

# Molecular Genetic Evidence of Bacterial Colonization of Cholesterol Gallstones

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**Background/Aims:** Cholesterol gallstone formation is believed to be unrelated to the presence of bacteria because attempts to culture potentially causative bacteria from surgically removed cholesterol stones have failed. However, the formation of gallbladder gallstones takes years. Embedded bacteria may be damaged or killed. The aim of this study was to search for bacterial DNA sequences in cholesterol stones with negative bacterial culture. **Methods:** Bacterial gene fragments were amplified in vitro from DNA extracted from cholesterol gallbladder stones. Comparative 16S ribosomal RNA sequence analysis was used for identification. **Results:** Gallstones with cholesterol content between 70% to 90% harbored bacterial DNA (16 of 17 patients). No bacterial DNA was found in the gallstones with cholesterol content of >90% (3 patients). Three bacterial groups typical for gallstone colonization were identified. Propionibacteria-related DNA was found in the stones of 9 patients (45%). Enterobacterial type sequences were obtained in 5 patients (25%). A more heterogeneous sequence collection was retrieved from 7 patients (35%) and could be assigned to the major bacterial line of gram-positive bacteria with a low DNA guanine and cytosine content. **Conclusions:** Most cholesterol gallstones harbor bacterial DNA. It is important to determine the actual role of these microorganisms in gallstone formation.

Gallstone disease remains a common cause of serious morbidity and mortality. Although several factors are known to trigger nucleation and/or growth of cholesterol crystals, our understanding of the natural history of gallstone propagation is poor. Previous studies suggested that bacteria are crucial in the pathogenesis of brown pigment gallstones.<sup>1,2</sup> Brown pigment stones are common in the Orient and rare in Western populations. They consist mainly of calcium bilirubinate and calcium palmitate and typically originate in the common bile duct rather than in the gallbladder. Bacteria are present in most cases. In contrast, a positive bile culture can be

obtained only from 10%–25% of patients undergoing surgery for cholesterol gallstones. The percentage is higher in cases complicated by acute cholecystitis.<sup>3,4</sup>

Marked differences in overall chemical composition, structure, morphology, microbiological findings, epidemiology, and localization of cholesterol and brown pigment gallstones suggest that there are two thoroughly different mechanisms underlying formation of these two types of gallstones. Cholesterol, the main component of a stone, has attracted major attention. The importance of bacteria in the development of cholesterol stone formation has been rejected,<sup>5–7</sup> but a few exceptions are known.<sup>8,9</sup> Vitetta et al. found infected bile in 10% of patients with cholesterol gallstones.<sup>8</sup> The associated bile showed a high degree of calcium bilirubinate precipitation; the precipitate was also detected in the center of the gallstones. These typical calcium bilirubinate deposits were present at the nuclear areas of approximately half of the patients with cholesterol gallstones, even when the gallbladder bile was shown to be sterile. Stewart et al. found bacteria in pigment gallstones but not in cholesterol gallstones using scanning electron microscopy.<sup>9</sup> Bacteria have also been found within the pigment portions of composite stones. These observations imply that the presence of bacteria might not be restricted to pigment stone disease. In fact, most cholesterol stones are composite stones, i.e., have a pigment center or section.

Our knowledge of many microbial systems is limited because it is currently not possible to grow the vast majority of bacteria and because bacteria recovered by cultivation are often not representative of the original bacterial population. Detection methods that do not rely on culturability are necessary. Comparative sequencing of 16S and 23S ribosomal RNA is now the golden standard for elucidation of bacterial phylogenies and bacterial identification.<sup>10</sup> For both of these molecules, large sequence databases have been established. These allow the rapid placing of a new sequence within the existing

framework. The aim of the present study was to detect bacterial DNA in cholesterol gallstones. The approach used was based on the amplification of 16S ribosomal RNA genes followed by comparative sequence analysis.

## Materials and Methods

The gallbladder stones were consecutively collected in February and March 1993. Three patients with positive bile cultures were excluded. The group for analysis consisted of 4 men and 16 women (mean age, 57.1 years; range, 40–70 years) who had symptomatic gallstone disease and underwent elective surgery at Friedrichshain Hospital, Berlin, Germany. Clinical information obtained on all patients included symptoms and physical signs, duration of illness, previous evidence of infection, presence of other medical illness, laboratory data, and pathological findings. All stones had diameters of more than 6 mm.

