Evaluation of the presence of Helicobacter species in the biliary system of Turkish patients with cholelithiasis

Kolelitiazisli Türk hastaların safra sisteminde Helikobakter türlerinin araştırılması

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Background/aims: Helicobacter genus and bile-resistant Helicobacter pylori are suggested to have a role in gallstone formation and epithelial cell proliferation in the gallbladder. The aim of this study was to evaluate the presence of Helicobacter species in the gallbladder tissue, bile and gallstones of Turkish patients with cholelithiasis. Methods: Forty-seven patients with calculous cholecystitis and 3 controls were evaluated for the presence of Helicobacter spp. by culture, polymerase chain reaction, and histological and immunohistochemistry methods. Results: Escherichia coli (10.6%), Enterobacter amnigenus (6.3%), Klebsiella planticola (2.1%), and Klebsiella ozaenae (2.1%) were isolated from the sample cultures of 8 patients. No other microorganisms, including H. pylori and other Helicobacter spp., were detected. Polymerase chain reaction was negative for Helicobacter spp. and H. pylori. No microorganisms resembling Helicobacter spp. were seen on the histological sections. The association between the presence of bacteria and epithelial cell proliferation index was not statistically significant (p=0.48). Conclusions: There was no association between the presence of Helicobacter spp. and development of cholelithiasis in our study group. The microorganisms found in the samples did not reveal any significant association with the underlying disease.

Key words: Helicobacter spp., cholelithiasis, polymerase chain reaction, histopathology, immunohistochemistry

INTRODUCTION

Helicobacter pylori is a spiral, microaerophilic, gram negative bacterium. It is usually found in the stomach of humans and is associated with acute and chronic gastritis, gastric and duodenal ulcer, gastric cancer, and gastric non-Hodgkin lymphoma (1). H. pylori DNA was also detected in human liver tissue samples of patients with primary sclerosing cholangitis and primary biliary cirrhosis (2).

In recent years, several authors have reported the presence of Helicobacter species such as H. pylori, H. bilis, ‘Flexispira rappini’ and H. pullorum in the human hepatobiliary system by molecular and histopathological methods. As a result, these species were regarded as being the cause of hepatobiliary diseases ranging from chronic cholecystitis and primary sclerosing cholangitis to gallbladder carcinoma (2-6). The presence of H. pylori DNA in...
the mixed bacterial population in cholesterol gallstones is proposed to reflect that either *H. pylori* is an indigenous part of the flora in a stone-containing gallbladder or that colonization of the biliary tract by the organism predisposes to stone formation (7). Despite these findings, there are also studies that could find no such association between *H. pylori* and hepatobiliary diseases (8-10).

In this study, we aimed at evaluating the presence of *H. pylori* and other Helicobacter spp. in the gallbladder tissue, bile and gallstones of patients with cholelithiasis and to determine the association between the presence of bacteria and epithelial cell proliferation in these patients.

**MATERIALS AND METHODS**

**Patients**

The study was carried out on patients who were referred to Ankara Türkiye Yüksek İhtisas Education and Investigation Hospital for cholecystectomy. A total of 51 subjects (14 male, 37 female) were enrolled in this study. Forty-seven of these patients were in the patient group with a diagnosis of chronic calculous cholecystitis. The control group consisted of 3 patients, two of whom had periampullary carcinoma and one gallbladder polyp. Patients with acute cholecystitis and those who had used antibiotics 4-6 weeks prior to cholecystectomy were excluded from the study. Ethical approval was obtained from the Ethics Committee of Ankara University School of Medicine (21.06.2004/54-134). Signed informed consent was obtained from each patient. The gallbladder tissue, bile and stone samples of the patients were taken under sterile conditions and anaerobe cultures were made in the operating room as soon as the samples were taken. The rest of the samples were transported to the laboratory of the Microbiology and Clinical Microbiology Department of Ankara University School of Medicine in order to make cultures for aerobic microorganisms and fungi.

**pH Measurement**

Bile pH was measured by pH meter (Hana, Portugal) immediately after obtaining the bile samples.

**Microbiological Culture**

All samples were subjected to macroscopic and microscopic examination. Gallbladder tissue and stones were prepared for culture on a sterile petri dish using sterile forceps and single-use sterile scalpel blade. The surface layer of each gallstone (envelope) was cut away; the nucleus of each gallstone was collected and put into a sterile vial. In the operating room, all the samples (gallbladder tissue, stones and bile) were inoculated onto plates containing Brucella agar (Becton Dickinson, France) with 5% sheep blood supplemented with vitamin K1 (Sigma, Holland) and hemin (Sigma, Holland), and put into an anaerobic jar. The samples were also inoculated into tubes containing brain heart infusion broth (Lab M, England) and thioglycolate broth (Merck, Germany) for transportation. Subcultures were made from these media onto plates containing 5% sheep blood agar (Merck, Germany) and MacConkey agar (Lab M, England) for aerobic culture and Sabouraud dextrose agar (Lab M, England) for the isolation of fungi.

