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Gastric *Helicobacter* species as a cause of feline gastric lymphoma: A viable hypothesis

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Abstract

Gastric *Helicobacter* spp. are associated with chronic inflammation and neoplastic transformation in humans as well as domestic and laboratory species. The present study examined the association of *Helicobacter heilmannii* (*Hhe*) infection in pet cats with feline gastric mucosa associated lymphoid tissue (MALT) lymphoma. Tissues were collected via gastric biopsy or at necropsy from 47 pet cats with clinical signs of gastrointestinal disease, including vomiting and inappetance, and classified as gastritis (14/47), lymphoma (31/37), or normal (2/47). Tissues positive for argyrophilic organisms with Warthin–Starry stain (29/47) were assessed by fluorescent in situ hybridization (FISH) for the presence of *Hhe* strains 1–4 as well as with a fifth probe that detected *Helicobacter salomonis*, *Helicobacter bizzozeronii*, or *Helicobacter felis*. A significant association of positive Warthin–Starry status with *Hhe* infection was found in cases of sick cats (22/29; $p < 0.05$ by Chi-square; $\chi^2 = 7.034$). Interestingly, a significant association between *Hhe* status and a diagnosis of lymphoblastic or lymphocytic lymphoma was observed as well in a subset of 24 Warthin–Starry positive lymphoma cases: of lymphoblastic lymphoma cases, 13/17 were positive for *Hhe* ($p < 0.05$; $\chi^2 = 4.854$). *Hhe* strains 2 and 4 were most commonly found (18/29 and 17/29, respectively) among sick cats, although a higher than expected number of cats was also positive for *Hhe*1, which initial reports have described as rare in cats and common in humans. The association found between a positive *Hhe* status with the presence of feline gastric lymphoma, especially lymphoblastic lymphoma, argues for the need to conduct prospective studies to better identify the frequency and strain distribution of *Hhe* infection in both healthy and clinically ill cats, particularly those cats with gastric lymphoma.

Keywords

Helicobacter heilmannii; Feline MALT lymphoma; Feline *Helicobacter* infection; *Hhe*; Gastric lymphoma; FISH

1. Introduction

Awareness of bacterial etiologies of gastric cancer is steadily increasing. In particular, gastric spiral organisms of the genus *Helicobacter* are emerging in association with both human and feline gastritis and gastric lymphoma. A 2001 study of 45 cats detected *Helicobacter* spp. in gastric tissues of 17; 9 of them were positive for *H. heilmannii* alone, 4/17 were positive for *H. felis*, 3/17 were positive for mixed *Helicobacter heilmannii* (*Hhe*) and *H. felis* infection and

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7/17 were positive for unclassified *Helicobacter* spp. *H. pylori* was not detected (Strauss-Ayali et al., 2001). In examining prevalence of *Helicobacter* spp. in pet cats, Neiger et al. (1998) found 91% of the cats in their study (38/49) were colonized by *Helicobacter* spp., 78% of which were found to be *H. heilmannii*.

Lymphoma remains the single most frequent type of cancer experienced by cats as well as the most common feline gastric neoplasm (Richter, 2003; Hartmann, 2006). Typically considered a disease of mature cats, gastrointestinal lymphoma comprises a very significant portion of overall disease and often has a worse prognosis than multicentric lymphoma. Primary gastric lymphoma of cats likely has a multifactorial etiology in which gastric infection with *Helicobacter* spp., especially *H. heilmannii*, may play a role.

Human studies have demonstrated a strong association between *H. pylori* infection and development of chronic inflammation and gastric cancer; indeed, epidemiological studies have shown that *H. pylori* may be an exacerbating or causative factor in 35–60% of human gastric cancer worldwide (Fox et al., 2006). While as much as 50% of the world population may be infected with *H. pylori*, less than 0.1% of that population will develop antibiotic-responsive MALT lymphoma. However, given the high prevalence of *H. pylori* infection as well as the potential for response and remission from *H. pylori*-negative and positive MALT lymphomas (Raderer et al., 2006), screening for and/or empiric antibiotic treatment of *H. pylori* has become standard practice in managing low-grade gastric MALT lymphoma in people. Infection with the gastric bacterium provides antigenic stimulation that can be sufficient to stimulate the lymphoproliferative processes of gastric MALT transformation. Current understanding of the disease process assumes that a neoplastic clone, usually with characteristics of marginal zone B cells, emerges from the background of bacterium-influenced gastritis, populates the MALT tissue, and displaces and eventually destroys the original follicles (Mueller et al., 2005). It is interesting to note that natural and experimental infection of *H. pylori* in cats induces a follicular gastritis, similar to the histology noted in humans, particularly children infected with *H. pylori* (Handt et al., 1995; Fox et al., 1995).

