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Detection of *Helicobacter pylori* in bile of cats

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Abstract

Lymphocytic cholangitis (LC) in cats is a biliary disease of unknown etiology. *Helicobacter* spp. were recently implicated in human primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC). Because of the similarities between PSC/PBC with LC, we hypothesized that *Helicobacter* spp. are involved in feline LC. A PCR with *Helicobacter* genus-specific 16S rRNA primers was performed on DNA isolated from feline bile samples. Four of the 15 (26%) LC samples were positive, whereas only 8/51 (16%) of non-LC samples were PCR positive ($p = 0.44$). Sequence analysis of the amplicons revealed a 100% identity with the *Helicobacter pylori* specific DNA fragments. Our data suggest an etiological role of *H. pylori* in feline LC and that cats are a potential zoonotic reservoir.

Keywords: *Helicobacter* spp.; Cholangitis; Liver diseases; Zoonose; Cats

1. Introduction

Cholangitis is a common biliary disease in cats. Based on histological examination this inflammatory disease can be classified into two distinct forms: (i) an acute or chronic exudative cholangitis caused by an ascending bacterial infection in which leucocytes predominate in the lumen and wall of the bile ducts. *Escherichia coli* can generally be cultured from the bile and the cats are usually cured by standard antibiotic treatment, and (ii) a chronic persistent cholangitis characterized by lymphocytic inflammation, subsequently referred to as lymphocytic cholangitis (LC). There are striking similarities between LC and primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) in humans with respect to the chronic nature and the macroscopic and microscopic lesions of the biliary system and the liver. In the cat, the chronic inflammation of the biliary tree causes an irregular severe dilatation of the extra- and intrahepatic bile ducts. Histological examination of the liver shows a consistent infiltration of small lymphocytes in and restricted to the portal areas, often associated with variable portal fibrosis and biliary ductular proliferation. Lymphocytes centering around the bile ducts or in the biliary epithelium may be present, but are not a specific disease hallmark. In chronic cases bridging fibrosis between the portal areas may develop [1]. Biochemical analysis of the blood of these cats reveals increased bile salts, liver enzymes and often also markedly increased gamma globulins. Signs of jaundice, nausea, decreased appetite and gradual weight loss are observed. Routine bile culture does not reveal a

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bacterial infection. The etiology of LC is unknown, but, like PSC and PBC in man [2], it is assumed that the disease has autoimmune or immune mediated features. The classical treatment of feline LC consists of the administration of immune suppressive drugs such as prednisone, but this never results in cure of the disease. The high level of gamma globulins in the blood of cats with LC may be interpreted as a very chronic persistent infection by a so far not identified virus or bacterium. Moreover, standard antibiotic treatment as used for exudative cholangitis in cats is not curative.

A potential candidate for a bacterial infection would be *Helicobacter* species. Since the discovery of *Helicobacter pylori* as a major causative factor in the pathogenesis of active chronic gastritis, and peptic ulcer disease, more than 25 additional *Helicobacter* spp. have been isolated from the stomach, intestinal tract and liver of various mammalian and avian species. Several groups have reported that *Helicobacter* DNA has been detected in livers of humans with PSC, PBC [3–5] chronic cholecystitis [6] and hepatocellular carcinomas (HCC) [7,8], as well as in the livers of some animals with hepatitis, such as mice, dogs and woodchucks [9–12].

Based on the similarities, feline LC may have a similar etiology as PSC or PBC in humans. This prompted us to test the association between a *Helicobacter* infection of the biliary tree and feline LC. Here, we describe the detection of a DNA fragment typical for *Helicobacter* spp. in the bile of cats that is identical to a *H. pylori* fragment found in humans with HCC. The presence of these Helicobacter-specific DNA fragments in the bile of cats suggests that cats may be a potential zoonotic hazard.

