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PCR based Detection of *Helicobacter* spp. in Saliva, Dental plaque, Vomitus and Feces of Dogs

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Abstracts : This study was aimed to determine the prevalence of *Helicobacter* spp. in privately owned pet dog's oral cavity samples (saliva, dental plaque, vomitus) and fecal samples in Korea and to evaluate the potential route for transmission. Total 100 patients dogs attending one Veterinary Medical Teaching Hospital were examined by *Helicobacter* genus-specific PCR assay and these dogs were divided into two groups whether they had gastrointestinal signs (vomiting, nausea and diarrhea) or not. The total detection rate of *Helicobacter* spp. by PCR in saliva, dental plaque and fecal samples was 23%, 1% and 68% respectively. The difference of prevalence with regarding the gastrointestinal sings was not significant. In vomitus, two of seven samples had positive results. These results suggested that *Helicobacter* spp. are present in the oral cavity although they were present in very low number and are not like to be normal oral flora of the oral cavity and *Helicobacter* spp. in dogs could be transmitted through oral-oral, gastro-oral and fecal-oral route.

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

Introduction

Helicobacter species are microaerophilic, spiral-shaped or curved motile gram negative bacterium that colonized stomach. They mainly inhabit mucus, glands and parietal cells of stomach and, although they are not invasive they have been reported as causative agents of inflammation of the gastric mucosa linked to gastritis and duodenal ulcer, gastric carcinoma and lymphoma (22,29).

Since *Helicobacter pylori* isolated from human gastric tissue by Warren and Marshall in 1983 these gastric spiral organisms have been reported in various animals including pigs with gastric ulcer (*Helicobacter heilmannii*), cheetahs with severe gastritis (*Helicobacter acinonychis*), ferrets with gastritis and peptic ulcer (*Helicobacter mustelae*), monkeys (*Helicobacter nemestrinae*), rodents (*Helicobacter muridarum*), domestic dogs and cats (3,6,8,13,18,20,23).

In human *Helicobacter pylori* represents the second most common bacterial infection throughout the world, involving 40% of the population in developed countries and up to 80% of the population in the developing ones (21) but knowledge about the transmission of the bacterium is still incomplete.

In dogs and cats various gastric spiral organisms have been reported since initial isolation of *Helicobacter pylori* but their presence has been ignored and understood as gastric commensals (9,31). However infection of *Helicobacter* spp. is highly prevalent in dogs and cats and the relationship between *Helicobacter* spp. and gastrointestinal diseases in dogs and cats have renewed attention. The prevalence of *Helicobacter* spp. in dogs has been reported to be between 61 to 100% and in cats, the prevalence of *Helicobacter* spp. has been similar to that of the dogs between 41 to 100% (10,12,14,16,18,19,32).

As above mentioned, despite the high prevalence of *Helicobacter* spp. in dogs and cats, the relationships between these bacteria and clinical manifestations have not been clearly understood with gastritis accompanying infection in some but not all infected dogs and cats (7,14,16,32) and their exact route of transmission is also unknown like *Helicobacter pylori*. Several reports of *Helicobacter* spp. infection in human have lead to speculation that animals, especially dogs and cats may serve as a source for human infection (5,15,26,28) and some studies have determined the prevalence of *Helicobacter* species in domestic pets.

In human *Helicobacter* spp. are known to be acquired in childhood, although exact transmission route is not understood, and these bacteria are also known to be existed in dental plaque, saliva and feces. So the speculation that the route of infection could be oral-oral or fecal-oral, gastro-oral route and dental plaque and saliva might harbor *Helicobacter*

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pylori and therefore might be a source of re-infection of gastric mucosa have been assumed (2,4,27). Current studies in human indicate that *Helicobacter pylori* is present in oral cavity although the number of organisms in individual samples is very low, and these numbers appear to vary from one site to another within the mouth (1,11,24,25,30).

The aim of the present study was to determine the prevalence of *Helicobacter* spp. in oral cavity (the dental plaque, saliva) and feces of privately owned pet dogs in Korea and to evaluate the potential route for transmission.

Materials and Methods

Sample collection

For evaluating the infection state of *Helicobacter spp*. in oral cavity in privately owned pet dogs, 100 patient dogs (58 males, 42 females) attending the Veterinary Medical Teaching Hospital of Seoul National University was chosen randomly with owner's acceptance. Each of the dogs had various diseases but was simply divided into two groups. One was a group that had no gastrointestinal signs and other was a group that had gastrointestinal signs (nausea, vomiting and diarrhea) for last one month. Three samples (feces, saliva and dental plaque) were taken by swabbing of rectum and oral cavity from each dog. Fecal samples were taken by swabbing of rectum with a sterile cotton swab and saliva samples were collected with sterile cotton swab rotated around the inside of the oral cavity. Pooled supragingival dental plaques were obtained with a sterile scaler passed around the tooth margins in two quadrants of the mouth. Each of three samples was submerged in a 500 μ*l* of phosphate buffered sterile saline for DNA isolation.