### Sample Preparation

**DNA extraction.** DNA (0.1–1 µg) could be obtained from stone samples with conventional purification methods as estimated from ethidium bromide staining of electrophoretically separated samples and references ( $\lambda$ -DNA). Spectrophotometrical analysis was not possible because of interference of gallstone components that could not be completely removed during purification. The extracts contained substances that severely inhibited the polymerase chain reaction (PCR). The interfering substances could successfully be removed by lithium chloride precipitation. Stone samples of approximately 200 mg were crushed in a 1.5-mL Eppendorf tube with a glass rod and incubated with 600 µL of 1% sodium dodecyl sulfate rotating overnight at room temperature. Lithium chloride solution (7 mol/L) was added to a final concentration of 1.5 mol/L. The DNA was then extracted twice with phenol, further purified using GeneClean kit (La Jolla, CA), and resuspended in 50 µL high-performance liquid chromatographic-purified water (Baker, Deventer, Holland).

### PCR Cloning and Sequencing

The 5' half of the 16S ribosomal RNA genes was amplified in vitro using a generic primer set: (5'-AGAGTTTGAT(c/t)(c/a)TGGCTCAG-3', 5'ACTAC(c/t)(a/c/g)GGGT-ATCTAA(g/t)CC-3'). To obtain higher amounts of DNA for the cloning step, the PCR product was reamplified with the same 5' primer and a new nested 3' primer (5'-ACCGC-(g/t)(a/g)CTGCTGGCAC). To prevent artifacts resulting from contamination, control experiments omitting template DNA were performed in parallel. Pre-PCR and post-PCR steps were performed in different buildings. If the PCR of the first gallstone (chosen at random) was positive, the rest of the stones of a given patient were usually not analyzed. Otherwise, all stones or at least four stones were analyzed. The 500-base pair DNA fragments were cloned into the vector p-Bluescript (Stratagene, La Jolla, CA) to isolate the various 16S RNA sequences amplified by PCR. The sequence of approximately 400 bases of each clone was determined applying the dideoxy chain termination technique.

**Table 1.** Mean Similarity Values of Ribosomal RNA Gene Fragment Sequences Retrieved From Gallstones and Reference Sequences From Public Databases

	Similarity group (mean $\pm$ SD)		
	I	II	III
<i>Propionibacterium</i>			
<i>acnes</i>	97.90 $\pm$ 1.79	69.02 $\pm$ 2.43	71.95 $\pm$ 1.17
<i>Streptococcus</i>			
<i>pyogenes</i>	73.26 $\pm$ 1.00	90.64 $\pm$ 1.75	77.68 $\pm$ 0.67
<i>E. coli</i>	73.10 $\pm$ 0.79	73.14 $\pm$ 1.29	97.42 $\pm$ 4.21
<i>Pseudomonas</i>			
<i>aeruginosa</i>	74.41 $\pm$ 0.7	74.05 $\pm$ 1.27	84.01 $\pm$ 2.81
<i>Clostridium</i>			
<i>difficile</i>	73.46 $\pm$ 1.10	72.19 $\pm$ 2.21	71.75 $\pm$ 1.42

### Sequence Data Analysis

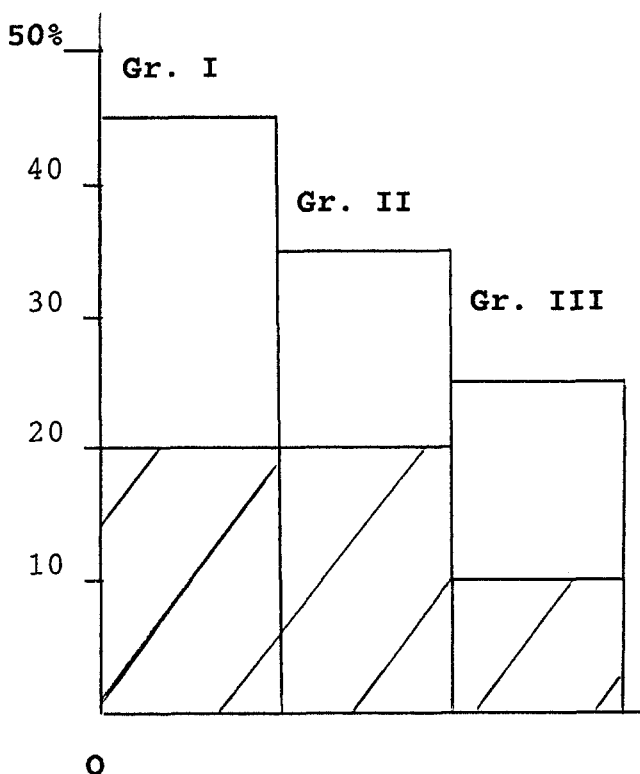
The new partial ribosomal RNA sequences were inserted in an alignment of 1300 bacterial 16S ribosomal RNA primary structures retrieved from public databases<sup>11,12</sup> according to primary and higher order structure similarity. Matrices of binary similarity and dissimilarity values (%) were established using the program SEQDIS (Ludwig et al., unpublished data, January 1993). A cluster analysis of the data set was performed applying Felsenstein's<sup>13</sup> program NEIGHBOR with the unweight-pair-group-method-using-arithmetic-average option to associate the partial sequences to a cluster of related organisms that had been defined by comparative analyses of complete reference sequences.