**H. pylori Culture**

For the isolation of *H. pylori*, the samples were transported to the microbiology laboratory in brain heart infusion broth and inoculated on *H. pylori* selective medium (Lab M, England) containing its supplement (X040 VCA, Lab M, England) within 4 hours (h). The plates were incubated under microaerobic conditions at 37°C for 3-7 days. *H. pylori* NCTC 11637 was used as the reference strain.

**Histopathological Examination**

Gallbladder tissue specimens were fixed in 10% buffered formalin immediately after cholecystectomy for histological examination. The samples were then embedded in paraffin blocks and 4 μm-thick histological sections were stained with hematoxylin-eosin for histological analysis and with tissue-Giemsa for examining microorganisms resembling *H. pylori*.

**Immunohistochemistry (IHC)**

Sections of 4 micron-thickness were cut, mounted on poly-L-lysine coated slides, and were stained with monoclonal antibody raised against Ki-67 (clone=SP6, 1:200, Neomarkers) using Ventana NexEs automated immunostainer for secondary visualization. Antibody detection was performed by using a biotinylated secondary antibody of Ventana (Ventana Medical Systems, Tucson, AZ, USA) and 3,3’-diaminobenzidine. Sections were counterstained with hematoxylin. Positive control tissues were used as recommended by the suppliers, whereas exclusion of the primary antibody served as negative control. The staining pattern of Ki-67 was considered as positive only in the presence of nuclear staining of epithelial cells in gallbladder mucosa. In most representative areas,
500 epithelial cells in the mucosa were counted, among them cells having positively stained nuclei were determined. Staining index (proliferation index) was calculated by dividing positively stained cells by 500 counted epithelial cells.

**DNA Extraction**

DNA was extracted by phenol-chloroform extraction method of Clayton et al. (11). Briefly, approximately 50 mg of tissue, 200 μl of bile samples and 50 mg of crushed stones were suspended in 500 μl Tris-EDTA and 60 μl 10% SDS. A total of 25 μl proteinase K solution (Fermentas, Lithuania) was added and incubated at 50°C for one night. The next day, DNA was extracted by adding equal volumes of phenol-chloroform. DNA was precipitated with ethanol and dissolved in 100 μl ddH2O. The positive control was prepared by mixing *H. pylori* NCTC 11637 standard strain to a portion of gallbladder tissue, stone, and bile sample of a control patient. These samples were subjected to the same extraction procedure, and used as the internal control of the polymerase chain reaction (PCR).

**PCR Amplification**

*Helicobacter* genus specific primers C97 (5’GCT ATG ACG GGT ATC C3’) and C98 (GAT TTT, ACC CCT ACA CCA3’) as well as C97 and C05 (5’ACT TCA CCC CAG TCG CTG3’) were used to amplify 423 and 1222 bp portions of the 16S rDNA gene of *Helicobacter spp.* as described previously with minor modifications (4). The reaction mixture contained 1X PCR buffer (750 mM Tris-HCl [pH:8.8], 200 mM (NH4)2SO4, 0.1% Tween20), 2.5 U Taq DNA polymerase (Fermentas, Lithuania), 1.5 mM MgCl2, 200μM each dNTP, 25 pmol of each primer, 4% (w/v) BSA (Fermentas, Lithuania) and 5μl of diluted DNA sample in a total volume of 50 μl. The samples were diluted by 1/10, and 4% BSA was added in order to reduce the PCR inhibitory effect of human bile (13). Positive and negative controls (PCR mix not containing any DNA) were used in each run. Cycling conditions were as follows: Initial denaturation at 94°C for 5 min, followed by 26 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and elongation at 72°C for 2 min. At last a final extension of 5 min at 72°C was performed. PCR products were electrophoretically separated in a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

**Statistical Analysis**

The association of bacteria with epithelial cell proliferation was analyzed by Mann-Whitney U test. p values less than 0.05 were considered as significant.

**RESULTS**

**Bile pH**

The pH of the bile samples was between 6.7 and 7.8, which was in the normal bile pH range (5.9-8.6) (14).

