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

Amaç: *Helikobakter cinsi ve safraya dirençli Helikobakter pilori'nin safra kesesinde taş oluşumu ve epitel hücre proliferasyonunda rolü olduğu düşünülmektedir. Bu çalışmanın amacı, kolelitiyazı olan Türk hastaların safra kesesi dokusu, safra sıvısı ve safra taşlarında Helikobakter türlerinin varlığının araştırılmasıdır.* ***Yöntem:*** *Kırk yedi taşlı kolesistit hastası ve üç kontrolde Helikobakter türlerinin varlığı kültür, polimeraz zincir reaksiyonu, histolojik ve immünhistokimyasal yöntemlerle araştırılmıştır.* ***Bulgular:*** *Sekiz hastanın kültür örneklerinde Escherichia coli (10.6%), Enterobacter amnigenus (6.3%), Klebsiella plantolica (2.1%), ve Klebsiella ozonea (2.1%) izole edilmiştir. H. pilori ve diğer Helikobakter türleri üretilmemiştir. Polimeraz zincir reaksiyonunda Helikobakter türleri ve H. pilori'ye ait amplikon tespit edilmemiş, histolojik kesitlerde Helikobakter türlerini andıran mikroorganizmalara rastlanmamıştır. Bakteri varlığı ile epitel hücresi proliferasyon indeksi arasındaki ilişki istatistiksel olarak anlamlı bulunmamıştır (p=0.48).* ***Sonuç:*** *Çalışma grubumuzda, Helikobakter türleri varlığı ile kolelitiyaz gelişimi arasında bir bağlantı bulunmamıştır. Örneklerden izole edilen mikroorganizmalar, altta yatan hastalık gelişimi açısından anlamlı değildir.*

Anahtar kelimeler: Helikobakter türleri, kolelitiyazis, polimeraz zincir reaksiyonu, histopatoloji, immünhistokimya

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

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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 Ihtisas 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 (21.06.2004/54-1334). 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 $(NH4)_2SO_4$, 0.1% Tween20), 2.5 U Taq DNA polymerase (Fermentas, Lithuania), 1.5 mM $MgCl_2$, 200 µM each dNTP, 20 pmol of each primer (C97 and C98 or C97 and C05), 1% (v/v) bovine serum albumin (BSA, Fermentas, Lithuania), and 5µl of diluted DNA sample in a total volume of 50 µl. PCR was performed in 30 cycles of denaturation for 45 seconds (s) at 94°C, annealing for 90 s at 55°C and elongation for 2 minute (min) at 72°C after an initial denaturation of 4 min at 94°C and followed by a final elongation for 5 min at 72°C. The products were run in 2% agarose gel electrophoresis and visualized under UV after ethidium bromide staining.

To determine the presence of *H. pylori,* another PCR reaction was performed by using *H. pylori*-specific primers HPU1 (5'GCC AAT GGT AAA TTA GTT3') and HPU2 (5'CTC CTT AAT TGT TTT TAC3'), and a 411 bp region of the *H. pylori ureA* gene was amplified as described previously (11,12). Briefly, PCR mix was prepared to contain 1X PCR buffer (750 mM Tris-HCl [pH:8.8], 200 mM $(NH4)_2SO_4$, 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. ozaenae* (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. ozaenae* + *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 cholesterolosis, 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).

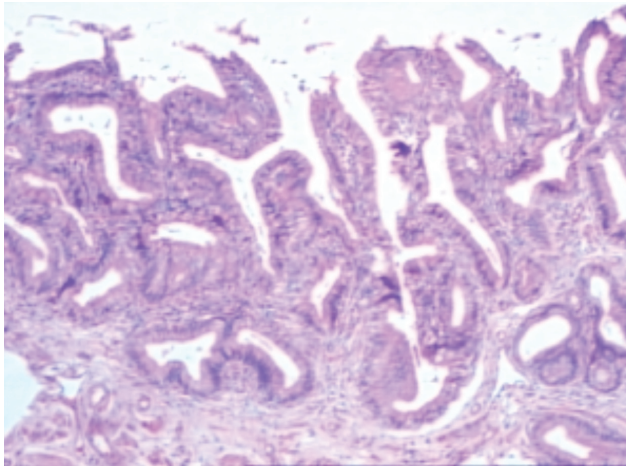

Figure 1. Chronic inflammation in the gallbladder epithelium in hematoxylin-eosin-stained sections.

