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

Vasundhara Tolia,* Hans-Olof Nilsson,† Kimberly Boyer,* Anne Wuerth,* Waleed Abu Al-Soud,† Raja Rabah‡ and Torkel Wadström†

*Department of Pediatrics, Children's Hospital of Michigan, 3901 Beaubien Blvd, Detroit, MI 48201 USA, †Department of Medical Microbiology, Dermatology and Infection, Lund University, Lund, Sweden, ‡Department of Pathology, Children's Hospital of Michigan, 3901 Beaubien Blvd, Detroit, MI 48201 USA

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], migrane [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*

Reprint requests to: Vasundhara Tolia, Department of Pediatrics, Children's Hospital of Michigan, 3901 Beaubien Blvd, Detroit, Michigan 48201, USA. Tel.: (313) 745–5585; Fax: (313) 745 5155; E-mail: vtolia@med.wayne.edu

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 preexisting 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 aminofransferase (ALT), alkaline phosphatase (ALP), prothrombin time (PT), partial thromboplastin time (PTT), total and direct bilirubin as well as other metabolic, infectious and autoimmune para-meters. 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′-TCGCCTTCGCAATGAGTATT-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 $MgCl_2$, 0.4% (wt/vol) bovine serum albumin, 1.25 U of r*Tth* 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 30 s; 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 $MgCl_2$, 1.0 U of Ampli*Taq* 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 and visualised by use of 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.hih.gov).

Sequencing of Helicobacter 16S-23S rDNA ISR regions

The16–23S 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 850-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′-CTCCTAAGCCCA CCAGAAATTG-3′), and GanR (5′-TTC-CCCATAATAGGGTAGTTTA-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

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GanF        5′-CTCCTAAGCCCACCAGAAATTG-3′      GanR 3′-ATTTGATGGGATAATACCCCTT-5′

H. ganmani 1  CTCCTAAGCCCACCAGAAATTG                TAAACTACCCTATTATGGGGAA
H. ganmani 2  ......................                ......................
H. canis      G.TTCT.T..T.T.CACGC..T                CCCCTGATAAGGG.GA..TC.G
H. hepaticus  T.AT.T.T.TT.A.CAC.GA.T                CCCCTGATAAGGG.GA..TCGG
H. mustelae   TATT.GTTTATCTTGAT..AAA                CCCCTGATAAGGG.GA..TC.G
H. pullorum   ..TTC...AGA.AT.ACT.AAT                CCCCTGATAAGGG.GA..TCGG
H. fennelliae TGTT..GA.AT...TA..CC.T                CCCCTGATAAGGG.GA..TCGG
C. coli       A.TTC..AGATTGAT..T.AA.                CCCCTGATAAGGG.GA..TC.C
C. jejuni     A.TTC..AGATTGAT..T.AA.                CCCCTGATAAGGG.GA..TC.C
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Figure 1 Alignment of the 16S-23S rDNA ISR region of different *Helicobacter* and *Campylobacter* species covering the region in which the *H. ganmani*-specific primers were located. The strains included are *H. ganmani* 1 (CCUG 43526), *H. ganmani* 2 (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.

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. pylori* CCUG 17874, *H. pullorum* CCUG 33840, *H. bilis* CCUG 38995, *H. canis* CCUG 33835, *H. hepaticus* CCUG 33637, *H. rappini* CCUG 23435, *H. cinaedi* CCUG 43521, *H. fennelliae* CCUG 18820, *H. pametensis* CCUG 29259, *H. salomonis* CCUG 37848, *H. felis* CCUG 28540, *H. muridarum* CCUG 29262, *H. acinonyx* CCUG 29263, *H. mustelae* CCUG 23951. Additional strains used were *H. bizzozeronii* AF 53 and *H. suncus* Kaz1. Genomic DNA of *H. rodentium* strains 1707 and 2060 were kindly provided by Dr Stephen L.W On, Danish Veterinary Institute, Copenhagen, Denmark.

