Association of *Helicobacter* with Cholangiohepatitis in Cats


Infection with *Helicobacter* spp. is increasingly linked with hepatobiliary inflammation and neoplasia in people and in a variety of animals. We sought to determine if *Helicobacter* species infection is associated with cholangiohepatitis in cats. Deoxyribonucleic acid was extracted from tissue blocks from cats with cholangiohepatitis (32), noninflammatory liver disease (13), and cats with normal liver histology (4). Deoxyribonucleic acid was polymerase chain reaction–amplified with 2 sets of *Helicobacter* genus–specific primers, gel purified, and sequenced. Polymerase chain reaction–positive hepatic tissue was further examined with Steiner’s stain, immunocytochemistry for *Helicobacter* species, and eubacterial fluorescent in situ hybridization. Gastric tissues of cats with known *Helicobacter* infection status served as controls for deoxyribonucleic acid extraction and sequence comparison. *Helicobacter* species were detected in 2/32 cats with cholangiohepatitis, and 1/13 cats with noninflammatory liver disease. Sequences had 100% identity with *Helicobacter species* liver, *Helicobacter pylori*, and *Helicobacter fenlleiucinaeii* in a cat with suppurative cholangitis, *Helicobacter species* liver, *Helicobacter pylori*, and *Helicobacter nemistrieneae* in a cat with mild lymphocytic portal hepatitis, and *Helicobacter bilis* in a cat with portosystemic vascular anomaly. In contrast, sequences from gastric biopsies showed highest homology (99–100%) to “*Helicobacter heilmannii*,” *Helicobacter bizzozeronii*, *Helicobacter felis*, and *Helicobacter salomonis*. Fluorescent in situ hybridization revealed a semicurved bacterium, with *Helicobacter*-like morphology, in an intrahepatic bile duct of the cat with suppurative cholangitis. This study has identified *Helicobacter* deoxyribonucleic acid in 2/32 cats with cholangiohepatitis and 1/13 cats with noninflammatory liver disease. Deoxyribonucleic acid sequences of hepatic *Helicobacter* species were distinct from those found in the stomach and are broadly consistent with those identified in cat intestine and bile, and hepatobiliary disease in people and rodents.

Key words: 16s rRNA; Fluorescence in situ hybridization; Liver.

Cholangiohepatitis/cholangitis complex in cats is an ill-defined inflammatory disorder of the hepatobiliary tree and is one of the most common hepatic disorders in cats.1,2 It has been subcategorized as suppurative and nonsuppurative to reflect the relative proportions of neutrophils to lymphocytes and plasma cells, and the degree of bile-duct hyperplasia and fibrosis.3,4 Suppurative cholangiohepatitis is frequently associated with a short duration of illness, moderate elevations in serum activity of liver enzymes, jaundice, fever, neutrophilia, and being male.3,4 Nonsuppurative cholangiohepatitis is similarly characterized by jaundice and elevated serum activity of hepatic enzymes, but a longer duration of clinical signs, hepatomegaly and a protein-rich abdominal effusion, hyperglobulinemia, and lymphocytosis are observed more frequently than in cats with suppurative cholangiohepatitis.3,5

Bacterial infections, most frequently enteric species, have been implicated in acute cholangiohepatitis, and a clinical response to antimicrobial therapy was observed in a cat with *Enterobacter*-associated cholangiohepatitis.6 Concurrent pancreatic or intestinal inflammation and cholestasis (intra- or extrahepatic) are also frequently diagnosed in cats with both suppurative and nonsuppurative cholangiohepatitis and may facilitate bacterial colonization, possibly by ascending infection of the biliary tree.3,7 However, a cause and effect relation of these potential etiologies to cholangiohepatitis has yet to be demonstrated.

