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Detection of Helicobacter ganmani-Like 16S rDNA in Pediatric Liver Tissue

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**ABSTRACT**

**Background.** To determine the presence of Helicobacter species in the liver biopsy specimens from children with various chronic liver diseases as data in adult literature suggests a possible role of these bacteria in their pathogenesis.

**Materials and methods.** Paraffin sections of 61 liver biopsies of pediatric patients with miscellaneous diseases and autopsy liver tissue from 10 control subjects with no evidence of preexisting liver disease were examined for the presence of Helicobacter species by a genus-specific seminested polymerase chain reaction (PCR) assay. PCR–products of positive samples were further characterized by denaturing gradient gel electrophoresis (DGGE) and DNA-sequence analysis. Based on those results, a seminested PCR assay for H. ganmani was developed and applied to the samples.

**Results.** On analysis, 40/61 patient samples were positive in the genus-specific Helicobacter PCR and 4/10 from the control group. The nucleotide sequences of 16S rDNA fragments were 99–100% similar to mainly Helicobacter sp. ‘liver’ and H. ganmani. PCR-products similar to H. canis and H. bilis were also found. The 16S rDNAs of control specimens showed similarity to Helicobacter sp. ‘liver’. In the H. ganmani-specific PCR analysis 19 patients, but none of the controls, were positive.

**Conclusions.** Amplified Helicobacter 16S rDNAs were related to Helicobacter sp. ‘liver’ or H. ganmani in liver biopsy specimens of pediatric patients. The possible significance of Helicobacter species in pediatric liver diseases needs to be evaluated further in prospective studies.

**Keywords.** Non-Helicobacter pylori Helicobacter, liver disease, children, polymerase chain reaction (PCR).

**Helicobacter pylori** is a gram-negative, spiral-shaped bacterium that colonizes the human stomach and causes gastric inflammation, gastric and duodenal ulcers, gastric cancer and mucosa-associated lymphoid tissue (MALT)-lymphoma [1–3]. Besides these gastroduodenal pathologies in adults and children [1,4,5], H. pylori has also been implicated in extra-gastric conditions such as ischemic heart disease [6,7], vascular and immunological disorders [10,11], halitosis [12], migraine [13], and poor growth in children [14].

Several Helicobacter species colonize the liver of animals and induce hepatitis [15]. Increasingly, it is being suggested that these nongastric Helicobacter species can cause hepatic tumors in certain strains of mice [16]. There is an ongoing debate as to whether some of these organisms should be considered indigenous gut microflora. Recently, the possible colonization of the human hepatobiliary system by these bacteria has been emphasized, and indeed, Helicobacters other than H. pylori have been found in the gallbladder of subjects suffering from chronic cholecystitis in Chile [17].

Patients with primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) have been reported to be positive for H. pylori in liver tissue by PCR and DNA sequence analysis, and the Helicobacter positivity was significantly more common in patients with cholestatic disease [18]. Recently, a Helicobacter strain, closely resembling H. pylori, was cultured from human liver tissue [19]. In contrast to these results, H. pylori was detected by PCR/DNA sequencing in only 1 of 29 patients with PBC [20], and Helicobacter
species were not detected in bile from German patients with bile duct disease [21].

A wide variety of pediatric liver diseases can be associated with cholestasis. While changes related to autoimmune diseases, fatty infiltration of liver and viral hepatitis may share similar pathophysiologic mechanisms as in adults, biliary tract disease in infancy presents an important challenge in that besides disturbance of hepatic structure and function, process of normal development may be altered [22,23].

As no data pertaining to the association of Helicobacter and liver disease exist for children, we sought to evaluate the presence of this genus of bacteria in liver biopsies of pediatric patients with miscellaneous liver diseases.

