Identification of *Helicobacter pylori* in Gallstone, Bile, and Other Hepatobiliary Tissues of Patients with Cholecystitis

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Background/Aims: Bacterial infection is accepted as a precipitating factor in cholesterol gallstone formation, and recent studies have revealed the presence of *Helicobacter* species in the hepatobiliary system. We utilized the polymerase chain reaction (PCR) to establish the presence of bacterial DNA, including from *Helicobacter* species, in gallstones, bile juice, and gallbladder mucosa from patients with gallstones.

Methods: At cholecystectomy, 58 gallstones, 48 bile samples, and 46 gallbladder mucosa specimens were obtained and subjected to nested PCR using specific 16S rRNA primers of *H. pylori* and other bacteria. Bacterial species were identified by DNA sequencing analysis. Bacterial 16S rRNA was detected in 25 out of 36 mixed-cholesterol gallstones, 1 out of 10 pure-cholesterol gallstones, and 9 out of 12 pigmented stones. Furthermore, 16S rDNA sequencing identified *Escherichia coli*, *Pseudomonas*, *Citrobacter*, *Klebsiella*, and *Helicobacter* species. **Results:** *Helicobacter* DNA was detected in 4 out of 58 gallstones, 6 out of 48 bile samples, and 5 out of 46 gallbladder specimens. Direct sequencing of *Helicobacter* amplicons confirmed strains of *H. pylori* in all four gallstones, five out of six bile samples, and three out of five gallbladder specimens. Almost all mixed-cholesterol gallstones appear to harbor bacterial DNA, predominantly *E. coli*. **Conclusions:** *H. pylori* was also found in the biliary system, suggesting that these bacteria are of etiological importance in gallstone formation. (*Gut Liver* 2010; 4:60-67)

Key Words: Gallstone disease; Pathogenesis; Bacterial infection; *Helicobacter*

INTRODUCTION

Previous studies suggest that bacterial infection plays an important role in the formation of brown pigmented gallstones and that the formation of pure cholesterol gallstones depends mainly on cholesterol saturation and solubility.¹⁻⁴ Attempts to culture potentially causative bacteria from cholesterol gallstones have failed probably because the formation of gallstones takes a very long time, thus embedded bacteria might be damaged or killed. However, there have been a few recent studies reporting on infectious agents contributing to the pathogenesis of cholesterol gallstones. Swidsinski *et al.*⁵ identified bacteria in cholesterol gallstones using PCR amplification and *E. coli* and *Pseudomonas* were suggested as the culprit pathogens in cholesterol gallstone formation by Lee *et al.*⁶ Consequently, bacterial infection is now accepted as a precipitating factor in the pathogenesis of mixed cholesterol gallstones. Although *Helicobacter pylori* is recognized as a human pathogen associated with gastric lesions, recent studies have revealed convincingly the presence of several *Helicobacter* species in the hepatobiliary system.⁷⁻¹⁸ A microorganism resembling *H. pylori* was firstly detected in resected gallbladder (GB) mucosa of patient with gallstone by Kawaguchi *et al.* in 1996⁷ and bile-resistant hepatic *Helicobacter* species such as *H. bilis*, *H. pullorum*, and *F. rappini* were extracted from GB mucosa and bile juice of patients with chronic cholecystitis, suggesting that these agents may be key elements in the development of various GB-related diseases, especially GB cancer.⁹ The pres-

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ence of *H. pylori* DNA in gallstones was established by PCR in several reports.¹²⁻¹⁴ Together with the discovery of *H. pylori* in bile juice,¹⁶⁻¹⁸ this has led to the suggestion that *Helicobacter* species are etiological agents in gallstone formation. However, another study from Germany reported no association of *Helicobacter* species with gallstone formation suggesting possible ethical and regional differences.¹⁹ In this study, we aimed to determine the frequency of bacterial infection in cholesterol gallstones, bile, and GB mucosa from Korean patients with cholecystitis. In addition, we attempted to ascertain the specific *Helicobacter* strains identified by PCR and DNA sequencing.