### Cholesterol Content Analysis

Cholesterol and DNA isolation from the same gallstone were performed independently. About 50 mg of native gallstone material was dried at 105°C to a constant weight in 2.5-mL polyethylene vials. The material was extracted with 1.0 mL *tert*-butylmethylether (Merck no. 818109; Merck, Darmstadt, Germany) on a vortex mixer for 5 minutes. After centrifugation at 12,000g (5 minutes), an aliquot of 50 µL supernatant was diluted by the factor 40 (1.95 mL) with a mixture of acetonitrile, 67 vol% (Merck no. 16), and *tert*-butylmethylether. For standard, 50 mg of cholesterol (BHD Chemicals Ltd., Dorset, England) was processed as described for the sample preparation. For the photometric test, the cholesterol analyses were performed with the enzymatic CHOD-PAP method<sup>14</sup> on a BM/Hitachi 717 (Mannheim, Germany) analyzer according to the manufacturer's instructions.

## Results

Ribosomal RNA gene fragments could be amplified from gallstones of 16 patients. The PCR products were cloned, and 10–24 clones were analyzed by sequencing for each gallstone. Twenty-nine different sequences were found. All but 4 of 223 retrieved sequences could be assigned to only 3 different groups of phylogenetically related organisms (Table 1). Figure 1 shows the patients whose stones



**Figure 1.** Percent of patients colonized with bacteria of each similarity group. Z, Percentage of stones colonized with more than one group of microorganism.

were colonized with bacteria of each group. Approximately half of them were colonized with gram-positive bacteria with a high DNA guanine and cytosine content (guanine and cytosine content of more than 55%) related to propionibacteria (similarity to group I). Seven gallstones (35% of patients) contained more heterologous sequence collection (similarity to group II) that could be assigned to the major bacterial line of gram-positive bacteria with a low DNA guanine and cytosine content (clostridia and relatives, guanine and cytosine content under 55%). Stones of 5 patients contained *Escherichia coli*-related enterobacteria (similarity to group III). The clinical characteristics of patients, cholesterol content, and bacterial ribosomal RNA sequences retrieved from gallstones are presented in Table 2.

In vitro amplification was not possible in four cases, even after several attempts of DNA enrichment, purification, and repeated PCR reactions. Remarkably, none of the 3 patients, whose stones contained more than 90% cholesterol, had detectable bacterial DNA. Lack of evidence of the presence of bacteria in the gallstones of patient 7 may be of methodical nature because PCR is very vulnerable towards impurities and bilirubin in particular.

Colonization was of a complex nature. Half of the patients carried bacteria mixed of two groups. Five of 8 patients whose gallstones were colonized with only 1

group of microorganisms were shown to have 2 or more closely related sequences. Two patients had one kind of sequence.

There was no striking difference in either cholesterol content of gallstones or in clinical and laboratory data between patients from various groups, probably because of the limited number of cases studied.

No traces of DNA could be extracted from 1–5 mL of the corresponding bile. All attempts at PCR were negative or unspecific, but the optimal PCR conditions for bile specimens are not yet known.

## Discussion

Maki postulated that bacterial  $\beta$ -glucuronidase catalyzes the hydrolysis of bilirubin conjugates in bile, leading to increased amounts of unconjugated bilirubin, which precipitates as calcium bilirubinate.<sup>1</sup> Bacterial species (especially *E. coli*, *Klebsiella*, and *Clostridium perfringens*) that show  $\beta$ -glucuronidase activity are present in most cases of calcium bilirubinate stones.<sup>15,16</sup> The role of bacteria in the pathogenesis of brown pigment stones is therefore generally accepted. In contrast, attempts to cultivate bacteria from bile or fresh cholesterol stones were usually unsuccessful. In the majority of reports, bacteria in bile were present in only 10%–25% of patients undergoing elective biliary tract surgery.<sup>4</sup> The presence of bacteria in these cases is therefore thought to be a secondary phenomenon.<sup>7</sup> Potential pathogens and their impact on human disease often remain unrecognized because of the lack of appropriate methods of detection and identification. *Helicobacter jejuni* is the latest example. The frequency of bacteria detected with PCR in this study is much higher and differs strongly from the culture results. However, pure cholesterol stones seem to be genuinely free of bacteria. Their bacterial load is not sufficient to be detected with PCR, but the cutoff for cholesterol stone definition must be increased more than 90%. Only a minority of gallbladder stones falls under this stringent criteria; others should be placed in the category of composite stones. There have been many reports on bacteria in the bile of patients with gallbladder stones.<sup>3,4,17–19</sup> Enterobacteria were especially searched for and occasionally found. *E. coli* was most frequent. Our findings support the culture data in the case of enterobacteria. The similarities among proteobacterial sequences found in gallstones (similarity to group III) were comparable with those derived from the corresponding regions of well-characterized, more complete database ribosomal RNA sequences of enterobacteria. However, propionibacterium-type sequences and sequences of group II represent the major fraction of the partial 16S ribosomal RNA sequence data set established in the present study. Unfor-

