Abstract: Chronic gastritis causes significant morbidity and mortality in captive cheetahs but is rare in wild cheetahs despite colonization by abundant spiral bacteria. This research aimed to identify the Helicobacter species that were associated with gastritis in captive cheetahs but are apparently commensal in wild cheetahs. Helicobacter species were characterized by PCR amplification and sequencing of the 16s rRNA, urease, and cagA genes and by transmission electron microscopy of frozen or formalin-fixed paraffin-embedded gastric samples from 33 cheetahs infected with Helicobacter organisms (10 wild without gastritis and 23 captive with gastritis). Samples were screened for mixed infections by denaturant gel gradient electrophoresis of the 16s rRNA gene and by transmission electron microscopy. There was no association between Helicobacter infection and the presence or severity of gastritis. Eight cheetahs had 16s rRNA sequences that were most similar (98 to 99%) to H. pylori. Twenty-five cheetahs had sequences that were most similar (97 to 99%) to "H. heilmannii" or H. felis. No cheetahs had mixed infections. The ultrastructural morphology of all bacteria was most consistent with "H. heilmannii," even when 16s rRNA sequences were H. pylori-like. The urease gene from H. pylori-like bacteria could not be amplified with primers for either "H. heilmannii" or H. pylori urease, suggesting that this bacteria is neither H. pylori nor "H. heilmannii." The cagA gene was not identified in any case. These findings question a direct role for Helicobacter infection in the pathogenesis of gastritis and support the premise that host factors account for the differences in disease between captive and wild cheetah populations.
Comparison of *Helicobacter* spp. in Cheetahs (*Acinonyx jubatus*) with and without Gastritis

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Chronic gastritis causes significant morbidity and mortality in captive cheetahs but is rare in wild cheetahs despite colonization by abundant spiral bacteria. This research aimed to identify the *Helicobacter* species that were associated with gastritis in captive cheetahs but are apparently commensal in wild cheetahs. *Helicobacter* species were characterized by PCR amplification and sequencing of the 16S rRNA, urease, and cagA genes and by transmission electron microscopy of frozen or formalin-fixed paraffin-embedded gastric samples from 33 cheetahs infected with *Helicobacter* organisms (10 wild without gastritis and 23 captive with gastritis). Samples were screened for mixed infections by denaturant gel gradient electrophoresis of the 16S rRNA gene and by transmission electron microscopy. There was no association between *Helicobacter* infection and the presence or severity of gastritis. Eight cheetahs had 16S rRNA sequences that were most similar (98 to 99%) to *H. pylori*. Twenty-five cheetahs had sequences that were most similar (97 to 99%) to “*H. heilmannii*” or “*H. felis*.” No cheetahs had mixed infections. The ultrastructural morphology of all bacteria was most consistent with “*H. heilmannii*,” even when 16S rRNA sequences were *H. pylori*-like. The urease gene from *H. pylori*-like bacteria could not be amplified with primers for either “*H. heilmannii*” or *H. pylori* urease, suggesting that this bacteria is neither *H. pylori* nor “*H. heilmannii*.” The cagA gene was not identified in any case. These findings question a direct role for *Helicobacter* infection in the pathogenesis of gastritis and support the premise that host factors account for the differences in disease between captive and wild cheetah populations.

Since the initial isolation of *Helicobacter pylori* from humans and its association with gastritis and peptic ulceration (23), *Helicobacter* spp. have been isolated from an ever-expanding range of host species (38). While *Helicobacter pylori* infection in humans has been associated with chronic gastritis, peptic ulceration, gastric adenocarcinoma, and lymphoma (23, 31, 32), *Helicobacter* pathogenicity in many species is less clear.

Worldwide, the majority of captive cheetahs (*Acinonyx jubatus*) have a progressive gastritis that causes vomiting, weight loss, and failure to thrive and is associated with *Helicobacter* infection (10, 25, 26). Moderate to severe gastritis was present in greater than 70% of cheetahs that have died since 1995 within the North American captive population. Many cheetahs develop systemic amyloidosis (type AA) secondary to gastritis that results in renal failure, a leading cause of death among captive cheetahs (30). Within the South African captive cheetah population, gastritis was a major cause of death or the reason for euthanasia in 69% of cheetahs (26).

In 1992, genetic and morphological analysis of spiral bacteria in cheetahs from a single captive facility identified a novel species, *Helicobacter acinonychis* (9). In these cheetahs, a second nonculturable spiral bacterium morphologically similar to “*H. heilmannii*” was identified by electron microscopy in some cases (11). However, there was no difference between the severity of gastritis in cheetahs colonized with *H. acinonychis*, “*H. heilmannii*,” or and coinfections.