MATERIALS AND METHODS

1. Clinical specimens

Gallstones, bile juice and GB mucosa specimens were obtained during therapeutic cholecystectomy from 58 patients (21 men and 37 women; mean age, 55.4 years; range, 31-77 years) presenting with cholecystitis at Inha University Hospital, South Korea. Bile was collected by intraoperative aseptic aspiration, and bacterial cultures were performed immediately. Bile samples and gallstones were stored at -20°C . To minimize false positive results, we eliminated culture positive specimens from the study. A 1.5 cm-sized piece of GB mucosa was snap-frozen in liquid nitrogen, and stored at -70°C .

2. Cholesterol content analysis

Cholesterol content was measured using a kit from Boehringer Mannheim (Mannheim, Germany). After adding 5 mg of crushed gallstone to 5 mL of DMF (N, N-Dimethyl Formamide)/DMSO (Dimethyl Sulfoxide) solution, a 200 μL aliquot was mixed with 2.5 mL of the reactive solution (ammonium phosphate buffer (pH7.0), methanol, catalase, acetylacetone, ethanol) and 20 μL of cholesterol oxidase, and then incubated at 37°C . One hour later, the absorbance of the reactant mixture was measured using the Beckman DU 650 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). For a standard cholesterol curve, the absorbance of standard cholesterol concentration (1 mg, 2 mg, 3 mg, 4 mg, and 5 mg) melted in isopropanol solution was analyzed and the percentage of cholesterol in gallstones was quantified in reference to the standard curve.

3. DNA extraction from gallstones

After washing with phosphate buffered saline (PBS), each gallstone was cut through the centers 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 *et al.*⁵ In brief, samples of 200 mg were crushed and incubated with 1 mL of lysis buffer containing 1% sodium dodecyl sulfate and allowed to rotate overnight at room temperature. The sample was then centrifuged at 10,000 g for 10 minutes. The supernatant was separated and lithium chloride solution (7 mol/L) was added to a final concentration of 1.5 mol/L. The DNA was then extracted with phenol-chloroform, and further purified using the QIAamp DNA kit (QIAGEN, Hilden, Germany). About 500 μL of bile samples were pelleted by centrifugation for 10 minutes at 14,000 g, and incubated with lysis buffer [10 mM Tris-HCl (pH8.5), 10 mM EDTA, 100 mM NaCl, 0.5% SDS] and proteinase K at 55°C for 8 hours.

4. DNA extraction from GB tissue

Firstly, 25 mg samples of GB mucosa were washed with PBS solution and the DNA was extracted by using QIAamp DNA kit. Each specimen was mixed with 180 μL of buffer ATL and 20 μL proteinase K. All samples were incubated at 56°C and slowly vortexed until completely lysed. After adding 200 μL of buffer AL, lysed sample was pulse-vortexed, then incubated at 70°C for 10 minutes and 200 μL of 100% ethanol was added again to each sample. The solution was applied to the QIAamp spin column in a 2 mL collection tube and was centrifuged at 8,000 rpm for 1 minute to allow the DNA to attach to the silica gel membrane. The collection tube containing the filtrate was discarded and the QIAamp spin column was transferred into a new collecting tube. With 500 μL buffer AW1 and 500 μL buffer AW2, the QIAamp spin column was washed twice by centrifugation, then was placed in a 1.5 mL microcentrifuge tube. To elute DNA, 20 μL buffer AE was added and the solution was centrifuged at 8,000 rpm for 1 minute.

5. DNA extraction from bile juice

Five hundred μL samples of refrigerated bile were pelleted by centrifugation for 10 minutes at 14,000 rpm, then incubated with lysis buffer [10 mM Tris-HCl (pH8.5), 10 mM EDTA, 100 mM NaCl, 0.5% SDS] and proteinase K at 55°C for 8 hours. The DNA was then extracted twice with phenol-chloroform, and further purified using a QIAamp DNA kit (QIAGEN, Hilden, Germany).

6. Polymerase chain reaction of DNA

The PCR method was used to amplify DNA of *Eubacteria*, *Helicobacter* species, and *E. coli* from the samples. The general genomic DNA sequence of eubacterial 16S rRNA is shown in Fig. 1. To obtain higher amounts of DNA for cloning and prevent artifact of amplification, the

PCR product was reamplified with nested PCR primers designed by Takara Shuzo Co. Ltd. (Shiga, Japan) (Tables 1 and 2).