**Culture**

*H. pylori* culture was negative for all the gallbladder tissue, stone, and bile samples. No anaerobic microorganisms or fungi were isolated in any of the samples. In the aerobic cultures of 8 patients (15.6%), aerobic microorganisms were recovered. The isolated strains were *E. coli* (in 5 patients), *K. planticola* (in 1 patient), *K. ozaena* (in 1 patient), and *Enterobacter* spp. (in 3 patients). In 2 patients, two different microorganisms were isolated (*E. coli + Enterobacter* spp in 1 patient, *K. ozaenum + Enterobacter* spp. in 1 patient). The same bacteria were recovered from the gallbladder tissue, stone, and bile samples of the same patient. The samples of the control group were sterile.

**Histopathology**

Hematoxylin-eosin-stained sections of the samples were investigated. Thirty-seven patients had chronic calculous cholecystitis, 12 had chronic calculous cholecystitis with cholelithiasis, and 2 had...
chronic calculous cholecystitis with evidence of active inflammation. None of the samples showed evidence of dysplasia. On tissue-Giemsa-stained sections, no microorganism was observed resembling *H. pylori* (Figure 1).

**IHC**

The number of positive-stained cells could not be calculated in 2 patients because the epithelial cells were lost. In the remaining 49 patients, the number of positive-stained cells determined by IHC was between 1 and 368 (Figure 2), and the proliferation indexes were between 0.4 and 73.6 (Table 1). The association between the above-mentioned aerobic bacteria and epithelial cell proliferation indexes as determined by IHC was evaluated by Mann-Whitney U test, and no statistically significant difference was found (p=0.48, Table 2).

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<th>Ki-67 Index (%)</th>
<th>Culture Results</th>
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*a*The number of positively stained cells could not be counted in these samples because the epithelial cells were exfoliated.
None of the PCR reactions performed by using primers specific for Helicobacter genus and H. pylori yielded any amplicons in the patient and control groups (Figure 3).

**DISCUSSION**

H. pylori is the etiological agent of gastroduodenal diseases such as acute and chronic gastritis, gastric and duodenal ulcer, gastric cancer, and gastric non-Hodgkin lymphoma (1). It is sensitive to bile acids, and 96% of the organisms are killed in duodenal fluid containing bile; an inverse relation between bile reflux and H. pylori presence was reported (12, 15, 16). However, H. pylori and other Helicobacter spp. such as H. bilis, H. rappini and H. pullorum were recently detected in diseased gallbladder tissue or bile (4, 5). The presence of Helicobacter spp. in the hepatobiliary system has been shown by molecular and histopathological methods, and Helicobacter spp. were associated with hepatobiliary diseases such as chronic cholecystitis, primary sclerosing cholangitis, primary biliary cirrhosis, and gallbladder carcinoma (2-6).

Proliferating cell nuclear antigen labeling index, which reflects the biliary cell proliferation activity, was found to be significantly higher in H. pylori-positive patients (5). In the development of gastric carcinoma, H. pylori is believed to act on the sequence of hyperplasia-dysplasia and carcinoma (17, 18). Gallbladder stones are proposed to be important in the pathogenesis of gallbladder carcinoma, and H. pylori is cited as having a role in the development of gallbladder stones (19). The presence of H. pylori DNA in the mixed bacterial population in cholesterol gallstones may reflect either that H. pylori is an indigenous part of the flora in a stone-containing gallbladder or that colonization of the biliary tract by the organism predisposes to stone formation (7). Kuroki et al. (5) investigated the tissue sections of 14 patients with hepatolithiasis by histopathology, PCR and cell kinetic study. H. pylori was detected in 37% and 29% of patients by histopathology and PCR, respectively. The proliferating cell nuclear antigen labeling index was significantly higher in H. pylori-positive patients (5). Silva et al. (19) investigated the presence of Helicobacter spp. by culture and PCR in the gallbladder tissue and bile from patients with cholelithiasis. Cultures were negative, but Helicobacter DNA was detected in 31.3% of gallbladder tissue and 42.9% of bile samples. Their data supported the association of Helicobacter spp. with the pathogenesis of cholelithiasis and cholecystitis (19). Chen et al. (20) also investigated the presence of Helicobacter spp. in the gallbladder of patients with gallstone diseases by genus- and species-specific PCR. Helicobacter DNA was detected in half of the gallbladder samples, and 39 were positive for H. pylori (20).

