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BRIEF ARTICLE

Helicobacter species and common gut bacterial DNA in gallbladder with cholecystitis

Peren H Karagin, Unne Stenram, Torkel Wadström, Åsa Ljungh

Peren H Karagin, Torkel Wadström, Åsa Ljungh, Department of Medical Microbiology, Lund University, Sölvegatan 23, SE-223 62 Lund, Sweden
Unne Stenram, Department of Pathology, Lund University, SE-22185 Lund, Sweden
Author contributions: Stenram U took part in designing the investigation, performed all histological examinations and participated in writing the paper; Karagin PH performed PCR analyzes and wrote the paper; Wadström T and Ljungh Å took part in designing the investigation and participated in writing the paper.
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Correspondence to: Peren H Karagin, Post Doc, Department of Medical Microbiology, Lund University, Sölvegatan 23, SE-223 62 Lund, Sweden. perenbaglan@yahoo.com
Telephone: +46-46-173298 Fax: +46-46-189117
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Abstract

AIM: To analyze the association between *Helicobacter* spp. and some common gut bacteria in patients with cholecystitis.

METHODS: A nested-polymerase chain reaction (PCR), specific to 16S rRNA of *Helicobacter* spp. was performed on paraffin-embedded gallbladder samples of 100 cholecystitis and 102 control cases. The samples were also analyzed for some common gut bacteria by PCR. Positive samples were sequenced for species identification.

RESULTS: *Helicobacter* DNA was found in seven out of 100 cases of acute and chronic cholecystitis. Sequence analysis displayed *Helicobacter pullorum* (*H. pullorum*) in six cases and *Helicobacter pylori* in one; *H. pullorum* was only found in cases with metaplasia. Control samples were negative for *Helicobacter* spp. and some common gut bacteria. There was a significant difference (P = 0.007) between cholecystitis and control samples for *Helicobacter* DNA.

CONCLUSION: A possible relationship was detected between *Helicobacter* DNA and cholecystitis. Further serological and immunohistochemical studies are needed to support these data.

Key words: *Helicobacter*; Gallbladder; Cholecystitis; 16S rRNA; Polymerase chain reaction

Peer reviewer: Zeinab Nabil Ahmed, Professor of Microbiology, Microbiology and Immunology Department, Faculty of Medicine (for girls), Al-Azhar University, Nasr City, 1047, Cairo, Egypt

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INTRODUCTION

The most well-known member of the *Helicobacter* genus, *Helicobacter pylori* (*H. pylori*), is classified as a type 1 carcinogen[1], and infects the human stomach and causes gastritis, peptic ulcer disease and gastric cancer. Besides *H. pylori*, the genus *Helicobacter* contains more than 25 species[2], many of which cause extragastric diseases in humans and animals[3-10]. These are named enterohepatic *Helicobacter* species (EHS) or EHS and colonize the hepatobiliary tract of humans, and include *Helicobacter hepaticus* (*H. hepaticus*), *Helicobacter bilis* (*H. bilis*), *Helicobacter rappini* (*H. rappini*), *Helicobacter ganmani* (*H. ganmani*) and *Helicobacter pullorum* (*H. pullorum*). Several of these EHS are associated with the pathogenesis of chronic biliary disorders, such as cholecystitis, cholelithiasis, gallbladder carcinoma and bile tract carcinoma and some liver diseases, such as primary

sclerosing cholangitis, primary biliary cirrhosis and hepatocellular carcinoma[11-14]. Moreover, chronic pancreatitis and pancreatic cancer, as well as inflammatory bowel diseases in humans have also been reported to be positive for EHS in various polymerase chain reaction (PCR)-based studies[15-17].

