoirs and transmission methods have not been thoroughly suggested at this time (5). While the study of gastric Helicobacters has been prioritized in experimental research due to their significance in causing gastric diseases in humans, identifying enterohepatic Helicobacters in both animals and humans caused interest in investigating the transmittable abilities of these organisms as well (6). Currently, approximately 53 validated species are in the *Helicobacter* genus (7). *H. felis*, *H. heilmannii*, *H. salomonis*, and *H. bizzozeronii* are among dogs and cats' most important gastric Helicobacters (4). Also, Enterohepatic Helicobacters, such as *H. canis*, *H. bilis*, and *H. cinaedi*, have been found in the mouth, stomach, intestine, liver, and pancreas of certain humans. These bacteria are often associated with dogs and cats, suggesting that there is a possible risk of transmission between animals and humans. *Helicobacter canis*, a species that has been related to several diseases in humans, is more commonly detected in dogs and cats compared to any other species. The presence of fecal history, ability to tolerate bile, and absence of urease activity in *H. canis* indicate that this particular species of *Helicobacter* primarily inhabits the lower bowel instead of the stomach (8, 9). The most common mode of transmission of *H. canis* to humans is through the fecal-oral route, which occurs during intimate contact with companion animals or through the intake of contaminated sheep milk. An additional contributing factor could be a compromised immune system in the patient (10). Studies conducted on animals, both by experimental infection and natural occurrence, have demonstrated that *H. bilis* has the potential to cause chronic active hepatitis, hepatocellular and biliary tract carcinoma, typhlocolitis, and cancer of the lower intestine. However, it should be noted that some individuals simply exhibited asymptomatic carriage of the bacteria. Recent *in vivo* studies have shown that *H. bilis*, along with other EHH, significantly increases the occurrence of cholesterol gallstone formation and intrahepatic cholelithiasis (11). *H. bizzozeronii* rarely occurs in animals, although it has been documented in dogs and cats, which could have clinical implications for humans. *H. salomonis* is a species of *Helicobacter* that infects the stomach of dogs. It has been found in the crypts of the caecum and colon of both healthy and sick dogs (12). The biochemical and tolerance characteristics of *H. salomonis* closely resemble the features of *H. bizzozeronii*, with the exception that one strain of *H. salomonis* lacks DNAse activity and another strain demonstrates resistance to metronidazole (13). Enterohepatic Helicobacters, which infiltrate the gastrointestinal tract and liver in humans and other animals, are linked to conditions such as gastroenteritis, hepatitis, cholangiohepatitis, and liver neoplasia (14). Furthermore, Non-*Helicobacter pylori Helicobacter* (NHPH) species have been found in the stomachs of 0.25 to 1.7% of individuals with gastric ulcers, suggesting that contact with animals like dogs, cats, or pigs may play a role in the transmission of this bacterium to humans (15). These data suggest that animals infected with enterohepatic *Helicobacter*, particularly cats, may play a significant role in transmitting zoonotic diseases. The precise mechanism of transmission of *Helicobacter* infection remains mostly unknown. Nevertheless, feces are the primary means by which enterohepatic Helicobacters are transmitted (16). This study aimed to examine the presence of *Helicobacter* spp. in the feces of both healthy and diarrheic cats, due to the growing global interest in researching enterohepatic Helicobacters and the insufficient knowledge on the prevalence of these species.

MATERIALS AND METHODS

Sample collection. The experiment was carried out under the supervision of the Iranian Society for Prevention of Cruelty to Animals, in accordance with the ethical guidelines for research involving laboratory animals in Iran.

This study examined a group of 40 cats that were brought to Tehran Pet Hospital in Tehran, Iran. The group consisted of 20 healthy cats (12 males and 8 females) and 20 cats with diarrhea (12 males and 8 females). The study only included mature felines aged between 9 months and 13 years, with an average age of 3.8 years. A written agreement was obtained from the cat owners before collecting rectal samples by gently rotating a swab 1.5 cm from the anal opening. The samples were collected using accurate aseptic techniques to perform molecular testing. Afterward, they were sent to the Microbiology Laboratory at the

University of Tehran.

DNA extraction. The DNA was isolated from the complete feces sample using a DNA extraction and purification kit manufactured by Qiagen, located in Valencia, CA. At first, 200 mg of fecal material from each sample was added to an ASL buffer and mixed thoroughly by vortexing. The solution was subsequently heated to a temperature of 95°C for 5 minutes in order to induce bacterial lysis. The DNA from all samples was extracted and purified following the guidelines provided by the manufacturer. The DNA concentration was quantified using a NanoDrop™ spectrophotometer (Thermo Scientific, Wilmington, DE). In order to ensure the precision of genome purification, a 5 microliter sample of the isolated DNA was subjected to electrophoresis on a 1% agarose gel.

