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Is There a Correlation between *Helicobacter Pylori* and Enterohepatic *Helicobacter* Species and Gallstone Cholecystitis?

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ABSTRACT

BACKGROUND

Cholecystitis is a common surgical condition. Recently, several authors have reported that DNA of bile tolerant *Helicobacter* spp. has been found in the human bile colonizing the biliary tract. The aim of this study was to evaluate the association between the presence of *Helicobacter* spp. and gallstone cholecystitis.

METHODS

In this case-control study, gallstones, bile, and gallbladder mucosa were collected from 25 patients without gallstone disease, 24 with acute cholecystitis, and 28 with chronic cholecystitis. The presence of *Helicobacter pylori* (*H. pylori*), *Helicobacter bilis* (*H. bilis*), *Helicobacter hepaticus* (*H. hepaticus*), and *Helicobacter pullorum* (*H. pullorum*) were investigated by polymerase chain reaction (PCR) using species-specific primers.

RESULTS

In this study, 77 subjects with acute and chronic cholecystitis and control groups with a mean age of 46.85 ± 14.53 years, including 58 (67.25%) women and 19 (32.75%) men were included. DNA of 10 *Helicobacter* spp. was detected in the bile of the patients with cholecystitis including eight *H. pylori* and two *H. bilis*. However, we could not detect *H. hepaticus* and *H. pullorum* DNA in the samples. Moreover, there was an association between *H. pylori* and acute cholecystitis ($p = 0.048$), which was found to be stronger in 31-40-year-olds group ($p = 0.003$).

CONCLUSION

We found an association between the presence of *H. pylori* DNA and acute gallstone cholecystitis. There is not statistically significant correlation between three enterohepatic *Helicobacter* spp. (*H. bilis*, *H. hepaticus*, and *H. pullorum*) and cholelithiasis. Given the low sample size of the patients, more studies are required to clear the clinical role of *Helicobacter* spp. in the gallstone disease and cholecystitis.

KEYWORDS:
H. pylori, *H. bilis*, *H. heopaticus*, *H. pullorum*, Gallstone, Cholecystitis

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a widespread gram-negative bacillus, which colonizes primarily the stomach and can probably cause the most common chronic bacterial infection worldwide.[1] During the last two decades, research on the *Helicobacter* genus has focused on *H. pylori* linked to duodenal and gastric

ulcers[1,2] gastric adenocarcinoma,[3-5] and non-Hodgkin's lymphomas of the stomach.[6,7]

Moreover, detecting *H. pylori* DNA in cholesterol gallstone may indicative *H. pylori* colonization as a predisposing factor for gallstone formation in gallbladder.[8,9] Some studies concluded that *H. pylori* infection in the gallbladder not only might lead to cholelithiasis but also was a risk factor for cholecystitis.[10-12] Contrary to mentioned research, Fallone and colleagues in the first North American investigation address the issue of *Helicobacter* in bile of the patients who underwent endoscopic retrograde cholangiopancreatography for hepatobiliary conditions. All 122 patients with hepatobiliary disease including 75 patients with gallstone disease had no *Helicobacter* DNA.[13] Moreover, two studies in human and animal model suggest that *H. pylori* infection dose not play a significant role in gallstone formation.[14-16]

In addition, some *Helicobacter* spp. are resistant to bile and recent interest has linked the presence of *H. pylori* and some of enterohepatic *Helicobacter* spp. in hepatobiliary diseases in human and animals.[17-18] Enterohepatic *Helicobacter* species (EHS) that colonize the hepatobiliary tract of humans 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, and cholelithiasis.[19-20]

Therefore, in this study we attempted to determine any potential association between *Helicobacter* species, particularly *H. pylori*, *H. hepaticus*, *H. bilis*, and *H. pullorum* and gallstone diseases in Iranian patients.

MATERIALS AND METHODS

Ethical approval

The study protocol and informed consent form were reviewed and approved by the Regional Research Ethics Committee of Isfahan University of Medical Sciences. Informed consent was obtained from all patients before their enrollment in the study according to the Helsinki's Declaration.

Sampling

Collection of samples was performed in Alzahra University Hospital in Isfahan, Iran. Patients were excluded if they were taking antibiotic courses (more than 5 days) within 3 months before sample collection. It is important to mention that all patients in acute and chronic cholecystitis groups had received between one to four doses of antibiotic before cholecystectomy. Serum samples were stored at -20°C until use.