Materials and methods

Patients

The liver tissue samples were obtained from pre-existing biopsies performed for the usual indications for diagnosing the etiology of liver disease in children with persistent biochemical and clinical abnormalities. Paraffin blocks of 61 liver biopsies from children and adolescents (Males = 34, Females = 27) with miscellaneous liver diseases were evaluated. These included patients with autoimmune chronic active hepatitis (AIH, n = 17), biliary atresia (n = 11), steatohepatitis (n = 7), hepatitis C (n = 5), neonatal giant cell hepatitis (n = 5), primary sclerosing cholangitis (n = 3), chronic hepatitis B (n = 3), primary familial intrahepatic cholestasis (n = 1), and other miscellaneous conditions (n = 9) including drug toxicity, ischemic injury and systemic diseases such as celiac disease and chronic inflammatory bowel disease. The ethnic distribution was: African Americans (n = 25), Caucasians (n = 20), Middle Easterners (n = 2) and Others (n = 14). The age range of the patients was from six weeks to 18 years (mean age 8.74 years). Autopsy liver tissue specimens from 10 control subjects with no evidence of preexisting liver disease were also examined. All liver specimens were collected from patients at the Children’s Hospital of Michigan. The sectioning by microtome was done at several sessions and the blade was wiped with 70% (v/v) ethanol after cutting each block to minimize any potential contamination.

Patient charts were reviewed for recording biochemical parameters of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), prothrombin time (PT), partial thromboplastin time (PTT), total and direct bilirubin as well as other metabolic, infectious and autoimmune parameters. An ethics committee exempt approval was obtained to conduct this study. The tissue extraction and DNA-analyses were performed in Sweden in a blinded fashion by investigators (HON, WAAS and TW) who were not aware of clinical or biochemical data of these patients.

DNA-extraction

The liver tissue samples were de-embedded by heating at 60°C for 10 minutes followed by washing in xylene for 2 × 5 minutes. The specimens were then rehydrated through graded ethanol (99% and 95% for 2 × 5 minutes and 70% for 5 minutes), and finally washed for 5 minutes in double-distilled water. DNA was extracted from the liver tissue using the QIAamp DNA mini Kit (Qiagen, Helden, Germany), according to the instructions of the manufacturer with the modification that 1–2 mg of de-embedded tissue was used. The DNA was stored frozen at −20°C.

Helicobacter genus-specific PCR

Amplification was carried out using a GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA) and Helicobacter genus-specific primers, previously published by Goto et al. [24] The sequence of the outer (first step) forward primer (1F) was 5′-CTATGACGGGTATCCGGC-3′ (positions 254–271 of H. hepaticus, GenBank accession no. L39122); reverse primer (1R) 5′-CTCACGACACGAGCTGAC-3′ (positions 1018–1035). In the second step, primer 1F and reverse primer 2R (5′-TCGCCTTCGCAAT-GAGTATT-3′, positions 667–686) were used. The reaction mixture of the first step (25 µl) contained 0.5 µM of each primer (1F and 1R), 0.2 mM of each deoxyribonucleotide triphosphate (Amersham Biosciences, Uppsala, Sweden), 1 × chelating buffer, 2.5 mM MgCl₂, 0.4% (wt/vol) bovine serum albumin, 1.25 U of rTth DNA polymerase (Applied Biosystems), and 5 µl of extracted DNA. The amplification conditions for the first step were 94°C for 2 minutes; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 minutes; and finally 72°C for 5 minutes. The reaction mixture of the second step (25 µl) contained 0.5 µM of each primer (1F and 2R), 0.2 mM of each deoxyribonucleotide triphosphate, 1 × buffer II,
2.5 mM MgCl2, 1.0 U of AmpliTag Gold DNA polymerase (Applied Biosystems), and 2 µl of 10× diluted PCR product from the first step. The amplification conditions for the second step were 95°C for 10 minutes; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and finally 72°C for 5 minutes. As a positive control, 10−10 g of H. pylori (CCUG 17874) DNA was added to the reaction mixture, while 5 µl of sterile Millipore-filtered deionized water was used as a negative control. The 0.43 kb PCR product was detected in a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide (0.2 µg/ml TAE) for 15 minutes, and visualized using a GelFotoStation (Techtum Laboratory, Klippan, Sweden). Several precautions were used to minimize the risk of PCR cross contamination. A buffer control without tissue or DNA added was included during each DNA extraction, and unless PCR-negative, the DNA extraction kit was discarded and specimens re-analysed. Each amplification contained positive (H. pylori genomic DNA) and negative (distilled water) controls to exclude cross-over contamination. DNA-extraction, preparation of PCR-mixtures, template addition, amplification, and gel electrophoresis were performed in separate rooms. Template addition and dilution of PCR-products were performed in a laminar air flow security hood.