All reactions were 50 μ L in volume and performed with an automated Gene Amp PCR system 9600 (Perkin Elmer, Norwalk, CT, USA). Reaction mixtures contained 0.2 mM of each oligonucleotide primer, 10x PCR buffer [25 mM TAPS (pH 9.3), 50 mM KCl, 2 mM MgCl₂, 1 mM 2-mercaptoethanol], 0.5 U of Taq polymerase, 0.4 μ L of each primer, and 5 μ L of template DNA. The PCR products were denatured at 94°C for 1 minute, annealed at 55°C for 1 minute, and elongated at 72°C for 1

minute. The PCR reaction ended at 35 cycles. Then, 50 μ L mixture containing 3 μ L of PCR product was reamplified by nested PCR.

Final nested PCR products were electrophoretically separated in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light in comparison with DNA molecular markers.

7. DNA sequencing

In order to verify the specificity of the PCR product, the DNA band from the agarose gel was cut, and the purified DNA was extracted with Gel extraction kit (QIAGEN) and sequenced using the BigDye Terminator Cycle Sequencing Kit (Perkin Elmer) on an ABI PRISM 310 Genetic Analyzer (Perkin Elmer). Resulting sequences were compared to databases accessed through the NCBI (National Center for Biotechnology Information) Geneinfo BLAST network server.

RESULTS

1. Gallstone analysis

Fifty-eight gallstones were classified by their gross appearance and composition of cholesterol, resulting in 10 pure cholesterol gallstones, 36 mixed cholesterol gallstones and 12 brown pigmented stones. Pure and mixed cholesterol gallstones contained 90-99% and 55-89% cholesterol respectively.

Po mod

1 CTTTATGGAG AGTTTGATCC TGG CTCAGAG TGAACGCTGG CGGCGTGCTT AATACATGCA
 61 AGTCGAACGA TGAAGCCTAG CTTGCTAGGT GGATTAGTGG CGCAGGGTG AGTAACGCAT
 121 AGATAACATG CCCTTTAGTT TGGGATAGCC ACTAGAAATG GTGATTAATA CCAATACTC
 181 CTACGGGGGA AAGATTATC GCTAAAGGAT TGGTCTATGT CCTATCAGCT TGGTGGTGAG

C97

241 GTAAAGGCTC ACNNAGGCTA TGACGGGTAT CCGGCTGAG AGGGTGAACG GACACACTGG
 301 AACTGAGACA CGGTCCAGAC TCCTACGGGA GGCAGCAGTA GGGAAATTG CTCAATGGGC
 361 GCAAGCCTGA AGCAGCAACG CCGCTGGAG GATGAAGTTT TTAGGATTGT AAATCCTTT

CH3

421 TGTCAGAGAA GATAATGACG GTATCTGACG AATAAGCACC GGCTAACTCC GTGCCAGCAG
 481 CCGCGGTAAT ACGGAGGGTG CAAGCGTTAC TCGGAATCAG TGGGCGTAAA GAGTGCCTAG
 541 GCGGGGTTGT AAGTCAGATG TGAATCCTA TGGCTTAACC ATAGAAGTGC ATTTGAAACT

C98

601 ACAACTCTGG AGTGTGGGAG AGGTAGTGG AATTCTTGGT GTAGGGGTAA AATCCGTAGA
 661 GATCAAGAGG AATACTCATT GCGAAGGCGA CCTGCTGGAA CAATACTGAC GCTGATGCR

CH2

721 GAAAGCGTGG GGAGCAAACA GGATTAGATA CCCTGGTAGT CCACGCCCTA AACGATGGAT

Fig. 1. Structure of bacterial 16S rRNA genomic DNA. Po mod, CH2, and CH3 are alleles for eubacterial 16S rRNA. C97 and C98 are alleles for *Helicobacter* specific 16S rRNA.

