

GASTRIC *HELICOBACTER* SPP. INFECTION IN CAPTIVE NEOTROPICAL BRAZILIAN FELINE

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ABSTRACT

Ten captive neotropical Brazilian feline were submitted to gastroscopic examination and samples of gastric mucosa from fundus, corpus and pyloric antrum were evaluated for the presence of *Helicobacter* species. Warthin-Starry (WS) staining and PCR assay with species-specific primers and enzymatic cleavage were applied for bacterial detection and identification. Histological lesions were evaluated by haematoxylin and eosin staining. All animals showed normal gross aspect of gastric mucosa. *Helicobacter heilmannii* was confirmed in 100% of the samples by WS and PCR assay. Mild lymphocytic infiltrate in the lamina propria was observed in eight animals, mainly in the fundus region. Small lymphoid follicles were seen in three animals. No significant association between *Helicobacter* infection and histological findings was verified. These observations suggest that gastric *Helicobacter* spp. could be a commensal or a eventual pathogen to captive neotropical feline, and that procedures, way life, and stress level on the shelter apparently had no negative repercussion over the integrity of the stomach.

Key words: *Helicobacter*, feline, gastric diseases, PCR, *Leopardus*.

INTRODUCTION

Helicobacter pylori infection in human beings has been associated with gastritis, gastric and duodenal ulcer, gastric adenocarcinoma, and MALT (mucosa associated-lymphoid tissue) lymphoma. While *H. pylori* is accepted as the dominant human gastric bacterial pathogen, a small percentage of human infections have been associated with *H. heilmannii*, which is more prevalent in pet and wild animals (3). The high prevalence of this species in animals has been suggested to have implication for public health (5). However, the subtypes

of *H. heilmannii* strain that colonizes humans and animals are different, indicating that animals represent a lower risk to man (17).

In several studies gastritis was associated with *Helicobacter* spp. infection in tigers, lions and cheetahs. *Helicobacter acinonyx* was implicated in gastritis in tigers (19). In a retrospective survey conducted with 69 captive cheetahs, 40% of the deaths were associated with severe gastritis induced by *Helicobacter* spp. infection (13). Moreover, the infection was also associated with gastroduodenal adenocarcinoma and rectal adenoma in a

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cougar (25). By contrast, others researches found no association between infection and gastric mucosal inflammatory infiltrate, suggesting that the bacteria are commensal or opportunist pathogens (7, 23). Also, the study of 28 semi captive cheetahs found out mild or no lesions in 27, concluding that gastritis was uncommon in these animals and proposing that stress could play a major role in the development of *Helicobacter*-associated gastritis (12).

In captive wild feline animals stress and host factors might be important issues in the development of gastritis associated with *Helicobacter* infection (23). The presence of *Helicobacter* spp. in the stomach of wild or captive feline has been reported in several countries (6, 10, 13); however the relationship between infection and gastric diseases remains controversial. Gross and histological analysis, and molecular characterization of *Helicobacter* species in captive neotropical Brazilian feline have not been previously described. Therefore, the purpose of the current report was to examine the presence of *Helicobacter* in gastric mucosa, and evaluate the association between infection, gross and histological findings in neotropical *Felidae*.

MATERIALS AND METHODS

Specimens

Samples of gastric mucosa from four female little spotted cat (*Leopardus tigrinus*), five female ocelots (*Leopardus pardalis*), and one male cougar (*Puma concolor*) were evaluated. The animals were housed at Brazilian Center to Conservation of Neotropical Feline – Mata Ciliar Association, São Paulo, southern Brazil. None of the feline had any previous signs of gastric disease. The animals were fed with fresh meat of chicken, cattle, and viscera. The study was approved by the institutional Ethics Committee for Animal Experimentation.

Food and water were withheld for 12 h before gastroscopy and gastric biopsy. The animals were anaesthetized with xylazine (1 mg/kg, *im*) and ketamine (10 mg/Kg, *im*). The biopsy procedure was performed with a flexible paediatric endoscope (2.3 mm diameter biopsy) and two samples of

gastric mucosa from pyloric antrum, corpus and fundus were collected. The mucosal aspect, presence of content, friability and bleeding at biopsy were evaluated at gastroscopy.