The study population consisted of 77 subjects who were examined prospectively. The subjects were categorized into three groups. The first group consisted of 25 patients without gallstone related biliary disease with the mean age of 46.24 ± 13.84 years (range 28-71 years) who underwent endoscopic retrograde cholangiopancreatography (ERCP) as control group, including 6 men and 19 women. The second group consisted of 24 individuals with the mean age of 47.94 ± 14.73 years (range 22-76 years) including 7 men and 17 women. These patients underwent laparoscopic cholecystectomy and were diagnosed as having gallstone related acute cholecystitis based on history, physical examination, and sonography, which were confirmed by pathological report. The third group consisted of 28 individuals with the mean age of 45.64 ± 11.33 years (range 22-79 years) including 7 men and 21 women. These individuals underwent laparoscopic cholecystectomy because of gallstone and repeated attacks of biliary colic and were diagnosed as having chronic cholecystitis in the pathological report.

The three groups were comparable in age, sex, and body mass index (BMI) composition. BMI was calculated in the day of surgery and divided into four categories including underweight (≤ 18.5), normal weight (18.5-24.9), overweight (25-29.9), and obese (≥ 30).

Serum and bile samples of the control group, as well as serum, gallstones, bile, and gallbladder mucosa samples of the patients with gallstone diseases undergoing laparoscopic cholecystectomy, were collected. To avoid contamination with the gastrointestinal bacterial flora, the bile samples were immediately obtained by direct aspiration from cholecystectomized specimens or by aspiration of bile after the first cannulation of common bile duct in ERCP. Aspirated bile samples and biopsies collected in phosphate buffer saline (PBS) were stored at -20°C until DNA was isolated. For *H. pylori* culture 0.5 mL of bile was used. An enzyme-linked immunosorbent assay (ELISA) test was also carried out on serum samples to determine the *H. pylori* infectious state of all patients.

Microbiological study

The bile were separately homogenized in 0.5 mL of broth in a glass tissue grinder and plated into Petri dishes containing freshly prepared Richard Agar (Merck, Germany) medium, optimized for growth of coccoid form of *Helicobacter*.[21] The plates were incubated under microaerophilic conditions at 37°C for up to 21 days.

DNA extraction

DNA was extracted from bile, biliary epithelium, and gallstones with a QIAamp DNA Mini kit (QIAGen GmbH, Hilden, Germany) according to the manufacturer's recommendations, with minor modifications.[22]

Preparation of samples

The bile samples were thawed at room temperature. About 200 µL of each bile was diluted with an equal volume of PBS with pH adjusted to 7.4-7.6 and washed three times with centrifugation at 18000 g for 15 min, and the supernatant was discarded in order to remove some of the inhibitors present in bile.

In the case of gallbladder tissue, it was washed with deionized distilled water for removing some of the bile. Then it was washed with PBS similarly for removing some of the inhibitors present in the bile. DNA was extracted from about 50 mg of the homogenized superficial cell layers and mucous of gallbladder tissue.

The supernatant of DNA extract was stored at -22°C, ready to be used for polymerase chain reaction (PCR) amplification. Purity was estimated spectrophotometrically at OD260/280. In order to determine the lower limit of detection of the extraction protocol for bile components, bile was spiked with tenfold serial dilutions of live *H. pylori* cell (ATCC strain 43504) suspension. These samples were processed and DNA extraction was performed in parallel with other samples.

In the case of gallstone, after washing with PBS, each gallstone was cut through the center and the inner matrix was obtained by scraping into a clean culture dish using a sterile blade. DNA was extracted by using a modified method of Swidsinki and colleagues.[23]

Bile inhibitory test

To test the inhibitory effect of bile components on PCR procedure, we spiked 10 randomly selected negative bile with pure *H. pylori* and prepared a standard suspension of bacteria according to the 0.5 McFarland tube. Tenfold serial dilutions of the *H. pylori* cell suspension were made and the bile samples were spiked with these concentrations. These samples were processed and DNA extraction was performed in parallel with other samples.

***Helicobacter* species-specific PCR amplification and PCR conditions**

The presence of *H. pylori* in DNA extracted from these samples was determined using *Helicobacter* hsp60 gene specific primers,[24] and the presence of *H. hepaticus*,[25] *H. bilis*,[26] and *H. pullorum*[27] in the DNA extracted from these samples was determined using species-specific 16S rRNA genes by PCR that is described in table 1. PCR amplification was performed in a total volume of 25 µL in 0.5 mL containing 1 µg of the extracted DNA sample, 1 µM of each of the primers, 2 mM MgCl2, 200 µM deoxynucleoside triphosphates, 2.5 µL of 10X PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl [pH 8.3]) and 1 unit of Taq DNA polymerase (Roche applied science, Germany). All oligonucleotide primers were synthesized by CinaGene (CinaGene Co, Tehran, Iran). Amplification was done by using Mastercycler Gradient Thermal Cycler (Eppendorf, Germany). Aliquots of 10 µL of PCR amplified products were separated electrophoretically. For this reason, PCR products were applied to the gel in parallel with DNA ladders (GeneRuler™ 100 bp Plus DNA Ladder Fermentas, Germany) to determine reaction product sizes. Constant voltages of 80 V for 30 min were used for products separation. After electrophoresis, the gel was stained with ethidium bromide and images were obtained by UVIdoc gel documentation systems (UK). The extracted DNA sample was replaced by distilled water as a negative control.