Statistical analysis

Demographic data was reported using proportions or means and standard deviations, relative to data scale. Differences in continuously scaled variables reported in Table 2 were examined using a parametric independent samples *t*-test. Statistically significant differences were considered achieved at a p-value of $\leq .05$. 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 1 Results of the *Helicobacter* genus-specific and *Helicobacter ganmani*-specific polymerase chain reaction (PCR) assays in the different patient groups

Diagnosis	*H.* genus PCR (%)	*H. ganmani* PCR (%)
Autoimmune hepatitis	10/17 (59)	7/17 (41)
Biliary atresia	8/11 (73)	1/11 (9)
Steatohepatitis	5/7 (71)	3/7 (43)
Hepatitis C	4/5 (80)	1/5 (20)
Neonatal hepatitis	2/5 (40)	2/5 (40)
Hepatitis B	2/3 (67)	0/3 (0)
Primary sclerosing cholangitis	2/3 (67)	2/3 (67)
Primary familial intrahepatic cholestasis	1/1 (100)	0/1 (0)
Other	6/9 (67)	3/9 (33)
Total	40/61 (66)	19/61 (31)
Controls	4/10 (40)	0/10 (0)

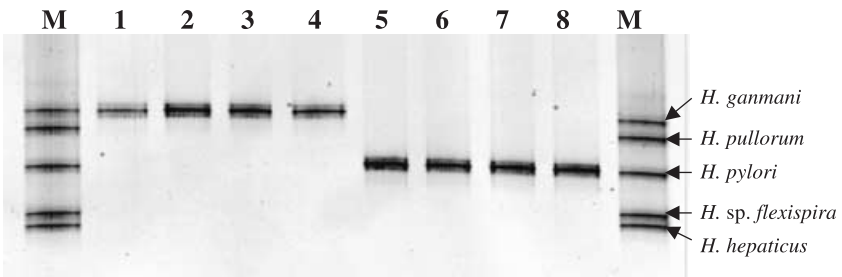

Figure 2 Denaturing gradient gel electrophoresis (DGGE) of a representative set of liver specimens amplified from the patient group. Lane M: marker DNA containing (from top to bottom) *Helicobacter ganmani*, *H. pullorum*, *H. pylori*, *H.* sp. *flexispira*, and *H. hepaticus*, lanes 1–4: Polymerase chain reaction (PCR)-products with a migration distance similar to that of *H. ganmani*, lanes 5–8: PCR-products with a migration distance similar to that of *H. pylori*.

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 *H. pylori* CCUG 17874 and nine samples were similar to the PCR-product of *H. ganmani* CCUG 43526. None of the specimens contained more than one PCR-product. Among the patients, bands comigrating with PCR-products of both *H. pylori* and *H. ganmani* were observed, whereas bands only matching *H. pylori* were found in liver specimens of the control group. A DGGE separation profile of amplified 16S rDNA products of the *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 *H. pylori* in the DGGE (n = 7), were closely related (> 99%) to *Helicobacter* sp. 'liver 3' (AF142585). Seven of nine 16S rDNA fragments that comigrated with *H. ganmani* in the DGGE-analysis, as well as two *Helicobacter*-positive samples that were directly sequenced, showed 99–100% similarity to *H. ganmani* strain UNSW H16 (average length of 387 bp, 348–413 bases overall). The two remaining sequences with a DGGE profile similar to *H. ganmani*, were related to *H. bilis* MIT 95-234-6 (402/402 bp) and to *H. canis* U65102 (388/388 bp), respectively.

H. ganmani-*specific PCR*

The specificity of the *H. ganmani* PCR-primers was evaluated using genomic DNA from 18 species of *Helicobacter* including two strains of *H. ganmani*. Only *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 *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 *H. ganmani* 16S rDNA determined by DNA sequence analysis were positive in the *H. ganmani* PCR and 13 additional samples, of which seven displayed a *Helicobacter* genus-specific PCR-product homologues to *Helicobacter* sp. 'liver 3' by sequence analysis, were also positive for *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 *Helicobacter* positive and negative groups, and although no significant differences were noted, the *p*-value for ALT in the *Helicobacter* positive and negative group just failed to reach significance as shown in Table 2.

