The Role of Bacteria in Gallbladder and Common Duct Stone Formation

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Debate continues as to the role that bacteria play in gallstone pathogenesis in Western countries. We, therefore, examined gallbladder and common duct stones from 67 consecutive patients undergoing cholecystectomy and/or common bile duct exploration. Bile was cultured and stone cholesterol content was measured. Stones were examined by scanning electron microscopy (SEM) for bacteria. Individual calcium salts were classified by windowless energy-dispersive x-ray microanalysis. Gallbladder stones in 65 patients were identified as cholesterol in 46 (71%), black pigment in 17 (26%), and brown pigment in 2 patients (3%). Common bile duct stones from ten patients were cholesterol in 4, black pigment in 2, and brown pigment in 4 patients. The five patients with brown pigment stones were significantly ($p < 0.05$) older, more likely to be men and to present with bile duct obstruction. Bile cultures were positive in 13% of patients with cholesterol stones, in 14% of those with black pigment stones, and in all of the patients with brown pigment stones ($p < 0.001$). By SEM, bacteria were observed only within the calcium bilirubinate-protein matrix of brown pigment stones ($p < 0.001$). In comparison to black pigment stones, brown stones were more likely to contain calcium palmitate ($p < 0.005$) and cholesterol ($p < 0.001$). We conclude that black and brown pigment stones have different pathogenic mechanisms and that bacterial infection is important only in the formation of brown pigment stones.

IN 1966 MAKI[1] proposed that bacterial infection plays a key role in the pathogenesis of pigment gallstones. Since that time, the association of bacterial infection with "calcium bilirubinate" or "stasis" pigment stones seen frequently in the Orient has been well accepted.[2-8] However, debate continues as to whether some of the gallstones that occur in Western countries are caused by bacteria. This debate has been further confused by differences in nomenclature, especially for pigment stones.[9]

Some of this confusion was dispelled by a 1982 National Institutes of Health International Workshop that classified most pigment gallstones as either black or brown.[10] In Western countries black pigment stones have been associated with hemolysis, cirrhosis, and old age, whereas brown stones are usually found as recurrent common bile duct stones.[10-16]

Recent scanning electron microscopic (SEM) studies have suggested that bacteria play a significant structural and functional role in the formation of pigment and cholesterol gallstones. Two recent studies from the United States have identified bacteria in the majority of pigment stones and in the pigment portion of "composite" stones.[17,18] These investigators did not find bacteria in cholesterol stones, but they concluded that bacterial infection was a primary factor in the pathogenesis of both black and brown pigment stones. Speer and his Australian colleagues[19,20] have also recently reported bacteria on the surface of cholesterol gallstones and have suggested a role for bacteria in cholesterol as well as brown pigment gallstone formation.

These recent SEM studies, however, have limitations. First, in these studies stones were obtained from selected patients and not from a consecutive series of patients with gallbladder and/or common bile duct stones. Second, the location of bacteria with respect to the stone's central areas, representing the earliest stage of gallstone formation, was not considered in the study by Speer et al.[19] Finally, the composition of the stone in the area where bacteria are seen was not determined. We, therefore, obtained gallbladder and common bile duct stones as well as bile for culture from 67 consecutive patients undergoing cholecystectomy and/or common bile duct exploration.

Supported by a Veterans Administration Research Grant.

Presented at the 100th Meeting of the Southern Surgical, Association, Boca Raton, Florida, December 5–7, 1988.

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TABLE 1. *Patient Characteristics*

	Galbladder Stones				Common Bile Duct Stones			
		Pigment				Pigment		
	Cholesterol	Black	Brown	All	Cholesterol	Black	Brown	All
n	46	17	2	65	4	2	4	10
Age, Sex, Race								
Age (years)	49 ± 3	37 ± 5*	67 ± 4§	47 ± 3	49 ± 9	35 ± 15	72 ± 5§	55 ± 7
Female (% patients)	67	65	50*	66	50	100	0*	40
Caucasian (% patients)	72	47	100	66	75	0	100	70
Black (% patients)	28	53	0	34	25	100	0	30
Diagnosis (% patients)								
Chronic cholecystitis	72	76	50§	72	25	50	0§	20
Acute cholecystitis	11	18	0	12	0	0	0	0
Bile duct obstruction	7	6	50§	8	25	50	75§	50
Gallstone pancreatitis	9	0	0	6	25	0	0	10
Acute cholangitis	2	0	0	2	25	0	25	20
Risk Factors (% patients)								
Obesity	30	6*	50	25	25	0	0	10
Estrogen	9	0	0	6	0	0	0	0
Hemolysis	2	41†	0	12	0	100	0	20
Ileal disorder	0	18‡	50	6	0	0	25	10
Parenteral nutrition	0	12*	0	3	0	0	0	0