In addition to *H. pylori*, more recent studies have provided evidence that a second *Helicobacter* species may be playing a role in the development of gastric MALT lymphoma in humans. Morgner et al. (2000) were the first to describe five human cases of *Hhe*-associated low-grade MALT lymphoma for which elimination of infection resulted in cancer remission. More recently, Okiyama et al. (2005) reported a case study in which *Hhe* was found in the stomachs of six patients with MALT lymphoma; eradication of the bacterium resulted in remission of disease. In addition to colonizing human stomachs, the bacterium also colonizes the stomachs of domestic species, including cats, dogs, and swine (Priestnall et al., 2004; Hwang et al., 2002). Overlap of *Helicobacter* spp. among animal species and humans (Dieterich et al., 1998; Meining et al., 1998) suggests potential for zoonotic transmission; however, additional research is required before these assertions are validated.

Mouse models have reproduced the findings of chronic gastritis and potential for neoplastic transformation of gastric mucosa that characterize *Hhe* infection in humans. In a prospective study, O'Rourke et al. (2004) infected BALB/c mice with 10 different *Hhe* isolates of both human and animal origin as well as *H. pylori* and *H. felis*. Gastritis was detected 6 months after infection and low-grade MALT lymphoma developed in 25% of the infected animals. Interestingly, *Hhe* isolates obtained from the bobcat (one isolate) as well as mandrills (two isolates) and cynomolgus macaques (two isolates) resulted in the highest incidence of neoplastic transformation and most severe pathological responses. Nakamura et al. (2007) described a prospective study in which the authors infected C57BL/6 mice with *H. heilmannii* strain 1 obtained from a cynomolgus monkey. Within 24 months of inoculation, the mice developed gastric MALT lymphoma.

While the relationship between *Hhe* infection and gastric cancer in domestic species has not been fully explored, a preliminary study has been performed that supports a relationship between MALT lymphoma and *Helicobacter* spp. in cats. A 2001 study examined gastric biopsies from 72 cats (25 with gastric lymphoma, 23 with gastritis, and 24 normal stomachs). The authors reported argyrophilic organisms in 92% of the lymphoma cases, 78% of gastritis cases, and 52% of normal cases (Marini et al.). These findings suggest that an association may exist between the presence of *Helicobacter* spp. in cat stomachs and development of gastritis and/or gastric lymphoma.

The purpose of this retrospective study was to better characterize the strains of *H. heilmannii* that colonize the gastric mucosa of domestic cats as well as to examine the potential link between *H. heilmannii* infection and the incidence of feline gastric lymphoma. Our results suggest that this link may be stronger than heretofore realized.

2. Materials and methods

2.1. Tissue sources

Gastric tissue was collected from pet cats with clinical evidence of gastritis (vomiting, inappetence) or suspicion of neoplasia based on physical examination or history. Tissues were collected as full-thickness surgical biopsy, gastric biopsy with forceps, or at necropsy. A total of 47 cases from 1995 to 2004 from Angell Memorial Animal Hospital and Tufts University School of Veterinary Medicine were examined. Cases were selected on the basis of a histopathologic diagnosis of gastritis or gastric lymphoma; *Helicobacter* status was not known at the time of selection.