Since the initial isolation of *H. acinonychis*, culture attempts have been unsuccessful from many cheetahs despite the presence of spiral bacteria histologically (K. Eaton, personal communication). This suggests that *H. acinonychis* may not be the most common *Helicobacter* sp. infecting captive cheetahs and that there may be additional unculturable species of *Helicobacter* important in the development of gastritis. Interestingly, gastritis is rare in wild cheetahs despite the presence of abundant spiral bacteria (L. Munson, unpublished data). Therefore, this study aimed to identify the *Helicobacter* spp. associated with gastritis in captive cheetahs and compare them with the apparent commensal organisms in wild cheetahs in order to understand the role of *Helicobacter* spp. in the pathogenesis of gastritis in this species. Furthermore, this study aimed to compare *Helicobacter* spp. within and among facilities to investigate whether geographic location determined bacterial type and could explain differences in gastritis severity within the captive cheetah population.

MATERIALS AND METHODS

**Animals.** Gastric samples from 33 cheetahs, 10 wild and 23 captive, that were infected with *Helicobacter* organisms and housed in different facilities and had different severities of gastritis were selected for this study. Wild cheetahs were located in south central Namibia, and samples were obtained opportunistically at necropsy (5 of 10) or by endoscopy (5 of 10) under general anesthesia. None (0 of 10) of the wild cheetahs had any histological evidence of gastritis. Gastric samples were obtained from captive cheetahs at necropsy (3 of 23) or by endoscopic biopsy (20 of 23) under general anesthesia during routine annual examination as part of the health surveillance program of the American Zoo and...
E. coli 16S rRNA sequences were amplified from gastric samples using the 8y F (AGAGTTTGATCCTGGCTCAG) and 8y R (1512–1492 CCGGGTTACCTTGTTACGACTT) primers (244f and 528r from Table 1). A GC clamp was attached to the 5′ end of the primer sequences most similar to H. hepaticus and E. coli (Table 2). Samples were screened for the urease gene was performed with primer sets specific for known sequences of H. pylori (clinical isolate 87A300, California State Health Department) and H. felis (ATCC 49179), DNA extracted from a cheetah isolate most similar to “H. heilmannii,” as well as a mixture of DNA from these three isolates. DNA was amplified along with cheetah samples and included DNA isolated from pure cultures of H. pylori (clinical isolate 87A300, California State Health Department) and H. felis (ATCC 49179), DNA extracted from a cheetah isolate most similar to “H. heilmannii,” as well as a mixture of DNA from these three isolates. DNA was stained with a fluorescent nucleic acid stain (GelStar nucleic acid gel stain, Cambrex, Rockland, Maine) and examined under UV light.

**RESULTS**

**Ultrastructural morphology.** Long, tightly coiled bacteria were present within the gastric glands and parietal cell canaliculi in all cases examined. The ultrastructural morphology of bacteria in all cheetahs was similar irrespective of the severity of gastritis (Fig. 1). Bacterial size and shape were more consistent with “H. heilmannii” than H. pylori. The bacteria with the 16S rRNA sequences most similar to H. pylori were helical, 4 to 6.4 by 0.25 to 0.54 μm with three to four polar flagella. Bacteria with 16S rRNA sequences most similar to “H. heilmannii” were helical, 3.4 to 7.9 by 0.35 to 0.64 μm with three to six polar flagella. There was no difference in the overall measurements between bacteria with different 16S rRNA sequences (P = 0.344). Mixed infections with bacteria exhibiting...
different morphological characteristics were not detected. In most cases, three to five polar flagella could be visualized along at least one pole. Periplasmic fibrils were not identified in any of the cases.

**16S rRNA, urease, and cagA gene analyses.** The 16S rRNA sequences were amplified by PCR from the stomachs of all 33 cheetahs. Sequences represented nearly the entire 16S rRNA gene with 1,188 to 1,427 bp (77 to 92% of the 16S rRNA gene) of readable sequence determined from bacterial DNA isolated from cheetahs. On the basis of these sequences, all 33 cheetahs were infected with bacteria that were consistent with the genus *Helicobacter*. The *Helicobacter* spp. infecting the cheetahs clustered into three groups irrespective of severity of gastritis (Fig. 2). Bacteria with identical sequences were present in cheetahs with and without gastritis. Bacterial types varied within and among captive facilities. Two facilities had multiple types of bacteria present within their population. Three facilities housing more than one cheetah had only a single type of bacteria.