2. Materials and methods

2.1. Feline bile samples

Bile samples were collected from 15 cats with histologically confirmed LC that were presented to our clinic for companion animals. Bile was aspirated under sterile conditions with ultrasound guidance directly from the gallbladder and was immediately snap-frozen in liquid nitrogen and stored at −80 °C until processing. For comparison, 51 bile samples were also obtained from cats with other liver diseases presented at our clinic and at the pathology department; the bile of cats presented at the latter department was collected at necropsy. In addition, bile of two groups of clinically healthy cats were included. These 12 cats were kept in two closed colonies (one family of stray cats, $n = 5$; and one group of SPF control cats that were sacrificed at the end of an unrelated study, $n = 7$) and the livers of these cats were histologically normal. All samples were tested blind.

2.2. Histological examination

At the time of bile collection from all cats also a liver biopsy was obtained for histological examination. Morphological classification of the biopsies was performed by one experienced board-certified veterinary pathologist (TSGAMvdI), who was blinded for the origin of the samples. The sections were scored according to the approach of the World Small Animal Veterinary Association (WSAVA) Liver diseases and Pathology Standardization Research Group [13].

2.3. DNA isolation

Approximately 1 ml of each bile sample was centrifuged at 14,000 rpm at 4 °C for 10 minutes [14] The supernatant was aspirated and stored at −20 °C. DNA was isolated from the pellet with the QIAamp DNA mini kit (Qiagen, Hilden, Germany; tissue protocol) according to the manufacturer's instructions.The pellet was lysed for 1.5 h at 56 °C under continuous shaking (∼150 rpm) with proteinase K. DNA was eluted in deionized water and stored at −20 °C.

2.4. PCR amplification

All PCRs were performed in a 50 μl volume with an MJ Research thermal cycler (MJ Research Inc., Watertown, MA, USA) Reaction mixtures contained 0.2 μM of each oligonucleotide primer (Isogen Life Science, Maarssen, The Netherlands), PCR buffer (Invitrogen Corporation, Carlsbad, CA, USA), 2.5 U of Platinum *Taq* polymerase (Invitrogen), 2 mM $MgCl_2$(Invitrogen) and 250 μM of each nucleotide. Primers *HelicoF* (5′-AAC GAT GAA GCT TCT AGC TTG CTA G-3′) and *HelicoR* (5′-GTG CTT ATT CGT TAG ATA CCG TCA T-3′) based on the 16S rRNA sequences of most gastric and enterohepatic members of the *Helicobacter* genus were used to generate a 400-bp amplicon [7]. *H. pylori* specific primers *HP-ACT-1* (5′-CTT GCT AGA GTG CTG ATT A-3') and *HP-ACT-2* (5'-TCC CAC ACT CTA GAA TAG T-3′) [15] were used to generate a 539-bp product. For both the *Helicobacter* genus PCR and the *H. pylori* PCR 5 μl of DNA was used in a first reaction. The amplicons were diluted 10 times and 5 μl of this dilution was used in a second reaction with the same primers and the same conditions. The PCR conditions were the following: an initial denaturation step at 95 °C for 4 min followed by a denaturation step for 30 s at 95 °C, annealing at 50 °C for 30 s, elongation at 72 °C for 1 min. A total of 40 cycles was performed and was followed by a final elongation step at 72 °C for 10 min. As controls, chromosomal DNA of *H. pylori* strain 1061 [16] and a DNA sample isolated from canine liver tissue that was positive in a PCR with the *Helicobacter* genus primers were used. In order to exclude that PCR

inhibition was the cause of negative PCRs, an additional PCR was performed in which the negative samples were spiked with different dilutions of *Helicobacter* DNA. Several samples containing water were always used as controls to exclude contamination.

2.5. Sequence analysis of the amplicons

The generated amplicons were directly sequenced using primers *HelicoF* and *HelicoR*. Sequencing was performed with an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The resulting nucleotide sequences were compared with the sequences present in the GenBank databases using the BLAST nucleotide program of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov\blast) and were aligned and analyzed using Lasergene (DNAStar Inc. Madison, WI, USA) and SECentral (Scientific & Educational Software, Durham, NC, USA) software.

2.6. Statistical analyses

All statistical analyses were performed with the SPSS 11.0.1 Software (SPSS Inc. USA). Significance was tested by means of the Pearson χ^2 test. Differences were regarded as significant if $p < 0.05$.

