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Research Article

Companion of Helicobacter Pylori Presence in Stomach and Biliary Tract in the Patients with Biliary Stones

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ABSTRACT

Background: Bacterial infections play a role in the formation of gallstones depends on cholesterol saturation and solubility. In the study we determine whether *Helicobacter pylori* (*H. pylori*) could be detected in bile obtained at ERCP (Endoscopic Retrograde Cholangiopancreatography) of patients with biliary stones and to evaluate the Companion with its gastric presence.

Patients & Methods: 150 consecutive patients undergoing ERCP for common bile duct (CBD) stones were asked to participate in this study. Bile juice was aspirated after selective cannulation of the CBD and stored at -20C. Each of the patient samples had been tested for *H. pylori* by PCR. Two specimens were obtained from the antrum of all patients for *H. pylori* histopathological examination.

Results: *Helicobacter* DNA was detected by PCR in 16 bile samples, 10 of 87 cholesterol gallstones, 4 of 41 black pigmented stones and 2 of 22 brown pigmented stones (p=0.383). Direct sequencing confirmed strains of *H. pylori* in all bile samples. Antral samples are positive for *H. pylori* in 103 subjects (68.7%). All of 16 positive bile samples for *H. pylori* were in patients with this bacterium of stomach ($p< 0.001$).

Conclusion: *H. pylori* was found in 10.6% bile juice samples of the patients with biliary stone diseases. It may be a just innocent bystander than etiological importance in biliary stone formation. The route of *H. pylori* infection in biliary diseases may be ascending through the sphincter of Oddi.

Keywords: Helicobacter pylori; biliary stone; PCR; Gastric infection.

BACKGROUND:

Helicobacter pylori (*H. pylori*) had been found by *Warren* and *Marshall* (1) in 1983. It is a gram-negative, spiral-shaped and motile micro-organism that plays a role in the pathogenesis of chronic gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma and development of gastric adenocarcinoma (2). *H. pylori* DNA had been detected in human liver tissue samples. Some reports confirmed the presence of *non-pylori species*, such as *H. pullorum, H. canis, H. cholecystus, F. rappini, H. hepaticus*, and *H. bilis* in the liver, bile samples and gallbladder tissues. Free bile acids of bile can kill *H. pylori*; however, the inhibitory effect of bile acids on the survival of *H. pylori* is still unclear (2). Certain pathological conditions such as bile duct obstruction, bile composition can be altered this inhibitory effect on the *H. pylori* growth (3).

Bacterial infections play a principal role in the formation of brown pigmented, mixed cholesterol and pure cholesterol gallstones depends on cholesterol saturation and solubility. Attempts to culture potentially causative bacteria from gallstones have failed because the formation of gallstones takes a very long time,

thus bacteria might be killed (4). Swidsinski, et al. (5) identified *E. coli* and *Pseudomonas* in cholesterol gallstones using PCR and these bacteria were suggested as the pathogens in cholesterol gallstone formation by Lee, et al. (6) *H. pylori* DNA in gallstone was detected by PCR in several reports (7-9). The discovery of *H. pylori* in bile juice has led to the suggestion that it is an etiological agent in gallstone formation (10-12). The16s rRNA is the most conserved (least variable) gene in all cells. Portions of the rDNA sequence from distantly-related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the differences easy to measure. For this reason, genes that encode the rRNA have been used to determine taxonomy, phylogeny and to estimate rates of species divergence among bacteria (13).

In this study, we investigated the frequency of *H. pylori* infection using PCR and DNA sequencing in bile obtained from Endoscopic Retrograde Cholangiopancreatography (ERCP) of patients with common bile duct (CBD) stones. We also evaluated any probable companion between the presence of *H. pylori* in stomach and bile samples.

PATIENTS & METHODS:

150 consecutive patients undergoing ERCP for CBD stones were asked to participate in the study from March 2014 to December 2016. Subjects were excluded if they were taking antibiotics during the previous 4 weeks. Two specimens were obtained from the antrum of all patients for *H. pylori* histopathological examination and bile was collected by aseptic aspiration after selective cannulation of the CBD. Bile samples stored at -20C and each bile sample was pelleted by centrifugation for 15 minutes at 12,000 rpm. DNAs from the bile samples were extracted by using phenol-chloroform method. To detect the bacterial DNA, *Helicobacter species* and *H. pylori* specific primer pairs were used, and generate amplicons of approximately 296 bases for *H. pylori* ureC genes (glm region). To obtain higher amounts of DNA, the products were re-amplified with nested PCR primers (Tables 1). Resulting sequences were compared to databases accessed through the NCBI (National Center for Biotechnology Information) server.