Chronic cholecystitis is the most prevalent disease in various populations in industrialized countries[18]. During the 20 years from 1965-1969 to 1985-1989, the mortality from gallbladder cancer increased by 30% in Sweden. However, not all high-risk European countries showed such an increase and the mortality decreased in some countries[19]. Chronic cholecystitis is commonly associated with gallstone disease[20] and some studies have shown that cholecystitis and gallstones can cause epithelial hyperplasia of the gallbladder mucosa or cancer, and various bacterial genomes have been detected in gallbladder carcinoma tissue[21]. Moreover, a recent study has shown that *H. pylori* can damage human gallbladder epithelial cells *in vitro*, and could be the key factor that leads to clinical cholecystitis[22]. Some studies have revealed the presence of bile-resistant EHS in the gallbladder mucosa and in gallstones. It has been shown that the presence of *H. pylori* and EHS in bile might represent a risk factor for bile stone formation[4,23-26]. One study has clearly demonstrated the presence of a mixed bacterial population in gallstones[4]. *Salmonella typhi* is another bacterial pathogen of the biliary tree in human gallstones and gallbladder cancer[27,28]. *Salmonella* biofilm has been shown on human gallstones[29]. Moreover, *Campylobacter* spp. have also been detected in bile and epithelial samples in cholecystolithiasis[30].

H. pylori, *H. pullorum* and *H. bilis* have been isolated from humans with gallbladder disease such as cholecystitis, cholelithiasis[9,31,32], gallbladder carcinoma and bile tract carcinoma[33]. A possible relationship between chronic cholecystitis and *Helicobacter* DNA has been shown by some investigators[9,31,32,34] but, as far as we are aware, there has been no study published on Scandinavian patients with cholecystitis. Therefore, we examined the relationship between *Helicobacter* spp. and some common gut bacteria in Swedish patients with cholecystitis.

MATERIALS AND METHODS

Patients and histological methods

We re-examined the gallbladders from 100 cholecystitis patients from 2006-2007 (mean age: 48 years; range: 20-84 years; 35 male, 65 female) and 102 control patients (mean age: 58 years; range: 11-85 years; 54 male, 48 female) from 1999 to 2009, taken from the files of the Department of Pathology, Lund University Hospital. Of the 100 cholecystitis samples, 50 were acute (mean age: 55 years; range: 23-81 years; 22 male, 28 female), and 50 were chronic (mean age: 44 years; range: 20-84 years; 13 male, 37 female). Among the 50 patients with acute cholecystitis, 34 cases (median age: 56 years; range: 23-79 years; 15 male, 19 female) were without metaplasia and 16 (median age: 54 years; range: 36-81 years; 7 male, 9 female) had metaplasia. Among the 50 patients with chronic cholecystitis, 27 cases (median age: 45 years; range 20-84 years; 8 male, 19 female) were without metaplasia and 23 (median age: 42 years; range: 20-71 years; 5 male, 18 female) had metaplasia. As control samples, we used 18 normal gallbladders from patients with pancreatic malignancies reported elsewhere[17], and 84 consecutive patients with normal gallbladders from 1999 to 2009 (median age: 61 years; range: 11-85 years; 44 male, 40 female). There was no metaplasia in these gallbladders. The diagnosis was: six hepatocellular carcinoma, 40 liver metastases (mainly colorectal), four intestinal carcinoids, three liver carcinoid metastases, seven focal nodal hyperplasias, three bile duct cysts, one gallbladder adenoma, three splenomegalies, two pancreatic neuroendocrine malignancies, one benign pancreatic cyst, one adrenal carcinoma, and 13 normal gallbladders with no other diagnosis.

Two to five sections were taken from each case, and one section from the ductus cysticus. Sections that showed mucosal metaplasia were stained with Alcian blue-periodic acid Schiff (AB-PAS), pH 2.5, and Warthin-Starry silver stain for *Helicobacter* spp. One section was immunostained with anti-*H. pylori* antibody (DAKO, Glostrup, Denmark; diluted 1:300) according to Apostolov *et al*[9]. Mucosa was cut from the paraffin blocks with the tip of a scalpel by careful comparison with the slides. Areas with gastric metaplasia, if present, were included in the samples. The Research Ethics Committee at Lund University approved this study (permit number 588/2006).

DNA extraction

DNA was extracted from approximately 5 mg of each paraffin-embedded gallbladder tissue sample. To ascertain that epithelium was included, two pieces, each of 2-3 mg, were taken from each case. Paraffin-embedded gallbladder samples were de-embedded as previously described[10]. Gallbladder tissue samples were de-embedded by heating at 60℃ for 10 min, followed by washing in xylene for 2 × 5 min. The specimens were rehydrated through graded ethanol (99% and 95% for 2 × 5 min and 70% for 5 min), and finally washed for 5 min in double-distilled water. DNA was extracted by a QIAamp DNA Mini Kit tissue protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracts (200 μL total volume) were combined, and 5 μL of the mixtures was analyzed by PCR.