**Denaturing gradient gel electrophoresis (DGGE)**

Patient specimens were used as templates in the Helicobacter genus-specific assay and subsequently analyzed with DGGE as previously described [25]. PCR products of H. ganmani (CCUG 43526, H. pullorum (NCTC 12825), H. pylori (CCUG 17874), H. hepaticus (CCUG 33637), and a canine isolate of H. sp. flexispira (kindly provided by M-L Hänninen, Helsinki, Finland), generated with the same primers, were used to construct a migration ladder. The gels were stained with ethidium bromide (0.2 µg/ml of 0.5 × TAE) for 15 minutes, and visualized using a GelFotoStation (Techtum Laboratory).

**Sequencing of 16S rDNAs**

Helicobacter genus-specific PCR products were purified either from agarose gels by Ultrafree DA centrifuge tubes (Millipore, Bedford, USA) according to instructions, or cut from DGGE gels with a scalpel and transferred to microcentrifuge tubes with 160 µl of sterile distilled water. The tubes were briefly centrifuged and placed at −80°C for 1 hour, then kept at room temperature for 1 hour, frozen again at −80°C for 1 hour, and finally incubated at 4°C for 2 hours. A 5-µl sample was used as a template in the second step of the Helicobacter genus-specific PCR. The resulting fragments were separated on agarose gels and purified with Ultrafree DA tubes. Sequence analysis was performed with an Applied Biosystems DNA sequencer (ABI 310), using the ABI PRISM BigDye v3.0 Terminator Sequencing Kit (Applied Biosystems). Both DNA strands were sequenced and aligned in Bio-Edit Sequence Alignment Editor (North Carolina State University, Raleigh, NC) (www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequence similarity comparisons were carried out using BLAST (NCBI, www.ncbi.nlm.nih.gov).

**Sequencing of Helicobacter 16S-23S rDNA ISR regions**

The 16–23S rDNA internal spacer region (ISR) of H. ganmani (CCUG 43526, CCUG 43527), H. canis (CCUG 32756), H. hepaticus (CCUG 33637), H. mustelae (NCTC 12198), H. pullorum (NCTC 12825), and H. fennelliae (CCUG 18820) was amplified using the primers fS1 and rS1 previously published by Miyajima et al. [26]. The 800-bp PCR products were separated by agarose gel electrophoresis, extracted and purified by Ultrafree DA centrifuge tubes (Millipore). Sequencing was performed using primers fS1 and rS1 as described above. The partial DNA-sequence of the ISR of H. ganmani CCUG 43526 and CCUG 43527 was submitted to GenBank under accession numbers AY277974 and AY277975, respectively.

**H. ganmani-specific PCR**

The 16S-23S rDNA sequences of different Helicobacter and Campylobacter species were aligned and primers GanF (5′-CTCCTAACGCCCA CCAGAAATTG-3′), and GanR (5′-TTC CCCATAATAGGTTAGTTA-3′) were selected using Vector NTI® Suite version 8.0 (InforMax, Frederick, Maryland) (Figure 1). The expected PCR-product of 166 bp was amplified and detected by agarose gel electrophoresis. A seminested PCR-assay, including primers GanF and rS1 in the first step and GanF and GanR in the second step was set up. The PCR-mixture used was similar to that described above for the Helicobacter genus-specific PCR. Amplification mixtures were denatured at 92°C for 2 minutes and run through
30 cycles of 92°C for 30 s, 48°C for 30 s and 72°C for 30 s. An extension at 72°C for 7 minutes was included. Diluted products were amplified through 35 cycles in the second step with an annealing of 50°C. The specificity of the primers was evaluated using strains obtained from the Culture Collection of the University of Gothenburg (CCUG); H. ganmani CCUG 43526, H. ganmani CCUG 43527, H. canis CCUG 32756, H. hepaticus CCUG 33637, H. mustelae (NCTC 12198), H. pullorum (NCTC 12825), and H. fennelliae (CCUG 18820). GenBank sequences of C. coli AF 146727 and C. jejuni AF 074835 were also included.

Statistically significant differences were considered achieved at a p-value of ≤ .05. Appropriate assumptions were checked (homogeneity of variance and normality) and verified. If violated, a nonparametric Mann–Whitney U-test was substituted. All analyses were conducted using SPSS Version 11.5.

