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Association of the Presence of *Helicobacter* in Gallbladder Tissue with Cholelithiasis and Cholecystitis

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The presence of *Helicobacter* DNA species has been investigated in the biliary epithelium of patients with biliary diseases. However, conflicting results have been observed that may have been due to the small number of subjects studied, difficulty in obtaining a healthy control group, absence of controlling for confounding factors, or differences among populations. Therefore, we investigated the presence of *Helicobacter* species by culture and nested PCR of 16S rRNA genes in gallbladder tissue and bile from 46 Brazilian subjects with and 18 without cholelithiasis. The control group was mainly composed of liver donors and of patients who had submitted to cholecystectomy as part of the surgical treatment for morbid obesity. No *Helicobacter* species were grown from the bile or gallbladder tissues. *Helicobacter* DNA was detected in the gallbladder tissue and bile from 31.3 and 42.9% of the patients, respectively. In a logistic regression model, cholelithiasis was positively and independently associated with the female gender ($P = 0.02$), increasing age ($P = 0.002$), and the presence of *Helicobacter* DNA in the gallbladder tissue ($P = 0.009$). The presence of *Helicobacter* DNA in the bile was not associated with cholelithiasis ($P = 0.8$). A significant association between the presence of *Helicobacter* DNA in the gallbladder epithelium and histological cholecystitis, even after adjusting for gender and age ($P = 0.002$), was also observed. The sequences of the 16S rRNA genes were >99% similar to that of *Helicobacter pylori*. In conclusion, our results support the hypothesis that *Helicobacter* is associated with the pathogenesis of human cholelithiasis and cholecystitis.

Helicobacter species isolated from the bile, gallbladder, or liver tissue of some animals, such as *Helicobacter pullorum* from poultry (20), *H. canis* from dogs (5), *H. cholecystus* from Syrian hamsters (6), "*Helicobacter rappini*" from sheep fetuses (9), and *H. hepaticus* and *H. bilis* from mice (4, 21), have been associated with hepatobiliary diseases. In the past few years, the presence of DNA of species of *Helicobacter*, including the well-known human pathogen *H. pylori*, has been identified in the bile, liver, and biliary epithelium obtained from patients with hepatobiliary diseases (3, 11, 14, 15). More recently, our group isolated (for the first time) a *H. pylori* strain from the liver of a patient with cirrhosis, demonstrating that bacteria of the genus *Helicobacter* may be viable in the human liver, as it is seen to be in animals (16).

In regard to the biliary diseases, few patients were evaluated in the first studies. In one of those studies, *ureB H. pylori*-specific DNA was detected in the gallbladder tissue of a Japanese patient with gallstone and cholecystitis (8). In another study evaluating the presence of *H. pylori ureA* genes in the bile by nested PCR, Lin et al. (11) observed a positive result in three patients with primary or metastatic pancreatic tumor but not in four patients with biliary diseases.

In studies of the same subject that included a larger number of patients, discordant results have been observed. In some of them, the presence of DNA of enterohepatic *Helicobacter* or *H. pylori* has been detected. Fox et al. (3) have found *H. bilis*, *H. pullorum*, or "*H. rappini*" DNA in bile or gallbladder tissue from Chilean patients with cholecystitis or cholelithiasis. More recently, the level of *H. bilis* DNA was seen to be higher in the bile of patients from Japan and Thailand with bile duct or gallbladder carcinoma than from those without malignant disease of the biliary tree (12). In another study from Yugoslavia, the presence of *H. pylori*-specific DNA in the bile was associated with biliary tract carcinoma but no association was seen between patients with gallstone and those without biliary disease (1).

Other studies from Germany (19) and Mexico (13) failed in detecting the presence of DNA of *Helicobacter* spp. in bile or gallbladder tissue from patients with biliary tree disease. In a Japanese study, furthermore, DNA of *Campylobacter* (rather than that of *Helicobacter*) was detected in the bile and biliary epithelium of patients with hepatolithiasis (7).

