Assessment of antimicrobial resistance of cultivable *Helicobacter*-like organisms in asymptomatic dogs

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Summary

This study was performed to detect cultivable canine gastric *Helicobacter*-like organisms (GHLO's) and to evaluate their sensitivity to common antibiotics in two groups of naturally infected dogs (pets and stray dogs). Gastric samples were taken from the body and antrum of 30 pets and 30 stray dogs. From each part of canine gastric mucosa, four gastroscopic samples were used for impression smear, rapid urease test (RUT), polymerase chain reaction (PCR) and culture examination. 88.5% and 95% of gastric samples were positive for the presence of GHLO's in cytological and PCR examination, respectively. From 60 canine gastric cultures, successful growth happened in 17 cases. Antimicrobial sensitivity test was performed by disk method. All isolates of helicobacters were highly susceptible to polymyxin B, ampicillin, tetracycline, clarithromycin, erythromycin and gentamicin. Two isolated GHLO's were resistant to metronidazole. One strain also was resistant to amoxiclav, ceftriaxone, norfloxacin and oxytetracycline. This matter could show the resistance of some strains of helicobacters to different antimicrobials in veterinary medicine. With regards to the results of this study, it is recommended that antibiotic sensitivity test or use of concurrent different antibiotics be tried in the case of treatment resistance.

Key words: Stray and pet dogs, *Helicobacter*, Antimicrobial sensitivity test, Culture and PCR

Introduction

The most common cause of chronic vomiting in dogs is thought to be chronic gastritis, although its histological verification is a poorly documented entity (Akhtardanesh *et al*., 2006). The underlying etiology of chronic gastritis is seldom determined, but immune-mediated, infectious agents and dietary factors may have some influences (Lee, 1989; Van der Gaag and Happe, 1989; Guilford, 1996; Hall and Simpson, 2000; García-Sancho *et al*., 2005). Meanwhile, *Helicobacter*-like organisms are also considered as one of the main causes of gastric inflammation. Nowadays, in human medicine the role of *Helicobacter pylori* in mucosal inflammation is documented, but in companion animals the exact role of these fastidious bacteria is not understood (Eaton *et al*., 1996; Robic *et al*., 2007). These bacterial agents are prevalent in gastric samples of dogs and other carnivores; they are detected in 61-80% of dogs with vomiting, 67-86% of clinically healthy pet dogs and almost 100% of laboratory Beagles and shelter dogs (Fox and Lee, 1997; Jalava *et al*., 1997; Simpson *et al*., 1999; Ettinger and Feldman, 2005; Prachasilpchai *et al*., 2007). These bacteria were detected in animals with visible clinical signs of gastritis (Yamasaki *et al*., 1998), but they were also present in clinically healthy dogs (Happonen *et al*., 1996; Hwang *et al*., 2002). Five cultivable species of helicobacters with similar length (5-15 μm) and width (Jalava *et al*., 1997; Simpson *et al*., 1999; Ettinger and Feldman, 2005; Robic *et al*., 2007) have been reported in canine stomach including

Helicobacter felis, *Helicobacter bizzozeronii*, *Helico-bacter salomonis*, *Helicobacter bilis* and *Flexispira rappini* (Eaton *et al*., 1996; Happonen *et al*., 1996; Jalava *et al*., 1997; Cattoli *et al*., 1999; Buczolits *et al*., 2003); It is not known whether these species represent all the gastric helicobacters in the canine stomach, or if some of them are more common (Cattoli *et al*., 1999). Some of these organisms can create chronic active gastritis in humans, but it seems that the degree of their gastric inflammation is milder (Van den Bulck *et al*., 2005).

Nowadays, numerous drug regimens (double, triple or quadrant therapy) are used in the control of *H. pylori* infections in humans because single-drug protocol proved not to be successful (Van den Bulck *et al*., 2005). Since in veterinary medicine all of these bacteria are not cultivable, similar treatment protocols have been used in the cases of *Helicobacter heilmannii* infections (Van den Bulck *et al*., 2005). Treatment of these animals is performed only when they are present in abundant number and accompanied by gastric mucosal inflammation and clinical symptoms.