### Results

#### Helicobacter genus-specific PCR

PCR analysis using Helicobacter genus-specific primers was positive in 40 of 61 (65.6%) samples with liver disease and in 4 of the 10 (40%) controls. The distribution of Helicobacter positive samples among the different patient groups is shown in Table 1.

### DGGE

DGGE analysis was performed on 30 of 40 Helicobacter genus-specific PCR products amplified from patients with following distribution of diseases: Autoimmune hepatitis (n = 8), biliary atresia (n = 6), steatohepatitis (n = 4), hepatitis C (n = 2), neonatal hepatitis (n = 2), hepatitis B (n = 1), primary sclerosing cholangitis (n = 1), and from six of the other miscellaneous conditions. By comparing the migration distance of

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>H. genus PCR (%)</th>
<th>H. ganmani PCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoimmune hepatitis</td>
<td>10/17 (59)</td>
<td>7/17 (41)</td>
</tr>
<tr>
<td>Biliary atresia</td>
<td>8/11 (73)</td>
<td>1/11 (9)</td>
</tr>
<tr>
<td>Steatohepatitis</td>
<td>5/7 (71)</td>
<td>3/7 (43)</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>4/5 (80)</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>Neonatal hepatitis</td>
<td>2/5 (40)</td>
<td>2/5 (40)</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>2/3 (67)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Primary sclerosing cholangitis</td>
<td>2/3 (67)</td>
<td>2/3 (67)</td>
</tr>
<tr>
<td>Primary familial intrahepatic cholestasis</td>
<td>1/1 (100)</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Other</td>
<td>6/9 (67)</td>
<td>3/9 (33)</td>
</tr>
<tr>
<td>Total</td>
<td>40/61 (66)</td>
<td>19/61 (31)</td>
</tr>
<tr>
<td>Controls</td>
<td>4/10 (40)</td>
<td>0/10 (0)</td>
</tr>
</tbody>
</table>
these PCR products with those amplified from reference strains, it was found that the migration of PCR products of 21 samples were similar to the migration of the PCR-product of \textit{H. pylori} CCUG 17874 and nine samples were similar to the PCR-product of \textit{H. ganmani} CCUG 43526. None of the specimens contained more than one PCR-product. Among the patients, bands comigrating with PCR-products of both \textit{H. pylori} and \textit{H. ganmani} were observed, whereas bands only matching \textit{H. pylori} were found in liver specimens of the control group. A DGGE separation profile of amplified 16S rDNA products of the \textit{Helicobacter} genus-specific PCR is shown in Figure 2.

**DNA sequencing of 16S rDNAs**

Partial 16S rDNA nucleotide sequences of 17 patients and one control, including PCR-products representing the two different DGGE migration profiles, were determined. The PCR-products (average length of 397 bp sequenced, 378–406 bases overall) that comigrated with \textit{H. pylori} in the DGGE (n = 7), were closely related (> 99%) to \textit{Helicobacter} sp. ‘liver 3’ (AF142585). Seven of nine 16S rDNA fragments that comigrated with \textit{H. ganmani} in the DGGE-analysis, as well as two \textit{Helicobacter}-positive samples that were directly sequenced, showed 99–100% similarity to \textit{H. ganmani} strain UNSW H16 (average length of 387 bp, 348–413 bases overall). The two remaining sequences with a DGGE profile similar to \textit{H. ganmani}, were related to \textit{H. bilis} MIT 95-234-6 (402/402 bp) and to \textit{H. canis} U65102 (388/388 bp), respectively.

**\textit{H. ganmani}-specific PCR**

The specificity of the \textit{H. ganmani} PCR-primer was evaluated using genomic DNA from 18 species of \textit{Helicobacter} including two strains of \textit{H. ganmani}. Only \textit{H. ganmani}-DNA was successfully amplified and the expected 166 bp PCR-fragment was detected by agarose gel electrophoresis. None of the remaining type strains were positive using the GanF and GanR primers (Figure 3). When the \textit{H. ganmani} PCR-assay was applied to the samples, 19 of the patients, but none of the controls, were positive. Eight of nine samples that harboured \textit{H. ganmani} 16S rDNA determined by DNA sequence analysis were positive in the \textit{H. ganmani} PCR and 13 additional samples, of which seven displayed a \textit{Helicobacter} genus-specific PCR-product homologues to \textit{Helicobacter} sp. ‘liver 3’ by sequence analysis, were also positive for \textit{H. ganmani} as determined by PCR.