2.2. Tissue processing

Tissues were fixed in formalin, paraffin embedded, and processed routinely for subsequent staining with hematoxylin and eosin as well as Warthin–Starry stains. Any case that was Warthin–Starry positive for argyrophilic organisms was further evaluated for the presence of *H. heilmannii* by fluorescent in situ hybridization (FISH) for *Hhe* strains 1–4 as well as a probe specific for *Helicobacter salomonis*, *Helicobacter bizzozeronii*, and *Helicobacter felis* (Probe 5). Probes in this study were synthesized by Integrated DNA Technologies (Coralville, IA) and labeled with Cy3. Probe sequences derive from the 16S rRNA gene and were described by Trebesius et al. (2001) for use in human gastric biopsies and subsequently used by Priestnall et al. (2004) for cat samples (Table 1).

Briefly, gastric tissue was deparaffinized by serial passage through xylene, 100% ethanol, and 95% ethanol, and air-dried. The hybridization buffer (0.9 M NaCl, 100 mM Tris–HCl, 0.1% SDS, 30% formamide) with probe was preheated for 10 min at 74.5 °C; 50 µl of hybridization buffer containing 10 ng/ml of a particular probe was applied to each slide. Slides were covered in parafilm, protected from light, and placed in a humidification chamber overnight at 48 °C. They were rinsed gently in double-distilled water to remove the parafilm and serially washed in prewarmed rinsing buffers for 15 min each (Buffer 1: 0.9 M NaCl, 100 mM Tris–HCl, 0.01% SDS. Buffer 2: 0.9 M NaCl, 100 mM Tris–HCl.). Slides were rinsed again with double-distilled water, allowed to air dry, and then mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and coverslipped. Slides for each probe were examined under a Zeiss Axioskop 2 fluorescent microscope. A slide was designated as positive for a particular strain of *H. heilmannii* if the distinctive spiral organisms could be viewed as fluorescent under the Rhod filter.

2.3. Statistics

Two-sided Chi-square analysis was performed on the data. Results were viewed as significant when $p < 0.05$. Calculations were performed on GraphPad Prism 4.0.

3. Results

All diagnoses of gastritis or lymphoma were made by veterinary pathologists. Of 47 cases of cats biopsied or necropsies performed for suspected gastritis or neoplasia, 31 were confirmed as having gastric lymphoma (66%), 14 were diagnosed with gastritis (30%), and 2 had normal gastric mucosa (4%). Of all cases evaluated, 29 (62%) were positive for argyrophilic organisms by Warthin–Starry stain (Fig. 1). Table 2 displays the results of *H. heilmannii* infection by strain.

Of a total of 29 Warthin–Starry positive cases, 22 (76%) were positive by FISH for *H. heilmannii*, regardless of strain (representing 47% of all 47 cases reviewed), demonstrating a significant association between the presence of the organism and disease status in Warthin–Starry positive animals ($p < 0.05$ by Chi-square analysis; $\chi^2 = 7.034$) (Fig. 1). Additional *Helicobacter* species other than *Hhe* and *H. pylori* were detected with Probe 5 and represented 8/29 cases (27% of W–S positive slides and 17% of all 47 cases reviewed).

One of 29 Warthin–Starry positive cats was positive for Probe 5 only, representing 3% of that cohort and 2% of all cases. No significant association was found between individual strains of *Hhe* and presence of lymphoma or gastritis. Of those cats colonized with *Hhe*, all but one cat were colonized by multiple strains of the bacterium, most often strains *Hhe2* and *Hhe4* (70 and 65% of Warthin–Starry positive cases of lymphoma reviewed). The cat identified as having only one strain of the five considered was positive for the non-*heilmannii* *Helicobacter* group. Of the 10 cases with Warthin–Starry associated gastritis and lymphoma reviewed for *H. pylori*, none was positive (data not shown.)

A subset of 24 gastric lymphoma cases were evaluated and diagnosed histologically as lymphocytic or lymphoblastic by veterinary pathologists; 17 were categorized as lymphoblastic (71%) and 7 as lymphocytic (Fig. 2). Sixteen of these 24 cats were Warthin–Starry positive and were included in the evaluation for the presence of *H. heilmannii* and other *Helicobacter* spp. (Table 3). The presence of *Hhe* species was significantly associated with lymphoblastic lymphoma ($p < 0.05$; $\chi^2 = 4.854$). It is important to note that while the distinction between MALT lymphoma and severe follicular gastritis is a difficult and controversial one, the feline lymphomas described in the current study meet the histological criteria for MALT lymphoma as defined in the human literature (El-Zimaity et al., 2005; Ruskone-Fourmes-traux et al., 2001). These criteria include descriptions of low-grade lesions with diffuse populations of centrocyte-like cells, tissue destruction, and formation of lymphoepithelial lesions. Additional criteria include accumulation of plasma cells in the upper mucosa, associated with more extensive infiltrates with an increased incidence of malignancy. High-grade MALT lymphoma tends to consist of a diffuse proliferation of larger lymphoid cells with large vesicular nuclei and one or more nucleoli. As described by El-Zimaity et al. (2005), it is important to note that low-grade MALT lymphomas are capable of transforming into high-grade, large-cell MALT lymphoma.

