Helicobacter infection in the hepatobiliary system and hepatic lesions: a possible association in dogs

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Abstract

Helicobacter infection has been associated with hepatobiliary diseases in humans and animals. The aims of this study were to identify *Helicobacter* species in the hepatobiliary tract of dogs and to elucidate the possible association of these bacteria in liver diseases. Twenty-seven gastric and hepatobiliary samples were collected from 33 dogs with hepatic lesions and 17 dogs with no liver histological changes. Warthin-Starry staining, immunohistochemical assay, and PCR were performed to detect the presence of *Helicobacter*. *Helicobacter* genus was detected in 21.2% of the samples with hepatic lesions. The main lesion was chronic hepatitis. Immunohistochemistry revealed infection in liver (1/5) and gallbladder (1/3) 32 samples. The sequence analysis of seven amplicons of the 16S rRNA gene of *Helicobacter* genus from hepatobiliary samples showed 97.8 to 100% of nucleotide identity with gastric *Helicobacter*. One amplicon of the ureA and ureB gene of *Helicobacter* genus from the stomach showed 89.1 to 90.7% nucleotide identity with *H. heilmannii*. The presence of *Helicobacter* genus in liver samples showing hepatic lesions suggests the involvement of these bacteria in the etiology of hepatobiliary disease in dogs. DNA sequences were similar to gastric *Helicobacter* species, reinforcing the hypothesis of bacterial translocation from the stomach to liver by the biliary pathway.

Keywords Canine · *Helicobacter* · Hepatobiliary diseases · Molecular biology

Introduction

Since the discovery of *Helicobacter pylori* as a cause of gastritis and gastric neoplasia in human beings [1], more than 40 additional *Helicobacter* species have been isolated from the stomach, intestinal tract, and liver of various mammalian and avian species. Gastric non-*H. pylori* *Helicobacter* species were originally referred to as *Gastrospirillum hominis* and later as *H. heilmannii*. *H. heilmannii* was subdivided in two taxa (type 1 and type 2). *H. suis* has been accepted as a new

gastric *Helicobacter* corresponding to *H. heilmannii* type 1 while the *H. heilmannii* type 2 is formed by a group of species that colonize the gastric mucosa: *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis*, and *Candidatus H. heilmannii* [2]. In addition, the name *Helicobacter heilmanni* sensu lato was proposed when histological or crude taxonomical data were obtained, whereas *Helicobacter heilmannii* sensu stricto should be used when definite identification is attained [3]. The *Helicobacter* species that colonize the intestine and hepatobiliary system are known as enterohepatic species [4].

Some inflammatory and neoplastic diseases of the hepatobiliary system have been associated with *Helicobacter* infection in humans, creating new interest in the role of *Helicobacter* spp. in hepatobiliary diseases [5, 6]. The association between *Helicobacter* infection and hepatic diseases has also been described in some animal species, involving both gastric and enterohepatic *Helicobacter* species. In murine species, *H. hepaticus* induces inflammatory disease and primary neoplasia in the liver, contributing to cholesterol gallstone formation [7, 8]. Chronic hepatitis was associated with

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H. bilis and *H. nemestrinae* infection in hamsters [9] (Fox et al., 2009) and in a dog [10] Portal fibrosis and biliary hyperplasia were also observed in infected hamsters [9]. Moreover, in ferrets (*Mustela putorius furo*), the presence of *Helicobacter* spp. was associated with cholangiocellular carcinoma and cholangiohepatitis [11].

In pets, there are few reports concerning hepatobiliary diseases and *Helicobacter* infection. *H. canis* was isolated from the liver of a puppy with multifocal necrotizing hepatitis [12]. Recently, the presence of *Helicobacter* spp. in the livers of dogs showing hepatic histological changes was associated with increased hepatocyte proliferation [13]. In cats, *Helicobacter* was associated with cholangiohepatitis [14]. On the other hand, *Helicobacter* spp. was identified in the liver and pancreas of healthy cats [15]. Translocation and ascending way are the proposed mechanisms for *Helicobacter* spp. penetration into the hepatobiliary system [16]. In mice orally infected with *H. hepaticus*, the bacterium was found first in the intestine and later in the bile duct and liver, demonstrating the capacity for bacterial migration [17]. Tissue invasion may be a prerequisite for or a consequence of *Helicobacter*-associated disease in the gastrointestinal tract, either as a primary event or secondary to other disease conditions [18].