* $p < 0.05$.
† $p < 0.001$.
‡ $p < 0.01$ *versus* cholesterol gallbladder stone patients.

§ $p < 0.05$ all five brown pigment stone patients *versus* cholesterol and black pigment gallbladder stone patients.

Stones were examined with scanning electron microscopy equipped with windowless energy-dispersive x-ray microanalysis. This newly modified technique[21] allows both morphologic identification of bacteria as well as determination of the composition of the surrounding stone matrix.

Methods

Patient Population

Gallbladder and common duct stones were obtained from all patients undergoing cholecystectomy and/or common bile duct exploration at the Johns Hopkins Hospital between August, 1987, and April, 1988. During this 9-month period, 65 patients underwent cholecystectomy. The indication for cholecystectomy was chronic cholecystitis and cholelithiasis in 47 patients, acute cholecystitis and cholelithiasis in 8 patients, and gallstone pancreatitis in 4 patients. Two of these 59 patients also underwent common bile duct exploration for stones. Six additional patients who presented with jaundice (five patients) or cholangitis (one patient) underwent both cholecystectomy and common bile duct exploration. Thus, eight of 65 patients (12%) undergoing cholecystectomy had their common bile duct explored for stones. Finally, two patients with common duct stones, who had previously undergone cholecystectomy and had failed endoscopic sphincterotomy, also underwent surgical duct exploration.

The mean age of the 65 patients with gallbladder stones was 47 ± 3 years, and 66% of these patients were women (Table 1). Sixty-six percent were white; 34% were black; and none were Oriental. Twenty-five percent of the patients with gallstones were obese, and 6% were taking estrogens (Table 1). Other risk factors for gallstone disease included hemolysis (sickle cell disease in 5 patients, prosthetic heart valve in 3 patients) in 12%, an ileal disorder (ileal resection in 3 patients, Crohn's disease in 1 patient) in 6%, and long-term total parenteral nutrition in 3% of patients. The mean age of the 10 patients with common bile duct stones was 55 ± 7 years, and four of them were women (Table 1). Seven of these ten patients were white; three were black; and none were Oriental. Five patients presented with jaundice, 2 with cholangitis, 2 incidentally, and 1 with gallstone pancreatitis (Table 1). Of these 10 patients with common duct stones, the only risk factors were hemolysis in two, an ileal disorder in one, and obesity in one (Table 1).

Stone Classification and Bile Cultures

Stones were initially classified on the basis of their visual appearance into one of four categories: cholesterol, "mixed" (cholesterol and pigment), black pigment, or brown pigment. By visual appearance, 41 of 65 gallbladder and 4 common bile duct stones were initially thought to consist primarily of cholesterol. Many cholesterol stones had pigmented centers, layers, or shells. Five gallbladder stones with relatively large pigment components were initially classified as "mixed" stones. All stones, or portions thereof, were dried, weighed, crushed, and extracted with chloroform/methanol. Cholesterol content was then measured by the method of Roschlau.[22] All "mixed" stones were found to have at least 55% cholesterol by weight and, therefore, were grouped with the cholesterol stones. Thus, 46 of 65 gallbladder stones (71%) and 4 of 10 common bile duct stones (40%) were cholesterol (Table 1).