PCR amplification and DNA sequencing. In order to identify *Helicobacter* species, the process of 16S rRNA gene identification has been used. Additionally, the urease gene has been utilized to specifically detect certain *Helicobacter* species, including *H. pylori, H. felis,* and *H. bizzozeronii*. The heat shock protein gene has been used for *H. canis* and *H. salmonis,* while the specific protein gene has been utilized for *H. bilis* (Table 1). PCR amplification was conducted using a reaction volume of 25 µL, consisting of 12.5 µL of PCR Master Mix 2x (80Test/25ul-MM2011, Sinaclon, IRAN), 1 µL of each primer, and 1 µL of DNA template. The polymerase chain reaction (PCR) was performed using an MJ MiniTM thermocycler (Bio-Rad, USA). The PCR protocol included an initial denaturation stage at 95°C for 5 minutes, followed by a single cycle consisting of a denaturation step at 95°C for 50 seconds, an annealing step at 50-58°C for 50 seconds, and an elongation step at 72°C for 60 seconds. The last elongation stage was conducted at a temperature of 72°C for 7 minutes. Subsequently, all PCR products were processed by electrophoresis on a 0.8% agarose gel. The gel documentation system was utilized to take the image. A primer with a distinctive design, created by Youssefi et al. (17), was used for the molecular investigation of the *Helicobacter* genus. In this study, seven pairs of primers were used to identify seven *Helicobacter* species, including *H. canis, H. bilis, H. bizzozeronii, H. salomonis, H. pylori, H. felis,* and *H. heilmannii* (Fig. 2).

Statistical analysis. The statistical analysis of the data was conducted using SPSS statistics software version 16.0 (USA). The association between the age (in years), sex (female or male), and *Helicobacter* spp. was evaluated using the Pearson Chi-Squared 2-tailed test. Statistical significance was set at $p<0.05$.

RESULTS

Identification of *Helicobacter* genus. The PCR product was electrophoresed on an agarose gel, and a 400 bp fragment was observed (Figs. 1 and 2). Out of

Table 1. Primers used to identify the genus and species of *Helicobacter*

Helicobacter species	Primer	Oligonucleotide sequence (5′-3′)	PCR product size (bp)
H. heilmannii	H276f	F- CTATGACGGGTATCCGGC	374
	H676r	R- ATTCCACCTACCTCTCCCA	
H. bilis	p17f	F-ATGGAACAGATAAAGATTTTAAAGCAACTTCAG	435
	p17r	R- CTATGCAAGTTGTGCGTTAAGCAT	
H. Felis	Fe1F	F-TTTGGTGCTCACTAACGCCCTC	434
	Fe3R	R-TTCAATCTGATCGCGTAAAG	
H. bizzozeronii	Bi1F	F-AACCAAYAGCCCCAGCAGCC	373
	Bi2R	R-TGGTTTTAAGGTTCCAGCGC	
H. Salomonis	HSALF	F-CATTTTCAAAGAGGGCTTGC	537
	HSALR	R-GCACACCCCTCAGTTTGTTT	
H. pylori	HP-FOR	F-TTATCGGTAAAGACACCAGAAA	132
	HP-REV	R-ATCACAGCGCATGTCTTC	
H. canis	canis3F	F-TAAGCGCGGTATGGATAAGG	254
	canis4R	R- TTAAGTAGCCGCGGTCAAAC	

