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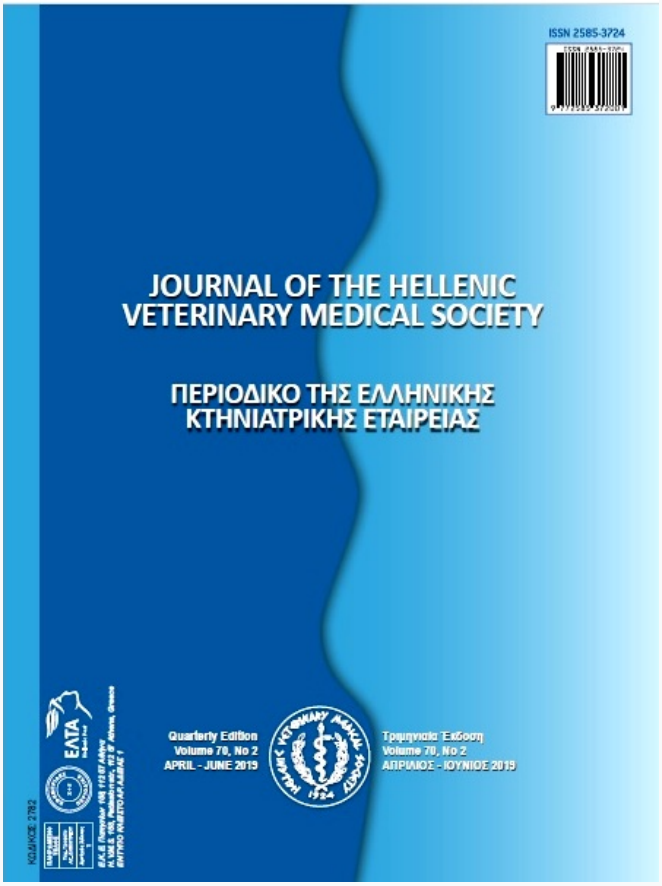

Investigation of some gastric Helicobacter species in saliva and dental plaque of stray cats by cultural and PCR methods

S. TARHANE, S. OTLU

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Research article
Ερευνητικό άρθρο

Investigation of some gastric *Helicobacter* species in saliva and dental plaque of stray cats by cultural and PCR methods

S.Tarhane[1], S. Otlu[2,*]

[1] *Institute of Health Sciences, Kafkas University, Kars, Turkey*

[2] *Department of Microbiology, Veterinary Faculty, Kafkas University, Kars, Turkey*

ABSTRACT. The purpose of this study was to explore the presence of gastric *Helicobacter* species in the oral cavity of stray cats in the Kars region. Saliva and dental plaque samples collected from 100 stray cats were evaluated by culture and PCR methods in terms of gastric *Helicobacter* species. For culture, samples were plated on 5% defibrinated-horse blood and 5% defibrinated-sheep blood enriched selective agar plates supplemented with Vancomycin (6 µg/ml), Polymyxine B (2.500 IU/l), Trimethoprim (20 µg/ml) and Amphotericin B (2.5 µg/ml). Molecular methods were also included to study by using the PCR targeting amplification of the 16S rRNA gene sequence for *Helicobacter* genus and *urease B* gene sequence for each *Helicobacter* species. As the results of cultural examination, *Helicobacter* spp. were isolated from 10 (10%) cats (10 saliva and 5 dental plaque samples) and these were further identified as *H. heilmannii* by PCR. Direct analysis of samples by genus-specific PCR revealed that a total of 70 (50 saliva and 20 dental plaques) samples from 65 cats were positive in terms of *Helicobacter* DNA. As the results of species - specific PCR analysis of these samples 34 (48.57%) (24 saliva and 10 dental plaque samples) were identified as *H. heilmannii*, while the remaining 36 (51.42%) were found to be negative in terms of related species (*H. heilmannii, H. pylori* and *H. felis*). It has been concluded that these bacteria, identified in the oral cavity of the cats, may play a role in transmission of infection to humans.