4. Discussion

The results of our study found that *Hhe2* and *Hhe4* were two of the most common strains of *Hhe* colonizing cats with gastritis and gastric lymphoma. *Hhe4* was found to occur in 59% of all our Warthin–Starry positive cases reviewed and in 65% of all lymphomas with spiral bacteria reviewed. These results are consistent with the results of a previous study that reported

that *Hhe4* is one of the most commonly found strains of *H. heilmannii* in domestic cats (Priestnall et al., 2004). Additionally, we found *Hhe2* occurring with a similar frequency: this strain was found in 62% of Warthin–Starry positive cats in the study and in 70% of all Warthin–Starry lymphomas. *Hhe3* occurred with an overall frequency of 21% in Warthin–Starry positive cases and was present in 30% of all lymphoma cases. Non-*Hhe* strains were also represented in the data, with 27% of all argyrophilic-positive cats infected with the *H. salomonis*, *H. bizzozeronii*, or *H. felis* group (40% of all such lymphoma cases). In a study by Trebesius et al. (2001), *Hhe1* was found in 66/84 (75%) of human cases of nonulcer dyspepsia; they concluded that *Hhe1* is likely the most prevalent strain infecting humans with gastritis. While more studies need to be done to expand upon this study, it is nonetheless interesting that the present study found that *Hhe1* occurred in cats with a higher frequency than previous studies have suggested: 4 of 29 animals (14%) with Warthin–Starry positive gastritis or lymphoma were positive for the organism, while 20% (4/20) of our lymphoma cases were colonized by this strain. These results differ from those obtained by Priestnall et al. (2004), who concluded that *Hhe1* is rarely found in the cat; their data suggested that this species is unlikely to be a reservoir for this strain of the bacterium. It is possible, however, that case selection for sick cats may have introduced some bias into the results described here; prospective studies with healthy control animals are needed to further explore the question of cats as a potential zoonotic reservoir for *Hhe1*.

Helicobacter species are increasingly linked to the development of gastric disease in many species. While the question of whether *Helicobacter* spp. find lymphomatous gastric mucosa permissive for colonization requires further exploration with prospective studies, studies exist in the human literature that demonstrate a causative role for *Helicobacter* spp. in development of gastric MALT lymphoma (Hsu et al., 2007; Fox et al., 2006). Additionally, at least five other reports of animal studies have demonstrated associations between infection with *Helicobacter* spp. and gastric MALT lymphoma. Erdman et al. (1997) described four ferrets with *Helicobacter mustelae*-associated gastric MALT lymphoma, two of which were high-grade and two low-grade cancers. Enno et al. (1995) as well as Mueller et al. (2005) reported *H. felis*-associated MALT lymphoma in BALB/c mice while Nakamura et al. (2007) and O'Rourke et al. (2004) both described development of gastric MALT lymphoma in laboratory mice inoculated with different strains of *Hhe*. The study by Nakamura examined *Hhe1* isolated from a cynomolgus monkey and is particularly interesting with regard to *Hhe1* and its zoonotic potential. Trebesius et al. (2001) concluded that *Hhe1* is more commonly found in humans, which raises intriguing possibilities with regard to zoonotic transmission and potential virulence of varying strains of the bacterium. While *Hhe1* does not appear to occur with the same frequency as *Hhe2* and *Hhe4* in the domestic cat, given the higher than expected frequency of *Hhe1* found in our study, we submit that zoonotic transmission could occur. Furthermore, other gastric *Helicobacters* strains in cats may also be zoonotically transmitted to humans (Dieterich et al., 1998; Meining et al., 1998).

