

## ORIGINAL ARTICLE

# Identification of *Helicobacter* spp. in bile and gallbladder tissue of patients with symptomatic gallbladder disease

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## Abstract

**Background:** This experimental study was designed to determine if *Helicobacter* spp. contribute to benign gallbladder disease using polymerase chain reaction (PCR) methods.

**Methods:** Patients with benign gallbladder disease scheduled for elective cholecystectomy at New York University Langone Medical Center were recruited from February to May 2008. Bile, gallbladder tissue and gallstones were collected. DNA was isolated from these specimens and amplified via PCR using C97F and C98R primers specific for *Helicobacter* spp. Appropriate positive and negative controls were used. Products were analysed with agarose gel electrophoresis, sequenced and results aligned using SEQUENCHER. Plasma was collected for detection of anti-*Helicobacter pylori* antibodies via enzyme-linked immunosorbent assay.

**Results:** Of 36 patients, 12 patients' bile and/or tissue were positive for *Helicobacter* spp. by PCR. Species were most homologous with *H. pylori*, although other *Helicobacter* spp. were suggested. Six of 12 patients demonstrated anti-*Helicobacter* antibodies in plasma, suggesting that the remaining six might have demonstrated other species besides *H. pylori*. Four of six plasma samples with anti-*Helicobacter* antibodies were anti-CagA (cytotoxin associated gene) negative.

**Discussion:** *Helicobacter* spp. can be detected in bile and gallbladder tissue of patients with benign gallbladder disease. The contribution of these bacteria to the pathophysiology of gallbladder disease and gallstone formation requires further study.

## Keywords

*Helicobacter*, gallstones, gallbladder, cholecystitis

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## Introduction

Bacterial colonization of tissue plays a critical role in human health and disease.<sup>1</sup> This interaction is well demonstrated by the role of *Helicobacter pylori* in peptic ulcer disease.<sup>2</sup> The recognition of this interaction dramatically changed the management of peptic ulcer disease and has led to a broader understanding of the

aetiology of benign and malignant disease of the stomach, duodenum and oesophagus.<sup>2-6</sup>

Gallbladder disease has a significant impact on health care in the USA. It is estimated that 750 000 cholecystectomies are performed annually in the USA (<http://www.ssat.com/cgi-bin/chole7.cgi>). Although the aetiology of gallbladder disease is multifactorial, bacteria are not traditionally thought to be a priming factor for the development of gallstones or gallbladder inflammation. In our own retrospective study of patients with gallbladder dysfunction, defined as a gallbladder ejection fraction of  $\leq 35\%$  on hepatobiliary iminodiacetic acid (HIDA) scan, 71%

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had pathological evidence of chronic cholecystitis and 40% of those patients had no evidence of gallstones. Furthermore, we found that 73.2% of 101 such patients also had gastro-oesophageal reflux disease (GORD), whereas 58.4% had gastritis.<sup>7</sup> This observation raised the question of whether bacterial colonization of the gallbladder may result in chronic inflammation similar to the association of *H. pylori* in chronic gastric inflammation. It is generally accepted that biliary obstruction and subsequent bile stasis can lead to bacterial overgrowth and to the development of pigmented gallstones. Stewart and colleagues have demonstrated this and have also suggested that 11–20% of cholesterol gallstones, which had been thought to be sterile, are colonized with bacteria.<sup>8,9</sup> These data indicate that bacteria may be important to the formation of all types of gallstones. Furthermore, recent evidence suggests that *Helicobacter* spp., which are fastidious spiral or rod-shaped Gram negative bacteria, can be found not only in gallstones<sup>10–12</sup> but also in bile<sup>13</sup> and gallbladder tissue of specimens demonstrating chronic cholecystitis.<sup>13–15</sup> This is particularly interesting in view of our finding that 58% of patients with gallbladder dysfunction had been diagnosed with gastritis, a disease associated with *H. pylori* infection. Stathopoulos *et al.* reported an association between gallstones and chronic gastritis.<sup>16</sup> In their series, 14 of 19 patients with symptomatic gallstones and moderate to marked gastritis had evidence of *H. pylori* in the stomach, although the authors did not investigate whether *H. pylori* could be detected in the gallbladder.

The purpose of this study was to determine if bacteria, particularly *Helicobacter* spp., play a role in benign gallbladder disease. To our knowledge, no study has evaluated all three elements of the gallbladder system (bile, gallbladder tissue and gallstones) in a single cohort of patients for the presence of *Helicobacter* spp., as we do here.