Keywords: stray cat, saliva, dental plaque, Gastric Helicobacter, *Helicobacter heilmannii*, isolation, PCR

Corresponding Author:
Salih Otlu, Department of Microbiology, Faculty of Veterinary Medicine, Kafkas University, 36100, Kars, Turkey.
E-mail address: sotlu@kafkas.edu.tr

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INTRODUCTION

Gastric *Helicobacter* spp. are microaerophilic, non-spore forming, Gram negative, helical bacteria which present in gastrointestinal system of humans and various animals (cat, dog, pig etc.) and can cause severe inflammatory changes in humans (Clyne et al. 1995; Wroblewski et al. 2010). Although the most common type of infection in humans is caused by *H. pylori*, the other Gastric *Helicobacter* species (*H. felis, H. heilmannii, H. bizzozeronii*) may lead to gastric diseases in a similar manner and *H. heilmannii*, which is commonly colonized in the stomach of cat and dogs, has zoonotic attribute (Joosten et al. 2016). It is known that *H. pylori* causes peptic ulcer, gastritis and gastric cancer in humans as well as non-digestive diseases (Ishaq and Nunn 2015). *H. pylori* infection is known to be the most common human infection worldwide with an approximately 50% of the world's populations are infected (Go 2002). The role of these agents in gastrointestinal disease in dogs and cats is uncertain. However, the presence of gastric Helicobacter species was reported in canines with and without clinical complaints (Handt et al. 1994; Jalava et al. 2001). In addition to stomach settlement, the presence of the Gastric Helicobacter in oral cavity, which has oral - oral, fecal - oral and gastric - oral transmission routes, also plays an important role in the infectious cycle. Invasive and non-invasive methods are used for the diagnosis of Gastric Helicobacter (Logan and Walker 2001) and of which culture is known as gold standard. However, the difficulties in growth conditions and being as rare cultivable bacteria (Glupczynski 1998) have led to the researchers to use of alternative diagnostic methods such as PCR which has higher sensitivity than culture based methods (Rahman et al. 2013). In such studies conducted in stomach biopsy, saliva, dental plaque and stool samples, 16S rRNA gene - based PCR for *Helicobacter* spp. (Ghil et al. 2009) and *urease B* gene - based PCR for species identification are used (Neiger et al. 1998).

The aim of this study was to investigate the presence of *H. heilmannii*, *H. felis* and *H. pylori* in oral cavity of stray cats with normal clinical appearance in Kars, Turkey. Saliva and dental plaque samples were used and subjected through culture and PCR methods.

MATERIALS AND METHODS

Animal material and sampling

A hundred stray cats with normal clinical appearance and behaviour were randomly selected from free-ranging area in Kars, Turkey and included to this study. The study was conducted with the approval of the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK - 2014/053). Saliva and dental plaque samples were taken in duplicate from the cats that were caught by a trap and restricted for a while. Saliva samples were collected by swabbing buccal mucosa with sterile cotton swaps and transferred into tubes containing 0.5 ml sterile saline. Dental plaque samples were removed from the tooth surfaces with steril periodontal curette and placed in 0.5 ml sterile saline. The samples were transferred in short time (approximately in 2 hours) to the Microbiology laboratories of the Veterinary Faculty of the Kafkas University, under cold conditions (2-8 ◦C). One copy of samples were subjected to culture processes immediately after the transfer and the others were kept at -20 ◦C till the PCR analysis.

Cultural analysis

For the isolation of Gastric *Helicobacter* species, swab samples were inoculated onto the 5% defibrinated - horse blood chocolate agar and 5% defibrinated - sheep blood agar plates which were supplemented with 2.5 μg/ml Amphotericin B (Sigma A2942), 20 μg/ml Trimethoprim (Sigma T7883), 6 μg/ml Vancomycin (Sigma 1404-93-9) and 2.500 IU/l Polymyxin B (Sigma P4932) (Norris et al. 1999). The plates were incubated at 37°C under microaerobic condition with using a kit (Anaerocult C, Merck 1.16275) for 3 to 7 days. Bacteria were identified as *Helicobacter* spp. by considering the microscopic morphology under light microscope and strong positive urease, catalase, oxidase activities (Bento-Miranda and Figueiredo 2014).

Genus and species-specific PCR analysis

PCR was used for confirmation of the isolates and for direct molecular analysis of the samples (saliva and dental plaque) tested. For this purpose, genus - specific PCR (Ghil et al. 2009) targeting the 16S rRNA gene of *Helicobacter* and species - specific PCR (Neiger et al. 1998) targeting the *urease B* gene of *Helicobacter* species including *H. pylori*, *H. felis* and *H. heilmannii* were applied (Table 1). DNA extraction from the isolates and samples was carried out with a commercially available kit in accordance with the instructions of the manufacturer (QIAamp DNA Mini Kit, 51306). A total of 25 μl PCR reaction was prepared for both PCR with the following contents: 200 mM deoxyribonucleoside triphosphates mixture (Sigma D7295), ×1 PCR buffer (Sigma P2192), 25 pmol of each primer (Biomers, Germany), 0.5 U Taq polymerase (Sigma D6677), and