In people, rodents, and dogs, there is evidence that inflammation and neoplasia of the liver and the biliary tract are associated with infection with *Helicobacter* spp. *Helicobacter* DNA or organisms, have been identified in the liver, bile, or gallbladder of people with chronic cholecystitis,8 cholestatic liver disease, and hepatocellular carcinoma,9,11 and cirrhosis.10,12 *H hepaticus* has been associated with hepatitis, hepatocellular carcinoma, and inflammatory bowel disease in mice. *H bilis* has been associated with hepatitis and typhlitis in mice.13,14 *H cholecystus* has been cultured from the gallbladders and pancreas of hamsters with cholangiohepatitis and pancreatitis.16 *H canis* has been cultured from the liver of a young dog with hepatitis.17 *H cinaedi* has been associated with hepatitis in rhesus monkeys.18 Because cats, like humans and other species, harbor *Helicobacter* in their stomachs, ie, *H felis*, *H heilmannii*, and *H bizzozeronii*, and *H canis*, *H bilis*, *H cinaedi*, and *Flexispira* have been cultured from cat feces,19 it is possible that *Helicobacter* species have a role in hepatic disease in cats. It is against this background that we examined the role of *Helicobacter* spp. in cholangiohepatitis in cats by evaluating archived hepatic tissue samples from cats with and without cholangiohepatitis for the presence or absence of *Helicobacter* spp with PCR, immunocytochemistry, silver staining, and fluorescence in situ hybridization.
**Materials and Methods**

**Samples**

Tissue blocks from 32 cats (16 F, 16 M, aged 2–17 years) with cholangiohepatitis (a total of 39 liver, 2 bile duct and 5 gallbladder samples), which had not been treated with antibiotics for a period of 1 month before biopsy, were identified in the pathology database for the period 1992 to 2001. Review of the histopathology (SPM) subcategorized these 32 cats as lymphocytic cholangiohepatitis (n = 10), mixed neutrophilic and lymphocytic cholangiohepatitis (n = 6), lymphocytic cholangitis (n = 6), mixed neutrophilic and lymphocytic cholangitis (n = 3), lymphocytic portal hepatitis (n = 3), neutrophilic cholangiohepatitis (n = 2), and neutrophilic cholangitis (n = 2). Tissue blocks were from a group of 13 cats (5 F 8 M, aged 0.4 to 10.5 years) with noninflammatory liver disease (8 portosystemic vascular anomaly, 2 lipidosis, 1 cyst, 1 hematoma, and 1 myelolipoma) and 4 cats with normal hepatic histology (4 M, >5 years) were used as a control group.

Paraffin-embedded stomach samples positive for *Helicobacter*-like organisms on modified Steiner stains were available for 2 cats from the cholangiohepatitis group and 1 from the control group. These stomach samples, along with paraffin-embedded samples from 3 uninfected cats, were used as internal controls to assess the recovery of *Helicobacter* spp. DNA from archival tissue embedded in paraffin and for sequence comparison.

**Deparaffinization and DNA Extraction**

In cases where several liver biopsies had been performed on 1 animal and specimens were fixed in different blocks, each block was evaluated separately. Liver tissue embedded in blocks together with gastrointestinal tissue were not evaluated to prevent potential false-positive results. Several 20-μm-thick slices of each block were cut by using sterile toothpicks and gloves (changed between each block). Slices were stored in sterile tubes at room temperature pending extraction.

Two slices (20 μm each) were deparaffinized by using the protocol described in the Quiagen Mini Kit. Samples were immersed in 1200 μL xylene, vortexed, and centrifuged for 5 minutes at 12,000 rpm. The supernatant was removed, and 1200 μL ethanol was added to the pellet. The samples were vortexed and centrifuged again, after which the supernatant was discarded. After a second wash with ethanol, the pellets were dried with the tubes opened for about 1 hour.

DNA extraction was performed as described in the Quiagen Mini Kit. DNA from the spin column was eluted 2 times in 50 μL Elution buffer, yielding 100 μL in total. DNA concentration was measured with a biophotometer. Negative controls were included in every batch of deparaffinization and DNA extraction to control for contamination.

