Helicobacter pylori in the Etiology of Cholesterol Gallstones

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Goals: We aimed to investigate the presence of bacterial structures in cholesterol gallstones and particularly presence of *Helicobacter spp/H. pylori* in gallstones by microbiologic cultivation, histopathologic staining, and polymerase chain reaction (PCR).

Background: Many studies suggest that different mechanisms are responsible for the formation of pigmented gallstones and cholesterol gallstones. Recently, studies showed that infection could have an important role in the formation of cholesterol gallbladder stones.

Study: We examined 77 mixed cholesterol gallstones. After cholecystectomy, gallbladder cultures were done for *H. pylori* and other bacterium. Gallbladder has also been examined by three histopathologic staining methods (Warthin-Starry, hematoxylin eosin, and gram staining) for *Helicobacter spp*. In addition, 16S rRNA-PCR amplification was performed for *Helicobacter spp* in gallstones. Twenty postmortem gallbladders without gallstones were investigated by the same histopathologic and PCR methods for *Helicobacter spp*. as a control group.

Results: Different bacterium were isolated from 22 gallbladder samples (12 *Escherichia coli*, 8 *Pseudomonas*, and 2 *clostridium*) and *H. pylori* was isolated in 6 gallbladder samples. *Helicobacter spp* was found in 7 gallstones by PCR amplification. *Helicobacter*-like organisms were demonstrated in 18 samples by three different histopathologic methods. *Helicobacter*-like organisms were also found in five samples by the same histopathologic methods (Warthin-Starry, hematoxylin-eosin, and gram staining). Only four samples were found positive for *Helicobacter spp/H. pylori* by all methods.

Conclusions: Bacterial population including *H. pylori* could have a possible role in the formation of cholesterol gallstones.

Key Words: *Helicobacter pylori*, cholesterol, gallstone, PCR

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Many findings obtained from microbiologic, morphologic, and chemical composition studies suggest that different mechanisms lead to the formation of pigmented gallstones and cholesterol gallstones.[1]

Previous studies have suggested that bacterium may have an important role in the formation of pigmented gallstones.[2,3] Because bacterial β-glucuronidase, phospholipases, and bile acid hydrolyzes catalyze biliary lipid hydrolysis, yielding calcium-sensitive anions and calcium salt precipitates, such precipitates are niduses for and constitutes of brown pigmented gallstones.[4] In fact, *Propionibacterium acnes* were suggested for having a role in the formation of gallstones.[5,6]

In contrast, the formation of pure cholesterol gallstone is thought to depend mainly on cholesterol saturation and solubility.[7] Recently, Swidsinski et al[5] documented DNA homology to bacterial rRNA in cholesterol gallstones. Microbiologic analyses of bile and electron-microscopic imaging of gallstones have implied that bacterium might also be responsible in the development of cholesterol gallstones.[1] Several studies have revealed the presence of bile-resistant *Helicobacter* spp. in bile samples and gallbladder mucosa.[8,9] *H. pylori* components and specific antibodies have been found in bile samples and this bacterial infection could constitute a putative cofactor of increased risk for gallstone formation.[10] *H. pylori* specific DNA was also found in cholesterol gallstones consisting mixed bacterial population.[11]

The aim of this study was to investigate the presence of bacterial structures, particularly presence of *H. pylori* in cholesterol gallstones by microbiologic cultures, histochemical staining, and polymerase chain reaction (PCR).

MATERIALS AND METHODS

Patients

We examined 77 mixed cholesterol gallstones removed aseptically from 49 women (mean age, 54 years; range, 24–80 years) and 28 men (mean age, 50 years; range, 25–73 years) operated in Surgery Department of Cukurova University Balcali Hospital. All patients were diagnosed that possessed gallstones. Sixty-nine of the patients had chronic cholecystitis, 3 with necrotizan cholecystitis, 4 with active chronic cholecystitis, and 1 had good differentiated adenocarcinoma. Seventy-six of patients were operated in elective conditions. Laparoscopic cholecystectomy and open cholecystectomy were performed to 73 and 4 patients, respectively. The gallbladders were aseptically transferred to the microbiology laboratory

urgently after operation. As a control group, we investigated 20 gallbladder samples without gallstones obtained from postmortem autopsy specimens (12 women, mean age, 42 years; range, 18–56 years; 8 men, mean age, 46 years; range, 24–60 years), which obtained under nonsterile conditions.

Cultivation of the Gallbladders

Gallbladder wall swaps were inoculated into bloody and endo agar for nonspecific bacterial cultures, *Brucella* agar (supplemented with 7% horse blood and bulltaler) for *Campylobacter* isolation and Colombia agar (with 7% horse blood, novobiocin, trimetoprime, and vancomycin) for *H. pylori* isolation. Mediums for *Helicobacter* and *Campylobacter* isolation were incubated in microaerophilic conditions for 72 hours. Classic bacteria identification was done by colonial morphology, gram staining, oxidase activity, and fermentation features of the bacterial strains. Certain isolates were identified by glycine, heat tolerance tests, and hippurat activity. Oxidase and fast urease activity were done at colonies grown on *H. pylori* specific media. Microorganism with suitable macroscopic and microscopic morphology and positive oxidase and urease activity were verified as *H. pylori*.