## Materials and methods

### Patients and specimen collection

During February–July 2008, 45 patients with benign gallbladder disease undergoing elective cholecystectomy at New York University Langone Medical Center were recruited. Immediately following gallbladder excision, the specimens were collected in a sterile specimen cup. Tissue, bile and gallstones (when available) were collected in a sterile manner and stored whole and unprocessed at –80 °C until the time of experimentation. Both bile and tissue samples were available for 36 of 45 (80%) patients; gallstones were not available for analysis in five (13.9%) of these 36 patients. Clinical history of antibiotic use within the past year, diagnosis of upper gastrointestinal (UGI) disease and specimen pathology (Table 1) were collected and stored in a Health Insurance Portability and Accountability Act (HIPAA)-compliant database.

### DNA extraction, polymerase chain reaction and DNA sequencing

DNA was isolated from bile using the Qiamp DNA Stool Minikit (Qiagen, Inc., Valencia, CA, USA) according to the company's

**Table 1** Clinical presentation of patients enrolled, including all patients and patients positive for *Helicobacter* by polymerase chain reaction technique

	Overall group	<i>Helicobacter</i> spp.+	
	<i>n</i> = 36	<i>n</i> = 12	
		Bile +	5
		Tissue +	4
		Bile and tissue +	3
Median age, years	48.5	52.0	
% female (median age)	52.8 (48.0)	50 (54.0)	
% male (median age)	48.2 (50.5)	50 (50.5)	
Took antibiotics within 1 year, <i>n</i> (%)	23 (63.9)	7 (58.3)	
Unknown antibiotic use, <i>n</i> (%)	5 (13.9)	2 (16.7)	
Pathology results			
Chronic cholecystitis, <i>n</i> (%)	34 (94.4)	11 (91.7)	
Cholesterosis, <i>n</i> (%)	10 (27.7)	4 (33.3)	
Clinical history, prior diagnoses			
GORD, <i>n</i> (%)	16 (44.4)	5 (41.7)	
Gastritis, <i>n</i> (%)	4 (11.1)	1 (8.3)	
Unknown, <i>n</i> (%)	3 (8.3)	1 (8.3)	
<i>Helicobacter pylori</i> serology			
Anti- <i>H. pylori</i> , <i>n</i> (%)	10 (27.8)	6 (50)	
Anti-CagA, <i>n</i> (%)	5 (13.9)	2 (16.7)	

GORD, gastro-oesophageal reflux disease

protocol. For DNA isolation from gallstones and tissue, the DNeasy Blood and Tissue Kit (Qiagen, Inc.) was used.

Forward and reverse primers were selected to amplify a conserved segment of the *Helicobacter* spp. 16s rRNA gene (C97F [5'-GCTATGACGGGTATCC] and C98R [5'-GATTTTACC CCT ACACCA]). An annealing temperature of 530 °C was used for 35 cycles. Polymerase chain reaction (PCR) was performed in duplicate on separate occasions for each DNA purified from the samples. Products were analysed with agarose gel electrophoresis. A patient sample was considered positive for *Helicobacter* spp. by PCR method only when there was a positive result on two separate occasions. If a sample demonstrated conflicting results within the two runs (i.e. one positive and one negative result), the sample was run a third time for a final result.

To confirm the presence of *Helicobacter* spp. DNA in bile and tissue, PCR samples were sequenced (Macrogen Corp., Rockland, MD, USA). The SEQUENCHER program was used for sequence alignment. Species identification was performed using the BLAST (Basic Local Alignment Search Tool) program from the National Center for Biotechnology Information.

## Results

### Patients

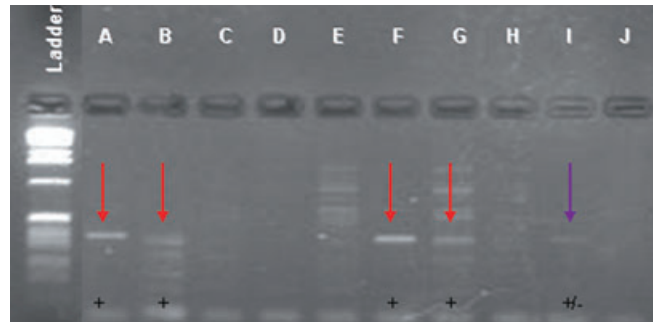
Of 45 patients recruited to this study, 36 (median age 48.5 years, range 24–79 years) had both bile and tissue specimens available for analysis. Nineteen were women (median age 48.0 years, range 25–70 years) and 17 were men (median age 49.0 years, range 24–79 years) (Table 1). Thirty-four of 36 demonstrated biliary colic or symptomatic gallstones, and two were diagnosed with gallbladder-related pancreatitis. One of these two patients had a common bile duct (CBD) and pancreatic duct (PD) stent placed before his scheduled cholecystectomy. Sixteen of 36 (44.4%) had a preoperative diagnosis of GORD, four (11.1%) were diagnosed with gastritis and in three (8.3%) the diagnosis was unknown.

