

Experimental infection of stray cats with human isolates of *Helicobacter pylori*

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Summary

In contrast to humans, in whom *Helicobacter pylori* infection predominates, the investigation of pathogenicity of this organism in cats is complicated based on the fact that they can be colonized by a variety of *Helicobacter* spp. To improve our understanding of *Helicobacter* infection in cats and to determine whether they are reservoirs for *H. pylori* and sources of zoonotic transmission to humans, we selected this animal as an experimental model for gastric colonization by *H. pylori*. Sixteen stray cats underwent *Helicobacter* eradication treatment followed by three consecutive oral inoculations of a cocktail of human *H. pylori* isolates. Four out of sixteen inoculated cats became colonized by *H. pylori* as confirmed by genus- and species-specific PCR. In conclusion, human *H. pylori* isolates are able to colonize the stomach of cats. It seems that these animals can be used as an experimental model in the future investigations of *H. pylori*-induced pathogenesis as well as evaluation of anti-*H. pylori* prevention and treatment regimens. Further experimental studies including additional animals are needed to identify the usefulness of the cat as an experimental model.

Key words: *H. pylori*, Colonization, PCR, Cat

Introduction

At present, more than 36 kinds of organisms with typical characteristics of *Helicobacter* spp. have been isolated from humans and different animals (Neiger, 2001). *Helicobacter felis* was the first species isolated and identified from the cat stomach (Lee *et al.*, 1988b) followed by *Helicobacter heilmannii* (formerly known as "*Gastrospirillum hominis*") (Heilmann and Borchard, 1991). These two species are collectively referred to as gastric *Helicobacter*-like organisms (GHLOs) (Hänninen *et al.*, 1996). GHLOs have been observed in gastric biopsies of 41-100% of clinically healthy and 57-100% of vomiting cats (Ettinger and Feldman, 2005). In Iran (Tehran), using 16S rRNA PCR assay, total prevalence of GHLOs infection in domestic and stray cats was reported as 100% and

56.7%, respectively (Akhtardanesh *et al.*, 2006).

Detecting no association between GHLOs and gastritis, some authors cited these organisms as gastric commensals (Perkins *et al.*, 1998). However, due to the tremendous interest in *Helicobacter pylori* as the major acquired factor in the pathogenesis of active chronic gastritis, peptic ulcer, and gastric neoplasia in humans (Marshall and Warren, 1983; Parsonnet *et al.*, 1994), a major focus has been on this gastric pathogen in experimental and natural models of infection. The aim of these models has been to explore the role of *H. pylori* in producing gastric pathology, as well as its zoonotic potential.

Some investigations have failed to isolate *H. pylori* from stray or pet cats (El-Zaatari *et al.*, 1997; Neiger *et al.*, 1998), whereas others have revealed the natural

presence of *H. pylori* in cat stomach (Handt *et al.*, 1994; Scanziani *et al.*, 2001; Sobhani *et al.*, 2002), saliva, gastric juice and/or faeces (Fox *et al.*, 1996). This pathogen has also been cultured from the gastric mucosa of experimentally infected cats (Perkins *et al.*, 1998). The lesions in the stomachs of experimentally infected cats mimic many of the features seen in human stomach infected with *H. pylori* (Perkins *et al.*, 1998). However, using specific pathogen-free (SPF) cats and ignoring the widespread prevalence of spiral bacteria in the gastrointestinal tracts of cats, limits the application of these animals as experimental models for *H. pylori* infection, not to mention the cost implications (Nedrud, 1999).

The principal aim of the present study was to establish gastric colonization of the human *H. pylori* isolates in cats following a routine anti-*Helicobacter* therapy regimen.

Materials and Methods

Pre-inoculation

Animals: Sixteen healthy asymptomatic male (n = 11) and female (n = 5) stray cats, 1-3 years of age, were obtained from different locations of Tehran, Iran. Cats were housed in separate cages for 4 weeks prior to initiation of the study. Each cat received vaccination against feline panleukopenia, FCV and FHV (Tri-cat; USA). Thereafter, twelve cats were randomly assigned to the experimentally inoculated groups (group 1 and 2) and four cats were kept as controls (control 1 and 2). All cats were individually housed during the entire course of the study.