DNA isolation

Each samples submerged in phosphate buffered sterile saline were added with phenol-chloroform-isoamylalcohol (volume ratio 25: 24: 1). These samples were vortexed and followed by centrifuging to 12,000 rpm for 5 minutes.

PCR assay

Helicobacter genus-specific PCR assay was performed with C97 (5' -GCTATGACGGGTATCC- 3') and C98 primers (5' –GATTTTACCCCTACACCA- 3') which amplify the 16S rRNA gene of *Helicobacter* species. The sensitivity and specificity of this assay was previously evaluated (17). Isolated DNA samples (3 μ*l*) were added to reaction mixture containing 400 μM dNTPs, 1X PCR buffer, 2.5U of *Taq* DNA polymerase(AB gene), 0.6 μM of each primer, and distilled water in total volume of 50 μ*l*. PCR samples were heated to 94 °C for 2.5min once, followed by 40 cycles of denaturation at 94 °C for 1min, primer annealing at 50 °C for 1min, and extension at 72 °C for 1min, with a final extension at 72 °C for 15min in Biometra personal thermocycler. PCR products were subjected to electrophoresis on a 2.0 % agarose gel containing 0.5 μg of ethidium bromide per m*l* and visualized over UV light.

Cloning and nucleotide sequence analysis.

To confirm the identity of the *Helicobacter* genus-specific PCR assay products with target genes, the PCR products were extracted by commercial gel extraction kit (Gel Extraction Kit-spin, NucleoGen, Ansan, Korea). The DNA fragment was ligated into PCR2.1-TOPOvector®, and transformed the recombinant vector into TOP10 *Escherichia coli* (E. coli; Invitrogen, Carlsbad, CA, U.S.A.). Transformed TOP10 cells were plated onto Luria-Bertani (LB) agar plates containing ampicillin (50 μg/m*l*) and incubated overnight at 37 °C. Picked 5 colonies were cultured overnight in LB broth medium containing 50 μg/m*l* ampicillin. Plasmid DNAs were extracted with a GENOMED plasmid kit (Genomed, Wielandstr, Germany). Both strands of plasmid inserts were sequenced using the dideoxy chain termination method with a Dye Terminator AmpliTaq kit (Applied Biosystems, Foster city, CA, U.S.A.) After amplification reaction, excess dye terminators were removed with G-50 column (Pharmacia Biotech, Uppsala, Sweden) then analyzed the nucleotide sequence of plasmid inserts with ABI auto sequencing analyzer (Applied Biosystems, Foster city, CA, U.S.A.).

The similarity of nucleotide sequences of PCR products with obtained by sequencing analyzer was calculated by BLAST program (http ://www.ncbi.nlm.nih.gov/BLAST, NCBI's sequence similarity search tool,).

Results

There was greater than 96 % identity between the sequence of *Helicobacter* genus-specific PCR products in the saliva (97 %) and dental plaque (96 %) and the GeneBank sequences of 16s rRNA gene of *Helicobacter* spp. (Accession No. U51874).

Helicobacter genus-specific PCR assay of feces, saliva and dental plaque in dogs showed that positive rates of the feces, saliva and dental plaque of dogs with gastrointestinal signs was not significantly higher than that of same site samples of dogs that showed no gastrointestinal signs. Total positive rate of the each sample was 68 % (feces), 23 % (saliva) and 1 % (dental plaque) (Table 1, Fig 1).

Two of seven vomitus samples collected from seven dogs having vomiting sign and positive result on their feces were also positive.

The proportion of patient dogs carrying *Helicobacter* spp. in at least one sampling site was 75 % (75 of 100).

In comparison of the positive results among the kind of samples, only 17 saliva samples from 68 feces positive dogs showed positive results. In addition, 6 saliva samples from feces negative dogs also showed positive results in the dental plaque samples; only one sample was positive and the dog showed negative results in his feces sample analysis (Table 2).

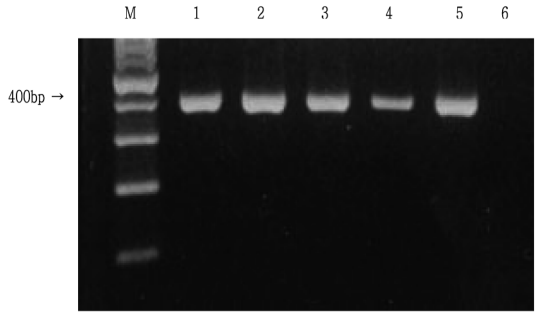

Fig 1. Detection of *Helicobacter* spp. in saliva, feces, dental plaque and vomitus of dogs by *Helicobacter* genus-specific PCR assay. Lanes: M, DNA ladder; 1, DNA from *H. felis* (ATCC 51211) as a positive control; 2~5, positive result in saliva, feces, dental plaque and vomitus respectively; 6, negative control, normal saline.