### Identification by PCR of *Helicobacter* spp. in bile and tissue specimens

*Helicobacter* spp. DNA was identified by PCR in eight of 36 (22.2%) bile samples and seven (19.4%) tissue samples for a total of 12 patients, or 33.3% of the total group (Table 1, Fig. 1). Only three patients demonstrated *Helicobacter* DNA in both bile and tissue. None of the 31 gallstones available for analysis demonstrated *Helicobacter* spp. DNA.

### *H. pylori* identification by DNA sequencing in bile and tissue specimens

Specimens positive for *Helicobacter* spp. DNA by PCR were confirmed by sequencing analysis in four bile and four tissue specimens (from seven patients [19.4%]). Of the four bile and four tissue specimens sequenced, BLAST results suggested that the bacteria isolated for each sample was most consistent with *H. pylori*. However, when evaluating results, other candidate bacteria include *Helicobacter acinonychis*, *Helicobacter pullorum*, *Helico-*



**Figure 1** Representative gel of bile samples after polymerase chain reaction (PCR) amplification using *Helicobacter*-specific primers C97F and C98R. Four water samples were used as a negative control; HP26, a known *Helicobacter pylori* sample, was used in duplicate as a positive control. In this gel, A, B, F and G were all considered positive and I was considered indeterminate. G and I were considered negative upon repeated PCR experiments. All other samples were considered negative for *Helicobacter* spp.

*bacter winghamensis*, *Helicobacter canadensis*, *Helicobacter bilis*, *Helicobacter cinaedi* and *Helicobacter hepaticus* because of the high homology in DNA sequence. Only one patient was found to have sequencing results demonstrating *H. pylori* in both bile and tissue samples.

### Overall assessment of *Helicobacter* in bile and tissue of patients with benign gallbladder disease

The median age of the 12 patients with *Helicobacter* spp. in bile and/or tissue was 52.0 years, which is similar to that of the total group (Table 1). Six were women (50%, median age 54.0 years) and six were men (50%, median age 50.5 years). Preoperative diagnosis of UGI disease included GORD in five (41.7%), gastritis in one (8.3%) and was unknown in one (8.3%). Only one patient was tested by esophagogastroduodenoscopy (EGD) to evaluate the presence of UGI disease.

### Antibiotic use before surgery

Antibiotic use by patients within 1 year prior to cholecystectomy was assessed as this could potentially alter the results. Data regarding antibiotic use were available for 33 of 36 patients. Twelve of 36 (33.3%) had taken antibiotics within 1 month of surgery, 17 (47.2%) had not and four (11.1%) could not recall (Table 1). A total of 23 of 36 (63.9%) had used antibiotics within 1 year of surgery, five (13.9%) had not and five (13.9%) could not recall. Antibiotics reported to have been used included clarithromycin, azithromycin, metronidazole, amoxicillin, levofloxacin, ciprofloxacin and xifaxin. Of the 12 patients with PCR evidence for *Helicobacter*, seven (58.3%) had taken antibiotics within 1 year of surgery. Two of the 12 (16.7%) patients with PCR evidence did not know whether they had used antibiotics in the year preceding surgery.

## Pathology

Pathological evaluation of specimens from the 36 patients demonstrated chronic cholecystitis in 34 patients (94.4%), cholesterosis in 10 (27.7%), acute on chronic cholecystitis in one (2.7%), eosinophilic cholecystitis in one (2.7%), hyperplasia in one (2.7%), lymphoid hyperplasia in one (2.7%), adenomyomatosis in one (2.7%), and intestinal and pyloric gland metaplasia in association with biliary intraepithelial neoplasia in one (2.7%). Of 12 *Helicobacter*-positive patients, 11 (91.7%) had pathological changes consistent with chronic cholecystitis and four (33.3%) demonstrated cholesterosis. One patient had acute on chronic cholecystitis (8.3%), and one demonstrated tissue hyperplasia (8.3%). Gallstone characterization was not recorded.

## Anti-*H. pylori* and anti-CagA antibodies in plasma

Of the 36 patients studied, 34 had plasma available for enzyme-linked immunosorbent assay (ELISA) analysis to evaluate for the presence of *H. pylori* antibodies and anti-cytotoxin associated gene (CagA) antibodies. Ten (27.8%) had detectable anti-*H. pylori* antibodies and five of the 10 had detectable anti-CagA antibodies. Of the 12 patients with *Helicobacter* spp., all had plasma samples available for ELISA analysis. Six demonstrated anti-*H. pylori* antibodies. This represents 60% of the 10 patients with these antibodies in the whole group. Interestingly, four of these six (66.7%) were anti-CagA- (Table 1).