Sampling via gastroscopy: In order to evaluate the status of *Helicobacter* spp. infection, gastroscopy was performed with

4.9 mm diameter pediatric bronchoscope (VET-VU/Swiss). For this purpose, fasting cats were anaesthetized with acepromazine (Neurotranq[®], 0.1 mg/kg, IM) and thiopental sodium (Nesdonal[®], 25-30 mg/kg, IV given until the signs of anaesthesia appeared). Biopsy forceps were used to prepare three (2 mm) pinch biopsies from each location of gastric cardia, body (greater curvature) and antrum (incisura to pyloric sphincter).

Quantitative urease test: Urease test was performed on biopsy specimens. One biopsy specimens from each location was placed into 5 ml of urea broth media (DIFCO/USA), incubated at 37°C for 24 h. Conversion to a pink-red colour within 24 h was considered as positive and the time was recorded. No colour change in the course of 24 h was considered as negative.

Cytology: Impression smears from three sites of gastric mucosa were prepared on a slide which was air dried, fixed with methanol, and stained with Giemsa for detection of GHLOs.

DNA extraction and PCR assays: Gastric mucosal biopsy specimens were frozen at -70°C until DNA extraction. The extraction of DNA was performed using DNeasy tissue kit (Qiagen, USA) according to the manufacturer's instructions. Gene-specific PCR was carried out in a final reaction volume of 20 µl containing 2 µl of PCR buffer, 0.6 mM of MgCl₂, 0.4 mM dNTP, 1 µl of specific primers (Table 1) and 0.1 U of Taq DNA polymerase (Fermentas/Lithuania) under conditions described in Table 2 (Camargo *et al.*, 2003). Gastric biopsies were first tested against genus-specific primers (16S rRNA), then assessed for colonization by *H. pylori*, *H. felis*, or *H. heilmannii* via strain-specific primers. PCR products were then electrophoresed on 2% (w/v) agarose gel with 0.3% ethidium

Table 1: Oligonucleotide primers

Target genes	Reference	Primer sequence (5'→3')	PCR product size (bp)
16S rRNA genes of <i>Helicobacter</i> spp.	Germani <i>et al.</i> (1997)	(F): AAC GAT GAA GCT TCT AGC TTG CTA (R): GTG CTT ATT CGT GAG ATA CCG TCA T	399
<i>ureA</i> and <i>ureB</i> genes (<i>H. felis</i>)	Germani <i>et al.</i> (1997)	(F): GTG AAG CGA CTA AAG ATA AAC AAT (R): GCA CCA AAT CTA ATT CAT AAG AGC	241
<i>ureB</i> gene (<i>H. heilmannii</i>)	Neiger <i>et al.</i> (1998)	(F): GGG CGA TAA AGT GCG CTT G (R): CTG GTC AAT GAG AGC AGG	580
<i>ureC</i> gene (<i>H. pylori</i>)	Labigne <i>et al.</i> (1991)	(F): GGA TAA GCT TTT AGG GGT GTT AGG GG (R): GCT TAC TTT CTA ACA CTA ACG CGC	294

Table 2: PCR conditions

Species	Denaturation step	PCR conditions (denaturation, annealing and extension)	No. of cycles and durations	Final extension step
<i>Helicobacter</i> spp.	94°C for 3 min	94°C, 60°C and 72°C	34 for 40 s	72°C for 3 min
<i>H. felis</i>	94°C for 4 min	94°C, 62°C and 72°C	34 for 50 s	72°C for 4 min
<i>H. heilmannii</i>	94°C for 3 min	94°C, 59°C and 72°C	32 for 50 s	72°C for 4 min
<i>H. pylori</i>	95°C for 5 min	94°C, 57°C and 72°C	34 for 1 min	72°C for 4 min

bromide in a 10% Tris-borate-EDTA (TBE) buffer. Amplified products were visualized via UV light illumination.