In the study of Roe et al. (12), 32 bile samples obtained from patients with biliary tract diseases were evaluated for the presence of H. pylori by PCR, bile pH measurement and culture. H. pylori DNA was detected in nearly one-third of the patients by PCR. Bile pH was not related to the presence of Helicobacter, and culture was not successful (12). A high prevalence of H. pylori positivity was also detected in the gallbladder and liver tis-

### Table 2. The associations between culture-positive materials and cell proliferation indexes as determined by Mann-Whitney U test

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<th>Mean ± Standard Deviation</th>
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<tr>
<td>Culture-positive</td>
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<td>14.00 (0.60 - 32.40)</td>
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**Figure 3.** A. PCR amplicons obtained by Helicobacter genus-specific primers C97-C98 and C97-C05. B. PCR amplicons obtained by H. pylori-specific primers HPU1 and HPU2. Lanes with numbers are the patient samples. PK represents positive control (H. pylori standard strain added patient sample), NK represents negative control. M is molecular weight marker (a. Mass Ruler; b. Gene Ruler 50bp DNA ladder; Fermentas, Lithuania). Corresponding molecular weights are shown on the left.
sue of Ukrainian patients with chronic cholecystitis by molecular and immunological methods, indicating a correlation between Helicobacter infection and chronic cholecystitis (3). Nilsson et al. (2) detected the presence of H. pylori DNA in the liver tissue of patients with primary biliary cirrhosis and primary sclerosing cholangitis. None of the samples was positive for H. bilis, H. pullorum or H. hepaticus (2).

Despite these studies supporting the relationship between the presence of Helicobacter spp. and hepatobiliary diseases, there are also studies that failed to find such an association. Bulajic et al. (8) investigated the bile samples of 72 patients with cholelithiasis. Half of the patients were found to carry H. pylori by PCR, but the prevalence of H. pylori infection in patients with benign biliary diseases did not show a statistically significant difference in relation to the patients with normal endoscopic retrograde cholangiopancreatography (ERCP) findings (8). On the other hand, Myung et al. (10) reported the presence of H. pylori in about 10% of the patients with hepatobiliary diseases by PCR. In the H. pylori-positive patients, bile pH was found to be significantly higher. They concluded that even if H. pylori DNA was present in the bile with lowered pH, bacteria were not able to colonize the bile duct epithelium, excluding the role of H. pylori in the development of hepatolithiasis (10). Another study that also failed to find an association between the presence of Helicobacter spp. in the hepatobiliary system and occurrence of gallbladder diseases was performed by Méndez-Sanchez et al. (9) in Mexico. They investigated 95 cholecystectomy specimens using histology, immunohistochemistry and PCR. Only two specimens were found to be positive for Helicobacter spp., suggesting a low incidence of Helicobacter in the Mexican patients with gallbladder diseases (9).

In our study, none of the patients with or without gallbladder stones carried H. pylori or other species of the same genus in the bile, gallstone or gallbladder tissue samples, and none of the pathological specimens showed evidence of dysplasia. As a result, we could not detect an association between the presence of Helicobacter spp., including H. pylori, and development of cholelithiasis in our patients. In all of our patients, bile pH was in the normal range, suggesting that the environment may not have been suitable for the invasion of Helicobacter spp. and proliferation of the epithelium, as suggested by Myung et al. (10). Genetic, environmental or dietary habits may have a more prominent impact on the development of gallbladder stones in the Turkish population. Despite our inability to show the presence of Helicobacter spp. in the patient group, we cannot exclude its role as a bystander in the pathogenesis of gallstone formation.

In the presence of acute or chronic cholecystitis and cholelithiasis, several bacteria including E. coli, Enterobacter spp. and Klebsiella spp. were found in the bile and gallbladder tissue. These bacteria usually enter the hepatobiliary system from the intestinal tract (21-25). Monstein et al. (7) found a mixed bacterial flora including H. pylori in cholesterol gallstones, suggesting that H. pylori may either be an indigenous part of the hepatobiliary flora or its colonization in the biliary tract may predispose to cholesterol gallstone formation (7). In our study, we also detected the presence of E. coli, Klebsiella spp and Enterobacter spp. in the hepatobiliary system. As these bacteria are also the members of the gastrointestinal flora, they may have entered the system from this route. Despite their presence, no statistically significant difference (p=0.48) was observed in the cell proliferation index of patients with and without these bacteria. Thus, these bacteria also do not seem to have a role in the development of gallstones in our patients. Helicobacter spp. may be found in the hepatobiliary system of some patients with benign disorders of the gallbladder such as cholelithiasis. However, a direct correlation between their presence and occurrence of these disorders cannot be made, at least with the present data. The conflicting results may be due to the small number of subjects studied or difficulty in obtaining a healthy control group. Different pathogenic mechanisms such as environmental factors, infectious agents, dietary habits, or genetic tendency may be responsible for the development of cholelithiasis in different populations (14, 25). In our study group, neither Helicobacter spp. nor the enteric bacteria (although their presence in the system was detected) were found to be responsible for the development of cholelithiasis. In order to support the relationship between Helicobacter spp. and cholelithiasis, more detailed, controlled and multi-centered studies involving more patients with different hepatobiliary diseases must be performed.

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