## Discussion

Evidence of *Helicobacter* spp. was found in 12 of 36 patients with benign gallbladder disease, suggesting that *Helicobacter* spp. were involved in 33.3% of patients studied. Only one patient whose tissue was positive for *Helicobacter* had a history of biliary tract manipulation, which may have resulted in direct inoculation of bacteria in the biliary system. Preoperative diagnosis of GORD (41.7%) or gastritis (8.3%) in the 12 positive patients was made by clinical judgement, not by endoscopy or imaging studies, in all but one patient. Therefore, no reliable conclusion can be drawn. The female : male distribution did not differ from that seen overall in the 12 patients and the age distribution was also similar. Of these 12 patients, six were seronegative for *H. pylori*, suggesting that a different *Helicobacter* species was probably present (such as *H. acinonychis*, *H. pullorum*, *H. winghamensis*, *H. canadensis*, *H. bilis*, *H. cinaedi* or *H. hepaticus* as mentioned earlier).

Sequencing analysis suggests that the bacteria identified in this group of seven patients were most probably *H. pylori*. Serology results further support this in five of these seven patients as they demonstrated anti-*H. pylori* antibodies in their serum. It is possible that the remaining two seronegative patients had different *Helicobacter* spp. present in bile or tissue.<sup>13</sup> Of the 12 patients who were positive by PCR only, six had antibodies against *H. pylori*, again suggesting that the other six had different *Helicobacter* spp. present in bile or tissue.

We also determined whether seropositive patients had evidence of exposure to CagA+ strains of *H. pylori*. CagA is a virulence factor found in *H. pylori* that translocates into gastric epithelial cells<sup>17</sup> and is associated with increased inflammatory response by gastric mucosal cells<sup>18,19</sup> as well as an increased risk of gastric adenocarcinoma.<sup>20</sup> In this study, 66.7% of patients whose bile or tissue were positive for *Helicobacter* spp. by PCR technique only and who demonstrated anti-*H. pylori* antibodies were anti-CagA-, whereas the remainder demonstrated evidence of infection with CagA+ strains. Although this did not reach statistical significance, perhaps secondary to small sample size, this questions whether CagA- strains of *H. pylori* are more likely to colonize or infect the gallbladder-biliary tract. This is particularly interesting as the *cagA* genotype is thought to protect against more severe forms of GORD, including the complication of Barrett's oesophagus.<sup>21,22</sup> This observation supports our hypothesis that benign gallbladder disease may be associated with GORD and gastritis, and it may involve infection with CagA- strains of *H. pylori*. Analysis of this hypothesis with a larger cohort of patients should be performed.

None of the gallstone specimens were found to have *Helicobacter* DNA, although bacterial DNA was detected in the bile of some patients. As *Helicobacter* spp. are fastidious organisms, it may be that the gallstone environment is not appropriate to support their growth. It is also possible that we did not detect *Helicobacter* spp. because we did not analyse gallstone samples from within the centre of the stones; instead, we took samples from the outer surface of each gallstone. Stewart and colleagues' founding work demonstrated bacteria within the whole gallstone as each gallstone studied was crushed before analysis.<sup>8,9</sup> Other groups who demonstrated *Helicobacter* spp. in gallstones took samples from the centre of the stones or used the whole stone for analysis.<sup>10-12,23,24</sup>

Overall, 58.3% of 12 patients with *Helicobacter* spp. in bile and/or tissue took antibiotics within 1 year prior to cholecystectomy. Overall, 69.6% of patients analysed reported antibiotic use within the previous year. In this preliminary study, we conclude that antibiotic use probably did not affect the results as we were able to detect *Helicobacter* spp. in patients who had used these medications.

Pathologically, 91.7% of patients with *Helicobacter* DNA by PCR had evidence of chronic cholecystitis. This does not differ from findings for the whole group, in which 94.4% of patients demonstrated evidence of chronic cholecystitis.

These results demonstrate that *Helicobacter* spp., particularly *H. pylori*, can be detected in the bile and gallbladder tissue of patients with benign gallbladder disease. The mechanism through which these bacteria contribute to the pathophysiology of gallbladder disease, particularly gallstone formation, is unclear at this time. This has been studied in more detail in a mouse model by Maurer *et al.*,<sup>25,26</sup> who suggest that *Helicobacter* spp. but not necessarily *H. pylori* may contribute to benign gallbladder disease. Based on our data, the paradigm of gallstone formation and the contribution of *Helicobacter* spp. should be evaluated critically.

### Conflicts of interest

None declared.

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