Anti-*Helicobacter* quadruple therapy: Every cat regardless of its status of GHLOs infection, received a 14-day oral course of quadruple therapy (Ettinger and Feldman, 2005) consisting of 0.7 mg/kg omeprazole (Zahravi Pharmaceutical Co., Tabriz, Iran) tid, 20 mg/kg amoxicillin (Farabi Pharmaceutical Co., Isfahan, Iran) bid, 20 mg/kg metronidazole (Tehran Chemie Pharmaceutical Co., Tehran, Iran) bid and 7.5 mg/kg clarithromycin (Tehran Chemie Pharmaceutical Co., Tehran, Iran) bid.

Inoculation

Bacterial strains, growth conditions, and sample preparation: A cocktail of five *H. pylori* strains isolated from dyspeptic patients with varying clinical diagnosis (gastritis and gastric cancer) plus the feline-adapted *H. pylori* strain (Lee *et al.*, 1997) were cultured on sheep blood-brucella agar plates under microaerobic conditions (37°C, 10% CO₂, 5% O₂, 85% N₂) for 3-5 days and then collected for oral inoculation of cats.

Dosing scheme: Two weeks after the end of quadruple therapy, the cats underwent starvation overnight, after which they were weighed and cimetidine (Tagamet[®], 7 mg/kg IM, bid for 7 days) was administered to induce hypochlorhydria. On days 3, 5 and 7 cats were sedated with ketamine 10% (Alfasan[®], 10 mg/kg IM) and orally inoculated with brucella broth containing 10⁹ CFU of each strain of *H. pylori* via an orogastric stomach tube. The control cats received brucella broth only.

Post-inoculation

Sampling via necropsy (group 1): Four weeks after completion of *H. pylori* inoculation, six infected cats (group 1) and two control cats (control 1) were euthanized with overdose injection of pentobarbital

sodium followed by necropsy. Gross lesions were recorded and samples were collected from gastric antrum, body and cardia of each cat for cytology, urease test and PCR.

Sampling via necropsy (group 2): Ten weeks after *H. pylori* inoculation, six infected cats (group 2) and two controls (control 2) were euthanized (as mentioned above). Gross lesions were recorded and samples were collected from antrum, body and cardia regions of the stomach of each cat for cytology, urease test and PCR

Results

Pre-inoculation

Clinical findings: Two GHLO positive cats (in group 1) showed anorexia and moderate vomiting after the first week of administration of drugs which resolved after three days.

Endoscopic findings: Endoscopic evaluation of gastric mucosa revealed gastric erosions in three cats (group 1). Three other cats were not evaluated by gastroscopy and were not included in the first part of the study (pre-inoculation assessment).

***Helicobacter* infection status:** Ten cats were positive for GHLO infection by urease test and eight were positive by cytology. Using 16S rRNA PCR assay, the total prevalence of GHLOs in this study was 69% (9 out of 13). The prevalence of natural *H. heilmannii* and *H. felis* infection was 69% (9 out of 13) and 46.6% (7 out of 13), respectively. Mixed infection was detected in 6 cats. None of the cats were found to be naturally infected with *H. pylori*.

Post-treatment

Following anti-*Helicobacter* quadruple therapy, none of the control cats were found infected with GHLOs by urease test or cytologic examination of gastric biopsies but using 16S rRNA PCR assay two control cats (control 2) were found positive. Therefore,

Helicobacter eradication therapy was not completely successful.

Post-inoculation

Clinical findings: One of the *H. pylori* inoculated cats (in group 1) died three weeks after inoculation due to severe pneumonia. No association could be made between tissue damages and oral intubation.

Gross necropsy findings: Gross lesions were detected in none of the cats.

GHLOs infection status: The cats which were found GHLO positive by urease test and cytology maintained their status at post-inoculation phase of the study. Using 16S rRNA PCR assay, the total prevalence of GHLOs infection was 50% (8 out of 16). The prevalence of *H. heilmannii* and *H. felis* was 50% (8 out of 16) and 46.6% (7 out of 16), respectively. Mixed infection was detected in 7 cats.