These discordant results may be explained by regional differences. However, it has to be emphasized that in most of the studies there was no control group or there were few patients included as controls (3). In other studies, patients that composed the control group had other disorders (such as pancreatic or gastric malignancies) that may have introduced bias (since the presence of *Helicobacter* DNA has been detected in

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the bile of patients with these diseases) (11). Furthermore, in the studies aimed to investigate the presence of *Helicobacter* in the biliary tree as a risk factor for biliary disease, no adjustment for confounding factors was done.

Thus, we investigated the presence of *Helicobacter* species in the bile and gallbladder epithelium from patients with cholecystitis and/or gallstone disease, including a control group mainly composed of liver transplantation donors and of subjects who had submitted to surgery for morbid obesity. We also evaluated (controlling for confounding factors such as gender and age) the association between the presence of *Helicobacter* DNA and that of biliary diseases.

MATERIALS AND METHODS

Patients. This study was approved by the Ethics Committee of each institution, and informed consent was obtained from all patients.

A total of 64 patients, 46 (31 females and 15 males; mean age, 51.5 ± 16.4 years [range, 21 to 80]) with cholelithiasis and 18 (6 females and 12 males; mean age, 38.3 ± 17.4 years [range, 18 to 70]) without cholelithiasis, were prospectively evaluated. The patients were referred to the Hospital Centenário de São Leopoldo and Irmandade Santa Casa de Misericórdia, Porto Alegre, Brazil. All of them had submitted to abdominal echography to confirm the presence of cholelithiasis before the cholecystectomy. Among the 18 patients without cholelithiasis, 9 were patients from whom the gallbladder was removed as part of the surgical treatment for morbid obesity and 4 were liver transplantation donors who did not have any other hepatic, pancreatic, gastric, or biliary disease. Two patients with gastric cancer and three with pancreatic cancer but without gallstone were also included in the analysis of the risk of gallstone disease.

Gallbladder tissue was taken from all patients, and bile specimens were taken from 56 of the patients immediately after cholecystectomy. The samples were immediately frozen at −80°C before processing for culture and DNA extraction was performed.

Histological study. Gallbladder tissue specimens for histology (available from 51 patients [41 of the test group and 10 controls]) were fixed in 10% buffered formalin immediately after cholecystectomy. The samples were then embedded in paraffin wax and 5-μm-thick histological sections were stained with hematoxylin and eosin for histological analysis. The samples were examined by a pathologist who was unaware of their origin. The diagnosis of cholecystitis was based on the presence of mono- or mono- and polymorphonuclear inflammatory cells in the lamina propria, fusion of the mucosal folds giving rise to buried crypts of epithelium, and the presence of Rokitansky-Aschoff sinuses (2).

Microbiological study. The gallbladder tissue and bile were separately homogenized in 0.5 ml of blood heart infusion broth in a glass tissue grinder and plated onto petri dishes containing freshly prepared Belo Horizonte medium (17). Plates were incubated under microaerophilic conditions at 37°C for up to 21 days.

DNA isolation. Gallbladder tissue or bile DNA was extracted with a QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations, with minor modifications. Briefly, approximately 25 mg of tissue and 500 μl of bile samples were suspended in 180 μl of lysis buffer (buffer ATL) and homogenized by vortexing. A total of 20 ml of a proteinase K solution (20 mg/ml) was then added, followed by an overnight incubation at 56°C. A second lysis buffer (buffer AL) provided in the kit was added, and the sample was incubated at 70°C for 10 min. Next, 200 μl of ethanol was added; this mixture was then loaded on the QIAamp spin column and centrifuged at 6,000 × *g* for 1 min. The QIAamp spin column was placed in a 2-ml collection microtube, and the containing filtrate was discarded. The column material was washed twice (250 μl each time) with the first buffer (buffer AW1) and twice (250 μl each time) with the second washing buffer (buffer AW2) provided in the kit. Finally, the DNA was eluted with 100 μl of distilled water (2 × 50 μl). The DNA concentration was determined by measuring the optical density at 260 nm.