In spite of these therapeutic regimens, resistance of *H. pylori* and some non-pylori helicobacters to some antimicrobial agents, such as trimethoprim, polymyxin B, metronidazole, clarithromycin and amoxicillin were reported (Yeh *et al*., 2002; Mohammadi *et al*., 2003; Falsafi *et al*., 2004; Megraud, 2004; Sherif *et al*., 2004; Jong Hwa *et al*., 2005; Kamoda *et al*., 2006; Testerman *et al*., 2006). In addition, recurrence of infection in human cases were also documented (O'Connor *et al*., 2001; Eisig *et al*., 2006; Gisbert *et al*., 2006). Recurrence of infection may occur by re-infection or by resistance of *helicobacters* to antimicrobial agents. This resistance can be related to the types of antibiotics used in routine eradication protocols (Kamoda *et al*., 2006), changing to coccoid form following antibiotic usage (Kamoda *et al*., 2006) and the role of *rdxA* gene (Kato *et al*., 2002).

This study was performed to detect GHLO's and to evaluate their sensitivity to common antibiotics in two groups of naturally infected dogs (pets and stray dogs).

Materials and Methods

Animals

Two groups of dogs were selected. In the first group, 30 pet dogs (aged 7-60 months, of both sexes) livings in different regions of East Azerbaijan province were admitted. Informed consent was obtained from each pet dog's owner and a detailed questionnaire was completed. Inclusion criteria for pet dogs were asymptomatic in terms of vomiting, diarrhea, anorexia, or no use of any drug treatment for at least 3 months prior to the study. 30 stray dogs (aged 7-60 months, including both sexes) were captured from different locations of East Azerbaijan province. All stray dogs were clinically healthy with no history of vomiting in the 2 days of their control. Systemic health condition was also controlled by cell blood count in both groups.

Sample collection

Sixteen hour fasting dogs were anesthetized with intravenous injection of acepromazine and ketamine, (0.03 and 22 mg/kg, respectively). Endoscopic examination was performed with an 1.1 cm diameter gastroscope (2.2 mm working channel, 1100 mm in length, ПУЧОК МТ-11; Russia). Biopsy forceps were used to obtain pinch biopsies from the body (greater curvature) and antral regions (incisura to pyloric sphincter). From each location four-biopsy samples were prepared (Hanninen *et al*., 1996; Jalava *et al*., 1997). One biopsy specimen was used for cytology and the other was wrapped up in normal saline and kept at -28°C for PCR studies. The third gastric sample was used for rapid urease test and the last gastric biopsy was utilized for culture in specific medium.

Diagnosis of canine gastric *Helicobacter*

Molecular diagnosis (PCR amplification of 16S rRNA)

The stomach samples were kept in normal saline and stored at -28°C for PCR evaluation. DNA extraction was performed by DNP™ KIT (CinnaGen, Iran). About 25-50 µl of gastric samples were used for DNA extraction. PCR on the 16S rRNA gene

(Germani *et al*., 1997) was performed in an Eppendorf Mastercycler (at Helicobacter Research Group, Biotechnology Research Center, Pasteur Institute of Iran). Primers were selected based on the 16S rRNA sequences available in the GenBank (*ureB* gene of *H. Felis*, *H. heilmanii*, *H. bizzozeronii* and *ureC* gene of *H. pylori*). PCR reactions consisted of chromosomal DNA, primers and Taq. The following conditions were used for amplification: denaturation at 94°C for 30 s, annealing at 62°C for 30 s, elongation at 72°C for 30 sec. A total of 32 cycles were performed followed by a final elongation step at 72°C for 3 min. The 16S rRNA sequences were determined with the diffusion in agarose gel electrophoresis.