**Liver function parameters**

The mean values of standard liver function tests such as AST, ALT, ALP, total and direct bilirubin and coagulation tests were compared between \textit{Helicobacter} positive and negative groups, and although no significant differences were noted, the \textit{p}-value for ALT in the \textit{Helicobacter} positive and negative group just failed to reach significance as shown in Table 2.

**Discussion**

The number of species in the genus \textit{Helicobacter} has rapidly expanded in the past decade [27]. Some preliminary evidence suggested a possible role of \textit{H. pylori} infection in the pathogenesis of hepatic encephalopathy in cirrhosis; however, it has not been proven [28,29]. \textit{Helicobacter pylori} vacA was incriminated as a contributing factor in elevated transaminases by causing hepatotoxicity through an initial direct and subsequent autoimmune process [30]. \textit{H. pylori} and \textit{H. pullorum} have also been implicated in the pathogenesis and progression of cirrhosis in individuals affected...
Helicobacters in pediatric liver disease

Presence of *H. pylori* and other *Helicobacter* species has also been reported in human liver tissue with several cholestatic diseases [18]. *H. hepaticus* and *H. bilis* cause chronic hepatitis and liver disease in laboratory rats, mice and possibly other laboratory and wild animals [15,16,25]. In the first human study in Chile, *H. bilis* and *H. pullorum* were diagnosed in chronic cholecystitis. However, *H. hepaticus* or other gastric or enteric *Helicobacter* species were not identified [17]. Interestingly, Avenaud and coworkers and Nilsson et al. reported on a novel *Helicobacter* species, very similar to *H. pylori*, from livers of patients with primary liver cancer [32,33]. *H. ganmani* is the first anaerobic species of *Helicobacter* and up to now it has only been detected in the murine gastrointestinal tract [34]. Whether this species can infect other animals, including primates, is unknown.

In view of these observations, it was a natural corollary to study pediatric liver tissue for detection of *Helicobacter* species. Biliary atresia may be defined as a progressive sclerosing inflammatory process of the extrahepatic and intrahepatic bile ducts, leading to complete obliteration of the biliary tree and obstruction to the flow of bile [35,36]. Biliary atresia is the most frequent cause of the entire spectrum of liver diseases in infants and children [37].


**Table 2** Comparison of liver function test results in *Helicobacter* polymerase chain reaction (PCR) positive- and negative patients

<table>
<thead>
<tr>
<th>Test</th>
<th>Helicobacter Positive patients</th>
<th>Helicobacter Negative patients</th>
<th>Significance</th>
</tr>
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<tbody>
<tr>
<td>AST IU/l</td>
<td>201</td>
<td>176</td>
<td>NS</td>
</tr>
<tr>
<td>ALT IU/l</td>
<td>259</td>
<td>165</td>
<td>0.06</td>
</tr>
<tr>
<td>ALP IU/l</td>
<td>597</td>
<td>397</td>
<td>NS</td>
</tr>
<tr>
<td>Total bilirubin mg%</td>
<td>1.6</td>
<td>2.03</td>
<td>NS</td>
</tr>
<tr>
<td>Direct bilirubin mg%</td>
<td>2.18</td>
<td>1.46</td>
<td>NS</td>
</tr>
<tr>
<td>PT (s)</td>
<td>10.5</td>
<td>12.2</td>
<td>NS</td>
</tr>
<tr>
<td>PTT (s)</td>
<td>26.3</td>
<td>29.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

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of liver disease producing obstructive jaundice in the first 3 months of life. The etiology of biliary atresia has not been defined. Because of the acquired, inflammatory nature of the biliary tract lesion in most patients, infection has been considered a possible cause. A seasonal clustering of cases provides support for theories that biliary atresia may be caused by an environmental exposure, possibly to a virus, during the perinatal period [37]. However, a more recent, large study failed to detect a seasonal variation in incidence or clustering [38]. Many viruses have been identified in these patients but have not been proven to cause biliary atresia including rubella virus, cytomegalovirus, and rotavirus. Considerable attention has been paid to reovirus type 3. Recent studies have identified reovirus RNA in approximately 50% of patients with biliary atresia and close to 80% of patients with choledochal cysts [39]. Thus, reovirus type 3 and possibly other infectious agents may have a role in the pathogenesis of the disorder, particularly in patients who may have a genetic predisposition to the disorder. We detected *H. ganmani* in a patient with biliary atresia and two patients with neonatal hepatitis. It appears that these bacteria should also be considered in the etiology of biliary atresia.