**PCR Analysis**

Primers for feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which yield an amplicon of approximately 180 base pair (bp) with genomic DNA, were used to evaluate the integrity of the DNA extracted from archival tissue blocks. DNA yielding a GAPDH amplification was further analyzed by using *Helicobacter* genus-specific 16s rRNA primers. Primers that produced amplicons ≤400bp were chosen to ensure optimal detection in archival formalin-fixed paraffin-embedded tissues. Primers C97 (GCT ATG ACG GGT ATC C) and C98 (GAT TTT ACC CCT ACA CCA) yield an amplicon of 400bp, whereas primers 8F (AGA GTT TGA TCC TGG CTC AG) and H16s6 (CAC CCT CTC AGG CCG GA) yield a 279bp amplicon. These primers have been previously validated with respect to specificity and sensitivity, and have been successfully used to amplify *Helicobacter* DNA in people and pigs.  

PCR was performed by using Quiagen Master Mix (MgCl₂ concentration 1.5 mM, reaction volume of 50 μL), and 1 μL of target DNA in a thermocycler. The cycle for GAPDH was 95°C for 5 minutes, followed by 95°C for 25 seconds, 60°C for 1 minute, 72°C for 30 seconds for 40 cycles. The C97/98 cycle was the following: denaturation step at 94°C for 4 minutes, followed by 94°C for 1 minute, 55°C for 90 seconds, 72°C for 2 minutes (35 cycles), with a final extension step at 72°C for 10 minutes. The 8F/H16s6 cycle was as follows: denaturation step of 3 minutes at 94°C, followed by 40 cycles of 1 minute of denaturation at 94°C, 1 minute of annealing at 65°C for the first cycle, reduced by 1°C per cycle to 55°C, and a 2-minute extension at 72°C. Elongation was completed at 72°C for 10 minutes for the *Helicobacter* genus specific primers. PCR products were subjected to electrophoresis on an agarose gel and visualized with ethidium bromide.

**DNA Sequencing**

PCR products amplified with *Helicobacter* genus-specific primers (stomach and liver samples) were purified from agarose gels with a QIAquick PCR purification Kit and directly sequenced in both directions, with the genus specific primers described above, at the DNA Sequencing Facility, Biotechnology Center, Cornell University by using the ABI 3700 Analyzer with Big Dye Terminator Chemistry and AmpliTaq-FS DNA Polymerase. Sequence comparison was carried out by using DNAstar, the Blast Program, and the GenBank databases.

**Histologic Identification of Helicobacter Spp.**

Histologic sections from cats with positive PCR reactions for hepatic tissue were evaluated by staining with (1) modified Steiner’s stain and (2) by immunocytochemistry with mouse monoclonal antibody to Campylobacter jejuni crossing with *H pylori*, clone 371/254.55, and (3) by FISH with a cubacterial probe.

For immunocytochemistry, sections were dewaxed, treated with 0.5% hydrogen peroxide in methanol for 20 minutes, and rehydrated. We have previously shown that MoAb 371/254.55 cross-reacts with *H felis*, "H heilmanni," and *H pylori* in formalin-fixed gastric tissue. Further tests indicated that this antibody also cross reacts with slides spotted with *H suis*, *H fennelliae*, and *H bilis*. Tissue sections were incubated with the primary antibody (1:1000) at 4°C for 18 hours. After washing, the sections were covered with a 1:200 dilution of goat anti-rabbit immunoglobulin biotinylated secondary antibody and were left at room temperature for 30 minutes. The peroxidase-conjugate ABC was allowed to react at room temperature for 30 minutes. Sections were incubated with 0.05% diaminobenzidine and 0.01% hydrogen peroxide substrate for 3 minutes, washed in tap water, and counterstained with Mayer’s hematoxylin. Negative controls for each sample were prepared by replacing the primary antibody with normal mouse serum. Known negative and positive control sections were included in each immunolabeling assay.

FISH with a cubacterial probe was performed as previously described. Briefly, paraffin-embedded gastric and hepatic biopsy specimens were sectioned at 4.0 μm and mounted on Probe-On-Plus slides. The sections were deparaffinized by passage through xylenes (3 × 20 minutes) and then 100% alcohol (20 minutes), 95% ethanol (20 minutes), and finally 70% ethanol (20 minutes). The slides were allowed to air-dry. The DNA probe Eub-338 (16S rRNA cy3-5’-GCT-GGC-TCC-CGT-3’) was synthesized by the Biotechnology Resource Center, Cornell University. The probe was reconstituted with sterile phosphate buffered saline (PBS) and then diluted to a working concentration of 5 ng μL⁻¹ and sections hybridized at 46°C for 4 hours. Washing was performed at 48°C for 15 minutes. Hybridized samples were washed in PBS, allowed to air-dry, and were mounted with ProLong Antifade Kit.
non-eub338-FAM was used as a negative control. Hybridized sections were examined with an Axioskop 2 plus epifluorescence microscope, and images were captured with AxioCam and AxioVision.

