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Original article

Detection of *Helicobacter* spp. in the saliva of dogs with gastritis

M. Jankowski[1], J. Spużak[1], K. Kubiak[1], K. Glińska-Suchocka[1], M. Biernat[2]

[1] Department of Internal Diseases with Clinic of Horses, Dogs and Cats, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Pl. Grunwaldzki 47, 50-366 Wroclaw, Poland
[2] Department of Microbiology, Faculty of Medicine, Wroclaw Medical University, Chałubińskiego 4, 50-368 Wroclaw, Poland

Abstract

The aim of this study was to identify the species and determine the prevalence of gastric *Helicobacter* in the saliva of dogs with gastritis. The study was carried out on 30 dogs of different breeds, genders and ages, which were diagnosed with gastritis. The nested-PCR method was used to detect *Helicobacter* spp. in saliva. *Helicobacter* bacteria were found in the saliva samples of 23 (76.6%) dogs. *Helicobacter heilmannii* was the most commonly detected species of gastric *Helicobacter* spp. in canine saliva, and was found in 22 (73.3%) cases. The results indicate that gastric *Helicobacter* spp. occurs relatively frequently in dogs with gastritis. Moreover, the saliva of dogs with gastritis may be a source of *Helicobacter* spp. infection for humans and other animals. However, further studies are needed to confirm this finding as the PCR method does not distinguish active from inactive infections.

Key words: *Helicobacter* spp., saliva, dog, PCR

Introduction

In 1983, the isolation of spiral bacteria from an inflamed human gastric mucosa by two Australian scientists – J. Marshall and J.R. Warren, was one of the most important events in gastroenterology. These bacteria were later named *Helicobacter pylori* (Kubiak 2006, Bakri 2012). Since then, this microorganism has been the focus of numerous studies, which revealed that *Helicobacter pylori* plays a role in the pathogenesis of chronic active gastritis, peptic and duodenal ulcers, gastric adenocarcinoma and gastric mucosa associated lymphoid tissue (MALT) lymphomas (Agüloğlu et al. 2006, Cellini et al. 2010, Abdel-Raouf et al. 2014, Jankowski et al. 2015).

Following the discovery of *Helicobacter pylori* in humans, several research teams have studied the existence of spiral bacteria in the stomachs of dogs and cats. This led to the isolation of the following *Helicobacter* species: *Helicobacter heilmannii*, *Helicobacter felis*, *Helicobacter salomonis* and *Helicobacter bizzozeronii* (Eaton et al. 1996, Jalava et al. 1997, Neiger et al. 1999, Bulck et al. 2005). However, the role of these species in the pathogenesis of gastric disease in companion animals remains unknown (Diker et al. 2002, Kubiak 2006, Amorim et al. 2015, Jankowski et al. 2015).

It is now widely acknowledged that the *Helicobacter* spp. is widely distributed. The World Health Organization estimates that these bacteria occur in 70%

Correspondence to: M. Jankowski, e-mail: jank1973@tlen.pl

of people in developing countries and 30% of people in developed countries (Downsett and Kowolik 2003, Bulck et al. 2005, Agüloğlu et al. 2006, Chung et al. 2014). A similar occurrence of the microorganism has been reported in companion animals. *Helicobacter* spp. was found in 67-86% of clinically healthy dogs, in 61-100% of animals with chronic vomiting and in 100% of laboratory beagle dogs and dogs from shelters (Henry et al. 1987, Eaton et al. 1996, Hwang et al. 2002, Bulck et al. 2005, Amorim et al. 2015). Despite the high prevalence of the microorganism, its transmission, including human-human, animal-human, human-animal and animal-animal paths, remains unclear (Recordati et al. 2007, Ekman et al. 2013).

Both invasive and non-invasive methods are used for the diagnosis of Helicobacter spp. infections. Invasive diagnostic methods are carried out on gastric mucosa samples obtained during gastroscopy, and include a rapid urease test, direct microscopic examination of Gram-stained samples, microbiological culture, histopathology, electron microscopy and polymerase chain reaction (PCR). Non-invasive methods do not require gastroscopy biopsy samples of the gastric mucosa. Instead, these methods use exhaled air (the urea breath test using C^{13} or C^{14}), saliva (PCR), feces (PCR, serological tests) and blood (serological tests) to determine the presence of *Helicobacter* spp. In animals, invasive methods are used more commonly than non-invasive ones to detect *Helicobacter* spp. (Swora et al. 2009, Urban 2010, Bakri 2012, Sowjanya et al. 2013, Amorim et al. 2015).