PCR amplification with *Helicobacter* genus-specific primers. The 16S rRNA gene of the genus *Helicobacter* was amplified by a nested PCR assay. The outer primer pair (B37 and C70) (4) was used to generate 16S rRNA amplicons of approximately 1,500 bp. The nested inner primer pairs, which are specific for the *Helicobacter* genus, amplified fragments of 1,200 bp (primer pair C97 and C05) or 400 bp (primer pair C97 and C98) (3). PCRs were performed in an Applied Biosystems thermal cycler in thin-wall tubes. A 10-μl amount of each DNA preparation was added to 100 μl of a reaction mixture containing 1% *Taq* polymerase buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.3]), a 0.5 μM concentration of each primer, a 200 μM concentration of each deoxynucleotide, and 2.5 U of *Taq* polymerase. The amplified product was identified by electrophoresis in a 1.0% agarose gel. The DNA was stained with ethidium bromide and examined under UV light. In the second round, 1 μl of the PCR product was added to the reaction mixture. The sequences of the primers and PCR conditions are shown in Table 1. An *Escherichia coli* strain (clinical isolate) and a *H. pylori* strain (TX30A) served as negative and positive controls, respectively, and distilled water was used as an internal reaction negative control.

TABLE 1. Oligonucleotide primers used for PCR amplification of the 16S rRNA gene

Primer[a]	Sequence (5′–3′)[b]	Position[c]
C70 (f)[d]	AGAGTTTGATYMTGGC	8–23
B37 (r)[d]	TACGGYTACCTTGTTACGA	1495–1513
C97 (f)[e]	GCTATGACGGGTATCC	262–277
C05 (r)[e]	ACTTCACCC CAGTCGCTG	1440–1458
C98 (r)[e]	GATTTTACCCCTACACCA	642–659

[a] f, forward; r, reverse.
[b] Base designations are standard International Union of Biochemistry designations for bases and ambiguity.
[c] *E. coli* numbering.
[d] PCRs were conducted under the following conditions: 95°C for 5 min followed by 24 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 3 min (with 5 s between cycles) and a final incubation at 72°C for 5 min.
[e] PCRs were conducted under the following conditions: 95°C for 5 min followed by 34 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min and a final incubation at 72°C for 5 min.

16S rRNA gene sequencing. The nested PCR products of 1,200 or 400 bp were purified using a Wizard PCR-Prep purification kit (Promega, Madison, Wis.) according to the manufacturer's directions. The purified amplicons were directly sequenced with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions by using sequencing primers B35, B36, C01, C31, and X91 for the amplicons with 1,200 bp or C97 and C98 for those with 400 bp (3, 4). The sequences were determined in an Applied Biosystems DNA automated sequencer (ABI PRISM 310; Applied Biosystems). The sequences were aligned using the CAP program at the INFOBIOGEN web server and compared (using the Blast Program at the National Center for Biotechnology Information computer server) with sequences listed in the GenBank database.

Statistical analysis. Data were analyzed with a statistical software package, version 10 (SPSS Inc., Chicago, Ill.). For the analysis of the risk of gallstone disease, a logistic regression model was created, adjusting for confounding factors such as gender and age. Variables that showed significance levels below 0.25 in the univariate analysis were selected for multivariate analysis. In the multivariate analysis, the level of significance was set at $P < 0.05$. The association between the presence of *Helicobacter* DNA and that of histologic cholecystitis was also adjusted for age and gender in a logistic regression model.

RESULTS

Culture. Despite prolonged incubation for up to 3 weeks under microaerophilic conditions, no *Helicobacter* species were grown from the frozen bile or gallbladder tissues.

PCR amplification with *Helicobacter* genus-specific primers. *Helicobacter* DNA was detected by nested PCR in the gallbladder tissue and bile from 20 (31.3%) of 64 patients and 24 (42.9%) of 56 patients, respectively. Among the gallbladder *Helicobacter* DNA-positive patients, 18 (90%) had cholelithiasis. Both the shorter (400-bp) and the longer (1,200-bp) amplicons were obtained in the samples of all positive patients.

A total of 55 (85.9%) patients received antimicrobial drugs before the surgery. Nine patients (three liver donors and six obese subjects), all of them from the control group, received no antibiotics. Previous use of antimicrobials was not associated with *Helicobacter* DNA detection ($P = 0.3$).