Gastric samples were fixed in Bouin's solution for 24 h, embedded in paraffin wax and processed routinely. Sections (3 µm) were stained with haematoxylin and eosin (HE) and Warthin-Starry (WS) method for histological evaluation and *Helicobacter* detection, respectively. For molecular analysis a further sample from each gastric region was placed into a tube DNase and RNase-free and frozen at -20°C until DNA extraction.

Histological analysis

Location and colonization density of *Helicobacter* were assessed. Colonization density was scored based in the number of bacteria per field (1000x magnification) as 0 (no infection); 1 (1 to 10 bacteria per field); 2 (11 to 30); 3 (31 to 50); and 4 (more than 50 bacteria). Histological changes were evaluated and scored as described elsewhere (20). Briefly, the number of lymphoid aggregates, the number of inflammatory cells and epithelial changes were evaluated. Gastritis was diagnosed when more than four inflammatory cells per field in the lamina propria were observed.

Polymerase Chain Reaction

Gastric biopsies were digested with 25 µl proteinase K (20 mg/ml) in 200 µl of lysis buffer pH 8.0 (100 mM NaCl, 100 mM EDTA, and 0.5% dodecyl sodium sulphate), agitated for 30 sec and then incubated at 56°C for 3 h. The DNA was extracted with a combination of phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate methods as described by Alfieri *et al.* (1). Sterile ultrapure water was included as negative control in all DNA extraction.

The genes targeted for *Helicobacter* genus and *Helicobacter* species, the primer sequences, the annealing temperatures, and the size (base pairs) of amplified fragments were described in a previous study (2). All gastric samples were tested for the species *H. pylori*, *H. heilmannii*, *H. felis*, and *H. bizzozeronii* or *H. salomonis*. PCR assay was

performed in a final volume of 25 μ l containing 1 μ l of extracted DNA, 0.4 mM of each deoxynucleotide, 0.5 μ l (20 pmol) of each primer, 1 \times PCR buffer (20 mM Tris HCl pH 8.4 and 50 mM KCl), 1.5 mM MgCl₂, 1.25 U *Platinum Taq* DNA polymerase (Invitrogen™ Life Technologies, USA), and ultra-pure water. A negative control was included in every amplification reaction. Amplifications were performed in a PTC-100™ thermocycler (MJ Research Inc, USA). The enzymes *Vsp*I (7) and *Hinf* I (4) (Life Technologies®) were

applied in the material amplified with specific pair primers, and the enzyme *Hinf* I in the species specific primers. Seventeen microliters from amplified material were added to 2 μ l of specific buffer and 1 μ l of enzyme, and maintained at 37 °C for 1 hr. Products from PCR and from enzymatic cleavage were analyzed by electrophoresis in 2% agarose gel with ethidium bromide in Tris-Borate-EDTA (TBE) buffer and visualized under UV light. The expected lengths products from enzymatic cleavage were disposed in Table 1.

Table 1. Species and expected length (in base pairs) from fragments according to the enzyme employed.

Species	Enzyme*	Expected length fragments
<i>Helicobacter</i> spp.	<i>Hinf</i> I	254 and 145 pb
	<i>Vsp</i> I	295 and 104 pb
<i>H. heimannii</i>	<i>Hinf</i> I	408 and 172 pb

* Invitrogen Life Technologies ®

Statistical Analysis

The association between colonization density, inflammatory infiltrate and *Helicobacter* infection in the gastric regions was evaluated by the Kruskal-Wallis test. The Spearman test was performed to verify the correlation between colonization density and inflammation (18). Significance was set at $p < 0.05$.

RESULTS

All animals showed a normal macroscopic aspect of esophagus mucosa, except in one *Leopardus tigrinus* that had no herring bone pattern at the distal segment of the esophagus. In three animals small patches of mucosal erythema and bilious fluid were observed in gastric mucosa. Plant fragments and hair coat were seen in the stomach of all animals.