The aim of this study was to identify the species and determine the prevalence of gastric *Helicobacter* in the saliva of dogs with *gastritis*.

Materials and Methods

The study was carried out on 30 dogs of different breeds, age and of both genders (17 males and 13 females), from 1 to 15 years old (mean 5.8 ± 4 years). The animals were included in the study based on their clinical symptoms of gastritis (different types of emesis, a decreased or lack of appetite, weight loss, stomach pain), gastroscopy results (macroscopic changes in the gastric mucosa), and the results of the histopathological examination of the mucosa samples obtained during endoscopy (inflammatory lesions assessed according to the Sydney system).

Saliva samples were obtained using sterile oral swabs, which were then placed in sterile tubes and frozen at -20°C for assessment using the PCR method.

Saliva DNA isolation

Saliva DNA was prepared using the Omega Bio-tek, Inc. „Forensic DNA kit" (catalogue no. D3591-01). Swabs were de-frosted, and their tips were cut-off. These tips were then placed in Eppendorf tubes. 200 µl of the STL buffer was then added and the tubes were incubated at 55°C for 15 minutes. After this time, 25 µl of the serine protease (protease OB) was added. Incubation at 60°C was continued for 45 minutes. Following this, 225 µl of the BL lysis buffer (buffer OB) was added to the lysate and the samples were further incubated for 10 minutes at 60°C. 300 µl of isopropanol was then added; the lysate was thoroughly mixed and packed into a previously prepared column according to the manufacturer's instructions. The column was washed with 500 µl of HB buffer and 700 µl of washing buffer. The membrane was dried and the column was saturated with 10 mM of Tris-HCl at pH 8.5 and centrifuged. A change in the pH led to the separation of the cleaned DNA, which could then be used in further stages of the process.

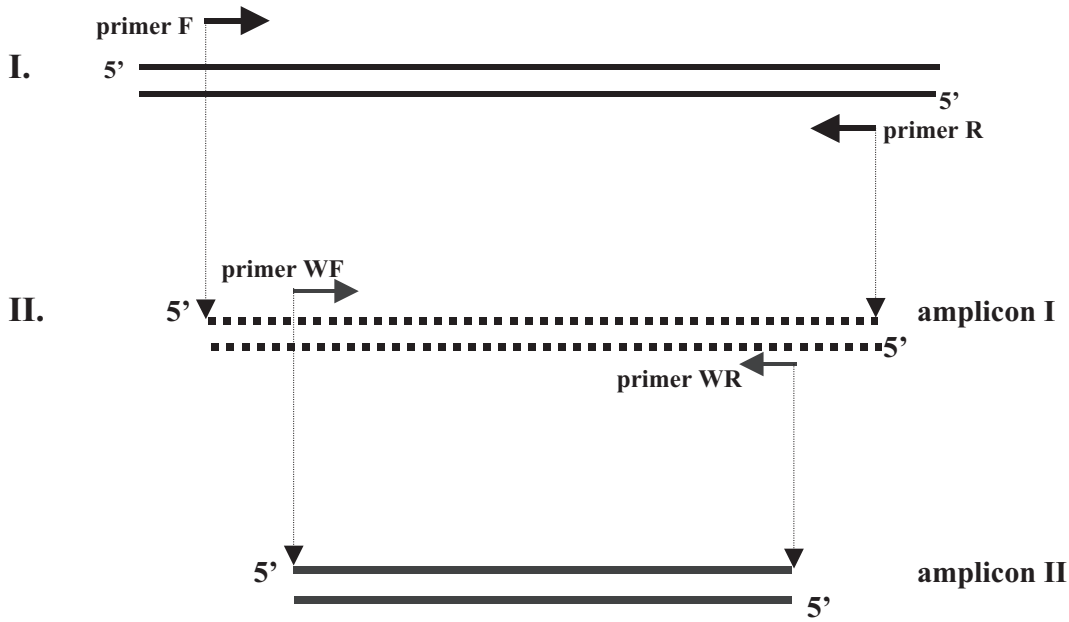
Specimens of the gastric mucosa used for PCR were obtained using biopsy forceps and were placed in storage containers and frozen at -20°C.