Non-molecular diagnosis (cytology and RUT)

Impression smears of gastric mucosa from the antrum and body were prepared on an air-dried slide, followed by methanol fixation, and stained with Giemsa and Gram for the detection of GHLO's.

Rapid urease tests were performed in gastric biopsy samples. Urease test can be positive within a few hours. The samples were checked for 24 h.

Culture

The stomach samples were sent to a microbiology reference laboratory in transport media (Stuart's media). In the laboratory, stomach samples were cultured in 5% defibrinated sheep blood brain heart infusion (BHI, Merck, Germany) and brucella blood agar (Merck, Germany) contained Skirrow's supplement (vancomycin, 5-10 µg/ml; trimethoprim, 2.5-5 µg/ml; polymyxin B, 1250 IU/l) and amphotericin B (2 µg/ml) or cycloheximide (50 µg/ml). Plates were incubated under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37°C for a maximum of 12 days (Cattoli *et al*., 1999). For the prevention of drying and the enhancement of *Helicobacter* growth, a few drops of BHI or brucella broth were added to the culture plates. Positive cultures resembled small pinpoint colonies or a spreading growth on the surface of the plates. Later, this was verified by Gram and Giemsa staining. Sub-cultures were prepared every 3 days on the same media containing selective supplements.

Antibiotic sensitivity test

Antibiotic sensitivity of isolated gastric helicobacters was evaluated with polymyxin B, amoxicillin, ampicillin, tetracycline, amoxicillin clavulanic acid, erythromycin, gentamycin, ceftriaxone, norfloxacin, clarithromycin, ciprofloxacin, vancomycin, oxytetracycline, cephalexin and metronidazole (manufactured in Iran, Padtan Teb Co.). Susceptibility of the isolated canine gastric helicobacters was evaluated by disk method (Table 1); according to the

Table 1: Antimicrobial agents and their concentration

Antimicrobial agent	Code	Concentration	Susceptible zone diameter		
			R (mm or less)	I (mm)	S (mm or more)
Amoxicillin clavulanic acid	AMC	20+10 µg/dl	13	14-17	18
Amoxicillin	AMX	25 µg/dl	13	14-17	18
Ampicillin	AM	10 µg/dl	13	14-16	17
Cephalexin	CN	30 µg/dl	14	15-17	18
Ceftriaxone	CRO	30 µg/dl	13	14-20	21
Ciprofloxacin	CP	5 µg/dl	15	16-20	21
Clarithromycin	CLR	15 µg/dl	13	14-17	18
Erythromycin	E	15 µg/dl	13	14-22	23
Gentamicin	GM	10 µg/dl	12	13-14	15
Norfloxacin	NOR	10 µg/dl	12	13-16	17
Metronidazole	MNZ	5 µg/dl	16	16-21	22
Oxytetracycline	OXY	30 µg/dl	14	15-18	19
Polymyxin B	PB	300 Iu/dl	8	9-11	12
Tetracycline	TE	30 µg/dl	14	15-18	19
Vancomycin	V	30 µg/dl	14	15-15	17

R: Resistant, I: Intermediate sensitivity and S: Sensitive

guidelines of the National Committee for Clinical Laboratory Standards (Bauer *et al*., 1996; NCCLS, 2003). The control strain was *H. pylori* (ATCC 43504) and all isolated canine samples were controlled and compared with this *Helicobacter* strain.

Results

In PCR examination, 95% of the gastric samples were positive (93% of pets and 97% of stray dogs, Fig. 1). There was no significant difference between the prevalence of gastric helicobacters in stray and pet dogs (P>0.05). *H. felis* and *H. heilmannii* were the identified strains in both groups. *Helicobacter pylori* and *F. rappini* were not detected in any of the samples by PCR. There was a difference between the prevalence of *H. heilmannii* in pets and stray dogs (P<0.05). Comparison of PCR results with other diagnostic procedures are listed in Table 2.