Tightly coiled bacteria, 5 to 12 μ m long, were detected in 100% of the samples stained by WS method (Figure 1). The bacteria were seen isolate or in clusters in superficial mucus layer and gastric pits. Scores of bacterial colonization ranged from 1 to 4 (Table 2). Although scores 3 and 4 were more

frequently observed in the corpus region, there was no significant difference in colonization density between the gastric regions ($p = 0.54$).

Histological evaluation showed mild lymphocytic infiltrate in fundus. Inflammatory infiltrate is significantly higher in this region than in corpus and pyloric antrum ($p < 0.001$). Two animals showed no inflammatory cells in gastric mucosa even with high colonization density (Table 2). No association was found between bacterial colonization and mucosal inflammation ($p = 0.330$). Epithelial alterations were rarely seen and when present were sparse and mild in magnitude.

In three animals lymphoid follicles were observed in the lamina propria. The lymphocyte aggregates appeared small and fewer reactive. No more than one lymphoid follicle per sample was observed. The frequency and distribution of lymphoid follicles are summarized in Table 2.

Samples from all animals generated a 399 bp amplicon with *Helicobacter* genus reaction. Cleavage of the amplified products by genus specific primer (399 bp) using *Hinf* I and *Vsp* I enzymes resulted in fragments with 254 and 145 bp, and

with 295 and 104 bp in length, respectively (Figure 2). The reactions with species specific primers were positive only for *H. heilmannii*, represented by a 580 pb amplicon. Cleavage of

H. heilmannii amplicon with *Hinf* I enzyme resulted in fragments with 408 and 172 bp in length (Figure 3). No mixed infection was detected.