Extraction of DNA from gastric mucosa specimens

An Omega Bio-tek, Inc. „Tissue DNA kit" (catalogue no. D3396-01) was used to extract the DNA from the gastric mucosa. The tissue sections were thawed and subjected to protease digestion at 55°C for 3 hours. The cells were then placed in a BL lysis buffer with detergent at 70°C. The cell lysate was then mounted on a silica gel column which selectively bound DNA at a pH lower than 7.5. The column was washed with 500 µl of HB buffer and 700 µl of washing buffer. After the membrane was dried, the column was saturated with 10 mM of Tris-HCl at pH 8.5 and was centrifuged. A pH change led to DNA extraction. DNA could then be used in further stages of the analysis.

Nested-PCR

The nested PCR method is used to detect *Helicobacter* microorganisms and to determine their species. This is a standard PCR method, which involves carrying out two subsequent PCR reactions. In the first reaction, DNA isolated from the analysed sample and an external pair of F (Forward) and R (Reverse)



First step (I.) In the first step, a specific DNA fragment (dotted line) is amplified using **F** and **R** external primers using a DNA template isolated from a given sample (continuous line). In the case of low-intensity infections, amplicon I is not detected in the control electrophoresis.
Second step (II.) In the second step, amplicon **I** produced in the first step (dotted line) is used as a template in the synthesis of the final product (blue line) using the **WF** and **WR** primers. In contrast to **amplicon I**, **amplicon II** is formed in large amounts. It is also easily identified during electrophoresis.

Fig. 1. Basis of nested-PCR method.

Table 1. Summary of the primers and PCR reactions used in the study.

Gene detected	Species detected	Type of PCR reaction	Stage of the PCR process	Primers	Sequence of primer nucleotides in 5'-3' orientation	PCR product (bp)	References
ureA	*H. pylori* M60398	nested	first	PylF PylR	CCA GAT GAT GTG ATG GAT GG TCA AGT CTG TAT CGC CCA ATC	607	Clayton et al. (1992)
			second	HPU1 HPU2	GCCAATGGTAAATTAGTT CTCCTTAATTGTTTTTAC	411	Lu et al. (1999)
ureB	*H. heilmannii* L25079	nested	first	HeilF HeilR	GGGCGATAAAGTGCGCTTG CTGGTCAATGAGAGCAGG	580	Neiger et al. (1998)
			second	WheilF WheilR	GGCATTTACAAAGCCGACAT ACCAAGGTAGCCAAGGTTCA	354	
ureB HSP60	*H. felis* X69080	nested	first	FelisF FelisR	ATGAAACTAACGCCTAAAGAACTAG GGAGAGATAAAGTGAATATGCGT	1150	Neiger et al. (1998)
			second	Fe1F Fe3R	TTT GGT GCT CAC TAA CGC CCT C TTC AAT CTG ATC GCG TAA AG	434	Baele et al. (2004)
	H. bizzozeronii AJ130881	nested	first	BizzF BizzR	GAA GTC GAA CAT GAC TGC AC GGT CGC ATT AGT CCC ATC AG	420	Baele et al. (2004)
			second	Bi1F Bi2R	AAC CAA YAG CCC CAG CAG CC TGG TTT TAA GGT TCC AGC GC	373	Jian et al. (2001)
	H. salomonis AJ558226	nested	first	HSALF HSALR	CATTTTCAAAGAGGGCTTGC GCACACCCCTCAGTTTGTTT	518	Baele et al. (2004)
			second	WSALF WSALR	TGGAGCTAATCCCATTGAGG CTAAGGTTGTGAGGGCTTCG	461	Mikkonen et al. (2004)

Table 2. DNA amplification conditions for various *Helicobacter* species.