In light microscopic examination (impression smears) 27 out of 30 stray dogs (90%) and 26 out of 30 pet dogs (86.6%) were positive for GHLO's. In most cases, organisms morphologically resembling *H. felis* and *H. heilmannii*-like organisms were present (Fig. 2). 87% of gastric samples were positive by RUT. Most samples became positive within 4-6 h, but it seems that in heavily infected samples, color change was rapid and occurred within one hour.

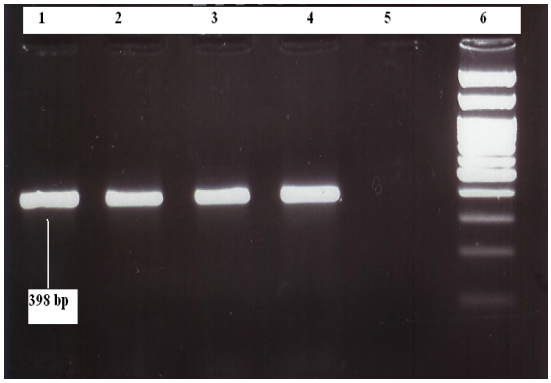

Fig. 1: PCR detection of 16S rRNA in gastric biopsy specimens. Line 1= positive control, Line 2-4= DNA samples, Line 5= Negative control, Line 6= 100 bp DNA marker

Table 2: Comparison of different *Helicobacter* diagnostic procedures

Method	Group	
	Pet dogs	Stray dogs
Cytology	86.6% (26/30)	90% (27/30)
RUT	86.6% (26/30)	86.6% (26/30)
Culture	20% (6/30)	36.6% (11/30)
PCR	93% (28/30)	97% (29/30)

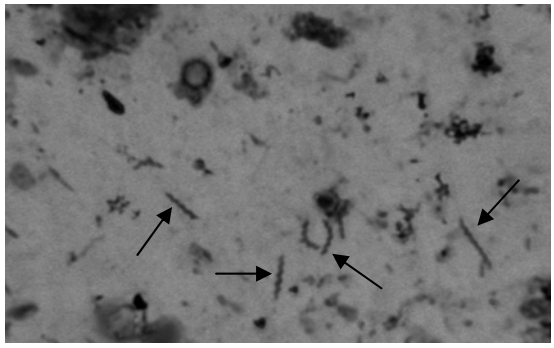

Fig. 2: *Helicobacters*-like organisms (arrows) in canine gastric impression smear. Giemsa staining (×1200)

From 60 canine gastric cultures, successful growth happened in 17 cases (28.3%). In 13 samples, there was a single strain, and the rest had mixed infections. Three distinct strains of *Helicobacter* were isolated. Cultured strains and their characteristics are listed in Table 3. Based on the biochemical and physiological characteristics of the cultured organisms, two were diagnosed as a *H. felis*-like organism (61.5%, n = 8) and *H. bizzozeronii*-like organism (30.75%, n = 4). The isolated *H. felis* organisms were proved by PCR. *H. heilmannii* did not grow in any samples in spite of the positive result by PCR. Neither *F. rappini*-like organisms nor *H. pylori* was detected in any samples by culture or PCR.

The results of sensitivity test are listed in Table 4. 12.5 percent of the *H. felis* isolates (n = 1) and 25% of the *H. bizzozeronii* isolates (n = 1) were resistant to metronidazole. 25% of *H. bizzozeronii* isolate (n = 1) was also resistant to amoxiclav, ceftriaxone, norfloxacin and oxytetracycline and all were resistant to vancomycin. Resistances to metronidazole were seen only in stray dogs.

Discussion

Based on cytological, culture and PCR evaluation, GHLO's can be considered as a

prevalent gastric organism in pets and stray dogs. Considering the low culture rate of GHLO's (17/60) in comparison with light microscopy and PCR, it can be concluded that a significant portion of the spiral organisms in canine gastric biopsies is not cultivable (Jalava *et al*., 1997); or we are not able to culture them. In spite of helicobacters' culture difficulty, it can also be considered as an important method in determination of helicobacters resistance or sensitivity to different antibiotics.