Results

DNA Extraction and PCR

The DNA extraction method used in this study gave final DNA concentrations from fixed-tissue samples that ranged from 3 to 180 μg/mL. PCR amplification with GAPDH primers yielded an amplicon in 3/3 *Helicobacter*-infected gastric samples, 3/3 *Helicobacter*-negative gastric tissue samples, and 38/39 hepatobiliary samples. DNA from samples with successful GAPDH PCR was amplified with both sets of genus-specific primers.

Genus-specific PCR was positive in the archival gastric tissue samples from cats with *Helicobacter* like organisms visible on Steiner staining. Analysis of DNA sequencing electrophoretograms showed the presence of single nucleotide polymorphisms, indicative of a mixed sample population, in DNA from gastric tissue of 2 cats. A single unambiguous trace was present in the 3rd cat. Sequence analysis of the 2 samples with nucleotide polymorphisms closely matched (99%, E values -119 to -121) *H heilmannii* AF506785, *H felis* AY686607, and *H salomonis* HSU89351. Sequences form the third cat gave a 100% match (E value -124) for *H bizzozeronii* HB16SRRNA.

These results are consistent with gastric *Helicobacter* spp. present in the stomachs of cats, many of which are coinfected with several different species\(^ {24-26}\) and serve to validate the extraction and amplification techniques used to examine formalin-fixed paraffin embedded tissues. PCR amplification of liver samples with *Helicobacter*-genus specific primers was positive in 2/32 cases with cholangiohepatitis, 1/13 cases with non-inflammatory histology, and 0/4 cases with normal histology.

Hepatic tissue from a cat (B1) with suppurrative cholangitis (Fig 1) was positive with both primer sets, yielding the expected 400bp band with C97/C98, but 3 different amplicons with the primers 8F/16s6 (the expected 279bp size, approximately 500bp and approximately 600bp) (Fig 2). Hepatic tissue from a cat with mild lymphocytic portal hepatitis (D15) was positive with primer sets C97/98 and 8F/16s6 (Fig 2). Hepatic tissue from a cat (C13) with a portosystemic vascular anomaly, that had histologic evidence of iron accumulation in Kupffer cells and rare parenchymal aggregates of neutrophils and histiocytes, was positive with the primers 8F/16s6, but not the C97/98 primers, and yielded an amplicon with the approximate size of 450bp, rather than the expected 279bp (Fig 2).

DNA sequences of PCR amplicons from the 3 positive liver samples were unambiguous (ie, free from nucleotide polymorphisms) and consistent with *Helicobacter* spp. (Table 1). Sequences from the cat with suppurrative cholangitis (B1) generated by the C97/98 and the 279bp amplicon of 8F/16s6 were most similar to *H pylori*, notably AF145285 “liver 3” isolated from a person with hepatocellular carcinoma. Sequencing of the 600bp amplicon yielded 100% homology with *H*
fennelliae and H. cinaedi. Sequencing of amplicons from the cat with lymphocytic portal hepatitis (D15) were also most similar to H. pylori, again AF145285 liver isolate 3, and H. pylori. Amplicons from the cat with a PSVA (C13) had highest homology with H. bilis.