### Table 2. Oligonucleotide primers used for amplification of the urease gene in *Helicobacter* isolates from cheetahs

<table>
<thead>
<tr>
<th>Species specificity</th>
<th>Primer$^a$</th>
<th>Sequence (5' to 3')</th>
<th>Size of expected product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;H. heilmannii&quot;</td>
<td>HhU3351F</td>
<td>CTATCAACTGCGGTTGCGAC</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>HhU3481R</td>
<td>TCGCCATAAGTGCGAGTCGAC</td>
<td>403</td>
</tr>
<tr>
<td></td>
<td>HhU3351F</td>
<td>CTATCAACTGCGGTTGCGAC</td>
<td>115</td>
</tr>
<tr>
<td>H. pylori</td>
<td>HpU1286F</td>
<td>ACGCAACATGCTACCTCG</td>
<td>297</td>
</tr>
<tr>
<td></td>
<td>HpU1400R</td>
<td>CTTGCATCGTTAGAAAGCTTA</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>HpU1204F</td>
<td>TCCCCCACAACACTCCCTAC</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>HpU1501R</td>
<td>TGTCGCAACATCTAACGC</td>
<td>115</td>
</tr>
</tbody>
</table>

$^a$ F and R designate forward and reverse primers, respectively.

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**FIG. 1.** Gastric histopathology, demonstrating the absence of gastritis in wild cheetahs (A and C) and severe lymphoplasmacytic gastritis with glandular destruction in captive cheetahs (B and D) infected with similar bacteria. Insets in each panel demonstrate the typical ultrastructural characteristics of the bacteria infecting that cheetah. The cheetahs in panels A and B were infected with *H. pylori*-like (based on 16S rRNA sequence) bacteria, while those in panels C and D were infected with "*H. heilmannii*"-like bacteria. In the large panels (hematoxylin and eosin stain), the bar equals 20 μm. In the insets (transmission electron microscopy), the bar equals 0.5 μm.
sequences resembled those that were morphologically indistinguishable, the 16S rRNA gene was most similar (98 to 99%) to sequences from eight cheetahs (five wild and three captive) were most similar (97 to 98%) to sequences previously isolated from cheetahs (9). Twenty-five cheetahs with bacterial 16S rRNA sequences most similar to *H. pylori* (U51870) were most similar (98%) to each other, while sequences from captive cheetahs were 97 to 99% similar to each other.

Despite the previous isolation of small numbers of coinfections in cheetahs (11), no coinfections were identified in this study either by electron microscopy or by denaturant gradient gel electrophoresis analysis of a 16S rRNA gene fragment. In addition to the strains identified in the current study, *H. acinonychis* was previously isolated from captive cheetahs at one facility (9, 10). Taken together, these data suggest that at least four different helicobacters are present in the stomachs of captive cheetahs with gastritis. Similar strains are apparently commensal organisms in wild cheetahs, bringing into question the role of specific *Helicobacter* spp. in the pathogenesis of gastritis in cheetahs.

Despite the previous isolation of *H. acinonychis* and naming of this organism after the cheetah, none of the cheetahs sampled in this study were infected by this *Helicobacter* spp. This observation is consistent with the negative results of more recent culture attempts from other cheetahs (K. Eaton, personal communication). It is possible that *H. acinonychis* was not representative of the *Helicobacter* spp. infecting the cheetahs.
H. acinonychis has been isolated from other species of exotic felids and may have been transmitted to other cheetahs in the previously studied collection (4, 35). Alternatively, these bacteria may have historically been more prevalent in cheetahs but become less so due to selective breeding efforts in captive institutions and maternal transmission of other Helicobacter spp. (15).

It is unlikely that the organisms with 16S rRNA sequences most similar to H. pylori truly represent H. pylori, as urease could not be detected with primers specific for conserved regions of the H. pylori urease gene. It is presumed that these organisms have urease genes, given their colonization of the gastric microenvironment. However, the urease sequence of these bacteria is likely different from that of either H. pylori or "H. heilmannii." Discrepancies between genetic and morphological characteristics further complicate the appropriate classification of these organisms in cheetahs. While H. pylori has been shown to assume the morphology of "H. heilmannii" under certain culture conditions (12), this phenomenon has not been reported in vivo.