Dogs are frequently affected by hepatobiliary disorders; however, data regarding the relationship with *Helicobacter* spp. infection in pets are limited. The aim of this study was to identify specific species of *Helicobacter* in the hepatobiliary tract of dogs diagnosed with histological liver lesions and to carry out the molecular characterization of these species to elucidate the etiology of these bacteria in liver diseases.

Materials and methods

Animals and sampling procedures

Samples of the gastric mucosa (corpus, fundus, and antrum regions), liver, and gallbladder were collected from 50 dogs submitted to routine necropsy examination. The cause of death was diverse, including disseminated neoplasia, trauma, and cardiac disease. The procedures were performed up until 1 h after death, to avoid autolytic changes in sampled tissues. Fragments were fixed in 10% buffered formalin solution, embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin (HE) and Warthin-Starry (WS) methods.

The criteria for inclusion in this study comprised one or more of the following hepatic histological changes: inflammatory infiltrate, portal fibrosis, vacuolar degeneration of hepatocytes, biliary ductular proliferation, and hepatocellular necrosis. Hematoxylin and eosin staining was performed in liver samples for histological evaluation according to the WSAVA

Liver Standardization Group [19]. (2006). In accordance with these criteria, from the initial 50 dogs, 33 animals (17 males and 16 females) were included in this study. Additionally, for comparison 17 dogs showing no liver histological changes were included (control group). WS staining was performed to evaluate the presence of organisms suggestive of *Helicobacter* spp. Various breeds were involved, and the ages of the dogs (3 months to 19 years) varied.

For PCR assay, one additional sample from each tissue, and also from bile, was placed into a DNase and RNase free microtube and immediately frozen at −20 °C until DNA extraction. Furthermore, to avoid cross contamination, sample collection were performed using individual sterilized scalpel blades. The study was approved by the institutional ethical committee for animal experimentation (number 35786). None of the animals were euthanized for inclusion in this study.

Molecular characterization

Bacterial DNA was extracted from gastric mucosa, liver, gallbladder mucosa and bile samples as described elsewhere [20]. Bile samples (100 µL) were diluted (1:5) with sterile distilled water before DNA extraction. PCR reactions were performed in a 25 µL final volume using 5 µL of DNA extracted, 0.5 µL (20 pmol) of each primer, 0.4 mM of each dNTP, 1 X PCR buffer (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 1.25 units of Platinum® Taq DNA polymerase (Invitrogen™ Life Technologies, São Paulo, Brazil), and 3 mM of MgCl₂. Specific primers pairs were used in PCR assays designed to amplify amplicons of specific genes. These included the 16S rRNA gene and the *ureA* and *ureB* genes of the *Helicobacter* genus (Table 1). Amplification was performed in a thermocycler under time and temperature conditions determined for each primer pair. The amplified products were analyzed by electrophoresis on a 2% agarose gel in TBE buffer, pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA), stained with ethidium bromide (0.5 µg/mL) and visualized under UV light. In all amplification reactions for 16S rRNA or *ureA* and *ureB* genes, samples of *Helicobacter* (*H. heilmannii*, *H. felis*, *H. bizzozeronii*, *H. baculiformis*, *H. salomonis*, and *H. cynogastricus*) were used as positive controls. The negative control consisted of ultrapure-free water (Invitrogen Corporation, Carlsbad, CA, USA).

Sequencing and phylogenetic analysis

The PCR amplicons were purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), quantified in a Qubit Fluorometer using Quant-iT dsDNA BR Assay Kit (Invitrogen Life Technologies, Eugene, Oregon, USA), and sequenced in an ABI3500 Genetic Analyzer sequencer with

Table 1 Sequences of the primers used in PCR assay to detect *Helicobacter* spp.

Primer	Gene amplified	Sequence 5' – 3'	Product size (bp)	Reference
H276F H676R	16S rRNA	CTATGACGGGTATCCGGC ATTCCACCTACCTCTCCCA	399	35
U430F U1735R	Urease gene	GCKGAWTTGATGCAAGAAGG CTTCGTGRATTTTAARRCCAAT	1224	27

the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) using the forward and reverse primers. Sequence quality analyses and contig assembly were performed using Phred and CAP3 software (<http://asparagin.cenargen.embrapa.br/phph/>) respectively, and the sequences were accepted if base quality was ≥ 20 . Sequence similarity searches were performed with sequences deposited in GenBank using the basic local alignment search tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic tree based on nucleotide sequences was obtained using the neighbor-joining method with the Kimura two-parameter model using MEGA software package (version 5.05) [21]. Bootstrapping was statistically supported with 1000 replicates. Sequence identity matrix was performed using the BioEdit software version 7.1.3.0.