Because of the presence of amplicons of unexpected size with the F8/H16s6 primer set, we reevaluated the specificity of both primer sets with DNA extracted from H. pylori (CS 1), H. felis (ATCC 49179), H. bizzozeronii (ATCC 700030), H. salomonis (CCUG 37845), H. helimanni (DNA from the stomach of an infected cat), H. fennelliae (ATCC 35684), H. bilis (ATCC 51630), H. cinaedii (ATCC 35683), H. hepaticus (ATCC 51450), H. canis (ATCC 51401), Campylobacter jejuni (dog isolate), and Proteus mirabilis (cat isolate). When visualized over ultraviolet light, primers C97/98 yielded an amplicon of 400bp with all Helicobacter spp. tested, whereas primers 8f/H16s6 yielded an amplicon of 279bp for most Helicobacter spp., except 600bp for H. cinaedi (ATCC) and H. fennelliae (ATCC) and 450bp for H. bilis (ATCC) (Fig 2). These findings concur with the results of gel electrophoresis and sequencing of the 3 positive liver samples.

### Table 1. DNA sequence analysis of archival hepatic tissue samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PCR Primers</th>
<th>Amplicon Size</th>
<th>%</th>
<th>E-Value</th>
<th>Species</th>
<th>Accession</th>
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<td>-178</td>
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<tr>
<td></td>
<td>8f/H16s6</td>
<td>279 bp</td>
<td>100</td>
<td>-140</td>
<td>H. pylori</td>
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<td>0</td>
<td></td>
<td>H. cinaedi</td>
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<tr>
<td>D15</td>
<td>C97/98</td>
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<td>100</td>
<td>0</td>
<td>H. sp liver</td>
<td>AF142585</td>
</tr>
<tr>
<td></td>
<td>8f/H16s6</td>
<td>450 bp</td>
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<td>0</td>
<td>H. bilis</td>
<td>AF054572</td>
</tr>
<tr>
<td>C13</td>
<td>8f/H16s6</td>
<td>450 bp</td>
<td>100</td>
<td>0</td>
<td>H. bilis</td>
<td>AF054572</td>
</tr>
</tbody>
</table>

* Samples B1, D15, and C13 were obtained from cats with suppurative cholangitis, lymphocytic portal hepatitis, and a portosystemic vascular anomaly respectively.

The Expect value (E) is a parameter that describes the number of hits one can expect to see just by chance when searching a database of a particular size; the lower the E-value, or the closer it is to “0” the more “significant” the match is.

The detection of DNA sequences amplified from archival hepatic tissue samples from cats was somewhat against feline GAPDH and Helicobacter spp. 16srDNA, the high homology of the DNA sequences detected in gastric tissues with those in the H. felis group, as would be expected for the cat stomach, and the ability of PCR to discriminate between different Helicobacter spp., despite the limitations of small amplicon size, dictated by the use of formalin-fixed tissue samples and the broad similarity of 16srDNA of different Helicobacter spp. However, it should be borne in mind that the direct sequencing of gel-purified PCR amplicons used in the present study will likely yield a chimeric sequence (as illustrated in 2 gastric samples) when more than 1 Helicobacter sp. is present. Cloning and sequencing of PCR products is required for precise speciation of mixed populations.

The Helicobacter sequences amplified from the livers of cats in the present study (Helicobacter liver 3, H. pylori, H. nemistrinae, H. fennelliae, H. cinaedi, and H. bilis) are clearly distinct from the Helicobacter spp. amplified from archival feline gastric tissue (present study) and those known to colonize the cat stomach, eg, H. felis and H. helimanni. The detection of DNA sequences with high similarity to Helicobacter sp isolated from “liver 3” (AF142585) and H. pylori in 2 cats with cholangiohepatitis is intriguing as these sequences have been found in the hepatobiliary system of people with cirrhosis, hepatocellular carcinoma, and sclerosing cholangitis.

Interestingly, H. pylori-like sequences have recently been reported in bile samples of Dutch cats with and without lymphocytic cholangiohepatitis. The prevalence of Helicobacter spp. DNA in bile samples obtained from those Dutch cats with lymphocytic cholangitis, nonlymphocytic cholangitis, and healthy cats was reported as 26, 24, and 58%, respectively, and is much higher than the 6% observed in the present study. This discordancy might reflect differences in the patient population, sample type (bile versus liver, formalin fixed versus frozen), PCR methodology, geographical location (United States vs. Europe), and perhaps unreported antibiotic utilization.