Organisms have been characterized in this study as H. pylori-like solely on the basis of the 16S rRNA sequences, which may not be the most accurate method of classification (19, 41). Despite proposals to utilize the 23S rRNA subunit (16, 18) or the urease gene (5), the 16S rRNA gene sequence currently remains the standard gene for classification of Helicobacter spp. (7). Because most of the bacteria in cheetahs are currently unculturable, information on the biochemical characteristics was unavailable. These results suggest that these H. pylori-like organisms may represent a distinct species. In some of the cheetahs with Helicobacter most similar to "H. heilmannii" or H. felis, the 16S rRNA sequences were phylogenetically equidistant from both of these organisms. Because the ultrastructural morphology of these organisms can be indistinguishable (8), many of these bacteria might be better classified as belonging to the H. felis-like clade of gastrospirilla.

In other species, the pathogenesis of Helicobacter gastritis is dependent on bacterial as well as host factors. It has been suggested that disease develops when either the host gastric microenvironment is altered or the bacteria acquire characteristics, such as the cag pathogenicity island, that may be evolutionarily beneficial to the bacteria (3). The cagA gene, a marker for virulence factors important in the induction of neutrophilic inflammation (33), could not be identified in any of the cheetah samples analyzed. This result was not surprising because neutrophils are an uncommon feature of gastritis in cheetahs (10, 25). Although it is possible that other, not yet identified, pathogenicity factors are present in the Helicobacter spp. associated with gastritis in cheetahs, it is more likely that host factors are responsible for the disparity in disease occurrence between captive and wild cheetahs.

Differences in occurrence and intensity of inflammation may be due to host genotypic differences (22, 24, 42). Cheetahs are homogenic for major histocompatibility complex (MHC) genes, a characteristic that has been proposed as an explanation for their unique susceptibility to some infectious diseases (28, 29). However, homogeneity is a feature of both captive and wild cheetah populations (28), yet only captive cheetahs commonly develop gastritis. Additionally, the founders of the captive population originated from the same region of Africa as the wild population in this study. The contributions of both MHC and non-MHC genes appear to influence the degree of inflammation in MHC-congenic mice infected with H. felis, as do polymorphisms in genes encoding inflammatory mediators in humans (24, 40). Therefore, genotypic differences in MHC are not likely the basis for the occurrence of inflammatory reactions only in captive cheetahs. Investigation of polymorphisms in other genes potentially important in the development of gastritis is warranted.

Another theory to explain inflammatory reactions to similar Helicobacter types is modulation of the host inflammatory response to Helicobacter spp. by enteric helminth infections (13). It is possible that enteric parasite infections in the wild cheetahs reduced the inflammatory response, whereas captive cats that receive regular anthelminthic medication as part of their routine health care lack this suppressive effect. However, of the five wild cheetahs from which samples were obtained at necropsy, only two animals had documented enteric cestode or nematode infections. Additionally, other species of captive and domesticated felids that receive anthelminthic treatment commonly have minimal to no inflammation associated with Helicobacter infections (20, 21, 27). These findings suggest that the gastric inflammatory reaction that occurs solely in captive cheetahs is likely due to aspects of captivity other than the absence of helmint infections.

Environmental differences between captive and wild cheetahs are almost certainly important in the development of gastritis. Diet alone is not likely the cause of gastritis, as captive cheetahs in South African facilities are fed a diet closely resembling that of wild cheetahs and yet gastritis is prevalent within this population (26). Cheetahs in the wild are generally spared many of the diseases afflicting captive cheetahs worldwide (L. Munson, unpublished data), suggesting that cheetahs are maladapted to some as yet unknown aspect of the captive environment. This maladaptation is evidenced by increased adrenocortical function in captive but not wild cheetahs (39). Because of the immunomodulatory affects of the glucocorticoids, it is possible that captive cheetahs have an altered systemic or local immune response that accounts for their reaction to otherwise commensal bacteria (2, 6, 34). This hypothesis would also explain the presence of gastritis in captive cheetahs infected with apparently different organisms. Ongoing research characterizing gastric cytokine profiles in captive and wild cheetahs aims to determine if elevated corticosteroids are affecting the local gastric immune response.

In summary, based on 16S rRNA sequences, urease sequences, and ultrastructural characteristics, multiple types of Helicobacter were identified in captive cheetahs with gastritis. Similar organisms were present in cheetahs with and without gastritis, suggesting that host factors are more important than bacteria in the pathogenesis of gastritis in cheetahs. The distinct differences in the occurrence of gastritis in captive and wild cheetahs, despite infection with similar Helicobacter organisms, provides an interesting natural disease model for analysis of host factors important in the development of gastritis.

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