Measurement of *H. pylori*-specific immunoglobulin G

Presence of *H. pylori*-specific immunoglobulin G was determined quantitatively by ELISAs in the serum samples of the patients using a commercial ELISA kit (Monobind®, Monobind, Inc., United States). Duplicates of 1:100 diluted serum samples were assayed according to the manufacturer's recommendations. The titer of 20 NTU/mL was given as cut-off value as recommended by

Table 1: **Primer sequences and PCR cycling conditions used for *Helicobacter* spp. identification**

Organism	Target	Sequence (5′ to 3')	Cycle	Amplicons (bp)	reference
H. hepaticus	16S rRNA	F:GCATTTGAAACTGTTACTCTG R:CTGTTTTCAAGCTCCCC	(95°C, 5 min; 94°C, 30 s; 59°C, 30 s; 72°C, 30 s; 72°C, 5 min) (30 cycles)	417	Fox et al.[28]
H. bilis	16S rRNA	F:CAGAACTGCATTTGAAACTAC R:AAGCTCTGGCAAGCCAGC	(95°C, 5 min; 94°C, 30 s; 56°C, 30 s; 72°C, 30 s; 72°C, 5 min) (30 cycles)	405	Hamada et al.[26]
H. pylori	Hsp60	F:AAGGCATGCAATTTGATAGAGGCT R:CTTTTTTCTCTTTCATTTCCACTT	(95°C, 5 min; 94°C, 30 s; 56°C, 30 s; 72°C, 30 s; 72°C, 5 min) (30 cycles)	501	Singh et al.[24]
H. pullorum	16S rRNA	F:ATGAATGCTAGTTGTTGTGAG R:GATTGGCTCCACTTCACA	(95°C, 5 min; 94˚C, 30 s; 60˚C, 60 s; 72˚C, 80 s; 72°C, 5 min) (40 cycles)	467	Stanley et al.[27]

Table 2: **Relation between age of the patients, cholecystitis, and number of samples positive for *H. pylori* and *H. bilis* in PCR assays**

	Chronic cholecystitis n (%)		Acute cholecystitis n (%)		Control group n (%)	
Patients age	***H. pylori***	***H. bilis***	***H. pylori***	***H. bilis***	***H. pylori***	***H. bilis***
< 20	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
21-30	1 (3.58)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
31-40	0 (0)	1 (3.58)	3 (12.5)	1 (4.16)	1 (4)	0 (0)
41-50	0 (0)	0 (0)	1 (4.16)	0 (0)	0 (0)	0 (0)
51-60	1 (3.58)	0 (0)	1 (4.16)	0 (0)	0 (0)	0 (0)
61-70	0 (0)	0 (0)	1 (4.16)	0 (0)	1 (4)	0 (0)
> 70	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	2 (7.14)	1 (3.58)	6 (25)	1 (4.16)	2 (8)	0 (0)

the manufacturer for serum samples. Thus any titer > 20 was considered positive.

RESULTS

In this study, 77 patients with acute and chronic cholecystitis and subjects of control groups with a mean age of 46.85 ± 14.53 consisting of 58 (67.25%) women and 19 (32.75%) men were included. They were divided into 6 age groups for statistical analysis. There was no significant difference in age, sex, and BMI ($p > 0.05$) between the case and control groups. In cholecystitis groups, 39 (75%) patients were overweight and 7 (13.5%) were obese. No *Helicobacter* species were detected in gallstone by PCR, and culture samples were all negative for *Helicobacter* species.

Evaluation of the bile samples for *Helicobacter* species among all groups (table 2) showed two (7.14%) *H. pylori* and one (3.57%) *H. bilis* among chronic cholecystitis group and six (25%) *H. pylori* and one (4.16%) *H. bilis* among acute cholecystitis group. Also,

Table 3: The prevalence of *H. pylori* immunoglobulin G positivity among the patients of the three groups

H. pylori-specific immunoglobulin G quantitative ELISAs	
	Positive* (%)
Acute cholecystitis (n = 24)	21 (87.5%)
Chronic cholecystitis (n = 28)	25 (89.2%)
Control (n = 25)	20 (80%)
*Border line values are included.	

two (8%) *H. pylori* was detected in the control group. Although statistical analysis revealed an association between *H. pylori* and acute cholecystitis ($p = 0.048$), the strong association was found in 31-40 year-olds group ($p = 0.003$). Laboratory examination for *H. pylori* IgG revealed no statistically significant differences among all groups ($p > 0.05$, table 3).