2.5 μl template DNA. Thermal condition of genus - specific PCR was adjusted as one cycle initial denaturation at 94°C for 2.5 min, 40 cycles amplification consisting of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, extension at 72°C for 1 min and one cycle final extension at 72°C for 15 min. A species - specific PCR analysis with thermal condition was applied as one cycle initial step consisting of denaturation at 94°C for 3 min, at 57°C for 2 min and at 72°C for 3 min, followed by 30 cycles amplification consisting of denaturation at 94°C for 30 sec, primer annealing at 57°C for 30 sec, extension at 72°C for 1 min and a final extension cycle at 72°C for 5 min. PCR reactions were carried out in the presence of positive controls obtained from characterized *Helicobacter* species of Kafkas University, Veterinary Faculty strain collection and negative control using RNase - DNase free water. A horizontal gel electrophoresis technique containing 1.5% agarose was used to visualize the amplified PCR products reported in Table 1.

RESULTS

Culture results

Following the incubation of samples on the aforementioned mediums, the oxidase, catalase and urease positive, Gram - negative helical shaped bacilli with S-type, transparent, white-grey colonies were evaluated as *Helicobacter* spp. From the 100 saliva samples tested, 10 (10%) were positive and from the 100 dental plaque samples tested 5 (5%) were positive for *Helicobacter* spp. culture. Consequently, from the 100 cats tested, 10 (10%) presented positive for *Helicobacter* spp. culture, of which 5 had positive culture from both saliva and dental plaque samples. According to the genus and species-specific PCR of these isolates all were identified as *H. heilmannii* by yielding 400 bp and 580 bp amplified product, respectively (Figs. 1, 2 and Table 2).

Genus and species - specific PCR results

Sequences obtained from PCR products were identified as *Helicobacter* spp. origin. Of the 100 saliva samples 50 (50%) were positive for Helicobacter and of the 100 dental plaque samples, 20 (20%) were positive (Fig. 1). Overall, of the 100 cats, 65 (65%) were positive for *Helicobacter* spp. of which 5 had positive PCR results in both saliva and dental plaque samples. Nineteen (38%) of the 50 PCR positive for Helicobacter saliva samples, and 10 (50%) of the 20 positive dental plaque samples were confirmed to be of *H. heilmannii* origin. Overall, of the 100 cats tested, 29 (29%) were positive for *H. heilmannii* (Fig. 2, Table 2). Neither culture method nor PCR were able to detect other Gastric Helicobacter species, *H. felis* and/or *H. pylori*, in any of the samples.

Table 1: Genus and species - specific primers used for PCR of Helicobacter identification

Primer sequences		Band size (bp)	Gene region
Genus - specific primers			
Helicobacter F	5' - GCT ACG ATC C - 3'	400	16S rRNA
Helicobacter R	5' - GAT TTT ACC CCT ACA - 3'		
Species - specific primers			
H. pylori F	5' - GGA ATT CCA GAT CTA AAA AGA TTA GCA GAA AAG - 3'	1.707	Urease B
H. pylori R	5' - GGA ATT CGT CGA CCT AGA AAA TGC TAA GTT G - 3'		
H. felis F	5' - ATG AAA CTA ACG CCT AAA GAA CTA G - 3'	1.150	
H. felis R	5' - GGA GAG ATA AAG TGA ATA TGC GT - 3'		
H. heilmannii F	5' - GGG CGA TAA AGT GCG CTT G - 3'	580	
H. heilmannii R	5' - CGT GTC AAT GAG AGC AGG - 3'		

Table 2: The overall results of culture and PCR of *Helicobacter* species from samples

Sample	The number of sample	Cultural method		Direct PCR analysis		
		Positive samples	Identified agent†	Genus - specific PCR	Species -specific PCR	Identified agent
Saliva	100	10	*H. heilmannii*	50	19	*H. heilmannii*
Dental plaque	100	5		20	10	
Total	200	15 (10)‡		70 (65)‡	34 (29)‡	

† The isolates were also confirmed by PCRs.
‡ The values in the brackets stand for the number of cats.