Immunohistochemical assay

Immunohistochemistry (IHC) was performed using polyclonal rabbit anti-*Helicobacter pylori* antibody (Dako, Denmark) on PCR-positive hepatobiliary samples. Histological sections were deparaffinized and subjected to antigen retrieval (citrate buffer, pH 6.0) on microwave. Endogenous peroxidase was blocked using hydrogen peroxide. Sections were incubated with primary antibody (1:100) overnight at 4 °C. The samples were incubated with the secondary antibody (HRP polymer, Zymed, CA). Finally, the antigen-antibody complex was visualized by its reaction with 3,3'-diaminobenzidine substrate-chromogen solution (Zymed, California, CA). Slides were counterstained with hematoxylin and coverslipped. Positive (tissue fragment from gastric mucosa infected with *Helicobacter* spp.) and negative (mouse serum substituted for the primary antibody) controls were used in all reactions according to the manufacturer's recommendations.

Results

Histopathological hepatic findings

Of the 50 animals, 33 presented liver histological lesions established as a criteria inclusion in this study. Seventeen animals with no histological changes were included as a control

group. The main histological finding was mild to moderate inflammatory infiltrate involving the portal tracts and lobular parenchyma. Chronic hepatitis (24/33) was predominantly composed of lymphocytes and plasma cells with a few neutrophils and macrophages associated with hepatocellular necrosis and fibrosis; in contrast, in acute hepatitis (4/33), there was neutrophilic inflammation and hepatocellular necrosis. A minimal to moderate quantity of fibrous connective tissue occurred in portal fibrosis (4/33) (Fig. 1a), with or without inflammatory cells. In some cases, the hepatocytes appeared with a swelling and pale staining cytoplasm or a vacuolar change of the cytoplasm, characterizing vacuolar degeneration (6/33) (Fig. 1b). Chronic cholangitis occurred in one dog. Microscopically, it was characterized by consistent lymphocyte and plasma cell infiltrate in portal areas, associated with portal fibrosis and biliary ductular proliferation (5/33). Hepatocellular necrosis (6/33) showed a focal or multifocal distribution in the parenchyma (Fig. 1c).

Warthin-Starry staining and immunohistochemical assay

In 29 (87.9%) dogs long spiral-shaped bacteria were detected in superficial mucus, fovea (Fig. 1d), and gastric pits. The bacteria were found in large clusters and also occurred isolated. None of the liver and gallbladder samples were positive by this method. A positive immunostaining was observed (Fig. 1e, f) in two samples (one from the liver and one from the gallbladder).

PCR assay

In the gastric mucosa, a 399 -bp amplicon that corresponds to the 16S rRNA gene of *Helicobacter* genus was detected in 90.9% of the samples (30/33). In one dog, a negative sample by WS staining was positive by PCR for *Helicobacter* spp.

In the hepatobiliary system, a 399-bp amplicon consistent with the *Helicobacter* genus was obtained in 15.2% (5/33) of the liver samples presenting histological changes. One dog presented a positive *Helicobacter* genus reaction for both the liver and gallbladder. None of the bile samples were positive for the *Helicobacter* genus. The positive samples for 16S rRNA *Helicobacter* genus were tested for the *ureA* and *ureB*

Fig. 1 **a** Portal fibrosis and biliary ductular proliferation in a chronic hepatitis case. HE. Bar, 100 μ m. **b** Diffuse vacuolar degeneration in a chronic hepatitis case. HE. Bar, 100 μ m. **c** Chronic cholangitis with biliary ductular proliferation and hepatocellular necrosis (arrow) in a chronic hepatitis case. HE. Bar, 100 μ m. **d** *Helicobacter* spp. colonization in the fovea of gastric mucosa. WS staining. Bar, 20 μ m. **e** Positive immunostaining to anti-*H. pylori* in liver (arrow). IHC for anti-*H. pylori*. Bar, 10 μ m. **f** Positive immunostaining to anti-*H. pylori* in gallbladder (arrow). IHC for anti-*H. pylori*. Bar, 10 μ m

gene. Only one dog was positive for 16S rRNA *Helicobacter* genus and negative for the *ureA* and *ureB* gene. Table 2 illustrates the positive results of the hepatobiliary system samples by PCR assay and their respective results for gastric mucosa. Hepatobiliary samples from animals with no histological changes were negative in PCR assays.