**Table 2.** Clinical Characteristics of Patients Compared With Cholesterol Content and Microbiological Features of Their Gallstones

Age (yr)/sex	Symptoms	Duration of illness before operation	No. of stones found/ no. of stones investigated	Cholesterol content (%)	No. of clones sequenced	No. of different clones found	Percentage of clones in similarity group			
							I	II	III	Other
62/F	C	3 yr	10/6	99						
63/F	C, N, F	5 yr	5/5	93						
48/F	N	6 mo	1/1	92						
56/F	A, N	4 mo	6/2	90	10	2	10	90		
65/M	C	4 yr	1/1	90	10	4	50	40		10
62/F	C	7 yr	1/1	88	20	2			100	
51/F	A, N	6 mo	1/1	88	10	1	100			
70/F	A	1 yr	3/1	87	12	3	84		16	
57/F	N, C	1 mo	3/1	86	10	2	20	80		
40/F	C, N, F	2 mo	1/1	85	12	5		92		8
47/M	A	1 yr	3/1	83	15	4		94		6
55/F	C	2 yr	5/1	82	10	3	80		20	
56/F	A	6 mo	7/4	81						
62/F	C	1 yr	9/1	80	10	2	100			
65/F	C	17 yr	3/1	78	12	3		100		
64/F	?	2 yr	9/1	77	20	3			100	
65/F	A	10 yr	1/1	75	14	2			100	
50/M	C	4 yr	32/1	75	10	2		100		
54/M	A	10 yr	4/1	73	24	1	100			
50/F	N, C	5 yr	7/1	70	24	2	96			4

A, flatulence and indefinite abdominal pain; C, biliary colic; F, fever; N, nausea and vomiting.

tunately, none of the ribosomal RNAs of similarity to group II is highly similar to any of the reasonable data set of reference sequences from gram-positive bacteria with a low DNA guanine and cytosine content. Thus, these sequences represent a group of organisms that has not been analyzed at the ribosomal RNA level.

The striking difference between cultural and molecular genetic findings is because of the differences in methodology. The optimization of conditions used can change the results dramatically. For example, a careful culture study by Goodhart et al. in 1978<sup>19</sup> showed growth of *Propionibacterium acnes* from the gallstone, bile, or gallbladder wall of approximately half of the patients with asymptomatic cholecystolithiasis but classified this bacterium as contaminant because of the low titer recovered and because of lack of biochemical activity. It remains unclear whether bacteria do indeed play a role in the development of gallstones. The presence of bacterial DNA shows only a colonization of the gallbladder stones. On the other hand, it seems likely that the colonization of the gallstone must modulate their formation in some way. Although we share the generally accepted opinion that cholesterol and pigment gallstones are not formed by the same mechanism, we are convinced that the actual role of bacteria in the formation of composite gallstones (70%–90% cholesterol) remains to be determined.  $\beta$ -Glucuronidase might not be the only way bacteria can influence gallstone formation. Other mechanisms are possible. Bacteria

could disturb gallbladder wall secretion and cause acute or chronic inflammation with resultant precipitation of bile. Bacteria show adhesion properties and can produce glycocalyx or other matrices<sup>20</sup> within which the pigment or cholesterol coalesces. They can produce calcium palmitate via a phospholipase.<sup>21</sup> Other biochemical properties could be involved as well. Many questions remain to be answered. What is the origin of these bacteria? How do bacteria enter the biliary tree? What is their interaction with other known pathogenetic mechanisms of gallstone formation? The identification of ribosomal RNA sequences alone is not sufficient to define the quantitative composition of the bacterial community. It is necessary to relate the sequences found to living bacteria and distinct pathogenetic mechanisms, which is the ultimate goal of these studies.

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