

Original Research Article

VacA, CagA, IceA and OipA Genotype Status of Helicobacter pylori Isolated from Biopsy Samples from Iranian Dogs

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Abstract

Purpose: To investigate the state of *vacA*, *cagA*, *oipA* and *iceA* genotypes of *H. pylori* isolated from gastric biopsy samples of dogs.

Methods: A total of 240 gastric biopsy samples were taken from 240 dogs using gastric endoscope. All the samples were cultured and *H. pylori*-positive samples were analyzed for the presence of various genotypes using polymerase chain reaction (PCR).

Results: Thirteen out of 240 (5.41 %) samples were positive for *H. pylori* with a higher prevalence of the bacterium in dogs with gastric ulcer (6.36 %). The most commonly detected genotypes were *vacA* s1a (53.84 %), *cagA* (46.15 %) and *vacA* m2 (38.46 %). Total prevalence of *iceA*1, *iceA*2 and *oipA* genotypes were 23.07, 10.8 and 10.81 %, respectively. M2s1a (30.76 %), m1am2 (30.76 %) and m1as1a (23.07 %) had the highest prevalence among other detected combined genotypes. Twenty-two different genotypic combinations were detected with higher prevalence of s1a/cagA+/iceA1/oipA- (23.07 %), m2/cagA+/iceA1/oipA- (23.07 %), and s1b/cagA+/iceA1/oipA- (15.38 %) genotypes.

Conclusion: The presence of similar genotypes in *H. pylori* strains of gastric biopsy samples of dogs and those of human clinical specimens suggest that dogs may be the source of the bacterium. Proper handwashing and avoidance contact with feces and saliva of dogs are recommended to prevent transmission of *H. pylori*.

Keywords: *Helicobacter pylori*, Genotype, Genotyping, Gastric biopsy, Iranian dogs

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INTRODUCTION

Throughout the years, dogs had been the most useful animal companion of humans. Dogs particularly can ease loneliness, reduce allergies, anxiety, depression, stress, and a variety of human diseases, and promote social interaction, encourage exercise and playfulness, and provide unconditional love and affection. In addition, dogs are good hunters, skilled guards, effective narcotic detectors and sharp probes. Company of dogs can increase the level of human

immunity against many kinds of zoonotic diseases but there are a lot of diseases that are transmitted from dogs to humans [1,2].

Helicobacter pylori (*H. pylori*) are gram negative, spiral shaped bacterium, with its main reservoir being humans, particularly the human stomach. The bacterium colonizes most of the population, making it one of the most controversial bacteria in the world that causes peptic ulcer, gastritis, lymphoma, duodenal ulcer, and gastric cancer [3]. Based on the documented data, dogs may

play an important role in transmission of *H. pylori* to humans [4-6]. Gastric *Helicobacter* infection is common in dogs, with reported high prevalence rates [4-7]. A small percentage (approximately 0.25 – 1.7 %) of human patients suffering from gastric disorders have been diagnosed with gastric *Helicobacter* spp. that are distinct from *H. pylori* and morphologically similar to the large spiral-shaped *Helicobacter* spp. of pet dogs [8]. Contact with dogs has been determined to be a risk factor for human *Helicobacter* infection [4-8]. To appraise the pathogenicity of *H. pylori*, evaluation of latent virulence factors and genotypes is essential. The most common but important virulence factors among *H. pylori* strains of different clinical outcomes of human and animal beings are vacuolating cytotoxin (*vacA*), induced by contact with the epithelium antigen (*iceA*), cytotoxin associated gene (*cag*) and outer inflammatory protein (*oip*) [9,10]. These genes are usually induced adhesion and invasion to the gastric epithelial cells [11,12]. The *vacA* belongs to the group of genes with variable genotypes or structures. This gene is associated with injury to epithelial cells. The *vacA* gene is polymorphic, comprising variable signal regions (type s1 or s2) and mid-regions (type m1 or m2). The s1 type is further subtyped into s1a, s1b and s1c subtypes and the m1 into m1a and m1b subtypes. The mosaic combination of s and m-region allelic types determines the particular cytotoxin and consequently, the pathogenicity of the bacterium [11,13]. The *iceA* gene has two main allelic variants *iceA1* and *iceA2* but their functions are not yet clear. Cag pathogenicity island (PAI) has been shown to be involved in persuading inflammation, ulceration and carcinogenesis [12]. The *cagA* gene has been detected in the specimens taken from severe cases of peptic ulcer [11,12]. The *oipA* gene plays a significant role in effective colonization of mucosa [11,12]. Genotyping using these virulence markers is considered as one of the best approaches for study of correlations between *H. pylori* isolates from different samples. Data on the epidemiology and transmission of *H. pylori* is extremely significant in order to prevent its distribution and to identify high-risk populations, especially in areas that have high rates of gastritis, peptic ulcers and gastric cancer such as Iran [9,10,13]. Considering the unclear epidemiological aspects of *H. pylori* in pets and according to the high prevalence of *H. pylori* all-around the world, the present investigation was carried out in order to study the exact status of *vacA*, *cagA*, *iceA* and *oipA* genotypes of *H. pylori* isolated from gastric biopsy samples of healthy dogs and those with gastric ulcer.