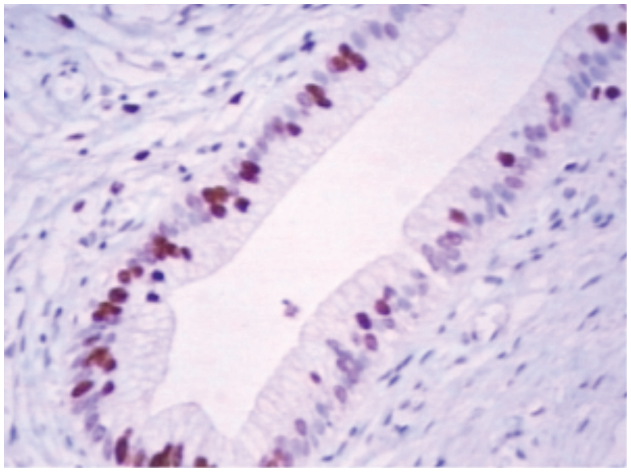

Figure 2. Gallbladder wall stained with monoclonal anti-Ki 67 immunohistochemistry.

Table 1. Proliferation indexes and culture results of the patients

Patient number	No. Positively Stained Cells (/500 cells)	Ki-67 Index (%)	Culture Results	Patient number	No. Positively Stained Cells (/500 cells)	Ki-67 Index (%)	Culture Results
1	13	2.6	Negative	**27**	279	55.8	Negative
2	37	7.4	Negative	**28**	38	7.6	Negative
3	78	15.6	Negative	**29**	125	25	Negative
4	130	26	Negative	**30**	101	20.2	***E. coli***
5	36	7.2	***E. coli***	**31**	122	24.4	Negative
6	368	73.6	Negative	**32**	6	1.2	Negative
7	2	0.4	Negative	**33**	3	0.6	***Klebsiella planticola***
8	162	32.4	***E. coli***	**34**	6	1.2	Negative
9	294	58.8	Negative	**35**	352	68.2	Negative
10	364	72	Negative	**36**	72	14.4	Negative
11	5	1	Negative	**37**	140	28	Negative
12	352	70.4	Negative	**38**	120	24	Negative
13	12	2.4	Negative	**39**	39	7.8	***E. coli* + *Enterobacter spp.***
14	129	25.8	Negative	**40**	78	15.6	Negative
15	314	62.8	Negative	**41**	124	24.8	Negative
16	117	23.4	Negative	**42**	59	11.8	Negative
17	50	10	Negative	**43**	113	22.6	***E. coli***
18	4	0.8	Negative	**44**	112	22.4	***K. ozaenae* + *Enterobacter* spp.**
19	3	0.6	Negative	**45**	14	2.8	***Enterobacter* spp.**
20	120	24	Negative	**46**	39	7.8	Negative
21	188	37.6	Negative	**47**	47	9.4	Negative
22	3	0.6	Negative	**48**	37	7.4	Negative
23	171	34.2	Negative	**49**	16	3.2	Negative
24	40	8	Negative	**50**	40	8	Negative
25	27	5.4	Negative	**51**	Not Counted*		Negative
26	Not Counted*		Negative				

*The number of positively stained cells could not be counted in these samples because the epithelial cells were exfoliated.

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

	Mean ± Standard Deviation	Median (Min - Max)
Culture-negative	21.98 ± 22.69	14.40 (0.40 - 73.60)
Culture-positive	14.50 ± 11.39	14.00 (0.60 - 32.40)
p	**0.48**	

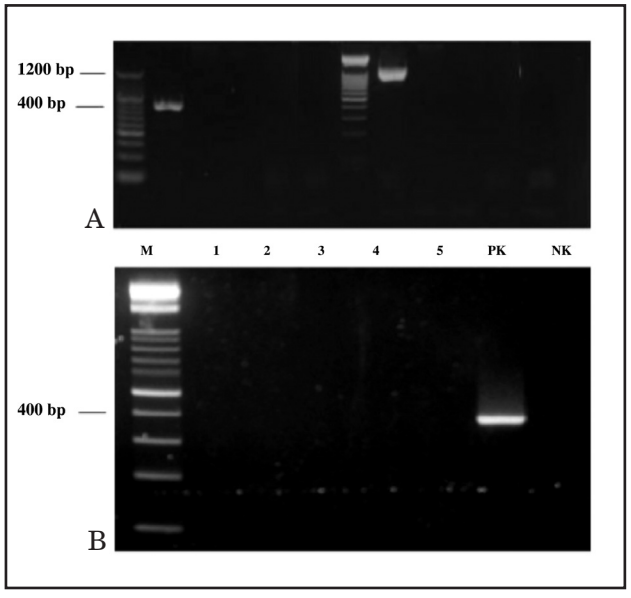

Figure 3. A. PCR amplicons obtained by *Helicobacter* genus-specific primers C97-C98 and C97-C05. **B.** PCR amplicons obtained by *H. pylori*-specific primers HPU1 and HPU2. Lanes with numbers are the patient samples. PK represents positive control (*H. pylori* standard strain added patient sample), 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.