Table-1 Primers for Helicobacter species

Bacterial Primers	Primer sequence (5'to3')	Genes	Size (bps)
Helicobacter species	F GGCTATGACGGGTATCCGGC R GCCGTGCAGCACCTGTTTTC	16S rDNA	764
H. pylori	F GGATAAGCTTTTAGGGGTGTTAGGGG R GCTTACTTTCTAACACTAACGCGC	ureC (glm)	296

All patients signed an informed consent form. This research was approved by the Ethical Committee of Golestan Research Center of Gastroenterology and Hepatology. Statistical analysis was performed with Chi-square test as well as Fisher's exact test, and one-way analysis of variance (ANOVA) test. *P* values of 0.05 or less were considered statistically significant. All the data were analyzed using SPSS 18 for Windows (SPSS Inc., Chicago, IL, USA) and the values were expressed as mean ± standard deviation (SD) for continuous variables and percentages for categorical variables.

RESULTS:

78 0f 150 patients (52%) were female, and the age ranged from 28 to 83 years (mean, 62.1 ± 16.3 years). 16 samples had positive PCR for *Helicobacter* species. All of them were positive for *H. pylori* ureC genes. 150 gallstones were classified by their gross appearance and composition of cholesterol, resulting in 58% cholesterol gallstones, 27.3% black pigmented stones and 14.7% brown pigmented stones. There were no statistical differences in age, gender, body mass index (BMI) and the detection of *H. pylori* DNA among the groups based on gallstone composition (Table 2,3).

Table-2 Diagnoses and characteristics of study subjects based on gallstone composition

Diagnosis	Frequency (%)	Female (%)	Age(years)	BMI(kg/m2)
Cholesterol gallstones	87 (58%)	47 (54.1%)	62.8 ± 14.7	24.36 ± 3.72
Black pigmented stones	41(27.3%)	20 (48.7%)	61.8 ± 16.5	23.67 ±3.82
Brown pigmented stones	22 (14.7%)	11 (50%)	58.4 ± 17.3	22.76 ± 2.77
p. value	-	0.324	0.213	0.297
Total	150	78 (52%)	62.1 ± 16.3	23.87 ± 3.61

Table-3 PCR for H. pylori among the groups based on gallstone composition

PCR for *H. pylori* in bile samples (n=150)	Cholesterol gallstones (n=87)	Black pigmented stones (n=41)	Brown pigmented stones (n=22)	*p. value*
Positive n=16 (10.7%)	10 (11.4%)	4 (9.7%)	2 (9.1%)	0.383

Antral samples are positive for *H. pylori* with histopathological examination in 103 subjects (68.7%). All of 16 positive bile samples for *H. pylori* were in patients with the presence of stomach ($p< 0.001$) against only 15.5% of patients with *H. pylori* presence in stomach had this bacterium in bile juices (Table 4).

Table-4 Correlation between existence of *H. pylori* in antral samples and *H. pylori* -PCR in bile.

PCR for *H. pylori* in bile samples (n=150)	*H. pylori* in antral samples	
	Positive n=103 (68.7%)	Negative n=47 (31.3%)
Positive n=16 (10.7%)	16	0
Negative n=134 (89.3%)	87	47

DISCUSSION:

Enter hepatic *Helicobacter* species have previously been found in adults with hepatobiliary diseases (14). The biliary system is thought to be sterile but this sterility could be broken under certain conditions. Two major routes of infection are ascending through the sphincter of Oddi and descending through the portal system (15). For example, bacterial infection was found in bile (20%) and the liver (17%) on post-operative individuals without any hepato-biliary abnormalities (16). Leung, *et al.* found bacteria in 84% of the inner cut surface of the pigmented gallstones using electron microscopy (17).