Based on the results of this study, all isolates of helicobacters were highly susceptible to polymyxin B, ampicillin, tetracycline, clarythromycin, erythromycin and gentamycin (Table 4). In this study 12.5% of *H. felis* and 25% of *H. bizzozeronii* isolates were resistant to metronidazole. 25% of *H. bizzozeronii* isolate was also resistant to amoxicillin clavulanic acid, ceftriaxone, norfloxacin and oxytetracycline. Oxitetracycline resistances were seen in 25% (n = 2) of *H. felis* isolates and 25% of *H. bizzozeronii*. 37.5% (n = 3) of *H. felis* and 75% (n = 3) of *H. bizzozeronii* isolates had intermediate resistance to this antibiotic. Resistance to cephalexin was present in only one isolated *H. felis*. All isolates were resistant to vancomycin.

In human medicine some authors reported the resistance of these organisms to clarythromycin and some other antibiotics (Hartzen *et al*., 1997; Yeh *et al*., 2002; Zhi-Jun *et al*., 2002; Nilius *et al*., 2003; Nahar *et al*., 2004; Jong Hwa *et al*., 2005; Authier *et al*., 2006; Edgie-Mark and Schiller, 2006; Kamoda *et al*., 2006; Testerman *et al*.,

Table 3: Biochemical and physiological characteristics of isolated gastric helicobacters (Heilmann and Borchard, 1991; Scanziani *et al*., 2001)

Light microscopic taxonomy	Isolated *H. felis*-like organisms	Isolated *H. bizzozeronii*-like organisms	Unknown isolated *Helicobacter* (probably *H. acinonychis* like-organism)
Long	Longer	Longer	Small
Shape of bacterial cell	long, with a tightly wound spiral shape	long, with a tightly wound spiral shape	Small with blunted shape
Biochemical tests			
Ureas	+	+	NP
Oxidase	+	+	NP
Catalase	+	+	NP
Nitrate reduction	+	+	NP
Physiological tests			
Growth at 25°C	-	-	NP
Growth at 37°C	+	+	+
Growth at 42°C	+	+	-
Motion	Fast and screw like	Fast and screw like	Slower than others

NP = Not performed

Table 4: Antibacterial sensitivity results to different antibiotics

Antibiotics	Samples												Undetected *Helicobacter*		
	Pet dogs						Stray dogs								
	H. felis			*H. bizzozeronii*			*H. felis*			*H. bizzozeronii*					
	S	R	I	S	R	I	S	R	I	S	R	I	S	R	NP
Polymyxcin	4	-	-	2	-	-	4	-	-	2	-	-	-	1	-
Amoxicillin	4	-	-	2	-	-	4	-	-	1	1	-	1	-	-
Ampicillin	4	-	-	2	-	-	4	-	-	2	-	-		-	1
Tetracycline	4	-	-	2	-	-	4	-	-	2	-	-	1	-	-
Amoxicillin clavulanic acid	4	-	-	-	-	2	4	-	-	2	-	-	1	-	-
Erythromycin	4	-	-	2	-	-	4	-	-	2	-	-	-	-	1
Gentamycin	4	-	-	2	-	-	4	-	-	2	-	-	-	-	1
Ceftriaxone	4	-	-	1	-	1	4	-	-	2	-	-	-	-	1
Norfloxacin	4	-	-	-	-	2	4	-	-	1	1	-	-	-	1
Clarythromycin	4	-	-	2	-	-	4	-	-	2	-	-	1	-	-
Ciprofloxacin	2	-	2	2	-	-	1	-	3	1	-	1	1	-	-
Vancomycin	-	4	-	-	2	-	-	4	-	-	2	-	-	1	-
Oxytetracycline	3	-	1	-	-	2	-	2	2	-	1	1	1	-	-
Cephalexin	2	-	2	2	-	-	1	1	2	-	1	1	-	-	1
Metronidazole	3	-	1	-	-	2	2	1	1	-	1	1	1	-	-