Black and brown pigment stones were differentiated by their color, surface characteristics, ability to be crushed, and cross-sectional appearance.[10-16] Black pigment stones tended to have a shiny surface, to resist manual crushing, and to have a uniform fractured surface. Brown pigment stones had a dull surface, were easily crushed, and on cross section usually had lighter and darker concentric layers. Using these criteria, 19 of 65 gallbladder stones (29%) were classified as pigment stones, 17 black and 2 brown (Table 1). Six of 10 common bile duct stones (60%) were pigment stones, 2 black and 4 brown (Table 1). Gallbladder or common duct bile from 59 of the 67 patients (88%) was cultured by standard techniques.[23]

Scanning Electron Microscopy (SEM)

Gallstones were obtained fresh, washed briefly in deionized water, and were dried under vacuum or prepared for critical point drying. Stones in the latter group were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.4 for 1 to 7 days, washed in 0.1 M sodium phosphate buffer, dehydrated through ascending grades of ethanol, and critical-point dried in carbon dioxide. Dried specimens were fractured, mounted on aluminum SEM stubs to expose a stone cross section, and sputter coated with a thin layer of silver. The interior of each stone was studied in the secondary electron imaging (SEI) and backscattered electron imaging (BEI) modes at 4 to 25 kV using a JEOL JSM-840 (JEOL, Peabody, Massachusetts) scanning electron microscope with 40Å SEI and 100Å BEI resolutions or a JEOL JSM 35C scanning electron microscope with 60Å SEI resolution.[21] Secondary electron imaging was used to identify morphologic evidence of infection. Several hundred fields of each stone cross section were scanned at magnifications of X4000 to X20,000. The findings of bacteria in fresh or fixed/critical point dried specimens or bacterial casts in air dried samples were considered as evidence of bacterial infection during gallstone formation.[17-20]

Windowless Energy-Dispersive X-ray Microanalysis (EDX)

In cholesterol gallstones calcium salts were localized by backscattered electron imaging, and calcium salts in all stones were identified by windowless energy-dispersive x-ray microanalysis (EDX). BEI produces an image with intensities dependent on the atomic mass of the sample. Material composed of heavier atoms with denser electron clouds scatter more electrons 180° (backscatter) and provide a brighter SEM/BEI image. Calcium salts, therefore, appear brighter than the surrounding organic gallstone material (Figs. 1A and B). Windowless EDX allows the specific identification of organic and inorganic calcium salts within gallstones by providing an elemental spectrum in which the x-ray data from light elements (carbon and oxygen) are not filtered. Each organic calcium salt has distinct ratios of calcium to oxygen and carbon.[21] Therefore, the composition of the gallstone where bacteria were structurally deposited was determined. A Princeton Gamma Tech System IV windowless EDX system (Gamma Tech, Princeton, NJ) was used for elemental analysis at 15kV accelerating voltage.