3. Results

3.1. PCR amplification with 16S rRNA primers

Of the 15 cats with LC, four cats were positive (26%) in the PCR and generated a product of the expected size of 400 bp. Of the bile samples of 51 cats with non-LC liver diseases eight had a PCR fragment of the expected size (24%). Of the 12 clinically healthy cats, seven were positive (58%) in the PCR with PCR fragments of the expected size. This is shown in Table 2. There was no significant difference between the percentage of positive cats in the PCR between the group of cats with LC and the group of cats with other diseases ($p = 0.44$).

3.2. Sequence analysis of the 16S rRNA amplicons

Sequence analysis of the PCR products of 12 *Helicobacter*-genus PCR positive cats revealed that they were all identical to the *H. pylori*-specific DNA sequence that Avenaud et al. [7] detected in liver tissue of human patients with HCC (deposited at GenBank; Accession No. AF 142585; *H. pylori* "liver 3") for all samples tested, as indicated in Table 1. We were unable to sequence the other fragments obtained in this study, because of their low yield. In addition, on four positive samples in the *Helicobacter* genus PCR of which we had enough material and four negative samples in this PCR we performed an additional PCR with *H. pylori* specific primers. The four genus PCR positive samples were all positive in the *H. pylori* specific PCR, whereas the four negative samples in the genus PCR were also negative in the *H. pylori* specific PCR.

Our *H. pylori*-like 16S rRNA sequences and the identical sequences (*H. pylori* "liver 3") found in the database all differed at two base pair positions in the 400 bp PCR fragment when compared to the sequenced *H. pylori* reference strain 26695 [17]. These differences were at positions 91 (G to A) and 129 (T to C), respectively, of the 16S rRNA gene sequence of *H. pylori* strain 26695 (Table 2). Our positive control did not have these typical basepair differences, which excludes contamination of the samples with the DNA of the positive control.

Table 1
The number of cats included in this study with their histological diagnosis, the results of the PCR with primers *HelicoF* and *HelicoR* (400 bp) and the number of amplicons that were sequenced. All sequences were confirmed to be *Helicobacter* specific DNA fragments homologous to the sequences reported by Avenaud et al. [7]

	No. of cats	Amplicon of expected size	Sequencing analysis
LC	15	4	2
Non-LC	51	8	3
Clinically healthy	12	7	7
Total	78	19	12

Table 2
16S rRNA sequence differences in the 400 bp *Helicobacter*-specific DNA fragments compared to the reference strain 26695 and to Helicobacter-specific DNA sequences detected in liver tissue in other studies

	nt 91[a]	nt 129[a]	Reference
Helicobacter pylori reference strain 26695 and other *H. pylori* strains ($n = 12$)	G	T	[17]
Verhoef et al. ($n = 9$)	A	C	[8]
Avenaud et al. ($n = 8$)	A	C	[7]
Magalheas Queiroz ($n = 1$)	A	C	[18]
Nilsson et al. ($n = 4$)	A	C	[4]
Cats with LC	A	C	This study
Cats with other diseases	A	C	This study
Clinially healthy cats	A	C	This study

A, adenine; C, cytosine; G, guanine; T, thymine.
[a] Numbering according to 16S rRNA of *H. pylori* strain 26695 [17].

4. Discussion

In this study, *H. pylori*-specific DNA fragments were detected by PCR analysis in the bile of 26% of cats diagnosed with LC. Based on the homology of these 16S rRNA gene products, they are most likely derived from members of the *Helicobacter* genus. A somewhat lower

number of cats (16%) with other liver diseases were positive in this PCR. All amplicons of which enough material could be obtained for sequence analysis were identical to *H. pylori* DNA fragments that were identified in the liver of humans with hepatocellular carcinoma [7], which have 2 base pairs difference with the *H. pylori* reference strain 26695. This indicates that they form separate clusters of Helicobacters, as all sequences differed two base pair positions with the conserved 400 base pairs part of the 16S rRNA gene of the *H. pylori* reference strains. These two typical base pair differences were also absent from 16S rRNA gene of other gastric Helicobacters, such as *H. felis*, *H. bizzozeroni*, *H. salomonis* and *H. heilmannii*, or any of the sequenced 16S rRNA sequences of enterohepatic *Helicobacter* species. Strikingly, these two mutations have thus far only been found in *Helicobacter*-specific sequences detected in liver tissue of human patients with different liver diseases, such as HCC, PSC and PBC (Table 2). Since we do not have the sequence data of all PCR fragments (only 12/19 could be sequenced), we cannot be 100% sure that all fragments have these mutations. In addition, all samples that were subjected to a *H. pylori* specific PCR were also positive in this test, indicating that we indeed detected *H. pylori* specific DNA fragments in the bile of these cats.