Preparation of DNA From the Gallstones and Gallbladder

Each gallstone was cut centrally on a sterile Petri dish using sterile forceps and a single-use sterile scalpel-blade. The matrix of the gallstone was obtained by crushing the cut parts in a sterile tube. Then, 1 mL of xylene was added to the matrix. The mixture was mixed on a vortex and boiled at 90°C for

(1 mmol/L Tris HCl, 0.1 mol/L EDTA, %0.5 SDS). 5 μL proteinase-K (100 mg/mL) was added to both gallstones and gallbladder tissue samples. All samples were incubated at 52°C for 2 hours and DNA was extracted by standard phenol/chloroform/isoamylalcohol extraction method. After that, PCR protocols were performed for *Helicobacter spp.*

16S rRNA-PCR Amplification

The amplification was done using 16S rRNA specific primers; sense (5′-CCCAAGAAGCCAATAAAACCCCAC-3′) and antisense (5′-CAAAGTCAAAACC GTAGAGCT GGC-3′), which resulted in a 349-bp product in a conservative region of *Helicobacter* genome. The reaction mixture (PCR core systems, Promega-M7660, Madison WI) was consisting 200 μmol/L of each dNTPs, 0.5 μmol/L of each primers, 2 mmol/L of $MgCl_2$, 2.5 U of Taq polymerase, 1 × PCR buffer, and 5 μL of sample DNA. The PCR reaction was performed; preheating at 94°C for 4 minutes, denaturation at 94°C for 1 minutes, annealing at 52°C for 1.5 minutes, extension at 72°C for 2 minutes for 35 cycles, and post elongation at 72°C for 5 minutes.

All PCR products were analyzed in 2% agarose gel by electrophoresis.

Histopathologic Examination of the Gallstones for *Helicobacter spp.*

The gallbladder was opened along a wall. A microscope slide was touched to the mucosa and the remained material smeared on the slide. Then the slide was fixed with 70% alcohol. Three different samples were also taken from each gallbladder and paraffin-embedded blocks were prepared. Then 5-μm histologic sections were prepared from these blocks. All histologic sections were stained with hematoxylin-eosin, Warthin-Starry, and gram staining. Mucosa samples prepared from gallbladder were stained with Giemsa stain and examined for *Helicobacter spp.* with light microscope. The same procedure was performed for 20 gallbladders without gallstones obtained from 20 postmortem cases as a control.

Cholesterol Detection and Quantification in Gallstones

Cholesterol quantification in gallstones was performed as previously described by Lee et al.[1] All stones were determined as mixed cholesterol stones.

Statistical Analysis

Values are expressed as mean (ranges) after analyzing nonparametric tests: Mann-Whitney *U* test and the χ^2 test were also used to determine the significance between groups. The *P* values were two-sided.

RESULTS

and *H. pylori* was detected in 12, 8, 2, and 6 patients, respectively (Tables 1, 2).

Helicobacter spp. Specific 16S rRNA-PCR Amplification of the Gallstones

Helicobacter spp. was detected in 7 mixed cholesterol gallstones by 16S rRNA-PCR amplification (Fig. 1). *Helicobacter spp.* did not detected in gallbladder samples of postmortem control patients by PCR.

TABLE 1. Helicobacter Positivity in Postmortem, Gallbladder and Stone Using Various Methodologies

Method	Gallstone (n = 77) [n (%)]	Gallbladder (n = 77) [n (%)]	Postmortem (n = 20) [n (%)]
Bacterial culture		6 (7.7)	
PCR	7 (9.09)		0
Histologic stainings			
Warthyn-Starry		10 (12.9)	0
Gram		10 (12.9)	0
HE		18 (23.3)	0

HE, hematoxylin-eosin; PCR, polymerase chain reaction.

TABLE 2. Demographics of Patients and Control Group

	Gallstone	Postmortem (Control Group)
Samples (n)	77	20
Male/female (n)	49/28	12/8
Mean ages (range) (yr)	52 (24–80)	44 (18–60)
Histopathologic diagnosis		
Chronic cholecystitis	69	
Necrotizing cholecystitis	3	
Active chronic cholecystitis	4	
Adenocarcinoma	1	
Isolated bacteria other than HP by culture		
E. coli	12	
Pseudomonas species	8	
Campylobacter jejuni	2	

Histopathologic Examination of the Gallbladders for *Helicobacter spp.*

We have found *Helicobacter spp.* in 18 patient samples by histopathologic method. *Helicobacter spp.* was detected in totally 10 histologic sections prepared from gallbladder with Warthin-Starry staining. It has also been detected in 18 samples with hematoxylin-eosin staining and in 10 samples with gram staining prepared from gallbladders. *Helicobacter spp.* was detected in five samples by all three histopathologic methods. No *Helicobacter* strains were detected in postmortem control samples by any of three staining methods and PCR. Demographics of the patients and postmortem control group are presented in Tables 1 and 2. *Helicobacter* positivity was higher in groups with gallstone compared with postmortem and differences were statistically significant ($P = 0.02$).