Table 1. *Helicobacter* genus-specific PCR assay results of dogs

Group (No. of dogs)	No. of positive (%)		
	Saliva	Dental plaque	Feces
None of GI signs (34)	8 (24)	0 (0)	23 (68)
GI signs (66)	15 (23)	1 (1.5)	45 (68)
Total (100)	23 (23)	1 (1)	68 (68)

Table 3. shows the relationship between *Helicobacter* spp. and the age of presentation. In saliva samples, 15 (65 %) of the 23 positive dogs were located in the 4-9 years age group. In fecal samples, prevalence of *Helicobacter* spp. of dogs was similar based on the age of the days.

Discussion

Helicobacter spp. represents the second most common bacterial infection throughout the world in human, involving 40% of the population in developed countries and up to 80% of the population in the developing ones. However exact routes of transmission have not been known. It has long been speculated that *Helicobacter* spp. may belong to the normal oral flora, maintaining a commensal relation with the host or *Helicobacter* spp. is not consistently present in oral cavity and when present, may be the result of occasional gastroesophageal reflux, so the route of infection could be oral-oral or fecal-oral, gastro-oral. In human, current studies indicate that *Helicobacter pylori* is present in oral cavity and although the number of organisms in individual samples is very low, oral cavity may harbor *Helicobacter pylori* and therefore be a source of re-infection or transmission of the gastric mucosa (4).

In human studies, it has been known that detection rates of *Helicobacter* spp. in oral cavity samples varies from 6.9 % to 68 % of cases. Probably it was thought that these differences of results were due to differences of techniques used in detection of *Helicobacter* spp. and difference of researched sample number. It has been known that *Helicobacter* genus-specific PCR assay is very useful method for diagnosis of *Helicobacter* infection because of higher sensitivity and specificity than other methods and lesser invasiveness. In our previous study using same PCR assay methods for developing non-invasive diagnostic test for *Helicobacter* infections in dogs showed that this *Helicobacter* genus-specific PCR assay had very high sensitivity (96 %) and specificity (100 %) (17).

Table 2. Deference of *Helicobacter* genus-specific PCR assay results among the sample groups

Sample	No. of Dogs	
	Feces (+)	Feces (-)
Saliva (+)	17	6
Saliva (-)	51	26
Dental plaque (+)	0	1

Table 3. *Helicobacter* spp. detection rate in age range by PCR assay

Age range (No. of dogs)	No. of PCR positive (%)		
	Saliva	Dental plaque	Feces
< 1 years (3)	2 (67)	0	2 (67)
1 – 3 years (15)	2 (13)	0	11 (73)
4 – 6 years (24)	8 (33)	0	18 (75)
7 – 9 years (32)	7 (22)	1 (3)	23 (72)
> 10 years (26)	4 (15)	0	14 (53)
Total (100)	23	1	68

In the present study, detection rates of *Helicobacter* spp. in oral cavity samples of dogs using *Helicobacter* genus-specific PCR assay was very low and especially in the dental plaque, only one sample of one hundred samples showed positive result. The difference of prevalence with regarding the gastrointestinal sings was not significant. Moreover present study showed that 18.7 % (6/32) of the dogs that showed negative result in feces samples had *Helicobacter* spp. in the saliva samples and 1.5 % (1/32) had in dental plaque sample. These percentages were not significantly different from feces positive group (saliva: 25 %, dental plaque: 0 %). However, these results suggest that the dogs without clinical gastrointestinal signs may have *Helicobacter* spp. in their oral cavity and also the dogs that don't have *Helicobacter* spp. in their stomach and intestine may have this bacterium in oral cavity. The finding that two of seven vomitus samples collected from seven dogs having vomiting sign and positive result on their feces were also positive suggest that gastroesophageal refulx is not always related to *Helicobacter* spp. present in oral cavity. More than anything else this study showed that *Helicobacter* spp. might be present in dog's oral cavity and so putting these results together, dog's oral cavity may be a source of transmission and re-infection.

Detection rate of *Helicobacter* spp. infection in dogs less than one year of age determined by fecal PCR assay was similar to the infection fate of older dogs and the tendency of increasing the infection rate with increasing age observed in *H. pylori* infection in humans was not detected. The positive rate in saliva samples from dogs less than one year old was higher than those of older dog groups but the number of samples evaluated was too small and further study is needed to evaluate it's significant difference.

In the above mentioned fact, detection rates of *Helicobacter* spp. in dog's oral cavity were low and these results suggest that *Helicobacter* spp. may not have commensal relationship with host dog in oral cavity but is occasionally present in oral cavity. Therefore transmission routes could be oral-oral or gastro-oral, fecal-oral and oral hygiene of domestic dogs may be the important tools that inhibit the infection or re-infection of *Helicobacter* spp.

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