TABLE 2. Results of univariate and multivariate analysis with variables associated with cholelithiasis

Independent variable	Results of[a]:			
	Univariate analysis (*P*)	Multivariate analysis		
		P	OR	95% CI
Age	0.007	0.002	1.07	1.31–1.12
Gender	0.03	0.02	5.68	1.38–23.49
H. pylori$^+$ DNA tissue	0.03	0.009	14.72	1.97–108.90

[a] Covariates with $P \leq 0.25$ in the univariate analysis were included in the full model. In the multivariate analysis, a value of $P \leq 0.05$ was considered significant.

Association between the presence of *Helicobacter* DNA and gallbladder diseases. In the univariate analysis, the presence of cholelithiasis was positively associated with old age (P = 0.007), female gender (P = 0.03), and the presence of *Helicobacter* DNA in the gallbladder (P = 0.03). When the logistic regression model was applied in the multivariate analysis, cholelithiasis remained independently associated with increasing age (P = 0.002; odds ratio [OR] = 1.07; 95% confidence interval [CI] = 1.03 to 1.12), female gender (P = 0.02; OR = 5.68; 95% CI = 1.38 to 23.49), and the presence of *H. pylori* DNA in the gallbladder tissue (P = 0.009; OR = 14.72; 95% CI = 1.97 to 108.90), as shown in Table 2. However, when the presence of *H. pylori* DNA in the bile was analyzed, no association between the presence of cholelithiasis and bile DNA positivity was seen (P = 0.8). The presence of histological cholecystitis was observed in 28 (54.9%) out of 51 tissue specimens. None of the gallbladder samples showed evidence of parasitic infection. Tests for the presence of *Helicobacter* DNA gave positive results with 15 (50.0%) gallbladder tissue samples and with 7 (23.3%) bile samples from 28 patients with cholecystitis. Inflammation was seen in 15 (93.8%) of 16 *Helicobacter* DNA-positive and in 13 (37.1%) of 35 *Helicobacter* DNA-negative gallbladder mucosa available for histological study. A significant association (P = 0.0003) was seen between the presence of *Helicobacter* DNA and that of histological cholecystitis even after adjusting for age and sex (P = 0.002). On the other hand, no association was observed between the presence of *H. pylori* DNA in the bile and inflammation of the gallbladder mucosa (P = 0.64).

***Helicobacter* genus identification.** Since association with gallbladder and cholecystitis was seen only in the presence of *Helicobacter* DNA in the gallbladder tissue, amplicons (of 1,200 bp from one patient and of 400 bp from the others) obtained from 18 gallbladders were sequenced to verify that they truly represented *Helicobacter* 16S rRNA and to determine species identity. Amplicons of 1,200 bp obtained from three bile samples were also sequenced. All the sequences were deposited in GenBank. The sequences of 19 strains were more than 99.3% similar to that of *H. pylori*. The levels of similarity to the sequence of *H. pylori* for the two other sequences were 98.9 and 97.8% (Table 3).

TABLE 3. Sequencing analysis of the 16S rRNA gene detected in gallbladder tissue and bile

Sample	Site	No. of bp sequenced	*H. pylori* similarity (%)	Accession no.
1	Gallbladder	403	100.0	AY304555
2	Gallbladder	402	100.0	AY304557
3	Gallbladder	399	100.0	AY304561
4	Gallbladder	399	100.0	AY304566
5	Gallbladder	383	100.0	AY304565
6	Gallbladder	357	100.0	AY304554
7	Gallbladder	382	100.0	AY304552
8	Gallbladder	402	99.7	AY304562
9	Gallbladder	400	99.8	AY304553
10	Gallbladder	400	99.8	AY304559
11	Gallbladder	400	99.7	AY304567
12	Gallbladder	389	99.7	AY304568
13	Gallbladder	372	99.7	AY304564
14	Gallbladder	400	99.5	AY304556
15	Gallbladder	335	99.3	AY304563
16	Gallbladder	387	98.9	AY304560
17	Gallbladder	333	97.8	AY304558
18	Gallbladder	1050	99.8	AY304551
19	Bile	1049	100.0	AY304571
20	Bile	1050	99.9	AY304569
21	Bile	1049	99.8	AY304570