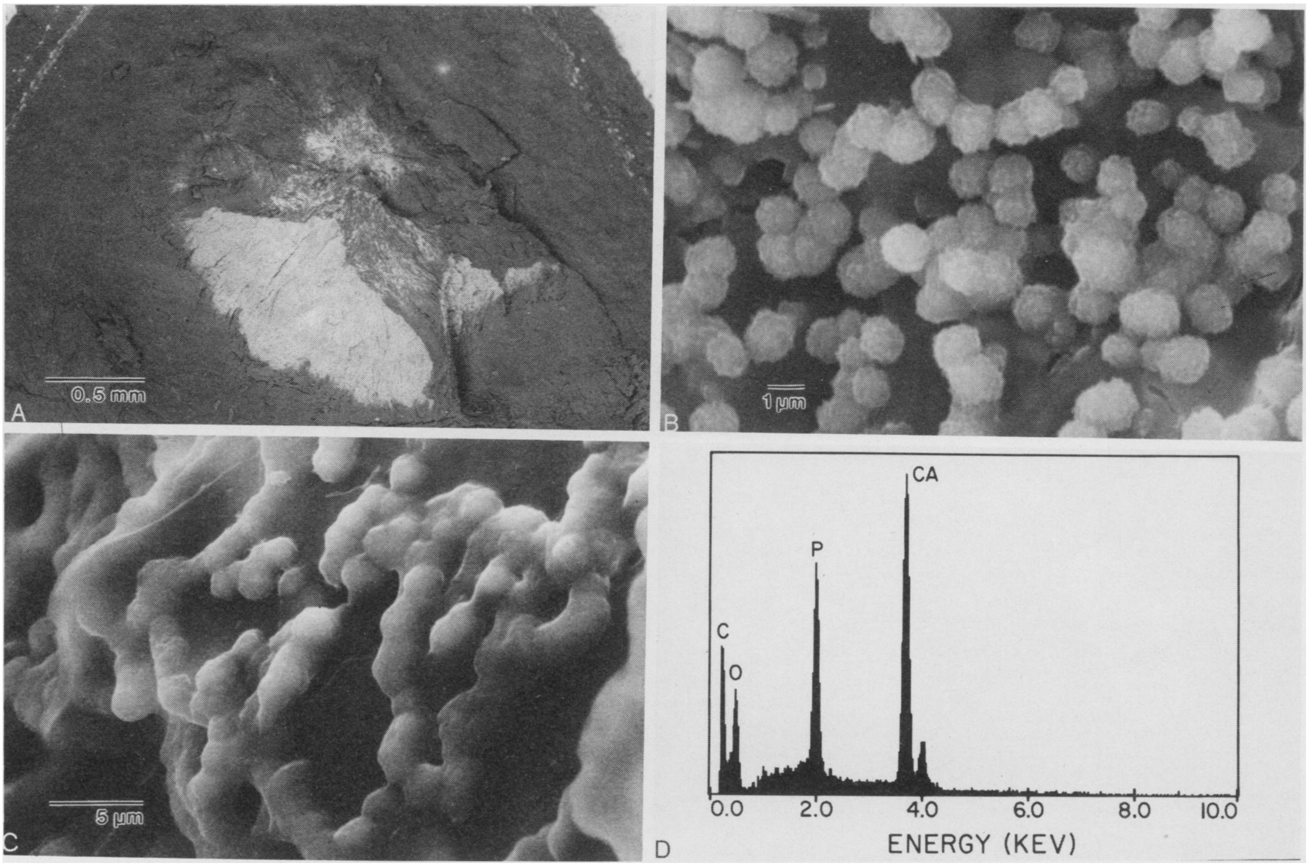
Statistical Analysis

All data are presented as percentage of appropriate groups or mean ± standard error of the mean. Statistical comparisons of most patient characteristics, bacteriology, and individual calcium salts were done with chi-square analysis. The mean ages and cholesterol contents of the various groups were compared with analyses of variance and Student's unpaired t-tests.

Results

Patient Characteristics

Forty-six patients had cholesterol gallbladder stones. Four of these 46 patients (9%) also had cholesterol common bile duct stones, while one patient had a brown pigment common duct stone with a cholesterol stone nidus. Seventeen patients had black pigment gallstones, and two of these 17 patients (12%) also had black pigment common bile duct stones. Two patients had brown pigment gallstones, and one of these two patients (50%) had a brown pigment common bile duct stone. Two additional patients, who had previously undergone cholecystectomy, had brown pigment common bile duct stones (Table 1). The five patients with brown pigment stones were significantly ($p < 0.05$) older (70 ± 5 years) than the 46 patients with cholesterol stones (49 ± 3 years) and the 17 patients with black pigment stones (37 ± 5 years) (Table 1). The patients with brown pigment stones were also more likely ($p < 0.05$) to be men and to present with bile duct obstruction. In comparison to patients with cholesterol gallstones, patients with black pigment gallstones were significantly younger ($p < 0.05$), less likely to be obese ($p < 0.05$), and were more likely to have a hemolytic ($p < 0.001$) or ileal disorder ($p < 0.01$) or to be receiving long-term parenteral nutrition (Table 1).



FIGS. 1A–D. (A) Low magnification BEI of cholesterol gallstone cross section demonstrating the localization of calcium salts in the core of this gallstone. (B) High magnification BEI of the core of this same stone showing calcium-containing microspheres. (C) High magnification SEI of similar microspheres. (D) Windowless EDX spectrum identifying microsphere composition as calcium phosphate.[21]