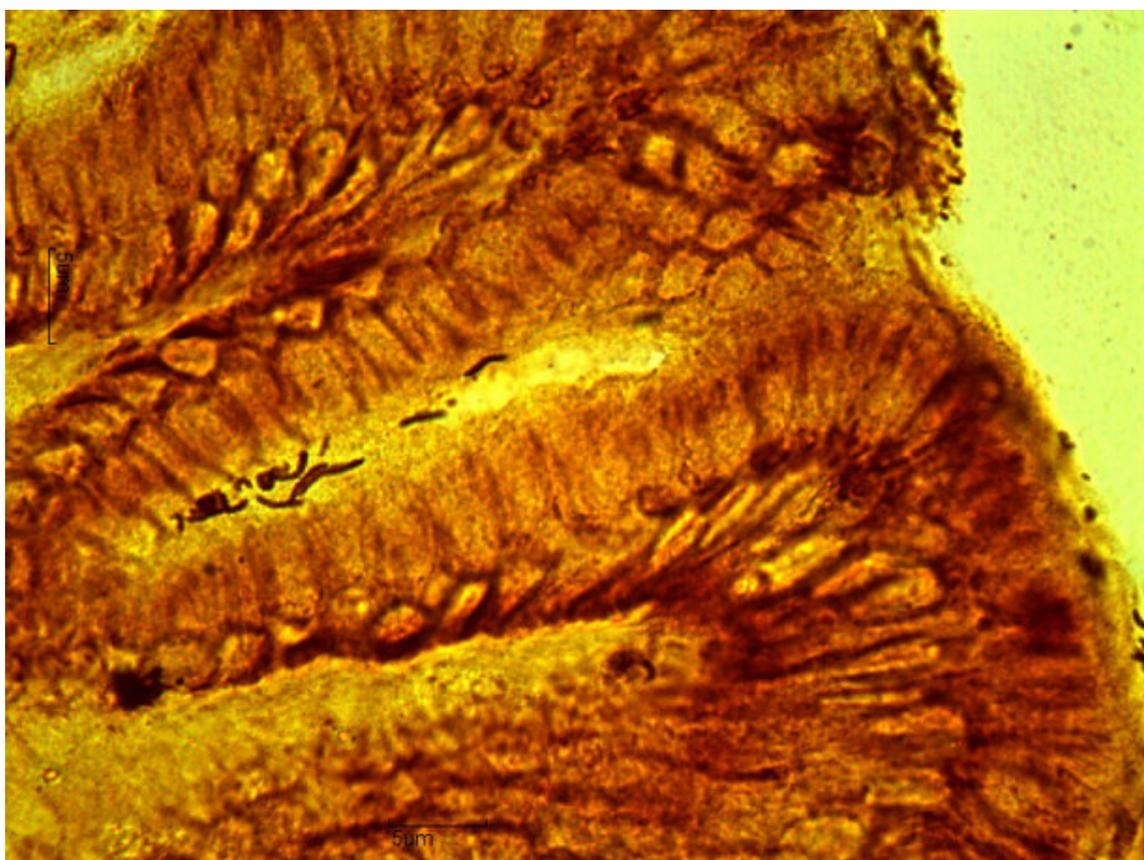


Figure 1. The gastric mucosa of a *Leopardus tigrinus*. *H. heilmannii* colonizing gastric pits. Warthin-Starry staining. Bar, 5 μ m.

Table 2. Scores of bacterial colonization and cellular inflammatory infiltrate observed in gastric mucosa samples of captive neotropical Brazilian feline.

Species	Animal	Bacterial and inflammatory cell infiltrate scores in gastric regions					
		Fundus		Corpus		Pyloric antrum	
		Bacterial	Cell infiltrate	Bacterial	Cell infiltrate	Bacterial	Cell infiltrate
<i>Leopardus tigrinus</i>	01	3*	1	4	0	2	0
	02	2	1	3	0	2	1
	03	2	1	3	0	4	0
	04	3*	1	3*	0	3	0
<i>Leopardus pardalis</i>	05	4	0	3	0	4	0
	06	1	0	4	0	1	0
	07	2	1	2	0	2	0
	08	3	1	2*	0	2	0
<i>Puma concolor</i>	09	2	1	2	0	3	0
	10	2	1	2	0	2	0