EXPERIMENTAL

Collection of gastric biopsy samples

From July to September 2014, a total of 240 biopsy samples were collected from the pyloric part of the stomach of dogs of various breeds (male and female, age 7 months to 11 years) that were referred for autopsy at the Department of Small Animal Internal Medicine, College of Veterinary Medicine, Islamic Azad University, Shahrekord, Iran. Samples were collected randomly from healthy dogs (n = 130) and those that had gastric ulcer (n = 110). The dogs with gastric ulcer had a history of gastritis, chronic vomiting and weight loss. Presence of gastric ulcer was diagnosed using the gastric endoscope. Samples were collected in a sterile manner, with care taken to avoid cross-contamination between different sites. Multiple gastric biopsies were taken from each dog. After each sampling, the endoscope and the biopsy forceps were thoroughly cleaned, then sterilized using an activated aldehyde solution. Samples were transported to the laboratory at 4 °C. All samples were kept under refrigeration in plastic bags.

Isolation of *Helicobacter pylori*

Twenty five milliliters of each homogenized sample were added to 225 mL of Wilkins Chalgren anaerobe broth (Oxoid, UK) supplemented with 5 % of horse serum (Sigma, St. Louis, MO, USA) and colistin methanesulfonate (30 mg/L), cycloheximide (100 mg/L), nalidixic acid (30 mg/L), trimethoprim (30 mg/L), and vancomycin (10 mg/L) (Sigma, St. Louis, MO, USA) and incubated for 7 days at 37 °C with shaking under microaerophilic conditions. Then, 0.1 mL of the enrichment selective broth was plated onto Wilkins Chalgren anaerobe agar (Oxoid, UK) supplemented with 5 % of defibrinated horse blood and 30 mg/L colistin methanesulfonate, 100 mg/L cycloheximide, 30 mg/L nalidixic acid, 30 mg/L trimethoprim, and 10 mg/L vancomycin (Sigma, St. Louis, MO, USA) and incubated for 7 days at 37 °C under microaerophilic conditions. For comparison, a reference strain of *H. pylori* (ATCC 43504) was employed.

DNA extraction and *Helicobacter pylori* 16S rRNA gene amplification

Suspected colonies were identified as *H. pylori* based on the PCR technique. Genomic DNA was extracted from colonies with typical characters of *H. pylori* using a DNA extraction kit for cells and

tissues (Roche Applied Science, Germany, 11814770001) according to the manufacturer's instructions and its density was assessed by optic densitometry. Extracted DNA was amplified for 16S rRNA gene (primers: HP-F: 5'-CTGGAGAGACTAAGCCCTCC-3' and HP-R: 5'-ATTACTGACGCTGATTGTGC-3') [14]. PCR reactions were performed in a final volume of 50 μ L containing 5 μ L 10 \times buffer + $MgCl_2$, 2 mM dNTP, 2 unit Taq DNA polymerase, 100 ng genomic DNA as a template, and 25 picomole of each primer. PCR was performed using a thermal cycler (Eppendorf Co., Germany) under the following conditions: an initial denaturation for 2 min at 94 $^{\circ}C$; 30 cycles of 95 $^{\circ}C$ for 30 s, 60 $^{\circ}C$ for 30 s, and 72 $^{\circ}C$ for 30 s and a final extension at 72 $^{\circ}C$ for 8 min.