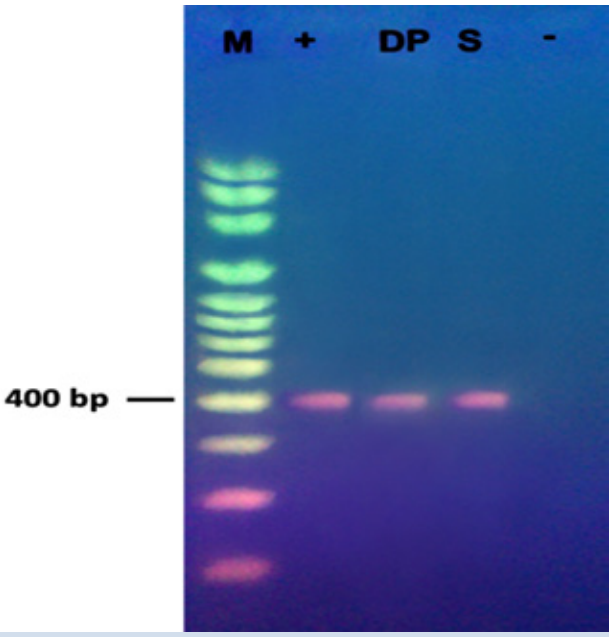

Figure 1. The gel electrophoresis images of genus - specific PCR of *Helicobacter* spp.: (M): Marker, (+): Positive control, (DP): Dental plaque, (S): Saliva, (-): Negative control

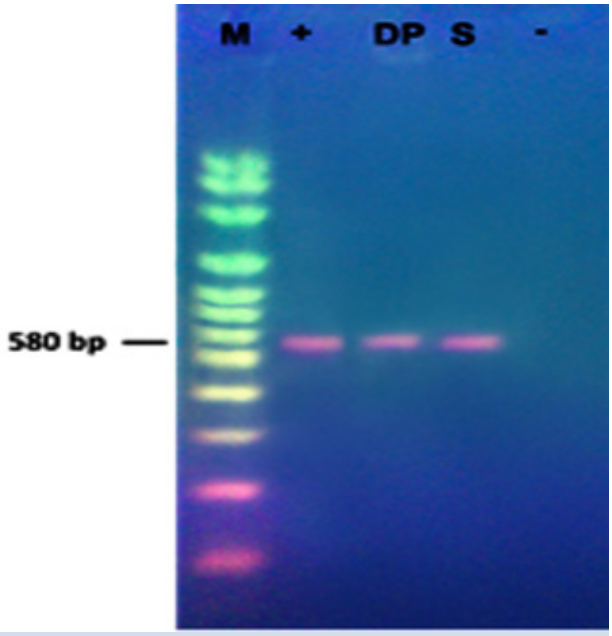

Figure 2. The gel electrophoresis images of species - specific PCR of *Helicobacter* spp.: (M): Marker, (+): Positive control, (DP): Dental plaque, (S): Saliva, (-): Negative control

DISCUSSION

The analysis methods such as rapid urease test (Berry and Sagar 2006) 13C-14C urea breath test (Gomes et al. 2002), histopathological examination (Lee and Kim 2015) and stool antigen test (Gulcan et al. 2005) are widely used in the diagnosis of Gastric Helicobacter as well as the cultural methods in which selective media are utilized. However, *Helicobacter* species are considered fastidious organisms which can be difficult to isolate and propagate (Handt et al. 1994; Yildirim 1997; Cantet et al. 1999; Sagnak and Ozgur, 2011) Optimization of methods, imparting selectivity of the culture media and sampling strategies (sampling region, transportation conditions) are important in increasing the isolation probability of *Helicobacter* species from heavy contaminated samples (Soltezs at al. 1992; Sturegard et al. 1998; Somily and Morshed 2015). In a study, *H. pylori* was able to be cultured from 3.44% of the gastric tissue of cats (Hantd et al. 1994). In a thesis study conducted on various animals (dogs, cats, chickens, rats and guinea pigs) in Turkey, *H. felis* was isolated in 2.6% of 75 dog and 25% of 16 cat stomach samples (Yildirim 1997). In this study, %5 defibrinated-horse blood and %5 defibrinated-sheep blood agar plates supplemented with 2.5μg/mL Amphotericin B, 20μg/mL Trimetoprim, 6μg/mL Vancomycin and 2.500 IU/L Polymixin were used. Helicobacter isolation was achieved in 10 (10%) of the 100 cats (10% of the saliva and 5% of the dental plaque) and these were further identified as *H. heilmannii*. The isolation rate is low when compared to the culture results yielded by the gastric mucosa of canine (Eaton et al. 1996; Jalava et al. 1998). But this can be explained as the antimicrobials participating in the medium cannot provide sufficient selectivity for Helicobacter isolation from oral cavity in which the microbial flora is much richer and more complex than the stomach flora has (Nigam 2015). However, the isolation rate has close similarity to those reported before (Handt et al. 1994; Yildirim 1997). The identified species, *H. heilmannii* is the sole species, is different as a reflection of some predictive factors. Even that *H. heilmannii* could be cultured at low rates suggests that Gastric Helicobacter protect the helical form in the oral cavity which makes it more recognisable unlike the stool samples have. In addition, the ability of Helicobacter to be cultured from the oral cavity of cats supports the views of that these agents, which are likely to have a zoonotic character, can contaminate food and many environmental samples (Neiger and Simpson 2000; van Duynhoven and De Jonge 2001; Guner and Telli 2012), transmit by oral - oral route (Jalava et al. 2001; Boyanova et al. 2007; Meining et al. 2009) and may be the most likely infection source of human beings (Ferguson et al. 1993; Megraud and Routet 2000).