Helicobacter-specific PCR

DNA extracts were amplified in a GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA, USA) using a semi-nested PCR assay specific for *Helicobacter* 16S rDNA, as previously described[11], using primers 1F (5'CTATGACGGGTATCCGGC3'), 1R (5'CTCACGACACGAGCTGAC3') and 2R (5'TCGCCTTCGCAATGAGTATT3'). Primers 1F and 1R were used in the first step, whereas primers 1F and 2R were used in the second step. The reaction mixture of the first step (25 μL) contained 0.5 μmol/L each primer (1F and 1R), 0.8 mmol/L

each dNTP (Amersham Biosciences, Uppsala, Sweden), 1 × chelating buffer, 2.5 mmol/L $MgCl_2$, 0.05% casein, 0.05% formamid, 1.25 U r*Tth* DNA polymerase (Applied Biosystems), and 5 μL extracted DNA. *H. pylori* (CCUG 17 874) was used as a positive control in all PCR reactions. The amplification conditions for the first step were 94℃ for 2 min; 30 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s; and finally 72℃ for 5 min. The reaction mixture of the second step (25 μL) contained 0.5 μmol/L each primer (1F and 2R), 0.2 mmol/L each dNTP, 1 × buffer Ⅱ, 2.5 mmol/L $MgCl_2$, 1.0 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems), and 2 μL 10 × diluted PCR product from the first step. The 416-bp PCR products were visualized by 1.3% agarose gel electrophoresis.

Amplification of non-Helicobacter bacteria

Enterobacteriaceae-, *Bacterioides-Prevotella* group- *and Enterococcus*-specific PCRs were performed. The reaction mixture and amplification conditions, except for annealing temperatures, for non-*Helicobacter* PCR assays were the same as in the first step of the semi-nested *Helicobacter* PCR. The annealing temperatures and primers used for detection of Enterobacteriaceae, *Bacterioides-Prevotella* group and *Enterococcus* were as described before[11]. Primers Eco1457F (5'CATTGACGTTACCCGCAGAAGAAGC3') and Eco1652R (5'CTCTACGAGACTCAAGCTTGC3') were used to amplify Enterobacteriaceae and primers Ent1F (5'TACTGACAAACCATTCATGATG3') and Ent2R (5'AACTTCGTCACCAACGCGAAC3') were used to amplify *Enterococcus,* whereas primers Bac303F (5'GAAGGTCCCCCACATTG3') and Bac708R (5'CAATCGGAGTTCTTCGTG3') were used to amplify the *Bacteroides-Prevotella* group. As positive controls, *Escherichia coli* (CCUG 17620), *Bacteroides fragilis* (CCUG 4856), and *Enterococcus faecalis* (CCUG 9997) were used in all PCR reactions. The 112-bp PCR product of *Enterococcus,* 418-bp product of *Bacteroides* and 195-bp product of Enterobacteriaceae were visualized by 1.3% agarose gel electrophoresis.

DNA sequence analysis

Helicobacter-specific PCR products were purified from agarose gels using the Montage DNA Gel Extraction Kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. DNA sequence reactions were performed using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems), as described by Tolia *et al*[10]. Products of the sequence reaction were aligned and the closest homologous DNA was identified by BLASTn-analysis.

Statistical analysis

Statistical analyses were done by χ^2 and Fisher's exact tests. $P < 0.05$ was considered to be significant.

RESULTS

Histology

Little metaplasia was detected in the sections and only a few glands or a few cells displayed gastric (antrum) metaplasia and/or acid mucin. Acid or neutral mucins were often seen only in parts of the epithelial cell cytoplasm. The AB-PAS staining method for metaplasia revealed among the chronic cases three with only gastric metaplasia (neutral mucosubstances), five with only non-gastric metaplasia (acid mucosubstances), and 15 with both types. For acute cholecystitis, these figures were two, five and nine, respectively (Table 1). The two types of metaplasia are displayed in Figure 1. Whartin-Starry staining and immunohistochemistry for *H. pylori* were negative in all studied specimens. The *H. pylori*-positive specimen was from a case of acute cholecystitis with extensive necrosis, but with a small area of preserved epithelium without metaplasia, from which the sample was taken.