PCR

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-

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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REFERENCES

1. Dunn BE, Cohen H, Blaser JB. *Helicobacter pylori.* Clin Microbiol Rev 2007; 10: 720-41.
2. Nilsson HO, Taneera J, Castedal M, et al. Identification of *H. pylori* and other *Helicobacter* species by PCR, hybridization and partial DNA sequencing in human liver samples from patients with primary sclerosing cholangitis or primary biliary cirrhosis. J Clin Microbiol 2000; 38: 1072-6.
3. Apostolov E, Al-Soud WA, Nilsson I, et al. *Helicobacter pylori* and other *Helicobacter* species in gallbladder and liver of patients with chronic cholecystitis detected by immunological and molecular methods. Scand J Gastroenterol 2005; 40: 96-102.
4. Fox JG, Dewhirst FE, Shen Z, et al. Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. Gastroenterology 1998; 114: 755-63.
5. Kuroki T, Fukuda K, Yamanouchi K, et al. *H. pylori* accelerates the biliary epithelial cell proliferation activity in hepatolithiasis. Hepatogastroenterology 2002; 49: 648-51.
6. Leung RWL, Sung JJY. Review article: *Helicobacter* species and hepatobiliary diseases. Aliment Pharmacol Ther 2002; 16: 1037-45.
7. Monstein HJ, Johnsson Y, Zdolsek J, Svanvik J. Identification of *Helicobacter pylori* DNA in human cholesterol gallstones. Scand J Gastroenterol 2002; 37: 112-9.
8. Bulajic M, Stimec B, Milicevic M, et al. Modalities of testing *Helicobacter pylori* in patients with nonmalignant bile duct diseases. World J Gastroenterol 2002; 8: 301-4.
9. Méndez-Sanchez N, Pichardo R, Gonzalez J, et al. Lack of association between *Helicobacter* sp colonization and gallstone disease. J Clin Gastroenterol 2001; 32: 138-41.
10. Myung SJ, Kim MH, Shim KN, et al. Detection of *Helicobacter pylori* DNA in human biliary tree and its association with hepatolithiasis. Dig Dis Sci 2000; 45: 1405-12.
11. Clayton CL, Kleandhous H, Coates PJ, et al. Sensitive detection of *Helicobacter pylori* by using polymerase chain reaction. J Clin Microbiol 1992; 30: 192-200.
12. Roe IH, Kim JT, Lee HS, Lee JH. Detection of *Helicobacter* DNA in bile from bile duct diseases. J Korean Med Sci 1999; 14: 182-6.
13. Al-Soud WA, Ouis IS, Li DQ, et al. Characterization of the PCR inhibitory effect of bile to optimize real-time PCR detection of *Helicobacter* species. FEMS Immunol Med Microbiol 2005; 44: 177-82.
14. Sanac Y. Safra Kesesi. In: Sayek I, ed. Temel Cerrahi. Turkey: Gunes Kitabevi, 2004; 1372-80.
15. Reysenbach AL, Giver LJ, Wickham GS, Pace NR. Differential amplification of rRNA genes by polymerase chain reaction. Appl Environ Microbiol 1992; 58: 3417-8.
16. Sobala GM, O'Connor HJ, Dewar EP, et al. Bile reflux and intestinal metaplasia in gastric mucosa. J Clin Pathol 1993; 46: 235-40.
17. Correa P. *Helicobacter pylori* and gastric carcinogenesis. Am J Surg Pathol 1995; 19: 37-43.
18. Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR, Isaacson PG. *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. Lancet 1992; 339: 745-6.
19. Silva CP, Pereira-Lima JC, Oliveira AG, et al. Association of the presence of *Helicobacter* in gallbladder tissue with cholelithiasis and cholecystitis. J Clin Microbiol 2003; 41: 5615-8.
20. Chen W, Li D, Cannan RJ, Stubbs RS. Common presence of *Helicobacter* DNA in the gallbladder of patients with gallstone diseases and controls. Dig Liver Dis 2003; 35: 237-43.
21. Keighley B. Micro-organisms in the bile. Ann Royal College Surg Engl 1977; 59: 328-34.
22. Lygidakis NJ. Incidence of bile infection in patients with choledocholithiasis. Am J Gastroenterol 1982; 77: 12-7.
23. Leung JWC, Ling TKW, Chan RCY, et al. Antibiotics, biliary sepsis and bile duct stones. Gastrointest Endosc 1994; 40: 716-21.
24. Williams RA, Wilson SE. Cholecystitis and cholangitis. In: Gorbach SL, Bartlett JG, Blacklow NR, eds. Infectious Diseases. USA: W.B. Saunders Company, 1998; 884-90.
25. Levison ME, Bush LM. Peritonitis and other intraabdominal infections. In: Mandell GM, Bennet JE, Dolin R, eds. Principles and Practice of Infectious Diseases. USA: Churchill Livingstone, 2000; 830-55.