Table 1. Primers for Bacterial 16S rRNA

Gene and DNA region	Primer	Primer sequence (5'to3')	Annealing tm (°C)	Size of PCR product
<i>Eubacteria</i> (16s rRNA)	First PCR	Pomod	AGAGTTTGATC(a/c)TGG	55
		CH2	ACTAC(c/t)(a/c/g)GGGTATCTAA(g/t)CC	55
	Second PCR	Pomod	AGAGTTTGATC(a/c)TGG	
		CH3	ACCGC(g/t)(a/g)CTGCTGGCAC	
<i>E. coli</i> (β -glucuronidase)	First PCR	uidA 858	ATCACCCTGGTGACGCATGTCGC	57
		uidA 1343	CACCACGATGCCATGTTTCATCTGCC	
	Second PCR	uidA 1047	TATGAACTGTGCGTCACAGCC	57
		uidA 1232	CATCAGCACGTTATCGAATCC	

Table 2. Primers for *Helicobacter* 16S rRNA

Gene and DNA region	Primer	Primer sequence (5'to3')	Annealing tm (°C)	Size of PCR product
<i>Helicobacter</i> species (16s rRNA)	C97	GCTATGACGGGTATCC	55	400 bp
	C98	GATTTTACCCCTACACCA		
<i>H. pylori</i> (26 kDa surface antigen)	HPF	TGGCGTGTCATTGACAGCGAGC	57	298 bp
	HPR	CCTGCTGGGCATACTTCACCATG		

2. Extraction of bacterial DNA from gallstones

In the group of mixed cholesterol gallstone, 25 of 36 stones (69.4%) had a positive PCR for bacterial 16S rRNA, whereas only 1 of 10 pure cholesterol gallstones showed a positive PCR (Table 3, Fig. 2). Positive PCR was also detected in 9 of 12 pigmented stones (75%).

3. Identification of bacteria by nested PCR and analysis of the DNA sequencing

The nested PCR using *E. coli* specific β -glucuronidase gene (*uidA*) was performed in the 26 cholesterol gallstones that had positive PCR for 16S rRNA. Twenty-three (88.4%) of them were positive for *uidA* (Fig. 3). Furthermore, DNA was sequenced in 17 available samples from these 23 positive nested PCR samples. As a result, 8 stones were found to contain sequence of *E. coli*, 5 stones contained *Pseudomonas* sequence, 2 stones contained *Citrobacter* sequence and 2 stones contained *Klebsiella* sequence (Table 4).

4. Extraction of *Helicobacter* species in bile juice, gallstones and GB tissue

Bacterial DNA was found in 35 (60.3%) of 58 gallstones. Additional PCR assays were run on each sample from the 35 gallstones with positive bacterial DNA using

Helicobacter genus-specific primers. Four samples had positive PCR for *Helicobacter* species. Bacterial DNA was also detected in 24 (50%) of 48 samples of bile juice by PCR assay, and 6 of these 24 samples were positive for *Helicobacter* species. Bacterial DNA was also discovered in 18 (39.1%) of the 46 GB mucosa specimens, and 5 of the 18 samples revealed positive findings concerning *Helicobacter* species (Fig. 4A). As a result, the positive rate of *Helicobacter* species was 4/58 (6.8%) in gallstone specimens, 6/48 (12.5%) in bile juice samples, and 5/46 (10.8%) in specimens obtained from the GB mucosa (Table 5). There was no difference in the identification of *Helicobacter* species among the groups based on gallstone composition; 2 samples of mixed cholesterol gallstones and 2 samples of pigmented stones had positive PCR results. Although there was no concurrent positive finding of the *Helicobact-*

Table 3. Bacterial DNA Detected in Crushed Gallstones

	Mixed cholesterol (n=36)	Pure cholesterol (n=10)	Brown pigment (n=12)
Bacterial DNA (%)	25 (69.4)	1 (10)	9 (75)
Cholesterol content, %	72.5	92.4	32.5

Fig. 3. Nested PCR for *E. coli* specific *uidA* gene in gallstones. M, DNA molecular marker; +, positive control; -, negative control; 1-3, positive samples; 4-5, negative samples.

Fig. 2. Bacterial 16S rRNA PCR products in gallstones. PCR products were amplified with the primers Pomod alleles (800 bp) for A, CH3 for B. (A) M, size marker; +, positive control; -, negative control; 1-3, positive samples; 4-5, negative samples. (B) M, DNA molecular marker; 1-2, positive samples; 3-5, negative samples.

er species in all of the three samples obtained from the same patient, we found 2 cases of simultaneous detection of *Helicobacter* species in two of the three samples; one was in gallstone and bile juice specimen, while the other was in the bile juice and GB tissue specimen.