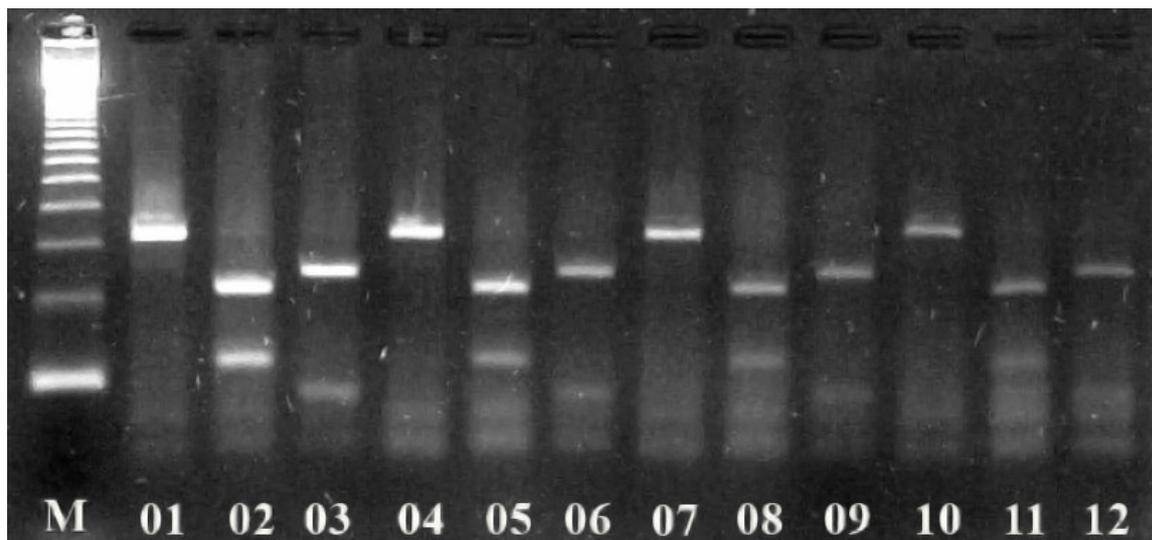


Figure 2. Ethidium bromide stained 2% agarose gel electrophoresis of restriction fragment length polymorphism (RFLP) with *Hinf* I and *Vsp* I enzymes of *Helicobacter* spp. genus amplicons. Lane M: 123 bp ladder (Invitrogen Life Technologies, USA); Lanes 1, 4, 7, and 10: *Helicobacter* spp. genus amplicons (399 bp) from captive neotropical Brazilian feline gastric mucosal samples; Lanes 2, 5, 8, and 11: *Helicobacter* spp. genus amplicons digested with *Hinf* I (254 and 145 bp). Lanes 3, 6, 9, and 12: *Helicobacter* spp. genus amplicons digested with *Vsp* I (295 and 104 bp).

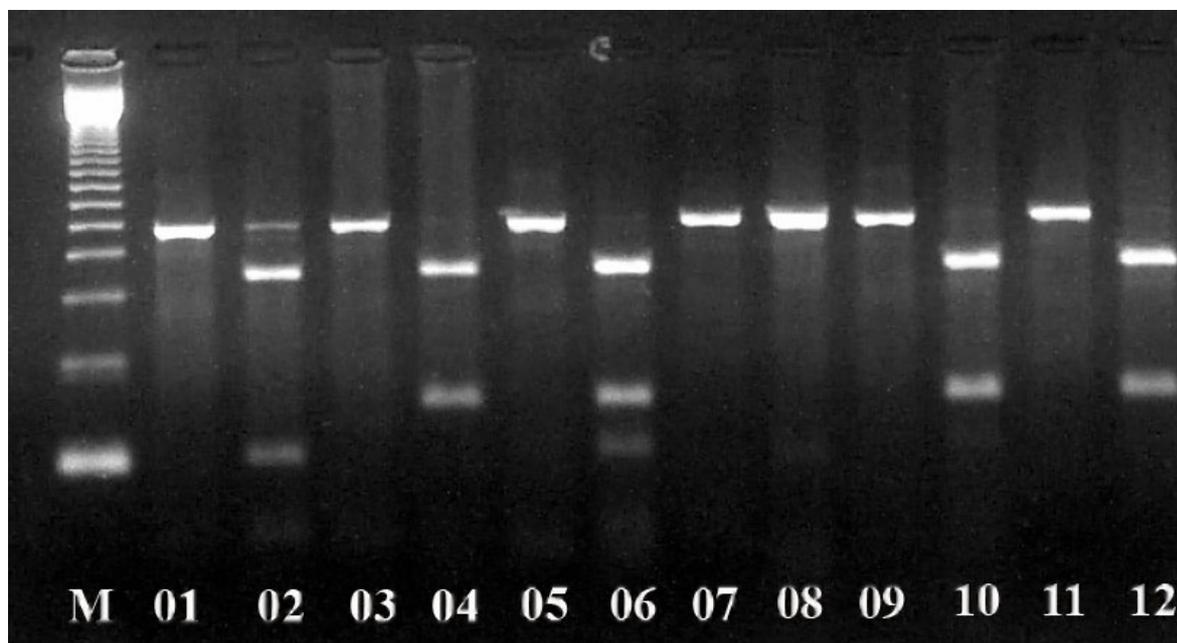


Figure 3. Ethidium bromide stained 2% agarose gel electrophoresis of restriction fragment length polymorphism (RFLP) with *Hinf* I enzyme of *Helicobacter heilmannii* amplicons. Lane M: 123 bp ladder (Invitrogen Life Technologies, USA); Lanes 1, 3, 5, 7, 9, and 11: *H. heilmannii* amplicons (580 bp) from captive neotropical Brazilian feline gastric mucosal samples; Lanes 2, 4, 6, 8, 10, and 12: RFLP of *H. heilmannii* amplicons digested with *Hinf* I (408 and 172 bp).