Table 1 Number of cases with metaplasia in patients with cholecystitis

	Acute	Chronic
Gastric	2	3
Non-gastric	5	5
Both	9	15
None	34	27
Total	50	50

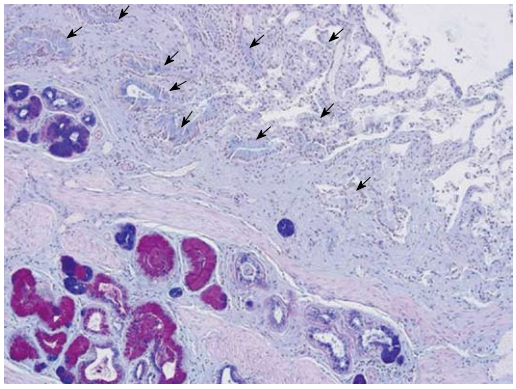

Figure 1 Histological section of ductus cysticus from a patient with chronic cholecystitis. Low-power view displaying antrum-type (red) and intestinal-type (blue) mucous metaplasia in glands. The ductus lumen is seen in the opposite corner of the photo with folds of the mucosa layer covered by epithelium without metaplasia, arrows (no intense color). Alcian blue-periodic acid Schiff staining.

Helicobacter-specific PCR assay and sequencing results

Using the *Helicobacter*-specific PCR assay and agarose electrophoresis, *Helicobacter* DNA was detected in 7/100 of gallbladder specimens of patients with cholecystitis. There were 4/50 (8%) and 3/50 (6%) samples positive for *Helicobacter* spp. among acute and chronic cholecystitis patients, respectively. Six samples showed 98-99% sequence similarity to *H. pullorum* and one to *H. pylori* (Table 2). *H. pullorum* was only found in cases with metaplasia, in six out of 39, as compared to none out of 61 without metaplasia. The difference was statistically significant ($P = 0.002$). All control samples were negative for *Helicobacter* spp. The difference between *Helicobacter* DNA prevalence in gallbladder

Table 2 Prevalence of *Helicobacter* spp. and some common gut bacteria *n* (%)

Patient group	*Helicobacter* PCR	Gut bacteria PCR	Sequencing results (No. of samples)
Acute cholecystitis	4/50 (8)	0/50 (0)	*H. pylori* (1) *H. pullorum* (3)
Chronic cholecystitis	3/50 (6)	0/50 (0)	*H. pullorum* (3)
Controls	0/102 (0)	0/102 (0)	-

Results are shown as the number of positive patients and the number of all patients in the group followed by the percentage in parenthesis. PCR: Polymerase chain reaction; *H. pylori*: *Helicobacter pylori*; *H. pullorum*: *Helicobacter pullorum*.

Table 3 Prevalence of *Helicobacter* DNA in cholecystitis mucosa in different studies from various geographical regions

Region	Prevalence (%)	Patients (*n*)	Ref.
Germany	2	1/57	Bohr *et al*[35] 2007
Japan	12-13	2/16	Murata *et al*[36] 2004
	27	4/15	Fukuda *et al*[37] 2002
China	27.2	22/81	Chen *et al*[34] 2007
Chile	39	9/23	Fox *et al*[31] 1998
Ukraine	73	16/22	Apostolov *et al*[9] 2005

of cholecystitis patients and controls was also significant ($P = 0.007$).

PCR and sequence detection of bacterial DNA other than Helicobacter

None of the tested patients' samples with acute and chronic cholecystitis and control samples was positive using the *Bacteroides*-, Enterobacteriaceae- and *Enterococcus*-specific PCR assays.

DISCUSSION

Helicobacter DNA was found in 7% of cholecystitis mucosa (8% acute, 6% chronic cholecystitis); none of the control samples was positive for *Helicobacter*. There are several reports on the presence of *Helicobacter* DNA in cholecystitis mucosa (Table 3). The studies in Germany, China and Japan with a prevalence of 2%-27% were more similar to our study[34-37] than was the study in Chile (39% prevalence)[31]. However, in a study from Ukraine (73%) the prevalence was much higher than in our study[9].