Genotyping of *vacA*, *cagA*, *iceA* and *oipA* genes of *Helicobacter pylori*

The presence of the *oipA*, *cagA* and the genotypes of *vacA* (*s1a*, *s1b*, *s1c*, *m1a*, *m1b* and *m2*) and *iceA* (*iceA1* and *iceA2*) alleles were determined using PCR technique. List of primers is shown in Table 1 [12,15-20].

PCR was performed on 50 μ L sample containing 1 μ M of each primer, 1 μ L of genomic DNA (approximately 200 ng), 1 mM of dNTPs mix (invitrogen), 2 mM of $MgCl_2$, and 0.05 U/ μ L Taq DNA polymerase (invitrogen). PCR amplifications were performed in an automated thermal cycler

(Biometra Co., Germany). The following cycle conditions were used for PCR amplification: for *vacA*: 32 cycles of 45 s at 95 $^{\circ}C$, 50 s at 64 $^{\circ}C$, and 70 s at 72 $^{\circ}C$; for *cagA*: 1 min at 94 $^{\circ}C$, 1 min at 56 $^{\circ}C$, and 1 min at 72 $^{\circ}C$; for *iceA*: 1 min at 94 $^{\circ}C$, 1 min at 56 $^{\circ}C$, and 1 min at 72 $^{\circ}C$ and finally, for *oipA*: 1 min at 94 $^{\circ}C$, 1 min at 56 $^{\circ}C$ and 1 min at 72 $^{\circ}C$. All runs included one negative DNA control consisting of PCR grade water and two or more positive controls (26695, J99, SS1, Tx30, 88-23 and 84-183).

Gel electrophoresis and sequencing

PCR amplification products (10 μ L each) were subjected to electrophoresis in a 1.5 % agarose gel in 1X TBE buffer at 80 V for 30 min, stained with ethidium bromide, and images were obtained in a UVIdoc gel documentation systems (UK). The PCR products were identified by 100 bp DNA size marker (Fermentas, Germany). In order to confirm the PCR results, the sequencing method was used. For this reason, PCR products of some positive samples were purified with high pure PCR product purification kit (Roche Applied Science, Germany) according to manufacturer's recommendations. Single DNA strands were sequenced with ABI 3730 XL device and Sanger sequencing method (Macrogen, Korea). The result of the sequence of each gene was aligned with the gene sequences recorded in the GenBank database located at NCBI.

Table 1: Oligonucleotide primers used for genotyping of *Helicobacter pylori* strains isolated from gastric biopsies of dogs

Genes name	Primer Sequence (5'-3')	Size of product (bp)
<i>vacA s1a</i>	F: CTCTCGCTTTAGTAGGAGC R: CTGCTTGAATGCGCCAAAC	213
<i>vacA s1b</i>	F: AGCGCCATACCGCAAGAG CTGCTTGAATGCGCCAAAC	187
<i>vacA s1c</i>	F: CTCTCGCTTTAGTGGGGYT R: CTGCTTGAATGCGCCAAAC	213
<i>vacA s2</i>	F: GCTAACACGCCAAATGATCC R: CTGCTTGAATGCGCCAAAC	199
<i>vacA m1A</i>	F: GGTCAAAATGCGGTCATGG R: CCATTGGTACCTGTAGAAAC	290
<i>vacA m1B</i>	F: GGCCCCAATGCAGTCATGGA R: GCTGTAGTGCCTAAAGAAGCAT	291
<i>vacA m2</i>	F: GGAGCCCCAGGAAACATTG R: CATAACTAGCGCCTTGCA	352
<i>cag A</i>	F: GATAACAGCCAAGCTTTTGAGG R: CTGCAAAAGATTGTTTGGCAGA	300
<i>iceA1</i>	F: GTGTTTTTAACCAAAGTATC R: CTATAGCCASTYTCTTTGCA	247
<i>iceA2</i>	F: GTTGGGTATATCACAATTTAT R: TTRCCCTATTTTCTAGTAGGT	229/334
<i>oip A</i>	F: GTTTTTGATGCATGGGATTT R: GTGCATCTCTTATGGCTTT	401