5. Identification of *H. pylori* using specific primers for 26 kDa surface

To identify *H. pylori* by PCR, *Helicobacter pylori*-specific primer pairs HPF and HPR were used to generate amplicons of approximately 298 bases in samples positive for *Helicobacter* 16S rRNA. Analysis of the PCR products showed the expected 400-bp amplicon in 4 of 4 gallstone

samples, 5 of 6 bile juice samples, and 3 of 5 GB mucosa samples (Fig. 4B).

6. Sequencing of *Helicobacter* 16S rRNA amplicons

All 400-bp amplicons produced by PCR with 16S rRNA primers were fully sequenced to verify that the amplicons were truly *Helicobacter* 16S rRNA, and their species identity was determined using the NCBI BLAST Network server.

Consequently, 4 of 4 gallstone samples, 5 of 6 bile juice samples, and 3 of 5 GB mucosa samples were identified as that of *H. pylori*, but the remaining samples had no concordance. These were consistent with the results of PCR using the *H. pylori* 26 kDa surface antigen (Table 5).

Table 4. Identification of Bacterial DNA by Direct Sequence Analysis in 17 Available Samples from 23 Nested PCR-Positive Cholesterol Gallstones

Bacteria	Number
<i>E. coli</i>	8
<i>Pseudomonas</i>	5
<i>Citrobacter</i> sp.	2
<i>Klebsiella</i> sp.	2

DISCUSSION

The role of bacterial infection in the formation of cholesterol gallstones is still unclear. Evidences for several bacterial species have been reported in mixed cholesterol stones, but those have been rarely reported in pure cho-

Table 5. Identification of *Helicobacter* Species in Specimens of Gallstone, Bile Juice, and Gallbladder Mucosa

	Gallstone	Bile juice	GB mucosa
Pomod-PC5 (Eubacteria)	35/58 (60.3%)	24/48 (50.0%)	18/46 (39.1%)
C97-C98 (genus <i>Helicobacter</i>)	4/35 (11.4%)	6/24 (25.0%)	5/18 (27.8%)
<i>Helicobacter</i> (+)/Total	4/58 (6.8%)	6/48 (12.5%)	5/46 (10.8%)
HPF-HPR (<i>H. pylori</i> surface Ag)	4/4 (100.0%)	5/6 (83.3%)	3/5 (60.0%)
Direct sequencing	<i>H. pylori</i> (4)	<i>H. pylori</i> (5) Unidentified (1)*	<i>H. pylori</i> (3) Unidentified (2)*

GB, gallbladder.

*No identical bacteria with BLAST network server of NCBI.

Fig. 4. (A) *Helicobacter* genus specific 16S rRNA PCR products in gallbladder mucosa. (B) PCR products of 26 kDa surface antigen for *H. pylori*. M, DNA molecular marker; +, positive control; -, negative control.

lesterol gallstones.^{5,6,12,13} Even though the human biliary system is thought to be sterile, this sterility can be broken under certain conditions.²⁰⁻²² The major routes of infection are ascending through the duodenal papillary sphincter and descending through the portal system. For example, bacterial infection was found in bile juice (20%) and the liver (17%) on post-operative individuals without any hepato-biliary abnormalities.²³ Also, patients who underwent non-biliary abdominal surgery had a 32% positive rate of bacterial infection in their portal blood.²⁴ Leung *et al.*² found bacteria in 84% of the inner cut surface of the pigmented gallstones but not cholesterol stones using electron microscopy. In this study, bacteria in gallstone were investigated by the amplification of 16SrRNA and DNA sequencing, as others have done recently.^{5,6} Our findings confirm the association of bacterial infection with mixed cholesterol stones but not pure cholesterol stones-bacterial DNA was positive in 69.4% of the mixed cholesterol stones compared with only 10% of pure cholesterol stones. Although the exact mechanism by which bacterial infections contribute to cholesterol gallstones is not known, bacterial biofilm, which is composed of glycocalyx, is suggested to play a role as a nucleation factor in cholesterol gallstone formation, like a mucin. Other causative factors are changes of bile juice composition by beta-glucuronidase and phospholipase produced by bacteria, excessive mucin production of GB epithelial cells triggered by lipopolysaccharides produced by gram-negative bacteria, and the promotion of nucleation process through the activation of the immune system by bacteria itself.