DISCUSSION

The presence of *Helicobacter* DNA has been investigated in the bile and biliary tissue of human beings with biliary diseases, but contradictory results have been observed. In some studies, the presence of intestinal *Helicobacter* or *H. pylori* DNA has been seen in bile and/or gallbladder tissue from patients with benign or malign biliary diseases. In contrast, other authors did not detect any *Helicobacter* DNA in the biliary trees of patients with the same diseases. Although regional variations might be considerable, it has to be mentioned that DNA from *H. pylori* was consistently detected in biliary specimens only when a more sensitive nested PCR method was used (1, 10, 11). The sensitivity of traditional 16S rRNA PCR analysis is quite low, giving positive results only when a bacterial concentration is higher than 10^3 CFU/μl. Conversely, intestinal species of *Helicobacter* have been detected by conventional PCR technique followed (or not followed) by hybridization (3, 12). These findings suggest that different *Helicobacter* species can be present in the human biliary tree but that the number of microorganisms can differ according to the species, with several intestinal *Helicobacter* species being present in higher numbers than *H. pylori.* This difference may be explained by the fact that intestinal *Helicobacter* species (such as "*H. rappini*," *H. bilis*, *H. canis*, *H. cholecystus*, and *H. pullorum*) are resistant to bile in vitro, a property that may confer protection against the deleterious effects of bile in vivo and adapt them better to the hepatobiliary milieu. In fact, all of these species have been seen in the liver of one or more animal species.

Why intestinal *Helicobacter* species have been identified in patients from Japan, Thailand, and Chile (3, 12) and *H. pylori* has been identified in patients from other geographical regions, as we observed in this study, deserves further investigations. We evaluated an urban population. We do not know whether the Asiatic or Chilean patients included in the studies cited above were from rural areas; if they were so, we speculate that they had more of a chance to acquire from animals (such as chickens) intestinal *Helicobacter* species that can colonize the hepatobiliary tract of human beings more easily than other gastric *Helicobacter* species.

Up to now, whether the *Helicobacter* species present in the human biliary tree play a role in the pathogenesis of biliary diseases has not been clearly proven. In this study, we observed an association between the presence of the microorganism in gallbladder and the presence of cholelithiasis as well as cholecystitis. Points that strengthen the validity of our results include the prospective study design, the accurate selection of controls, the adjustment for confounding factors, and the more accurate diagnosis of the presence of *Helicobacter* DNA. Our control group was mainly composed of morbid obesity patients who neither had gallbladder disease nor were previously submitted to any other gastric surgery that would interfere with *H. pylori* gastric status. In addition, there are no differences between obese populations and the general population with regard to gastric *H. pylori* infection (18). Furthermore, in this study the presence of the bacterium DNA was closely associated with histological mucosal inflammation. This kind of association is seen in *Helicobacter* infections that are really associated with the presence of a disease such as human gastric *H. pylori* infection, which is a factor linked to the genesis of gastric carcinoma and peptic ulcer. Although these facts point to a real association, we may not rule out the possibility that the bacterium colonized a previously damaged epithelium. Even if this were the case, we may assume that a persistent colonizer such as *Helicobacter* might be an additional factor in human biliary system tumorigenesis. In fact, Kuroki et al. (10) recently demonstrated that the level of epithelium proliferation (a condition that predisposes to carcinogenesis) was higher in *Helicobacter*-positive biliary epithelium than in bacterium-negative epithelium.

In similarity to the findings of other studies on this subject, we were unable to isolate the bacterium by culture; that may have been due to the fact that the DNA we detected was from nonviable organisms. Conversely, there are different ways to explain our inability to isolate viable bacteria. Firstly, most (85.9%) patients had received antimicrobial therapy before surgery. In addition, the samples were maintained frozen without any protective solution, which may have compromised bacterial viability. Finally, as discussed above, it is possible that the number of bacterium is very few and that they may have been partially inhibited by adverse conditions in the biliary milieu. In addition, we can speculate that these strains might have had some distinct requirements that are as yet unknown. These facts highlight the need to improve the conditions for the growth of *Helicobacter* species from the biliary tree to better characterize the microorganism and to allow the development of experimental models for studying the role of *Helicobacter* in the genesis of biliary diseases.

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