Gastric colonization with *H. pylori*: Four experimentally-infected cats became colonized with human isolates of *H. pylori* confirmed by urease test and PCR. Two of these cats were in group 1 and the other two cats belonged to group 2. Although the gastric body was colonized in all four infected cats, the cardia and antrum were sporadically colonized. All four control cats remained *H. pylori*-negative via cytology, urease test and *H. pylori*-specific PCR

throughout the study. Two of the *H. pylori* positive cats had mixed infection with *H. heilmannii* and *H. felis* (Table 3 and Fig. 1).

Discussion

Studying the effects of *Helicobacter* infection on the gastric mucosa of cats is limited by some factors, including the variability in the host genetic make up, the infecting *Helicobacter* spp. and strain variation. In contrast to humans, in whom *H. pylori* infection predominates, the investigation into the pathogenicity of this organism in cats is complicated based on the fact that they can be colonized by a variety of *Helicobacter* spp. and simultaneous colonization with multiple species has been frequently observed (Simpson, 2006). To improve our understanding of cat-*Helicobacter* interactions and to determine whether the cat can be a reservoir host of *H. pylori* for zoonotic transmission to humans, this animal was selected in our study as a potential experimental animal model for gastric colonization by *H. pylori*.

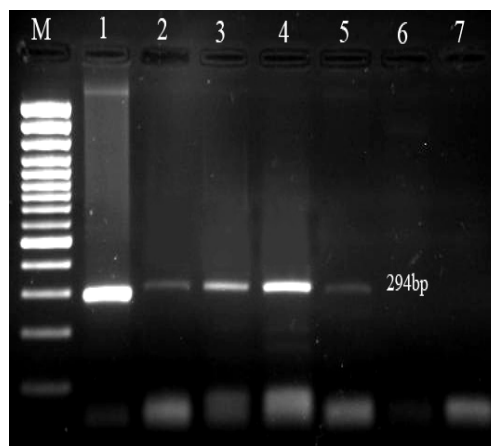
The rate of GHLOs infection in cats in the present study (69%; nine out of thirteen cases) confirms a recent report from stray cats in Tehran, Iran (56.7%) (Akhtardanesh *et al.*, 2006). The prevalence of GHLOs in

Table 3: Comparison of four *H. pylori*-infected cats (pre-inoculation vs. post-inoculation)

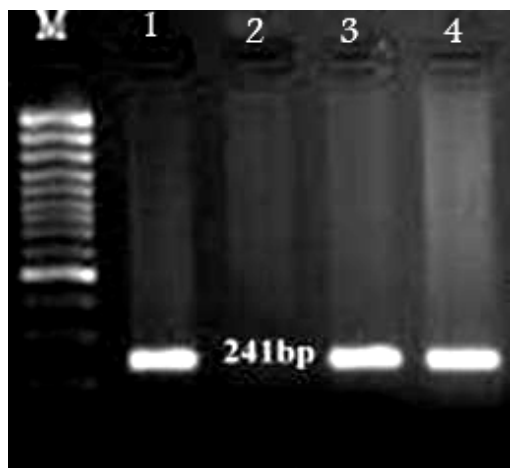
		Pre-inoculation					
Group		Urease test	Cytology	<i>Helicobacter</i> -genus	<i>H. felis</i>	<i>H. heilmannii</i>	<i>H. pylori</i>
Group 1							
	Cat # 3	+	+	+	+	+	-
	Cat # 8	+	+	+	+	+	-
Group 2							
	Cat # 13	+	+	+	+	+	-
	Cat # 15	+	+	-	-	-	-
		Post-inoculation					
Group		Urease test	Cytology	<i>Helicobacter</i> -genus	<i>H. felis</i>	<i>H. heilmannii</i>	<i>H. pylori</i>
Group 1							
	Cat # 3	+	+	+	+	+	+
	Cat # 8	+	+	+	-	-	+
Group 2							
	Cat # 13	+	+	+	+	+	+
	Cat # 15	+	+	+	-	-	+