Helicobacter pylori		
PCR conditions	First PCR	Second PCR
Initial denaturation	temp.-95°C, time-5 min	temp.-95°C, time-5 min
Appropriate denaturation	temp.-94°C, time-45 s	temp.-94°C, time-45 s
Primer connection	temp.-50°C, time-45 s	temp.-59°C, time-45 s
Appropriate elongation	temp.-72°C, time-3 min	temp.-72°C, time-45s
Number of cycles	24	34
Final elongation	temp.-72°C, time-5 min.	temp.-72°C, time-5 min.
End of reaction	temp.-4°C (until removal of sample from thermocycler)	temp.-4°C (until removal of sample from thermocycler)
Helicobacter heilmannii		
PCR conditions	First PCR	Second PCR
Initial denaturation	temp.-94°C, time-3 min	temp.-95°C, time-5 min
Initial primers connection	temp.-57°C, time-2 min	–
Initial elongation	temp.-72°C, time-3 min	–
Number of cycles	4	–
Appropriate denaturation	temp.-94°C, time-30s	temp.-94°C, time-3 min
Primer connection	temp.-57°C, time-30 s	temp.-58,5°C, time-45 s
Appropriate elongation	temp.-72°C, time-1 min	temp.-72°C, time-1 min
Number of cycles	31	35
Final elongation	temp.-72°C, time-5 min	temp.-72°C, time-5 min
End of reaction	temp.-4°C (until removal of sample from thermocycler)	temp.-4°C (until removal of sample from thermocycler)
Helicobacter felis		
PCR conditions	First PCR	Second PCR
Initial denaturation	temp.-95°C, time-5 min	temp.-95°C, time-5 min
Appropriate denaturation	temp.-94°C, time-1 min	temp.-94°C, time-3 min
Primer connection	temp.-52°C, time-1 min	temp.-57°C, time-45 s
Appropriate elongation	temp.-72°C, time-1 min	temp.-72°C, time-1 min
Number of cycles	28	35
Final elongation	temp.-72°C, time-7 min.	temp.-72°C, time-5 min.
End of reaction	temp.-4°C (until removal of sample from thermocycler)	temp.-4°C (until removal of sample from thermocycler)
Helicobacter bizzozeronii		
PCR conditions	First PCR	Second PCR
Initial denaturation	temp.-95°C, time-5 min	temp.-95°C, time-5 min
Appropriate denaturation	temp.-94°C, time-1 min	temp.-94°C, time-1 min
Primer connection	temp.-57°C, time-1 min	temp.-60°C, time-45 s
Appropriate elongation	temp.-72°C, time-1 min	temp.-72°C, time-1 min
Number of cycles	35	35
Final elongation	temp.-72°C, time-10 min.	temp.-72°C, time-10 min.
End of reaction	temp.-4°C (until removal of sample from thermocycler)	temp.-4°C (until removal of sample from thermocycler)

cont. table 2

Helicobacter salomonis		
PCR conditions	First PCR	Second PCR
Initial denaturation	temp.-95°C, time-5 min	temp.-95°C, time-5 min
Appropriate denaturation	temp.-94°C, time-30 s	temp.-94°C, time-30 s
Primer connection	temp.-55°C, time-30 s	temp.-62°C, time-30 s
Appropriate elongation	temp.-72°C, time-1 min	temp.-72°C, time-1 min
Number of cycles	30	30
Final elongation	temp.-72°C, time-10 min.	temp.-72°C, time-10 min.
End of reaction	temp.-4°C (until removal of sample from thermocycler)	temp.-4°C (until removal of sample from thermocycler)