DISCUSSION

Pathways of *H. pylori* penetration into the bile have not been completely understood but there are possible routs of *H. pylori* migration and colonization in the biliary tract such as *H. pylori* translocation from the duodenum via sphincter of Oddi and/or its hematogenous spread to the liver and then excretion into the bile.[29,30]

Moreover, HP infection affects the pathophysiology of gallbladder stone formation and its complications, including cholecystitis, cholangitis, pancreatitis, and biliary cancer. One mechanism is releasing large amounts of proinflammatory and vasoactive substances, such as interleukins (IL)-1, IL-6, and tumor necrosis factor (TNF)-α involved in gallbladder inflammatory disorders and pathogenesis of cholelithiasis.[31,32] In addition, producing oxidative stress and free radical reactions in the gallbladder wall and bile can induce gallstone formation.[33] Finally, HP infection affecting the apoptotic process, is also involved in chronic cholecystitis.[34]

A study from Greece indicates positive HP serology in 51.3% of patients with calcular biliary and pancreatic disease versus 19.3% in histology of gallbladder tissue, which is the gold standard for diagnosis of HP infection.[35] Another investigation in Germany showed that patients with gallstones were 3.5 times more likely to have *H. pylori* in the bile compared with patients in a control group.[30]

The *H. pylori* infection rate is very high in Iran. The overall reported infection rate is about 60-80% in various parts of Iran and is correlated with age.[36] It is compatible with high frequency of positive *H. pylori* antibody in case and control groups without significant difference.

H. pylori is very difficult to grow on culture media because of the microaerophilic characteristics of this organism as it dies if it has any contact with air.[37] Also, *H. pylori* exists predominantly in a non-culturable coccoid form outside the stomach. This is because bile has a chemorepellent effect on *H. pylori* as well as an oxygen concentration under the optimum value (< 7%).[38] This may explain why we could not detect *H. pylori* among the studied cases using culture methods. This finding is compatible with previous studies.

Using sequencing of PCR-amplified 16S rRNA gene fragments, DNA from *H. bilis* was also detected in the gallbladders of five out of eight Chileans with chronic cholecystitis.[39] In our study, 10 (19.2%) *Helicobacter* spp. DNA were detected in the bile of the patient with cholecystitis, of them eight (15.3%) were *H. pylori* and two (3.9%) were *H. bilis*.

Despite detecting *H. pullorum* by PCR from the bile of the patients with chronic cholecystitis,[20] no *H. pullorum* was detected in the bile samples of the patients with cholecystitis in our study.

One study from Nepal showed that there was a common association (76.66%) between *H. hepaticus* and cholelithiasis[40] but in our study, *H. hepaticus* was not detected in the bile samples of the patients with cholecystitis.

These discrepancies between the results of investigations about the association of gallstone cholecystitis and *Helicobacter* species in different countries or areas may be due to differences in the epidemiology of *Helicobacter*, used PCR methods, and inappropriate control groups.

There are limitations to our study. All the patients in acute and chronic cholecystitis groups should have received antibiotic, in the first group as standard of care and in the second group, a single dose of antibiotic as prophylaxis before surgery. All patients who had received antibiotic courses (more than 5 days) were excluded from the study. Despite this limitation, it seems that antibiotic use has not influenced the results, because we found more *H. pylori* infection in acute cholecystitis group despite more doses of antibiotic prescribed before surgery.

Although, there are multiple studies about *Helicobacter* and gall stone disease, there is limited data about

relation between *H. pylori* and several enterohepatic *Helicobacter* species with calculus cholecystitis especially in Iran. Due to diversity and epidemiological differences of *Helicobacter* species based on geographic areas, this research may be considered important from epidemiological and pathophysiological points of view.

CONCLUSION

In summary, we found an association between the presence of *H. pylori* DNA and acute cholecystitis with gallstone, especially in 31-40-year-olds group. Moreover, there is not statistically significant correlation between the three enterohepatic *Helicobacter* species (*H. bilis*, *H. hepaticus*, and *H. pullorum*) and cholelithiasis. Given the low sample size of the patients, more studies are required to clear the clinical role of *Helicobacter* spp. in the gallstone disease and cholecystitis.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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