Until now, more than 25 species of *Helicobacter* have been found, and these microorganisms have been confirmed to be causative agents in various diseases of the stomach, intestine, and the liver in human and mammals.²⁵⁻²⁹ Hepatitis and hepatomas are caused by *H. hepaticus* in mice,^{27,29} and *H. pulorum*, *Flexispira rappini*, and *H. canis* are isolated in diarrheal patients, showing the possibility of zoonosis.^{25,26,30,31} This strongly suggests that *Helicobacter* may be a triggering agent in liver diseases as well. *H. pylori* is very sensitive to bile acid, so it is difficult to grow in bile juice.²⁶ But, by extracting bile-resistant *Helicobacter* species from bile juice and GB of many animals, evidence is provided that many bacteria such as *F. rappini*, *H. hepaticus*, *H. bilis*, *H. canis*, *H. cholecystus*, and *H. pullorum* may be able to grow and survive in bile juice.^{32,33} Mice infected with *H. hepaticus* can develop chronic hepatitis, hepatic edema, and bile duct hyperplasia.²⁹ Hamsters infected with *H. cholecystus* can have pancreatitis and bile duct fibrosis.³⁴ Also, there have been several studies on the *Helicobacter* species on biliary diseases. In a

study investigating the presence of urease A gene by PCR in bile juice 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.¹⁶ Another study by Figura *et al.*⁸ implicated *H. pylori* as a precipitating factor in gallstone formation by identifying *H. pylori* antibodies in the bile juice of gallstone patients. *H. pylori* has also been reported to be present in bile refluxed in the stomach, and its association with gallstones is found in some studies but not in others.^{35,36}

Recently, there have also been many studies on the extraction of *Helicobacter* species from the different organs of the human body. Of the 23 Chileans patients with chronic cholecystitis, *Helicobacter* species were found in 9 cases of GB mucosa and 13 cases of the bile juice. Here, the *Helicobacter* was analyzed by the method of DNA sequencing and was found to be *H. bilis*, *H. pullorum*, and *F. rappini*. These are bile-resistant hepatic *Helicobacter sp.* that are closely associated with GB cancer.⁹ In a similar German study no *Helicobacter* species were found in bile juice, suggesting that there may be a racial and demographic differences.¹⁹ Nilson *et al.*¹¹ have also studied the relationship between *H. pylori* and primary biliary cirrhosis and primary biliary sclerosing cholangitis. As a result, *H. pylori* was found in the liver tissue but *H. bilis*, *H. pullorum* and *H. hepaticus* was not. Although there have been some reports in which several *Helicobacter* species were found in animal hepatobiliary and gastrointestinal organs, this holds uncertain clinical significance. In this study, we extracted bacterial DNA from the specimens of gallstones, bile juice, and GB mucosa 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 using *Helicobacter* genus-specific primers. Most of them were confirmed as that of *H. pylori* by DNA sequencing. We found the *H. pylori* from one of three samples in the most of patients, and only 2 patients showed simultaneous positive results in more than 2 samples. To elucidate further the inflow pathway of *H. pylori*, a prospective and expanded study with gastric mucosa tissue will be needed. Although this method is very precise and accurate, there are many limitations such as the inability to identify all the organisms in cases of infection with multiple *Helicobacter* subtypes. Hopefully, in the future there will be new technology which would enable us to rectify this problem. Although disadvantage of lack of comparative control group, we could identified bacteria including *Helicobacter* species in bile juice, GB mucosa, and gallstones of Korean patients with cholecystitis, most of them have cholesterol gallstones. In summary, our data

suggest that it maybe a just innocent bystander, but bacterial infection including *H. pylori* may have a role in the formation of cholesterol gallstones. Future studies focusing in the route of *H. pylori* infection and the effect of bacterial eradication on the development of gallstone will be needed.

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