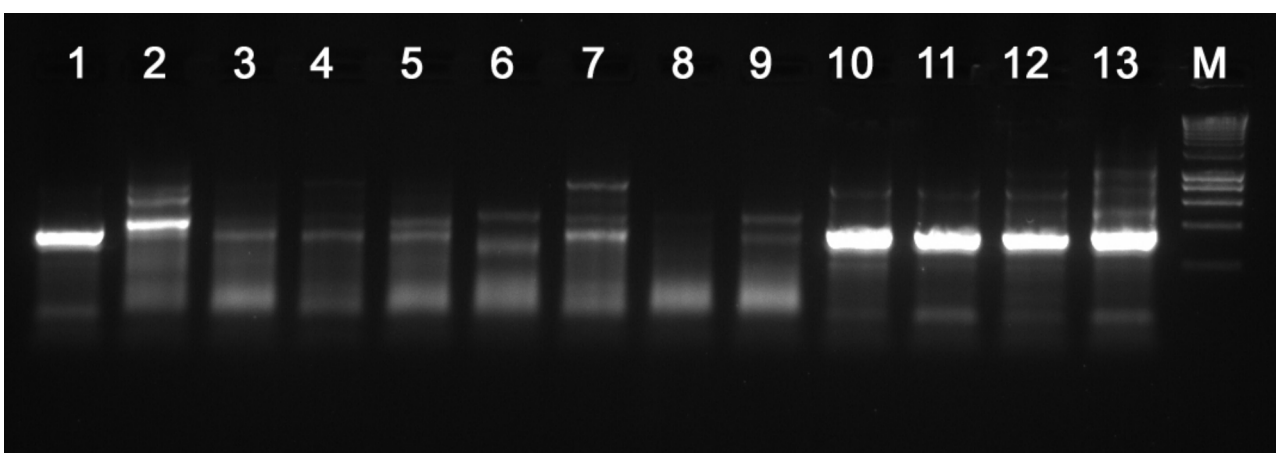


Fig. 2. Gel electrophoresis of nested-PCR products using primers for *ureB* gene from *Helicobacter heilmannii* (primers WheilF and WheilR), specific product (lines 1, 10, 11, 12, 13) is 354 bp.

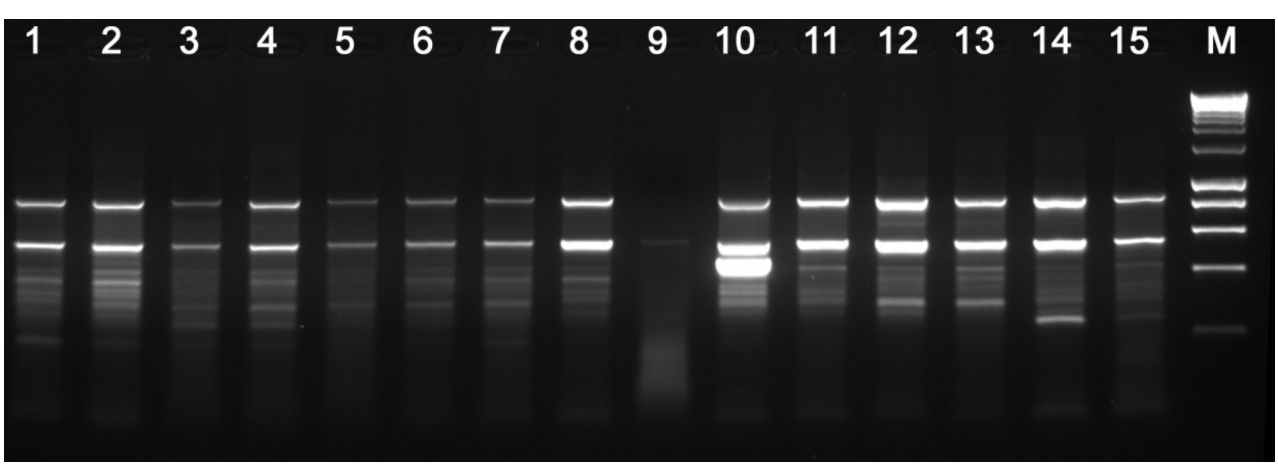


Fig. 3. Gel electrophoresis of nested-PCR products using primers for ureB gene from *Helicobacter salominis* (primers WSALF and WSALR), specific product (lines 10) is 461 bp.

primers is used as the matrix. After adding polymerase and a new pair of WF (Internal Forward) and WR (Internal Reverse) starters, the product of the first reaction is used as the matrix for the second reaction (Fig. 1). This methodology renders the PCR more sensitive and theoretically allows the identification of up to two xenogenic cells in the analysed material. At the same time the use of two pairs of primers ensures the high specificity of this method and makes it possible to reject false positive results (Singh et al. 2008, Hong et al. 2015). The selection of primers and PCR reaction schemes are presented in Table 1.

Thermo Scientific™ DreamTaq DNA Polymerase (catalogue number EP0703) was used to synthetize DNA. *Helicobacter* DNA amplification conditions used in the study are presented in Table 2.