To our knowledge, this is the first description of the detection of *H. pylori*-specific DNA fragments from bile of cats. Over the past years, many groups have described the amplification of *Helicobacter*-specific DNA in the intestines, bile and liver of various mammals. However, efforts to culture Helicobacters from these samples were mostly unsuccessful. In this study, limited cultivation efforts failed to demonstrate growth of *Helicobacter*-like organisms on both selective and non-selective media. The only published successful attempt to culture *H. pylori*-like bacteria in bile and liver samples is from a liver biopsy sample of a patient with Wilson's disease [18], who also harbored *H. pylori* in the stomach. Therefore, it was suggested that the organism most likely colonized the already diseased liver as a secondary event and that the hepatic abnormality was necessary for colonization. In our study some cats that were positive in the *Helicobacter* genus PCR were severely ill and therefore likely immuno-compromised, thus generating the option for Helicobacters to colonize these hosts a as secondary event. In a recent report [8] a *Helicobacter* specific DNA fragment with the exact same sequence as observed by Avenaud [7] and in this study, was found in both liver and gastric specimens of patients with non-cirrhotic HCC. Unfortunately it was not possible to collect gastric specimens of our cats, thus it was not possible to test for the presence of gastric *Helicobacter*-specific fragments, which are very common in cats, in order to see whether these would be identical to those found in the bile.

The clinically healthy cats had a remarkably high fraction of positive samples. Their PCR products had exactly the same typical basepairs differences that were also present in the PCR fragments of the diseased cats in this study. These healthy cats originated from two colonies in which they were kept in close association. The high incidence in these groups indicates transmission and subsequent colonization of the bacterium between members of the groups. This would indicate that the Helicobacters found in the cats in this study are indeed potentially transmittable pathogens. Consequently, this does not allow the comparison of LC plus non-LC cats with healthy cats.

Although no significant difference in prevalence between the LC and non-LC cats ($p = 0.44$, χ^2 test), it may be an indication that there is indeed an etiological association between the presence of *Helicobacter* specific DNA sequences and LC in cats. For definite proof, however, studies in much larger series will be required. Moreover, in a pilot study a treatment consisting of direct injection into the gallbladder of antibiotics aimed at *H. pylori* eradication (i.e. amoxicillin, gentamycin and metronidazole) has been applied to three cats in our clinic, thus far with a good clinical response. Recently, Nilsson et al. [4] identified *H. pylori* specific sequences in liver samples of patients with PSC and PBC (Table 2). The *H. pylori* DNA sequences found in liver of patients with PSC and PBC have identical sequence characteristics as the *H. pylori* specific DNA fragments detected in the bile of cats. Moreover, there are more assumptions that PSC and PBC are of infectious origin or that the bacterium may trigger an autoimmune response [2,5]. This may also be the case in feline LC.

The knowledge that gastric Helicobacters may be present in the stomach without causing any clinical symptoms was long thought to indicate that these Helicobacters are opportunistic pathogens. It cannot be excluded that the *H. pylori*-specific sequences observed in this study represent such opportunistic pathogens and possible zoonoses, like *H. heilmannii* [19]. In the light of our current knowledge, and given that Handt et al. [20] detected *H. pylori* in the stomach of cats, it may well be possible that (i) *H. pylori* is the causative agent of feline LC, and (ii) cats represent a reservoir for this bacterium. To our knowledge this is the first report on the presence of *H. pylori* DNA sequences in the bile of a significant proportion of individuals from a non-human species. The presence of these DNA sequences in the bile of cats suggests that cats may be a potential zoonotic hazard.

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