Ethical consideration

This work was approved by the ethical committees of Department of Small Animal Internal Medicine, College of Veterinary Medicine, Azad University, Shahrekord, Iran and Research Adjunctancy of the Islamic Azad University of Shahrekord (Ref number 1102371AU). Written informed consent was obtained from the owners of the examined dogs. All stages of sampling was done in such a way as to minimize pain and harm to the dogs.

Statistical analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), Chi-square and Fisher's exact tests were performed, and differences were considered significant at $p < 0.05$. Distribution of *H. pylori* genotypes isolated from food stuff was also statistically analyzed.

RESULTS

All of the gastric biopsy samples were examined using the culture method. The results of the culture method was confirmed using the PCR technique. Table 2 shows the total distribution of *H. pylori* in the gastric biopsy samples of dogs. Of 240 gastric biopsy samples, 13 (5.41 %) were positive for *H. pylori*. Higher prevalence was seen in dogs with gastric ulcer (6.36 %). There were statistically significant differences amongst the incidence of bacteria and type of samples ($p = 0.047$).

Distribution of *vacA*, *cagA*, *iceA* and *oipA* genotypes of the *H. pylori* strains of gastric biopsy samples is shown in table 3. We found that the most commonly detected genotypes were *vacA* s1a (53.84 %), *cagA* (46.15 %) and *vacA* m2 (38.46 %). Total prevalence of *iceA*1, *iceA*2 and *oipA* genotypes were 23.07 %, 10.81 % and 10.81 %, respectively. Significant differences were found between the incidence of s1a and s1b ($p = 0.038$) and also between *iceA*1 and *iceA*2 ($p = 0.025$), m2 and m1b ($p = 0.029$), s1a and *oipA* ($p = 0.022$) and m2 and *iceA*2 ($p = 0.031$).

Distribution of combined genotypes of *H. pylori* strains isolated from gastric biopsies of dogs is shown in table 4. The most commonly detected combined genotypes were m2s1a (30.76 %), m1am2 (30.76 %) and m1as1a (23.07 %). Significant differences were found between the incidence of m2s1a and m1bs1b ($p = 0.020$), m2s1a and m1as2 ($p = 0.033$) and m1am2 and m1as2 ($p = 0.031$).

Twenty two different genotypic combinations were detected in the *H. pylori* strains of gastric biopsy samples (Table 5). The most commonly detected combined genotypes were s1a/cagA+/iceA1/oipA- (23.07 %), m2/cagA+/iceA1/oipA- (23.07 %), s1b/cagA+/iceA1/oipA- (15.38 %), s2/cagA+/iceA1/oipA- (15.38 %), m1a/cagA+/iceA1/oipA- (15.38 %) and m2/cagA-/iceA1/oipA- (15.38 %).

Table 2: Distribution of *Helicobacter pylori* in gastric biopsies of dogs

Types of gastric samples	No. of samples collected	Positive results for <i>H. pylori</i> (%)
Biopsies from healthy dogs	130	6 (4.61)
Biopsies from dogs with gastric ulcer	110	7 (6.36)
Total	240	13 (5.41)

Table 3: Distribution of various genotypes in *Helicobacter pylori* strains of gastric biopsies of dogs