Helicobacter pylor strain ATCC 700392/26695, *Helicobacter felis* strain ATCC 49179, *Helicobacter bizzozeronii* CCUG 35545 and *Helicobacter salomonis* CCUG 37845 DNA were used as a control in the nested-PCR reaction. In the case of *Helicobacter heilmannii,* a strain cultured by the Department of Microbiology of the Faculty of Medicine of the Wroclaw Medical University was used as a positive control.

Results

Based on molecular studies carried out using the nested-PCR method, the presence of *Helicobacter* spp. was found in saliva samples from 23 (76.6%) dogs. This gastric bacterium was not found in the saliva samples of seven (23.4%) dogs. Twenty-one (70.0%) animals were infected with a single species, while nine were infected with two *Helicobacter* species (30.0%). *Helicobacter heilmannii* was the most commonly identified species and was found in 22 (95.7%) cases (Fig. 2). The dogs were also infected with other species, such as Helicobacter *felis* – 1 (4.4%) case, *Helicobacter salomonis* – 4 (17.4%) cases (Fig. 3), *Helicobacter pylori* – 2 (8.7%) cases and *Helicobacter bizzozeronii* – 3 (13.0%) cases. The following combinations were found in animals infected with two *Helicobacter* species: *Helicobacter heilmannii* + *Helicobacter pylori* – 2 (22.2%) cases, *Helicobacter heilmanii* + *Heliconacter salomonis* – 3 (33.3%) cases, *Helicobacter felis* + *Helicobacter salomonis* – 1 (11.2%) cases and *Helicobacter heilmannii* + *Helicobacter bizzozeronii* – 3 (33.3%) cases.

In all dogs, *Helicobacter* spp. DNA was detected in sections of the gastric mucosa more often than in saliva samples. *Helicobacter heilmannii* was the most frequently detected species in saliva samples and gastric mucosa specimens. It was found in 29 (96.7%) of the gastric samples. Other species occurred much less frequently. *Helicobacter felis* was found in 4 (13.3%) cases, *Helicobacter bizzozeronii* was detected in 12 (40%) cases, *Helicobacter salomonis* was found in 11 (36,7%) cases and *Helicobacter pylori* was detected in two cases (6.7%). Nine animals (30.0%) were infected with a single *Helicobacter* species, fifteen (50.0%) animals were infected with two species, five animals (16.7%) were infected with three species and one (3.3%) animal was infected with four species.

Discussion

There are numerous non-invasive methods by which *Helicobacter pylori* infections are detected in humans. These include the detection of IgG in blood and *Helicobacter pylori* antigens as well as DNA in the stool and saliva using the polymerase chain reaction (Swora et al. 2009, Urban 2010, Bakri 2012). In veterinary medicine, there are limited reports concerning the use of non-invasive methods for the detection of gastric *Helicobacter* spp. in dogs and cats. In our study, using the nested-PCR method, we identified the presence of *Helicobacter* in saliva samples of more than 76% of dogs with gastritis. Similar findings have been confirmed by Recordati et al. (2007), who also used the nested-PCR method. They recorded *Helicobacter* spp. in the oral cavity of 71.1% of dogs. On the other hand, Ekman et al. (2013) noted a 100% incidence of *Helicobacter* spp. in canine saliva. The difference in the prevalence of *Helicobacter* spp. in the oral cavity of dogs may be attributed to the fact that dogs in our study, as well as those in the study of Recordati et al. (2007), were kept in various environments and did not have close contact with other dogs. Ekman et al. (2013) used laboratory beagles that were kept together in one kennel, thus facilitating the transmission of the bacteria. This finding has been confirmed by Henry et al. (1987) and Eaton et al. (1996) who recorded the presence of *Helicobacter* spp. in all the animals they studied.

There was a difference in the incidence of *Helicobacter* spp. between saliva and gastric samples in 23.4% of animals. Recordati et al. (2007) obtained similar results, which showed different *Helicobacter* spp. in the saliva and stomach of 23.6% of animals. On the other hand, Ekma et al. (2013) obtained a larger difference in the incidence of gastric *Helicobacter* spp. between saliva and gastric samples, which was found in 35.7% of the studied animals.