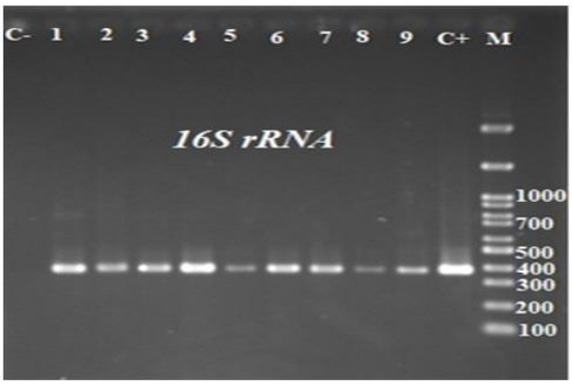

Fig. 1. Bands of agarose gel electrophoresis for 16S rRNA gene PCR produce

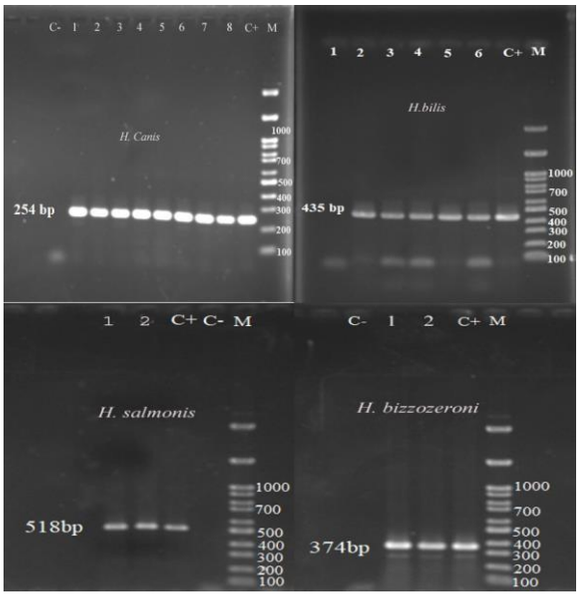

Fig. 2. Electrophoresis of PCR product of *Helicobacter* species. Lane M: a 100 bp DNA ladder; lane 1-8: PCR product of *Helicobacter* species; lane C+: Positive control; lane C-: Negative control.

the 40 fecal samples, 16S rRNA genes were detected in 26 samples, indicating that 65% of the cats were infected with *Helicobacter* spp. The diarrheic group had a higher prevalence of *Helicobacter* spp. positive cases (95% and 19 out of 20) compared to the healthy group (35% and 7 out of 20) (Table 2).

***Helicobacter* species identification through PCR analysis.** The prevalence of *Helicobacter* species between diarrheic and healthy groups in stool samples is indicated in Table 2. The epidemiologic data, including the age and gender of *Helicobacter*-positive cats, are presented in Table 3. The infection rate of *Helicobacter* spp. in fecal samples was found to be significantly higher ($P \leq 0.05$) in cats over 2 years compared to those less than 2 years. However, the statistical analysis indicated that there was no significant association between sex and the occurrence of *Helicobacter* infection in domestic cats ($P > 0.05$).

Table 2. Isolation numbers of enteric *Helicobacter* spp. In feces of healthy and diarrheic cats*

Helicobacter species	Fecal samples from cats		
	Total (n=40)	Healthy (n=20)	Diarrheic (n=20)
H. canis	18 (69.2%)	6 (85.7%)	12 (63.1%)
H. bilis	5 (19.2%)	1 (14.2%)	4 (21%)
H. bizzozeronii	2 (7.6%)	0	2 (10.5%)
H. salomonis	1 (4%)	0	1 (5.2%)
Total	26	7	19

* *H. pylori*, *H. felis*, and *H. heilmannii* were not detected in the feces

DISCUSSION

While studies on stomach *Helicobacter* infections have provided significant findings, this subset of the genus represents just a third of all cases. The remaining two-thirds are known as enterohepatic *Helicobacter* and primarily inhabit the intestines, bile ducts, and liver of animals and humans. Enterohepatic *Helicobacter* spp. is receiving more attention due to its probable involvement in both human enterohepatic disorders and their ability to transmit infections between animals and humans (10, 18). Unlike *Helicobacter pylori*, there are no commercially accessible noninvasive diagnostics for diagnosing human non-*Helicobacter pylori* infections. Moreover, these microscopic organisms are never identified by conventional culture methods, leading to a possible underestimation of their frequency and clinical significance (19). Different *Helicobacter* species have been associated with gastritis, gastric and duodenal ulcers, and lymphoma in humans who have had close contact with animals. This finding provides evidence to support the concept of zoonotic transmission (3, 10, 20). There have been no epidemiologic studies conducted, thus it is surprising that the normal reservoir of these organisms is not thoroughly characterized (5, 21). On the other hand,

Table 3. Frequency distribution of *Helicobacter* species among cat groups

Group of cats	*Helicobacter* species	*Helicobacter* infection distribution			
		Sex		Age	
Diarrheic (n=20)		**Male**	**Female**	**2≤**	**2≥**
	H. canis (n=12)	7	5	9	3
	H. bilis (4)	2	2	3	1
	H. bizzozeronii (2)	2	0	1	1
	H. Salomonis (1)	1	0	1	0
healthy	*H. canis* (n=6)	3	3	4	2
(n=20)	*H. bilis* (1)	1	0	1	0
Total	26	16	10	19	7