Phylogenetic analysis

The seven nucleotide sequences of the 16S rRNA *Helicobacter* genus from the liver and gallbladder samples displayed high nucleotide identity (97.8 to 100%) with *H. felis*, *H. bizzozeronii*, *H. cynogastricus*, *H. salomonis*,

Table 2 PCR results for 16S rRNA and *ureA* and *ureB* genes and histological liver changes from dogs with *Helicobacter* spp. positive samples from the alimentary system

Animal	PCR assay						Hepatic histological changes
	Liver		Gallbladder mucosa		Gastric mucosa		
	16S rRNA	Urease A/B	16S rRNA	Urease A/B	16S rRNA	Urease A/B	
Dog 4	KC007543*	+	—	n/a	+	+	Chronic hepatitis
Dog 6	KC007544*	+	KC007547*	—	+	+	Chronic hepatitis
Dog 8	—	n/a	KC007545*	+	+	+	Chronic hepatitis
Dog 13	KC007541*	+	—	n/a	+	+	Vacuolar degeneration
Dog 21	KC007546*	+	—	n/a	+	KT288228*	Chronic hepatitis
Dog 32	—	n/a	KC007542*	+	+	+	Chronic hepatitis, vacuolar degeneration
Dog 33	+	+	—	n/a	+	+	Chronic hepatitis, biliary ductular proliferation, fibrosis

Fig. 2 Neighbor-joining phylogenetic tree based on partial sequences with 331 bp (nt 459–789) of the 16S rRNA gene of *Helicobacter* spp. from the liver and gallbladder samples of dogs. The numbers adjacent to

the nodes represent the percentage of bootstrap support (1000 replicates) for the clusters. Bootstrap values less than 50% are not shown. ◆ Sequences from this study

H. baculiformis, and *H. heilmannii* (*H. heilmannii* sensu lato) and clustered in the phylogenetic tree with these strains (Fig. 2). The positive sample from the stomach by the *ureA* and *ureB* gene (dog 21) showed high nucleotide identity (89.1 to 90.7%) with strains that belong to *H. heilmannii* (*H. heilmannii* sensu lato and sensu stricto) and clustered with them in the phylogenetic tree (Fig. 3). The 16S rRNA gene nucleotide sequences of the liver from dog 33 and all the samples of liver and gallbladder

ureA and *ureB* gene tested were not included in the phylogenetic analysis due to the low quality of the sequences.

Discussion

Helicobacter spp. have been reported to infect the gastric mucosa of pets in many countries, with a prevalence ranging from

◀ **Fig. 3** Neighbor-joining phylogenetic tree based on partial sequences with 795 bp (nt 422–1216) *ureA* and *ureB* gene of *Helicobacter* spp. from the stomach sample of dog 21. The numbers adjacent to the nodes represent the percentage of bootstrap support (1000 replicates) for the clusters. Bootstrap values less than 50% are not shown. ♦ Sequences from this study

41 to 100% [22, 23]. However, data about *Helicobacter* infection in the liver and gallbladder are scarce in pets [12–14]. *Helicobacter* infection and hepatobiliary diseases [24–26]. In our study, *Helicobacter* was detected in 21.2% of the hepatobiliary samples (15.2% in the liver and 9.1% in the gallbladder). Moreover, only liver samples with histological changes were positive for *Helicobacter* genus by PCR.

The main lesion observed in infected animals was chronic hepatitis, associated or not with degenerative or proliferative changes. Similar findings were reported for cats [14], prairie dogs [27], and a ferret [11] in which *Helicobacter* DNA fragments were detected. An association between *H. bilis* infection and chronic hepatitis, fibrosis, and biliary hyperplasia was also reported in hamsters [9]. In contrast, *Helicobacter* spp. was identified in liver and pancreas samples from healthy cats [15]. In a study using liver samples from human patients with cholangitis and cirrhosis, an association with the presence of *H. pylori* and *Helicobacter* spp. DNA was observed, suggesting the involvement of bacteria in liver diseases [25]. In addition, in a previous study, we have showed in dogs an association between liver infection by *Helicobacter* spp. and increased hepatocyte proliferation [13].