Six samples (three from acute and three from chronic cholecystitis) were positive for *H. pullorum*. Fox *et al*[31] have reported a link between EHS infections and chronic cholecystitis. *H. bilis* was the most common but *H. pullorum* was also reported[31]. Apostolov *et al*[9] have developed a first generation of enzyme immunoassays and immunoblotting to serodiagnose EHS infections in mice and humans. *H. pullorum* was found in 18% of patients with hepatitis C virus by immunohistochemistry in one of our previous studies[38]. However, *H. pullorum* is most commonly seen in poultry[39]. There is most likely a zoonotic transmission between humans and chickens by undercooked chicken.

One sample with a similar sequence to *H. pylori* was detected. Other studies on gallbladders or gallstones from patients with cholecystitis and cholelithiasis have shown the presence of *H. pylori*[9,32,40]. Other *Helicobacter* species have also been detected in different studies such as, *H. rappini*, *H. ganmani*[35] and *H. hepaticus*[41].

Kawaguchi *et al*[42] were the first to demonstrate *Helicobacter* spp. in cholecystits mucosa that displayed gastric metaplasia. Metaplasia was seen in all cases of cholecystitis in a Chilean study[31], in 15% of cases in a British study[43], and in 14% of cases in a Ukrainian study[9]. In the British study, no *Helicobacter* was found by immunostaining. Our results confirm the importance of gastric metaplasia for detection of *Helicobacter* DNA. Misra *et al*[40] have detected *Helicobacter* only in areas with gastric metaplasia, with a prevalence of 45%, but could not detect *Helicobacter* DNA in paraffin blocks or formalin-fixed mucosal tissue.

None of the gallbladder samples was positive for *Bacteroides* and *Enterococcus* spp. in our study. Enteric bacteria have been detected from gallstones and bile samples by culturing and PCR methods in some studies[44-47], but not by fluorescence *in situ* hybridization[48].

Apart from geographical differences, the variation in *H. pylori*, EHS and some gut pathogens between countries could be due to the use of different PCR methods. Our PCR technique was evaluated as a highly reliable method for genus level identification of *Helicobacter* spp.[49], and inhibitors that might influence the PCR results have been discussed in our other studies[50]. Moreover, some of the studies have used inappropriate control groups. We selected 102 normal control gallbladders from patients diagnosed with diseases other than cholecystitis.

In cholecystitis, *Helicobacter* DNA might preferentially or only be found in epithelial cells or on their surface, thus, much care has to be taken when selection the samples from the paraffin blocks.

In conclusion, several *Helicobacter* spp. infect a range of hosts (most probably, certain species are pathogens in some animals and humans). Divergent results might be due to different geographical areas, different PCR methods, using different control groups or lack of control groups, and sampling from different areas of the biopsy. The present study shows the possible relationship between *Helicobacter* spp. and cholecystitis in Swedish patients. Further studies are needed to determine the possible role of EHS and other pathogens in biliary tract infections and the possible relationship to various hepatobiliary malignancies such as cholangiocarcinoma.

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COMMENTS

Background

Helicobacter genus has nearly 25 species and many of them cause extragastric diseases in humans and animals.

Research frontiers

Helicobacter DNA in gallbladder mucosa has been reported with different prevalence and is associated with several biliary tract diseases, but there are still doubts about the relationship between enterohepatic *Helicobacter* species (EHS), *Helicobacter pylori* and hepatobiliary diseases.

Innovations and breakthroughs

Recent reports have highlighted the presence of *Helicobacter* in the biliary tract in different regions. However, this is believed to be the first study to report the possible relationship between chronic cholecystitis in Scandinavian patients.

Applications

By understanding the relationship between *Helicobacter* and cholecystitis, this study could represent a future strategy for further pathological studies of patients with cholecystitis.

Terminology

EHS are species in the genus *Helicobacter* that colonize the hepatobiliary tract and can cause extragastric diseases in humans or in animals.

Peer review

The authors have tackled a newly developing area of interest to many researchers. The work is a contribution to the study of the association between *Helicobacter* spp. and some common gut bacteria in patients with cholecystitis. They concluded that there is a possible relationship between *Helicobacter* DNA and cholecystitis, and recommended further serological and immunohistochemical studies to support their data.

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