DISCUSSION

No previous studies of gastric endoscopic pattern in Brazilian neotropical feline were reported. The findings were compared to the patterns registered for domestic cats. On the endoscopic evaluation the most unexpected alteration was the absence of transversal rings observed upon distal esophagus in an adult *Leopardus tigrinus*. Others animals of the same species that were evaluated, showed a herring bone pattern. This pattern is typically seen in domestic cats, and is expected in *Felidae* family (21). The ingestion of grass and plants is considered a normal pattern between feline, and bilious reflux probably is related to pyloric relaxation mediate by anesthetic drugs (22). Mucosal erythema could be seen in domestic cats and dogs as a result of local blood flow variations in physiologic conditions (8). The endoscopic aspects observed were not associated with gastric disease, as animals presented normal behavior and activity level, good nutritional status and showed no alterations on physical examination. Furthermore, the feline had no previous signs of gastric disease, such as weight loss, variable appetite or vomit.

This study had characterized that Brazilian neotropical captive feline were commonly infected by *Helicobacter*, as free-ranging and captive cheetahs from South Africa and North America (14). Gastritis accompanied by vomit and weight loss was reported as a major cause of death in captive cheetahs, meanwhile the association with *Helicobacter* infection still controversial (23). Differently from cheetahs, captive *Leopardus tigrinus*, *L. pardalis* and *Puma color* showed no signs of gastric disease, supporting the hypotheses of commensal bacteria in these species.

In none mucosal sample bacteria resembling *H. pylori* or *H. acinonyx* were observed as previously reported (6, 19, 24). The bacterial morphology and distribution on the mucosa was similar to previously observed in Brazilian pet cats (20). The results of PCR and enzymatic cleavage confirmed the infection by *H. heilmannii* which was the most frequent species detected in cheetahs (23) and cats (16). Nevertheless, to the best of the authors' knowledge, this report represents the first cases of

Helicobacter infection in captive neotropical feline confirmed by PCR assay in Brazil.

Gastritis has been associated with *Helicobacter* infection in a variety of wild carnivores (9, 10). In the present study, mild lymphocytic infiltrate was frequently observed, mainly in the corpus region. However, no association between colonization density and the degree of inflammation was verified. Furthermore, mucosal samples with no sign of inflammation were taken from two infected animals. It has been suggested that gastric pathology develops when the bacteria acquire characteristics such as the *cag* pathogenicity island. The *cagA* gene is a marker for virulence factors in *H. pylori*, inducing a neutrophilic inflammation (15), however this genomic region was not identified in samples from cheetahs with gastritis (23). Although it is possible that other pathogenicity factors could be involved in *Helicobacter* spp. gastritis, it is more likely that *H. Heilmannii* in captive neotropical feline are commensals or opportunistic organisms.

The presence of lymphoid aggregates was reported as the main histological change associated with the *Helicobacter* spp. infection in semi or captive feline (10, 19). However, in our study small lymphoid follicles were seen in three animals, despite the presence of *Helicobacter* in all samples. These structures are normally seen on gastrointestinal tract, increasing in number and dimension in response to antigenic stimulation. Thus, apparently no relationship exists between the presence of bacteria and inflammatory infiltrate or lymphoid aggregates in wild feline.

Extrinsic factors, stress and genetic impoverishment had an important role in the pathogenesis of gastritis (23). The fact that captive feline had a greater occurrence of gastritis than wild animals (14) strengthen the effect of stress in this lesion. Our results had demonstrated that, differently from captive cheetahs, captive feline from the Atlantic Forest (São Paulo, Brazil) had no significant gastritis. Differences in conservation management and adaptability to environmental changes could explain the absence of gastric disease in this captive population.

In conclusion, the present study shows that captive

neotropical Brazilian feline are commonly infected by *H. heilmannii*. The infection was not associated with clinical signs, gross or histological changes, suggesting that these gastric bacteria are just commensals or occasional pathogens. Also, the practices employed to maintain the animals seem adequate to minimize stressful conditions, contributing to gastric mucosal homeostasis.

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