Due to some difficulties encountered in cultural processes of Helicobacter, it is emphasized that PCR

techniques are more advantageous for investigation of the bacteria from materials such as stool and saliva (Dunn et al. 1997). There are many researches on identification of Gastric Helicobacter by PCR, especially from gastric and stomach specimens of cats. Handt et al. (1994) investigated 29 gastric tissues of cats sampled from pet shops and identified *H. pylori* in only one sample by culture and PCR methods.Strauss-Ayalia et al. (2001), reported that *Helicobacter* was detected in 17 of the 45 gastric biopsy specimens taken from a group of sick and healthy cats and identified that 9 of them were *H. heilmannii* and 4 were *H. felis* (*H. heilmannii* and *H. felis* are common in 3 samples), while 7 were not typed. Ghil et al. (2009) investigated the saliva and stool samples of 165 cats in Korea by PCR and obtained Helicobacter positivity in 91.1% of stray cats and in 56.3% of owned cats. However, none of the samples were identified as *H. pylori* or *H. felis*. The most striking examples of the colonization of Gastric Helicobacter in cats have been reported by Dag et al. (2016) and Neiger et al. (1998) in which Gastric Helicobacter-like organisms were detected in 93.3% of the stomach biopsy samples of stray cats by immunohistochemically and PCR and *H. heilmannii* was detected in 78% of the stomach biopsy samples of cats with using PCR, respectively. In this study, *Helicobacter* DNA was detected in 65 (65%) of 100 stray cats. While this ratio shows that cats are *Helicobacter* carriers as that can be seen in many of the above mentioned studies, it supports the idea that the differences in the prevalence values may be related to the determinants such as regional, genetic and age as mentioned by Kuipers (1999). Moreover, the prevalence in our study is affected by the fact that we know nothing about the medical record of these cats.

Several *Helicobacter* species (*H. heilmanii, H. felis*,*H. pylori, H. pametensis* and *H. baculiformis* sp. nov.) are naturally found in cats, while the host preferences of Gastric Helicobacter were different (Handt et al. 1994; Nieger et al. 1998; Cantet et al. 1999; Baele et al. 2008). In contrast to the high prevalence in humans, *H. pylori* and *H. felis* are reported to be very low in cats (Norris et al. 1999; Goh et al. 2011). Similar to this *H. felis* and/or *H. pylori* was not identified from the samples either culture method or PCR method. In many studies, *H. heilmannii* was identified as the dominant species in cats (Neiger et al. 1998; Cantet et al. 1999; Neiger and Simpson 2000). In this study, 19 (38%) of 50 saliva and 10 (50%) of 20 dental plaque samples were detected as *H. heilmannii* with the PCR using the *urease B* gene primers specific for Gastric Helicobacter. This result is similar to other studies (Neiger et al. 1998; Cantet et al. 1999; Neiger and Simpson 2000) and indicating that *H. heilmannii* is the predominant species in cats studied in this region. Unidentified species are thought to be useful for analysis of presence of the other *Helicobacter* species (such as *H. bizzozernii, H. salomonis,* etc.) or unknown new species reported to be present in cats.

CONCLUSIONS

The identification of *H. heilmannii* at certain ratios from saliva and dental plaque of cats suggests that cats may play a role in the transmission of *H. heilmannii* in humans. Furthermore, to investigate the Gastric Helicobacter such as *H. felis, H. pylori* and other possible species will be important to identify the bacterial habitat of oral cavity of cats in terms of Helicobacters.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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