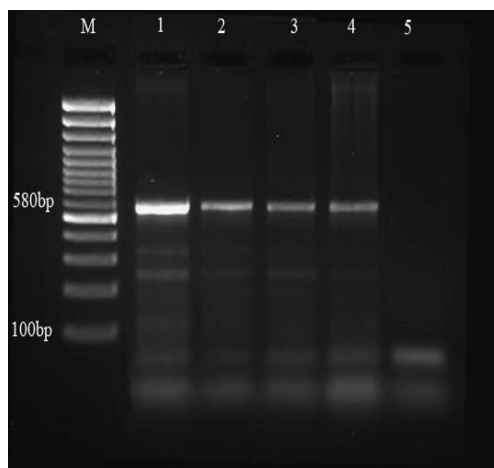
A: 16S rRNA gene-*Helicobacter* genus; M: 100 bp marker, Lane 1: positive control, Lane 10: negative control, Lane 2, 3, 6 and 7: DNA extracted from gastric body of four *H. pylori*-colonized cats



B: *ureC* gene-*H. pylori*; M: 100 bp marker, Lane 1: positive control, Lane 7: negative control, Lane 2, 3, 4 and 5: DNA extracted from gastric body of four *H. pylori*-colonized cats



C: *ureB* gene-*H. felis*; M: 100 bp marker, Lane 1: positive control, Lane 2: negative control, Lane 3 and 4: DNA extracted from gastric body of two *H. pylori*-colonized cats



D: *ureB* gene-*H. heilmannii*; M: 100 bp marker, Lane 1: positive control, Lane 5: negative control, Lane 2, 3 and 4: DNA extracted from gastric body (Cat # 3), cardia (Cat # 3) and cardia (Cat # 13) of *H. pylori*-colonized cats

Fig. 1: Genus-specific and strain-specific PCR in four *H. pylori*-infected cats (gastric body)

cats is also reported by other investigators ranging from 41 to 100% (Happonen *et al.*, 1996; Papisoulitis *et al.*, 1997).

Natural GHLOs colonization in cat stomach may compete with *H. pylori* colonization in the same niche (Handt *et al.*, 1994). In this study, all experimental cats must be treated irrespective of their infection status as sub-threshold GHLO infections (undetected by our applied detection methods) to possess the identical condition prior inoculation; other *Helicobacter*-unrelated infections of the host may affect the outcome of experimental infection. In an

attempt to eradicate pre-existing GHLO infection in stray cats, we employed a 14-day quadruple anti-*Helicobacter* therapy. Despite the fact that such regimen for seven days led to a complete eradication in 74% of the GHLOs infected cats (Ettinger and Feldman, 2005), our eradication therapy for 14 days failed to eliminate GHLO infection in the majority (10/16) of the studied cats.

In the present study experimental inoculation with *H. pylori* resulted in gastric colonization in four cats, which was detected by species-specific PCR assay. This finding indicates that previous infection with

GHLOs (due to resistance of these organisms to such intensive anti-*Helicobacter* treatment and/or the possibility of recrudescence following treatment) can not interfere with subsequent *H. pylori* colonization.

In the only similar study (Perkins *et al.*, 1998) using *cagA*+ human strain of *H. pylori*, infection was detected in all four SPF cats. Failure of *H. pylori* to colonize more number of cats in our study may indicate a distinctive sterile niche required by *H. pylori* which may be absent or less hospitable in the gastric epithelium of stray cats.

Our study revealed co-colonization of GHLOs (*H. felis* and *H. heilmannii*) with *H. pylori* in two infected cats. This agrees with data reported by Perkins *et al.* (1998) in non-human primates and humans, where *H. pylori* was occasionally able to co-colonize with large gastric spirals. However, simultaneous infection by GHLOs and *H. pylori* was rarely found in another study possibly resulting from competitive exclusion of one or the other (Stolte *et al.*, 1994).