Discussion

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

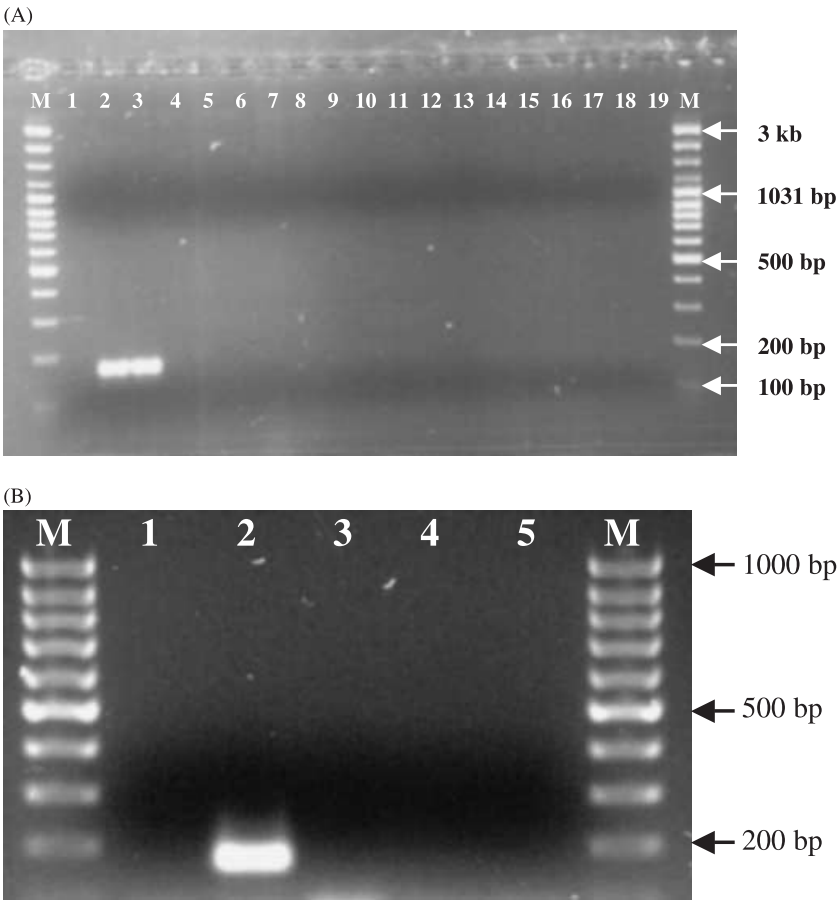

Figure 3 Specificity test of the *Helicobacter ganmani* polymerase chain reaction (PCR)-assay. (A) Lane 1: water control, lanes 2 and 3: *H. ganmani*, lane 4: *H. pylori*, lane 5: *H. pullorum*, lane 6: *H. bilis*, lane 7: *H. canis*, lane 8: *H. hepaticus*, lane 9: *H. rappini*, lane 10: *H. cinaedi*, lane 11: *H. fennelliae*, lane 12: *H. pametensis*, lane 13: *H. salomonis*, lane 14: *H. felis*, lane 15: *H. muridarum*, lane 16: *H. suncus*, lane 17: *H. acinonyx*, lane 18: *H. mustelae*, lane 19: *H. bizzozeronii*, and M: Generuler™ 100 bp DNA Ladder Plus. (B) Lane 1: water control, lane 2: *H. ganmani* CCUG 43527, lane 3: *H. bilis*, lane 4: *H. rodentium* 1707, lane 5: *H. rodentium* 2060, and M: Generuler™ 100 bp DNA Ladder.

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

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

with hepatitis C and possibly in hepatocellular carcinoma [31]. Presence of *H. pylori and* other *Helicobacters* 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 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 overexpression 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 superadded infections in liver diseases in children and adults may clarify the significance of *Helicobacter* spp. in liver tissue.

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