S = Sensitive, R = Resistant and I = Intermediate

2006). Jong Hwa *et al*. (2005) reported that 20.2% of human isolates are resistant to clarithromycin. Nahar *et al*. (2004) also determined the resistance rate of 77.5, 15, 10 and 6.6% in *H. pylori* isolate to metronidazole, tetracycline, clarithromycin, and amoxicillin, respectively. Based on the results of different studies, antibiotic sensitivity of *Helicobacter* strains could be changed with time and the type of antibiotics (Hirschl *et al*., 2000; Megraud, 2004; Nahar *et al*., 2004; Sherif *et al*., 2004), so it is reasonable to use triple or quadrant antibiotic therapy in the cases of symptomatic *Helicobacter* infections. Based on some reports, *Helicobacter* morphology will change to coccoid following antibiotic therapy (*in vivo*) and this may affect their antibiotic resistance (Kamoda *et al*., 2006).

Regarding the mechanism of antibiotic resistance in *H. pylori*, especially for metronidazole, some authors have investigated the role of the *rdxA* gene (Kato *et al*., 2002). Some studies emphasize that the resistance rate of *H. pylori* isolates are significantly different in dissimilar geographical areas (Nahar *et al*., 2004; Sherif *et al*., 2004), which may be related to routine use of some antibiotics. Resistance of *H. pylori* to clarithromycin in Iranian patients was present even before its usage (Mohammadi *et al*., 2003). Furthermore, recurrence of *Helicobacter* infection after eradication was reported (Sekine *et al*., 1999; Khor *et al*., 2000; Eisig *et al*., 2006; Gisbert *et al*., 2006). Eisig *et al*. (2006) reported a 5.7% recurrence rate in Brazilian patients.

Meanwhile, some canine gastric helicobacters are zoonotic and can produce active chronic gastritis in human patients (Otto *et al*., 1994; Van den Bulck *et al*., 2005). Different studies proved the clinical importance of these bacteria in human and veterinary medicine (Buczolits *et al*., 2003; Lekunze Fritz *et al*., 2006). Although this study was performed in asymptomatic dogs, the results could be used in human cases infected with GHLO's. Lekunze Fritz *et al*. (2006) reported 23.3% of human patients with *H. felis* origin. With regards to the occurrence of canine gastric helicobacters in human gastritis and their clinical importance, there is a need for more evaluation of these organisms in different countries and their sensitivities against different antimicrobial agents.

Like human medicine, the present study may show the resistance of helicobacters to metronidazole. The study of Van den Bulck *et al*. (2005), showed acquired resistance of *H. felis* to metronidazole but in the same study, different isolated helicobacters were susceptible to ampicillin, clarithromycin, tetracycline, tylosin, enrofloxacin and neomycin. In the present study, there was no difference between the prevalence of helicobacters in the two groups of dogs, also there was no difference in the antibiotic resistance between the pets and the stray dogs.

In human medicine the role of *H. pylori* in mucosal inflammation is documented, but in companion animals the exact role of these bacteria is not known. They seem to be commensal and therefore, the usage of antibiotics must be performed in the case of *Helicobacter* gastritis. Lack of previous antibiotic therapy in the history of these stray dogs may suggest helicobacters innate or acquired resistance to some antibiotics. Finally, with attention to the zoonotic aspect of these bacteria and their resistance to some antibiotics, concurrent use of different antibiotics would be of importance in preventing gastric *Helicobacter* insensitivity. Extensive study with a larger population is recommended for further researches. It is recommended that antibiotic sensitivity test should be tried in patients with resistance or recurrent infections. Because of the need for long term therapy, some drugs are not suitable in GHLO therapy (like gentamycin). In addition to GHLO's sensitivity to different antibiotics, drug availability, cost and safety must be considered.

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