In the present study, PCR assay indicated that the gastric body (colonized in all 4 infected cats) is better suited for *H. pylori* colonization than the gastric cardia (colonized in 2 infected cats) or the antrum (colonized in only one cat). This finding is consistent with the study by Perkins *et al.* (1998) in which *H. pylori* was detected in body and cardia of all four cats. Some studies in cats showed that body and cardia (areas rich in parietal cells) were more heavily colonized with GHLOs and *H. pylori*, than the antrum (Erginsoy and Sozmen, 2006). Different patterns of colonization in cardia and antrum were reported in BALB/c mice infected with *H. felis*; these differences may be attributed to varying gastric pH in different regions of stomach (Danon *et al.*, 1995). This suggests that host-specific factors may be involved, and one possible mechanism that has been described is the ability of some *Helicobacters* to directly inhibit the secretory function of parietal cells (Vargas *et al.*, 1991).

Chronic *Helicobacter* infection results in gastrointestinal complications, including a variety of gastritis. Therefore, in every

Helicobacter-related experimental animal model sufficient time is required for occurring such tissue damage. Accordingly, the main goal of this study was to establish a long-term colonization of *H. pylori* in the cat stomach. Further studies on more chronically infected cats are needed to measure the development of host inflammatory and immune responses, and their relationship with the putative bacterial virulence factors in combination with bacterial colonization density in feline model of *H. pylori* infection. Changes in gastric acid secretion and serum gastrin levels which are known to occur in *H. pylori*-infected patients must also be assessed in the infected cats.

It seems that group housing in the study by Perkins *et al.* (1998) was responsible for group colonization and persistence of *H. pylori* in each cat. Isolated housing of cats in our study could influence the degree of gastritis or the ability of *H. pylori* to persist in the stomach. This model can be used in the future to ascertain *H. pylori* status in domestic animals. A systematic and detailed analysis using several diagnostic criteria is required to determine the true susceptibility of cats in regards to *H. pylori* colonization.

Similar to the study by Perkins *et al.* (1998), *H. pylori* was not detected in the gastric cytological smears in our study. It may be more difficult to visualize them than GHLOs by special tissue stains, and may be missed in a perfunctory manner (Heilmann and Borchard, 1991). In addition, *H. pylori* can simply be missed due to its smaller size in comparison to other gastric *Helicobacter* spp. (2.5 μm vs. 4-10 μm).

The route of transmission for members of the genus *Helicobacter* is the topic of much controversy. Isolation of *H. pylori* from human and cat dental plaques, saliva and faeces (Kelly *et al.*, 1994; Fox *et al.*, 1996) prompted some authors (Fox *et al.*, 1996) to propose oral-oral and faecal-oral route of transmission.

Several case reports of GHLOs infection in human have suggested animals as a possible source of infection (Lee *et al.*, 1988a; Heilmann and Borchard, 1991). One epidemiological study supported the hypothesis that cats were considered as a source of zoonotic spread of GHLOs;

however, data focused on *H. felis* and *H. heilmannii*, not *H. pylori* (Stolte *et al.*, 1994). In contrast, several other epidemiological studies do not support animals as potential reservoirs of *H. pylori* infection in humans (Ansorg *et al.*, 1995). Recently authors, who could not demonstrate *H. pylori* in large numbers of cats, suggested that *H. pylori* in cats may be an anthroponosis (El-Zaatari *et al.*, 1997). They also argued that *H. pylori*-infected cats from one commercial source (Handt *et al.*, 1994) could have been originally infected by *H. pylori* from a human (El-Zaatari *et al.*, 1997). The experimental infection of cats with human strains of *H. pylori* supports the hypothesis that anthroponosis is a distinct possibility in the only known colony of *H. pylori*-infected cats. Additional surveys in various geographic locations are needed to ascertain prevalence of *H. pylori* in pet cats as well as in feral cats living in close proximity to humans in urban settings.

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