Lee, *et al.* (4) extracted bacterial DNA from the specimens of gallstones, bile juice and Gall-bladder (GB) mucosa. Bacterial DNA was positive in 69.4% of the mixed cholesterol stones compared with 10% of pure cholesterol stones. *Helicobacter* DNA was detected in 4 out of 58 gallstones, 6 out of 48 bile samples and 5 out of 46 gallbladder specimens. Almost all mixed-cholesterol gallstones appear to harbor bacterial DNA, predominantly *E. coli* and identified the DNA of *Helicobacter* species in 27.7% of the GB mucosa, 25% of the bile juice, and 11.4% of the gallstones. Similarly, in our study, we extracted *H. pylori* DNA from the specimens of bile juice samples in 10.6%.

More than 25 species of *Helicobacter* have been found and these microorganisms have been caused various diseases in mammals. Hepatitis and hepatomas are caused by *H. hepaticus* in mice (18-20). *H. pulorum*, *F. rappini*, and *H.canis* are isolated in diarrheal patients, showing the possibility of zoonosis (21). The extraction of bile-resistant *Helicobacter* species from animals' bile showed that *F. rappini*, *H. hepaticus*, *H. bilis*, *H. canis*, *H. cholecystus*, and *H. pullorum* may be able to grow and survive in bile juice (22). Lin, *et al.* investigated the presence of urease A gene in bile sampled by the percutaneous transhepatic route, 3 out of 7 cases showed positive findings, suggesting that *H. pylori* might be the cause of subclinical cholangitis (10). Figura, *et al.* implicated *H. pylori* as a precipitating factor in gallstone

formation by identifying *H. pylori* antibodies in the bile juice of gallstone patients (23).

Hamada, *et al.* collected 126 bile samples from patients with cholelithiasis, cholecystitis, gallbladder polyp and other non-biliary diseases. *H. hepaticus* was detected in bile samples with nested PCR whereas *H. bilis* was not. IgG antibodies to *H. hepaticus* were detected by western blotting. *Helicobacter hepaticus* was detected in 32% of total samples. Patients with cholelithiasis (41%) and cholecystitis with gastric cancer (36%) had higher prevalence of *H. hepaticus* infection than samples from patients with other diseases (24). Kobayashi, *et al.* examined 57 bile samples from 30 patients with benign biliary diseases, 6 malignant biliary diseases and 21 non-biliary diseases. *Helicobacter* genus DNA was statistically frequently detected in bile samples from 53% and 86% of benign and malignant biliary diseases, compared with 9% of non-biliary diseases. The *H. pylori* urease A gene was also frequently found in bile, whether benign, malignant, or control, though neither 16S rRNA nor the 26K protein gene was detectable in any bile samples. *H. bilis*-16S rRNA genes were detectable in only two cases. *H. hepaticus* was not detectable in any samples (25). In a similar German study, no *Helicobacter* species were found in bile juice, suggesting that there may be racial and demographic differences (26).

The relationship between the *H. pylori* infection and cholelithiaisisstill remains controversial. Some studies have supported a cause-and-effect association while some others even failed to confirm the existence of *H. pylori* in bile specimens. There is a lack of evidences to determine the entry routes of *H. pylori* to the hepatobiliary tree including either the ascending duodenum infection or the portal system circulation (27-30). Zhou, et al in a meta-analysis of 18 case-control studies published showed that higher presence of *H. pylori* in cholelithiasis patients than control group and this trend was significant in the regions with higher prevalence of this agent. Evidences supporting the association between *H. pylori* and cholelithiasis could be found by using different tests but the gold standard for the identification of these bacteria in biliary system has yet to be established. Considering obvious heterogeneity, a large multicenter study will facilitate us to further clarify the association between the *Helicobacter* infection and cholelithiasis (31).

CONCLUSION:

In summary, there are many limitations to identify all the organisms in cases of infection with multiple *Helicobacter* subtypes. Although disadvantage of lack of comparative healthy control group, we could identify *H. pylori* in 10.6% bile juice samples of the patients with biliary stone diseases. It may be a just innocent bystander than etiological importance in biliary stone formation. All positive bile samples for *H. pylori* were in patients with the bacterium presence in stomach, maybe the route of it in biliary diseases is ascending through the sphincter of Oddi. In future, researches will be needed to show the effect of *H. pylori* eradication on the development of these diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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