Previous studies on the role of *Helicobacter*-induced inflammation in B-cell proliferation, transformation, and tumorigenesis in feline MALT lymphoma have been conducted: Esteves et al. (2000) detected gastritis as early as 8 months in cats naturally infected with *H. pylori*; although no ulcers developed, gastric lesions worsened with age and demonstrated preneoplastic changes. Immunophenotypic analysis of older infected cats revealed that lymphocytes within lymphoid nodules and follicles were predominantly CD45+ B cells, whereas lymphocytic infiltrates of lamina propria and mucosal epithelium were CD3+ T cells. Similarly, a study by Simpson et al. (2000) noted lymphoid follicular hyperplasia and mild gastritis develop in cats experimentally infected with *H. felis*.

A study by Peterson et al. (2001) demonstrated that the host immune system not only charts the development of disease, it also plays a substantial role in development of *Hhe*-associated gastritis and lymphoproliferative lesions in BALB/c mice. Indeed, the study concluded that

CD4 immunocompetence is integral in development of gastric inflammation, mucosal hypertrophy and gastric nodular hyperplasia, all of which may represent precursor steps to the development of MALT lymphoma. Further exploration of the role of the immune system in gastric tumor formation was also done by Mueller et al. (2005), who performed immunotyping of *H. felis*-induced MALT lymphoma in BALB/c mice and saw that accessory infiltrating CD4⁺ T cells expressed CD28, CD69, and IL-4 but not INF- γ , suggesting that the tumor B-cell population was driven by immunocompetent Th2- polarized T cells. It is interesting that the current study found that 14 of a subset of 16 Warthin–Starry positive lymphoma cases (87%) were described as lymphoblastic. O'Rourke et al. (2004) found a B-cell immunotype in the MALT lymphoma that developed in *Hhe*-infected BALB/c mice; however, immunologic labeling for B or T cells was not done in this study. Therefore, we can only speculate that the predominance of lymphoblastic lymphoma in our Warthin–Starry positive, *Hhe*-positive cases are of B-cell origin. A study by Mueller et al. (2005) demonstrated a proliferation of marginal zone B cells with coincidental MALT lymphogenesis in BALB/c mice infected with *H. felis*. Treatment for 2 weeks with bismuth, metronidazole, and tetracycline was administered, with histologic resolution effected in 75% of the cases. Transcriptional profiling revealed the presence of residual B cells in gastric tissue. Reinfection with *H. felis* resulted in a rapid reemergence of aggressive MALT lymphoma. Immunoprofiling of the tumor cells showed them to be IgM^{high}, B220⁺, CD40^{low}, CD79^{α+} B cells.

Despite the role that *Helicobacter* spp. may play in the development of feline lymphoma, a plethora of other infectious and environmental factors in addition to *Helicobacter* infection exist. One such factor includes the lentivirus FIV; the virus's role in feline lymphoma may be either direct or indirect, depending in part on the integration of provirus into the genome; however, its overall prevalence in cats with lymphoma is still low in the United States (Hartmann, 2006). Studies have shown the percentage of FIV-infected cats that had lymphoma range from 0 to 15% (Milner et al., 2005), while a study from Australia reported that 50% of cats with lymphoma were also FIV-infected. The reason for this is unknown, although it may be due to a higher prevalence of the disease in Australia (Gabor et al., 2001). Feline leukemia virus (FeLV), a γ -retrovirus, has been historically considered responsible for up to 75% of feline lymphomas, with infection rates estimated at 1–8% of the feline population. In recent years, however, there is clear evidence that the prevalence of the disease has declined markedly; recent reports from Tufts Veterinary Diagnostic Laboratory indicate a decline from 8% positivity from practitioner-submitted tests in 1989 to 4% in 1995 (Hartmann, 2006). Mediastinal lymphomas are very often associated with FeLV and typically affect cats at a younger age (Milner et al., 2005), whereas gastrointestinal lymphomas are far more variable in their association with FeLV. Despite the declining prevalence in the disease, new evidence is emerging that suggests that tumors negative for FeLV antigen in serologically negative cats may be PCR positive for viral DNA. Although FeLV infection may be less of a primary cause in development of gastrointestinal lymphoma, it may orchestrate a pre-disposition for lymphocytic cell transformation that acts in synergy with other infectious agents such as *Helicobacter* or other potentially carcinogenic factors to lower the threshold for neoplastic development.