Types of gastric samples (No. positive results)	Distribution of various genotypes (%)										
	<i>VacA</i> s1a	<i>VacA</i> s1b	<i>VacA</i> s1c	<i>VacA</i> s2	<i>VacA</i> m1a	<i>VacA</i> m1b	<i>VacA</i> m2	<i>CagA</i>	<i>IceA</i> 1	<i>IceA</i> 2	<i>OipA</i>
Biopsies from healthy dogs (6)	2 (33.33)	1 (16.66)	-	1 (16.66)	1 (16.66)	-	1 (16.66)	1 (16.66)	1 (16.66)	-	-
Biopsies from dogs with gastric ulcer (7)	5 (71.42)	2 (28.57)	-	2 (28.57)	3 (42.85)	1 (14.28)	4 (57.14)	5 (71.42)	2 (28.57)	1 (14.28)	1 (14.28)
Total (13)	7 (53.84)	3 (23.07)	-	3 (23.07)	4 (30.76)	1 (10.81)	5 (38.46)	6 (46.15)	3 (23.07)	1 (10.81)	1 (10.81)

DISCUSSION

The human is known as the main host of *H. pylori*; however pathological examination

contrasts with that of many other gastric *Helicobacter* where the natural host has mild or absent inflammatory response so it seems that *H. pylori* has originated from another mammalian host in the distant past [21]. The present investigation revealed that dogs, especially those suffering from gastric ulcer, may be the reservoir of *H. pylori* and/or might be the original host of this bacterium. This finding has been documented previously from all-around the world including Belgium [22], Thailand [23], Italy [4], Denmark [24], Egypt [5] and Iran [6]. The prevalence of *H. pylori* in dogs from our investigation was 5.41 % which was entirely lower than those of Thailand (17.3 %) [25], Iran (7%) [6] and Italy (78.9 %) [4]. *Helicobacter*-like organisms are highly prevalent in dogs, with 100 % of random-source dogs [26], 67–100 % of clinically healthy dogs [26–30] and 100 % of laboratory beagles and infected shelter dogs [30,31]. *Helicobacter* spp. have also been demonstrated in gastric biopsies from 61 – 95 % of dogs [32,33].

The low prevalence rate of *H. pylori* in our investigation may be due to the fact that the gastric biopsy samples of our study were taken from dogs who were kept in houses. On the other hand, all the dogs in our study had owners. Therefore, they had high levels of health.

Table 4: Distribution of combined genotypes of *Helicobacter pylori* isolated from gastric biopsies of dogs

Genotype	Prevalence (%)*
M1as1a	3 (23.07)
M1as1b	2 (15.38)
M1bs1a	-
M1bs1b	1 (7.69)
M1as1c	-
M1bs1c	-
M2s1a	4 (30.76)
M2s1b	2 (15.38)
M2s1c	-
M2s2	2 (15.38)
M1as2	1 (7.69)
M1bs2	-
M1am2	4 (30.76)
M1bm2	-
CagA+	6 (46.15)
CagA-	7 (53.84)
IceA1	3 (23.07)
IceA2	1 (7.69)
IceA1 IceA2	2 (15.38)
OipA+	1 (7.69)
OipA-	12 (92.30)

*From a total of 13 positive strains of *H. pylori*

Table 5: Combined *vacA*, *cagA*, *iceA* and *oipA* genotypes of *Helicobacter pylori* isolated from gastric biopsies of dogs