DISCUSSION

The role of the infection in the formation of gallstones is still controversial. Recently, bacterial infections were accused for the formation of brown gallstones.[3] Bacterial DNA from *Propiobacter* and *Enterobacter* has been detected in mixed cholesterol gallstones by using molecular biologic methods.[5]

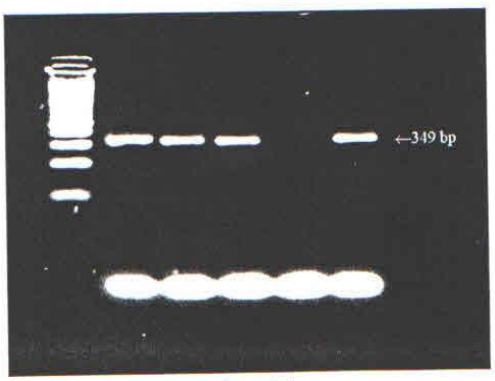

FIGURE 1. PCR products of *H. pylori*.

Bacterial DNA sequences were also shown in mixed cholesterol stones in choledoc, brown gallstones but rarely shown in pure cholesterol gallstones.[1]

The biliary tree is normally sterile, but when gallstones are present, bacterium can be isolated from bile or gallbladder wall.[12,13] We had isolated microorganisms in 22 samples, mostly gram-negative, aerobic bacteria (*E. coli, Pseudomonas*, rarely *C. jejuni*). These findings are similar with the findings of Lee et al; all of these organisms are closely related with normal intestinal flora.[1] Products from the species implicated could plausibly contribute to gallstone initiation or growth. *E. coli* β-glucuronidase can produce calcium bilirubinate from conjugate bilirubin[14] and *Pseudomonas* phospholipases A1 and C[15] produce calcium palmitate and calcium stearate from bilier lecithin.[4] The cleavage of biliary lecithin might decrease cholesterol solubility and accelerate nucleation.[16] Lee et al demonstrated that some bacteria has lithogenic enzyme activity in biliary system.[1]

H. pylori infection has important roles in pathogenesis of peptic ulcer,[17] chronic gastritis,[18] and gastric cancer.[19] It has also been demonstrated that it could be responsible for the pathogenesis of skin diseases[20] and coronary heart diseases.[21] Some microorganisms closely resembling *H. pylori* have been detected in resected gallbladder[9] and gall samples.[10,22] It has also been proposed that the presence of *H. pylori* in gall samples could be a risk factor for the formation of gallstones.[10]

Generally, different techniques such as 16S rRNA-PCR amplification, DNA sequencing, and southern blot have been using for identification of *H. pylori* and other bacteria in human cholesterol gallstones. Temporal gradient gel electrophoresis of DNA sequencing products is quite useful to differentiate the strains.[11]

In our study, *Helibacter spp.* was detected in seven cholesterol gallstone samples by 16S rRNA PCR. *H. pylori* could also have been isolated from gallbladder tissue samples of 6 patients. Four of the 6 culture-positive patients also had positive results by PCR at the same time. *Helicobacter spp.* was demonstrated in 18 patients by three different histopathologic stains. *Helicobacter* was demonstrated in 18, 10, and 10 samples by hematoxylin-eosin, Warthin-Starry, and Gram staining, respectively. Five patients were positive with each histopathologic methods. In four samples, *Helicobacter* was isolated or detected with each methods in which gallbladder culture, gallstone PCR, and three different histopathologic methods. On the other hand, *Helicobacter* could not have been demonstrated by histopathologic methods or PCR in any control samples. *Helicobacter* presence was higher in the group with gallstone compared with the control group, and this was statistically significant ($P = 0.02$).

There is always a false-negative or -positive result of any method. Use of conventional Warthin-Starry, hematoxylin-eosin, Giemsa staining and additional PCR technique with identification of *H. pylori* in gallstones and/or gallbladder tissue by microbiological cultures, more sensitive results could be achieved.

H. pylori may cause a possible chronic inflammation in the gallbladder mucosa. This inflammation may impair acid secretion with reducing the solubility of calcium salts in bile and increasing the risk of precipitation in the lumen. In

addition, this bacterium may act as a nidus for stone formation. We have detected *Helicobacter spp.* in the nucleus of the gallstones. In a previous study, Monstein et al reported that they have detected *H. pylori* in the nucleus but not in the envelope.[11] These findings imply that *H. pylori* may have a role in the formation of nucleus.

In our study, one sample with differentiated carcinoma has been found positive for *Helicobacter spp.* by hematoxylin-eosin staining, so that *H. pylori* incidence should be studied in large series of gallbladder carcinomas.

The incidence of *H. pylori* is high in the stomach and low in the gallstones, implying that the ascendant route is important in a minority of cases. Absence of *H. pylori* in postmortem control cases without gallstones implies that bacteria might be responsible for the development of gallbladder stones. The probability of decreasing gallstone formation with *Helicobacter pylori* eradication will be the target of future studies.

In summary, our data suggest that bacterial population including *H. pylori* could have a possible role in the formation of cholesterol gallstones.

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