The results described above provide an association between *H. heilmannii* and the development of feline gastritis and gastric lymphoma. The study by Marini et al. began the exploration of this connection in cats; the current study expands upon it and strengthens that association. Additionally, given the association of presence of *Helicobacter* spp. infection with concurrent neoplastic disease demonstrated in this study in conjunction with reports of resolution of antibiotic-treated gastric MALT lymphoma in *H. pylori* and *Hhe*-infected humans, we hypothesize that a subset of cases of feline gastric lymphoma may respond to eradication of gastric *Helicobacter* spp. with regression of the neoplasm comparable to that described in humans. Studies conducted with this hypothesis in mind would undoubtedly provide insight

into the role of *Hhe*-induced lymphoma in cats and help to better define the role of gastric *Helicobacters* as causative, exacerbating, or coincidental factor in the genesis of feline gastritis and gastric lymphoma.

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Fig. 1.

The image on the left depicts a Warthin–Starry positive stain of gastric tissue positive for *Helicobacter heilmannii* infection. The image on the right is from the same cat and shows a section of gastric mucosa positive for *Hhe4* as detected by FISH. Inset: the distinctive, tight spiral of *Hhe4* in the gastric pits is clearly visible in red. This cat had been diagnosed with gastritis by veterinary pathologists.

Fig. 2.

Lymphomas were of two major morphologic types, lymphoblastic (10× and 40×, upper left and right) and lymphocytic (10× and 40×, lower left and right). 87% of lymphoblastic lymphomas were positive for *Hhe* organisms, while the percentage positivity for lymphocytic lymphoma was 12%.

Table 1Probes used in FISH assay to determine *Hhe* status (Trebesius et al., 2001)

<i>Hhe1</i>	5'-CCC-ACA-CTC-CAG-AAG-RAT-AG-3'
<i>Hhe2</i>	5'-CCC-ACA-CTC-TAG-GGT-KGC-AG-3'
<i>Hhe3</i>	5'-CCC-ACA-CTC-TAG-AAA-GAT-AG-3'
<i>Hhe4</i>	5'-CCC-ATC-TAG-CTT-GCC-ACT-CCG-3'
Probe 5	5'-CCC-ACA-CTC-CAG-AGT-TGT-AG-3'

Table 2
Lymphoma and gastritis in cats infected with *Helicobacter* spp.

	W-S positive	Hhe1 positive	Hhe2 positive	Hhe3 positive	Hhe4 positive	Probe 5 ^a positive
Total cases reviewed: 47	29/47 (62%)	4/29 (14%)	18/29 (62%)	6/29 (21%)	17/29 (59%)	8/29 (27%)
Lymphoma: 31 cases	20/31 (64%)	4/20 (20%)	14/20 (70%)	6/20 (30%)	13/20 (65%)	8/20 (40%)
Gastritis: 14 cases	9/14 (64%)	0/9	4/9 (44%)	0/9	4/9 (44%)	0
Normal stomach: 2 cases	0/2	0	0	0	0	0

^aProbe 5 detects *H. salomonis*, *H. felis*, and *H. bizzozeronii*.

Table 3
Association of *H. heilmannii* and lymphoblastic lymphoma in cats

	W-S positive	Hhe1 positive	Hhe2 positive	Hhe3 positive	Hhe4 positive	Probe 5 positive
Subset of LSA cases considered: 24	16/24 (68%)	4/16 (25%)	12/16 (75%)	6/16 (37%)	12/16 (75%)	6/16 (37%)
Lymphoblastic LSA: 17/24 (71%)	14/16 (87%)	3/14 (21%)	11/14 (78%)	5/14 (36%)	11/14 (78%)	4/14 (28%)
Lymphocytic LSA: 7/24 (29%)	2/16 (12%)	1/14 (7%)	1/14 (7%)	1/14 (7%)	1/14 (7%)	2/14 (14%)