Combined genotype	Total distribution (%)*
s1a/cagA+/iceA1/oipA+	1 (7.69)
s1b/cagA+/iceA1/oipA+	-
s1c/cagA+/iceA1/oipA+	-
s1a/cagA+/iceA2/oipA+	1 (7.69)
s1b/cagA+/iceA2/oipA+	-
s1c/cagA+/iceA2/oipA+	-
s1a/cagA-/iceA1/oipA+	1 (7.69)
s1b/cagA-/iceA1/oipA+	1 (7.69)
s1c/cagA-/iceA1/oipA+	-
s1a/cagA-/iceA2/oipA+	-
s1b/cagA-/iceA2/oipA+	-
s1c/cagA-/iceA2/oipA+	-
s1a/cagA+/iceA1/oipA-	3 (23.07)
s1b/cagA+/iceA1/oipA-	2 (15.38)
s1c/cagA+/iceA1/oipA-	-
s2/cagA+/iceA1/oipA+	1 (7.69)
s2/cagA+/iceA2/oipA+	-
s2/cagA-/iceA1/oipA+	1 (7.69)
s2/cagA-/iceA2/oipA+	-
s2/cagA-/iceA2/oipA-	1 (7.69)
s2/cagA+/iceA2/oipA-	1 (7.69)
s2/cagA+/iceA1/oipA-	2 (23.07)
m1a/cagA+/iceA1/oipA+	1 (7.69)
m1b/cagA+/iceA1/oipA+	-
m1a/cagA+/iceA2/oipA+	-
m1b/cagA+/iceA2/oipA+	-
m1a/cagA-/iceA1/oipA+	1 (7.69)
m1b/cagA-/iceA1/oipA+	-
m1a/cagA-/iceA2/oipA+	1 (7.69)
m1b/cagA-/iceA2/oipA+	-
m1a/cagA+/iceA1/oipA-	2 (15.38)
m1b/cagA+/iceA2/oipA-	1 (7.69)
m2/cagA+/iceA1/oipA+	1 (7.69)
m2/cagA+/iceA2/oipA+	-
m2/cagA+/iceA2/oipA-	1 (7.69)
m2/cagA+/iceA1/oipA-	3 (23.07)
m2/cagA-/iceA1/oipA+	1 (7.69)
m2/cagA-/iceA2/oipA+	-
m2/cagA-/iceA2/oipA-	1 (7.69)
m2/cagA-/iceA1/oipA-	2 (15.38)

*From a total of 13 positive strains of *H. pylori*

As it is obvious the prevalence of *H. pylori* in household dogs was low. This fact can be proved by two possible reasons: First, household dogs are usually fed healthy and probably cooked foods which were not sources of *H. pylori*. Second, household dogs are less in contact with stray dogs.

To our best knowledge, the present study was the first report of the genotyping of *H. pylori* isolated from the gastric biopsy samples of household dogs. As it showed, the most commonly detected genotypes were *vacA* s1a (53.84 %), *cagA* (46.15 %) and *vacA* m2 (38.46 %). *CagA*-positive strains of the *H. pylori* have been considered more virulent than *cagA*-negative strains [34]. In the current survey, some of *H. pylori* isolated from dogs regardless to clinical outcomes harbor the *cagA* gene ($P > 0.05$), therefore, our finding did not support the

role of the *cagA* gene as a predictive marker for increased virulence feature of *H. pylori* in healthy dogs. Similar deduction can also be made for *vacA* s1a genotype. According to our findings for the *vacA* alleles, the *vacA* s1am2 and *vacA* m1am2 positive strains were predominant in gastric biopsy samples of dogs. Based on statistical analyses there was a significant correlation between s1a/m2 and m1am2 genotype of *H. pylori* in healthy and sick dogs. High prevalence of the *vacA* s1am2 and *vacA* m1am2 genotypes of *H. pylori* have been reported previously in the clinical samples of human beings in Iran [35-37]. This may indirectly prove that dogs can be sources of *H. pylori* for human population. Low prevalence of *oipA* and *iceA* showed that these two genotypes were not specific for *H. pylori* strains recovered from the gastric biopsy samples of Iranian dogs.

Limitations of the study

The high costs of research and also difficulty in convincing owners of dogs to allow samples to be taken from their dogs posed caused some limitations in the number of samples used in this study.

CONCLUSION

Low prevalence of *H. pylori* in the gastric biopsy samples of healthy and sick dogs in Iran as well as high presence of *vacA* and *cagA* genotypes in both groups of dogs have been detected. The findings also show that Iranian household dogs harbor *H. pylori* in their gastric tissue similar in genotype to the *vacA* and *cagA* alleles of isolates recovered from humans suggesting that dogs may be the ancestral host of the bacterium and that it entered the human population over a period of time. Due to the low prevalence of the virulent strains of *H. pylori*, basic household hygiene measures such as proper handwashing and avoiding contact with feces and saliva of dogs should be adequate to prevent the transmission of *H. pylori*.

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