In humans, the incidence of *Helicobacter pylori* in the oral cavity ranges from 0% to 100% (Majmudar et al. 1990, Bernander et al. 1993, Agüloğlu et al. 2006, Cellini et al. 2010). Such a discrepancy may be caused by various factors. These include different methods used to detect *Helicobacter pylori*, different socio-economic status of the patients, oral hygiene and environmental factors (Kilmartin 2002, Dowsett and Kowolik 2003, Agüloğlu et al. 2006). There is disagreement among scientists regarding the prevalence of *Helicobacter pylori* in the oral cavity of humans. Some consider the flora to be normal and believe it maintains a symbiotic relationship with the host flora while others believe it enters the oral cavity during a gastroesophageal reflux (Savoldi et al. 1998, Checchi et al. 2000, Song et al. 2000).

To date, 25 species of *Helicobacter* have been detected and have been divided into gastric and enterohepatic species (Diker et al. 2002, Kubiak 2006, Chung et al. 2014). In our study, *Helicobacter heilmannii* was the most commonly detected species (> 70% of cases). In descending order, *Helicobacter salomonis, Helicobacter bizzozeronii*, *Helicobacter pylori* and *Helicobacter felis* were detected much less frequently. Ekman et al. (2013) found that the enterohepatic *Helicobacter canis* occurred most commonly in canine saliva, while *Helicobacter salomonis* (50% cases) was the most commonly found gastric species. *Helicobacter bizzozeronii* (21.4% cases) was also detected. *Helicobacter felis* and *Helicobacter pylori* were not found in their study. The identification of species depends on the geographical area where the study is performed. Studies assessing the prevalence of various species of *Helicobacter* in the canine stomach seem to confirm this finding. The most common species identified in Finland, the United States and Belgium is *Helicobacter bizzozeronii* (Jalava et al. 1997, Priestnall et al. 2004, Bulck et al. 2005). *Helicobacter salomonis* is the most commonly found species in Sweden (Ekman et al. 2013), while *Helicobacter heilmannii* is the most recognized species in Poland, Portugal and South Korea (Hwang et al. 2002, Kubiak 2006, Amorim et al. 2015). *Helicobacter pylori* rarely occur in dogs. Taking this into consideration, Abdel-Raouf et al. (2014) found these bacteria in saliva samples of 45.3% of cases of dogs kept at home and 35.3% stray dogs. The authors did not explain the possible cause of the high incidence of *Helicobacter pylori* in dogs.

The nested-PCR method used to detect gastric *Helicobacter* offers high sensitivity and specificity (Bamford et al. 1998, Neiger et al. 1999, Kubiak 2006, Cellini et al. 2010, Chung et al. 2014). This method also has some limitations, which can hamper the detection of *Helicobacter* spp. The nested-PCR method detects *Helicobacter* spp. DNA, it does not indicate whether the infection is active or not (Farrugia er al. 2010, Sjödin et al. 2011).

The mode of transmission of *Helicobacter* spp. between dogs and cats remains unclear. It is suspected that animals infect one another orally, for example through licking and grooming of pups by female dogs, by eating stool or regurgitated food (Recordati et al. 2007, Ghil et al. 2009, Shojaee Tabrizi et al. 2010). In humans, oral-oral, fecal-oral and gastro-oral routes of transmission are suspected, while the oral cavity is thought to serve as the reservoir of the bacteria (Agüloğlu et al. 2006, Cellini et al. 2010, Abdel-Raouf et al. 2014). Other species of *Helicobacter* may be the cause of gastritis in 0.25 – 4% of humans (Priestnall et al. 2004). Therefore, it is thought that dogs and cats are risk factors of a *Helicobacter* spp. infection in humans, whereby the bacteria are transmitted through licking animals. This theory is supported by our own observations and the results of Recordati et al. (2007) and Ekman et al. (2013), who found *Helicobacter* spp. in numerous saliva samples.

In conclusion, gastric *Helicobacter* spp. occurs relatively frequently in the saliva of dogs with gastritis. The most commonly identified species is *Helicobacter heilmannii*. The results obtained indicate that canine saliva may be a potential source of *Helicobacter* spp. infection for other animals and humans. Moreover, although *Helicobacter pylori* has been detected in few dogs, its reservoirs may be located in the oral cavity of dogs. The exact mode of animal-animal and animal-human transmission requires further study since the PCR method does not distinguish active from inactive infections.

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