Majority of patients with biliary atresia develop the process after birth. There is considerable interest in the immune and inflammatory response as a trigger for the pathogenesis of biliary atresia which is possibly related to an infectious process. For example, microarray analysis of liver tissue from infants with biliary atresia has shown a coordinated activation of genes involved in lymphocyte differentiation [40]. The over-expression of osteopontin and interferon gamma points to a potential role of Th-1-like cytokines in the disease pathogenesis. In other studies, the bile duct cells in biliary atresia patients showed aberrant expression of ICAM-1 and HLA-DR [41]. The biliary tract has connection with the gut in the second portion of duodenum from where ascending infections may occur. *H. pylori* DNA has been detected in bile samples of patients with gastric cancer suggesting a role for asymptomatic cholangitis [42]. It is possible that as with *H. pylori*, only a subset of individuals with other *Helicobacter* infections will develop disease complications related to bacterial virulence factors, environmental exposure and host responses.

Despite considerable study, the etiopathogenesis of AIH and PSC is not understood. Several theories have been proposed, including genetic predisposition, immunologic dysfunction, infection (portal bacteremia, endotoxins, viruses), toxicity from bile acid metabolites, and vascular/ischemic insults [42]. Although *H. ganmani* was detected in several patients with AIH and PSC in our study, whether such infection may be an initiating or perpetuating factor in the occurrence of the actual disease is not known.

*Helicobacter* sp. ‘liver 3’ was detected by DGGE and/or sequence analysis in seven samples that also were positive for *H. ganmani* by species-specific PCR. If a sample contains more than one *Helicobacter* species the competitive amplification of the genus-specific PCR possibly favors the template of highest amount, leading to detection of this particular PCR product in DGGE and sequence analysis, whereas a species-specific PCR also would detect the template of lower concentration.

Among closely related species of *Helicobacter*, large portions of the 16S rRNA genes are similar. However, some species, such as *H. cinaedi* and *H. trogontum*, show substantial (4–5%) infraspecific divergence of 16S rDNA [25] that may lead to anomalous results in DGGE and sequencing. Nevertheless, such diversity is restricted to few species and we found that DGGE was efficient to discriminate between *Helicobacter* species based on differences in the V6-7 regions of the 16S rRNA.

The results of the present investigation of pediatric liver tissues were similar to several previously published studies, which reported that the amount of *Helicobacter* PCR-positive liver tissue specimens of biliary tract cancers and chronic liver diseases were in the range of 50–100% [18,19,32,33]. Positive samples were equally distributed among the specimens, even though the sectioning was performed at several occasions, except from the control subjects, in which fewer positive cases were detected. The harsh de-embedding procedure, including heating at 60°C, repeated xylene and ethanol treatment, should wash off loosely associated microorganisms or DNA, possibly introduced by the microtome. In addition, at least two different *Helicobacter* DNA-sequences (four were identified in total, i.e. *Helicobacter* sp. ‘liver’ [H. pylori], *H. ganmani*, *H. bilis*, and *H. canis*), were identified from each sectioning occasion, thus excluding the element of contaminating DNA.

We attempted to correlate biochemical markers to the presence of *Helicobacter* DNA but found no identifiable patterns. In a previous study, ALP and prothrombin values were higher in the
presence of Helicobacter sp. [18]. We could not confirm this observation; however, a trend of higher ALT levels was observed in the PCR positive group.

Detection of H. ganmani in a variety of pediatric liver diseases is unexpected and exciting. Recently, a H. pylori-like organism were isolated from an adult patient with liver disease [19]. It was not possible to culture Helicobacter from the studied samples because of the harsh fixation and embedding procedures. However, fresh liver tissue should be used to optimize culture conditions to enhance the growth of these and other fastidious enteric microaerophilic species. Future research to determine the possible role of super-added infections in liver diseases in children and adults may clarify the significance of Helicobacter spp. in liver tissue.

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