Identification of *Helicobacter* spp. in the gastric mucosa using WS staining showed a high association with PCR assay, but for hepatobiliary system, no bacteria were observed in histological slides, even for PCR positive specimens. Similar results were reported in the liver of cats infected with *Helicobacter*-like organisms that were PCR positive but negative by WS or Steiner staining [14]. In contrast, some authors have demonstrated the presence of *Helicobacter* spp. in the hepatobiliary system using WS staining [12, 24]. Nevertheless, even with special stains, the bacterial colonization observed is low [28]. Another point to be considered is that as a consequence of the high number of wavy reticular fibers in the liver, identifying spiral shaped bacteria using histochemical methods in this tissue could result in false negatives. Even with the use of immunohistochemical assay the bacteria was detected only in two samples. It seems that the microscopic analysis for observing organisms that suggest *Helicobacter* spp. in liver sections by WS staining or IHC is much harder than that in gastric mucosa samples. This difference between the IHC, WS staining and PCR results may be explained by a heterogeneous colonization in the liver, as has been demonstrated in gastric mucosa [20].

In our study, the species detected in hepatobiliary samples by 16S rRNA phylogenetic analysis were clustered with

gastric helicobacters, such as *H. baculiformis*, *H. cynogastricus*, *H. salomonis*, *H. felis*, *H. bizzozeronii*, and *H. heilmannii* (*H. heilmannii sensu lato*). Detection of *ureA* and *ureB* gene was performed in hepatobiliary samples, and the amplicons generated were sequenced; however, the quality was insufficient to identify the infecting species [29]. The quality of the amplicons generated may be related to mixed infections that, similarly as in gastric mucosa, may occur in hepatobiliary system [30]. Most likely, the origin of infection or the presence of *Helicobacter* spp. DNA in liver and gallbladder samples was from gastric mucosa. Gastric helicobacters may be present in the stomach without causing any clinical symptoms, but they could be opportunistic pathogens in the biliary tract. Other studies, which have detected *H. pylori* DNA in bile and liver samples in human beings, have also suggested the enterohepatic transport as the origin for hepatobiliary infection [25, 31].

Bacterial translocation occurs both in humans and animals, and three primary mechanisms have been identified: intestinal bacterial overgrowth, increased permeability of the intestinal mucosal barrier, and deficiencies in host immune defenses [32]. In the case of *Helicobacter* spp., there are two proposed pathways for liver colonization: translocation from the stomach to the liver through the duodenum and biliary tract or through the hepatic portal vein. Data indicate the biliary pathway as the most plausible route [33]. In this study, the isolate sequenced from gastric mucosa (*ureA* and *ureB* gene, dog 21) displayed high nucleotide identity with *H. heilmannii* (*H. heilmannii sensu lato* and *sensu stricto*). The liver sample from the same animal (16S rRNA gene, dog 21) also shared high nucleotide identity with *H. heilmannii* (*H. heilmannii sensu lato*), but also with other gastric *Helicobacter*. These results reinforce the hypothesis of bacterial translocation from gastric origin, since both species are reported in samples from gastric mucosa [23, 34].

The detection of *Helicobacter* spp. in bile samples was reported in humans and rodents with hepatobiliary disorders [7, 31, 35]. These data indicate a possible role for *Helicobacter* infection in the development of hepatobiliary diseases in humans and animals, nevertheless more studies in pets are necessary. In our study, no bile samples were positive by PCR. Data indicate that when a nested PCR was used, a higher occurrence of *Helicobacter* spp. infection was observed in bile samples [26, 35]. Another point that can explain the negative results in bile samples is the low DNA quantities [26] and the presence of inhibiting substances [36]. Additionally, considering the physiology of the gallbladder, the biliary flow could inhibit some bacterial colonization.

Conclusion

In the present study, *Helicobacter* was detected in dogs showing hepatic histological changes. In the literature,

hepatobiliary diseases were associated with infection by different species of *Helicobacter* in humans and animals. These results suggest a possible role for *Helicobacter* spp. in the etiology of hepatobiliary disease in dogs. In addition, the DNA sequences were most similar to gastric *Helicobacter* species, reinforcing the hypothesis of bacterial translocation from the stomach to the liver by the biliary pathway. More studies have to be performed to elucidate the pathogenicity of *Helicobacter* spp. infection in the hepatobiliary tract of dogs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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