The detection of H. pylori-like DNA sequences in hepatobiliary tissue samples from cats is somewhat

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**Silver staining, immunohistochemistry and FISH**

No Helicobacter-like organisms were identified in hepatic tissues by Steiner staining or immunohistochemistry. FISH revealed a semicurved bacterium (2-μ long), with Helicobacter-like morphology, in an intrahepatic bile duct of the cat with suppurative cholangitis (Fig 1 inset).

**Discussion**

This study has documented the presence of Helicobacter DNA in archival formalin-fixed paraffin-embedded liver samples from 2/32 cats with cholangiohepatitis and 1/17 cats with normal hepatic histology or non-inflammatory liver disease. The validity of this PCR-based approach to detect Helicobacter spp. in archival formalin-fixed tissues is supported by the successful amplification of intact DNA with primers directed against feline GAPDH and Helicobacter spp. 16srDNA, the high homology of the DNA sequences detected in gastric tissues with those in the H. felis group, as would be expected for the cat stomach, and the ability of PCR to discriminate between different Helicobacter spp., despite the limitations of small amplicon size, dictated by the use of formalin-fixed tissue samples and the broad similarity of 16srDNA of different Helicobacter spp. However, it should be borne in mind that the direct sequencing of gel-purified PCR amplicons used in the present study will likely yield a chimeric sequence (as illustrated in 2 gastric samples) when more than 1 Helicobacter sp. is present. Cloning and sequencing of PCR products is required for precise speciation of mixed populations.

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The detection of H. pylori-like DNA sequences in hepatobiliary tissue samples from cats is somewhat
surprising, because *H pylori* is not considered part of the gastric microflora of domestic cats. However, cats are susceptible to infection with *H pylori*, and the infection is able to become established and propagate within closed research colonies. The ability of *H pylori*, a bacterium adapted to life in the acidic environment of the stomach, to survive in the biliary tract of people and cats is unclear, particularly because bile acids have an inhibitory effect on its growth in vitro. However, it is reported that *H pylori* survives better in taurine-conjugated bile acids than glycine-conjugated bile acids, and this could facilitate colonization of the feline hepatobiliary tree.

DNA of *Helicobacter* spp., other than *H pylori* was also present in feline hepatic tissues. Additional DNA sequences detected in the cat with suppurative cholangitis were consistent with *H fennelliae* or *H cinaedi*. *H cinaedi* has been detected in cat feces and has been associated with liver disease in monkeys. *H fennelliae* has not been associated with liver disease. *H bilis* was amplified from the liver of a cat with a portosystemic vascular anomaly and minimal hepatic inflammation. *H bilis* has been found in the feces of cats from commercial suppliers and is associated with cholecytitis in people and hepatitis in aged inbred strains of mice. It has also been identified in the stomachs of dogs.

The clinical significance of positive PCR results for *Helicobacter* spp. DNA in liver or bile is unclear. Whereas, *H bilis* and *H hepaticus* have been cultured from bile and localized within the hepatobiliary system, discordancy between PCR positivity and bacterial isolation by culture and in-situ localization is frequent in studies of non-rodent hepatobiliary disease. Failure to culture may reflect the fastidious nature of many *Helicobacter* spp., and PCR may be more sensitive than in-situ localization. Alternatively, it is possible that PCR positivity reflects enterohepatic circulation of intestinal DNA rather than true colonization, or perhaps transient colonization, and this might explain the overrepresentation of cholestatic disorders and those with intrahepatic shunting in human hepatobiliary disease. We addressed this problem in PCR-positive cats by using silver staining, immunocytochemistry, and FISH, and were only able to detect a single bacterium in the bile duct of a cat with suppurative cholangitis by using FISH.

Acknowledgment

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References


Footnotes

* Qiagen, Valencia, CA
* Eppendorf,Westbury, NY
* Biometra Personal Cycler, Biometra, Goettingen, Germany
* Applied Biosystems, Foster City, CA
* DNASTar, Genetics Computer Group, Madison, WI
* MoAb 371/254.55, Novocastra Laboratories, Newcastle upon Tyne, UK
* Fisher Scientific, Pittsburgh, PA
* Molecular Probes Inc, Eugene, OR
* Carl Zeiss Inc., Thornwood, NY