H. canis is the predominant *Helicobacter* in the intestines of both well- and sick cats and dogs, according to two separate investigations (22). Therefore, this study aimed to examine the prevalence of *Helicobacter* species in fecal samples by the PCR technique to determine the frequency and potential transmission risk between cats and humans. Currently, there has been no research carried out in Iran to examine fecal samples from both healthy and diarrheic cats to identify enterohepatic and stomach *Helicobacter* species. This study conducted a comparative analysis between cats that were in good health and cats that were experiencing diarrhea, specifically focusing on *Helicobacter* infection. As previously mentioned, the incidence of *Helicobacter* infection in this study was greater in the group with diarrhea compared to the group without any health issues. *H. bizzozeronii* and *H. salomonis* were found only in the group of cats that had diarrhea. Nevertheless, it remains uncertain whether there is a correlation between these two *Helicobacter* species and the incidence of diarrhea. Therefore, more investigations are necessary to acquire a greater understanding of this topic. Consistent with prior research by Shen et al. (23), The findings of the present study revealed that *H. canis, H. bilis,* and *H. bizzozeronii* were successfully isolated from cat feces. Rossi et al. found the DNA of *H. canis* and *H. bilis* in the feces of both diarrheic and healthy cats. The infection rate was reported to be 28.6% for *H. canis* and 4.8% for *H. bilis* (24). The results correlate with our findings in terms of the prevalence and species detected. Moreover, scientists discovered the presence of *H. canis* DNA in every single sample of dog feces. In addition, the researchers conducted a PCR analysis to examine the presence of *H. bilis* in dogs' feces. The results revealed a remarkable similarity between cats and dogs in this regard (25). In a study done by Ghil et al. (26), The presence of *H. felis* and *H. pylori* infections in domestic and wild cats in Korea was examined using PCR analysis of stool and saliva samples. However, none of the samples tested positive for these specific species. Our study showed identical results in fecal specimens. In the study conducted by Taillieu et al. none of the stool samples tested positive for gastric *Helicobacter* species in any of the tests they performed (7). However, in the current research, *H. salomonis* and *H. bizzozeronii* were found in our fecal test results.

Based on previous studies, *H. canis* is considered an EHH species and has been associated with several diseases in humans, including gastroenteritis, inflammatory bowel disease (IBD), as well as liver and gallbladder diseases (10, 27-29). *H. bizzozeronii* is the second most significant *Helicobacter* species that can be transmitted between humans after *H. pylori* and it was found in 5% of human fecal samples. *H. bizzozeronii* was shown to be the most common stomach NHPH in dogs who were spontaneously infected. A recent case report has documented an instance of *Helicobacter bizzozeronii* infection in a 20-month-old girl in México. The girl experienced severe gastrointestinal issues and had regular and intimate interaction with the household dog. The dog received proper vaccination and displayed no evident clinical signs (19). Furthermore, according to previous investigations, *H. felis* and *H. heilmannii* species are more common in cats, which is contrary to the findings of the present study (30). Research has shown that *Helicobacter bizzozeronii, H. salomonis,* and *H. heilmannii* have the potential to be transmitted between humans and animals, and are potentially clinically relevant in humans (20). Due to the increasing number of domestic cats in Iran and the possibility of these cats coming into close contact with

both people and children, it is crucial to thoroughly investigate their potential for transmitting diseases to humans. While there is currently no evidence of zoonotic risks associated with *Helicobacter* infection in cats, the findings of this study indicate that it may be necessary to reconsider this perspective. To prevent potential outbreaks in cats, it is important to utilize appropriate techniques for managing cat stool and preventing the spread of germs. Special focus should be placed on eliminating these bacteria from the intestines of pets to prevent the possibility of diseases being transmitted from pets to humans.

Furthermore, the detection of *Helicobacter* species in the feces of both healthy and diarrheic cats suggests that they could be a normal part of the intestinal microbiota, and there is a possibility of contamination spreading from the bile duct and leading to infection in the liver. Hence, further investigations are required to compare intestine samples with liver and bile specimens.

CONCLUSION

Currently, there has been no research conducted in Iran to examine fecal samples from both healthy and diarrheic cats to identify the presence of *Helicobacter* species. This study shows *H. canis* as the predominant *Helicobacter* species found in the feces of both groups of cats. Given its high prevalence, there is an increased risk of transmission to humans and a greater chance of contracting the disease, particularly in individuals with compromised immune systems. Hence, adhering to appropriate hygiene protocols is essential in this instance.

In addition to *H. canis* and *H. bilis*, which are important enterohepatic *Helicobacter* species found in cats, two gastric *Helicobacter* species, namely *H. salomonis* and *H. bizzozeronii*, have also been identified in fecal samples. The official classification of these species as Enteric *Helicobacter* is still uncertain and requires further inquiry to determine if they are derived from the stomach *Helicobacter* population.

Additionally, our data indicate the need for additional research on the potential harmful involvement of *Helicobacter* species in the onset of diarrhea in cats. Furthermore, the progress in understanding the significance of EHH in human and animal pathology has been impeded by several knowledge limitations that need to be resolved in future research. Acquiring a comprehensive understanding of different kinds of animals that serve as reservoirs for EHH is imperative. This knowledge will help us to find out their epidemiology, clarify probable transmission pathways, and potentially facilitate intervention and prevention of human infection.

As previously mentioned, there is a difference in the occurrence of *Helicobacter* infection in the gastrointestinal tract and stool samples of cats in different studies. This difference may be attributed to variations in the sensitivity of the techniques used, the number of samples analyzed, differences in hygiene levels between developed and developing nations, and the increasing population of stray cats in third-world countries.

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