Bacteriology

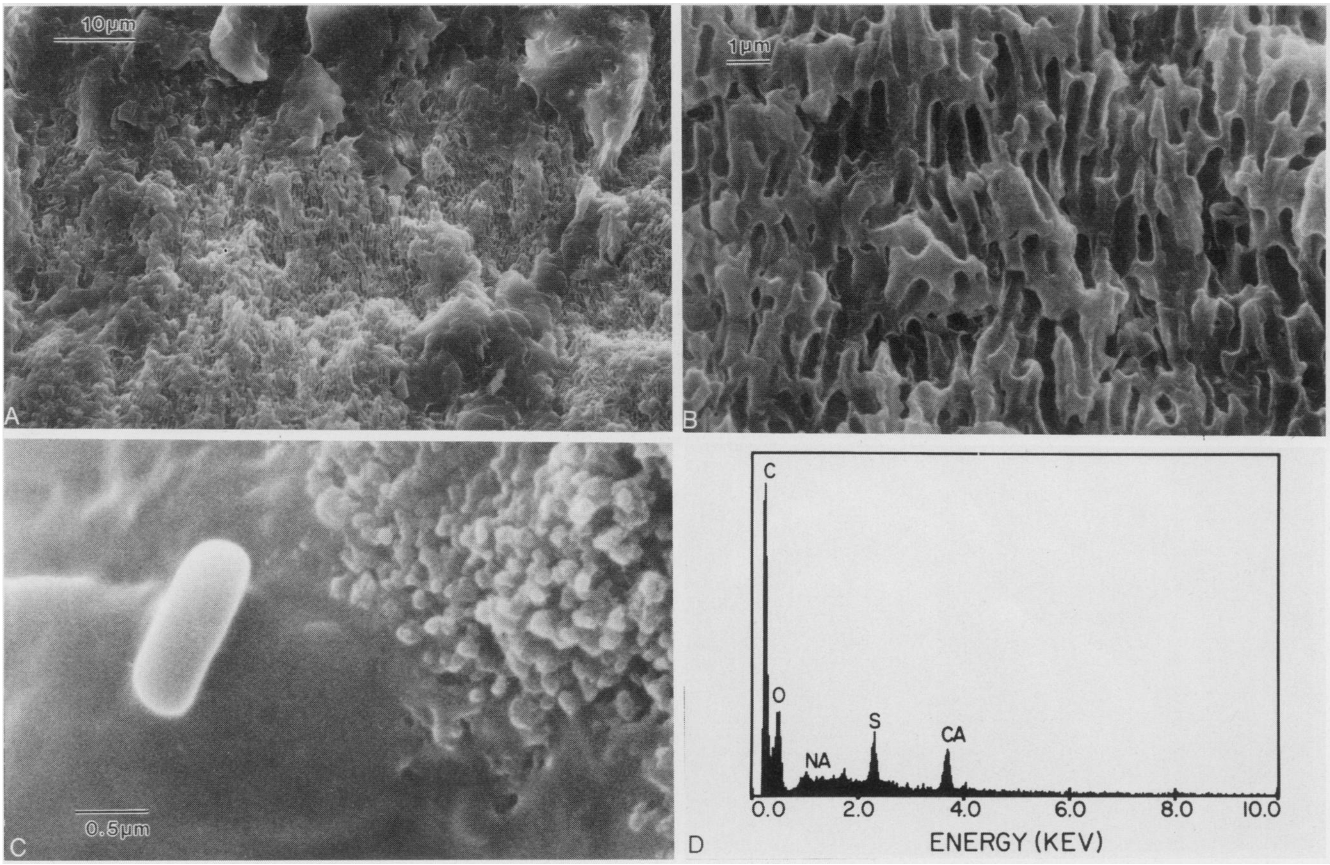
By scanning electron microscopy, bacteria or bacterial casts were identified in the two brown pigment gallbladder stones and in all four brown pigment common duct stones (Table 2, Figs. 2 and 3). Bacteria were observed only within the calcium bilirubinate-protein matrix of these brown stones and not in the calcium palmitate or cholesterol phases of the stones. Evidence of bacteria was not demonstrated in cholesterol or black pigment stones despite the presence of calcium bilirubinate in 50% of the cholesterol stones and in 88% of the black pigment stones. Calcium phosphate microspheres (Fig. 1), which could possibly be mistakenly identified as bacterial cocci, were identified in 30% of the cholesterol stones and in 47% of the black pigment stones. Thus, by SEM, bacteria were only found in brown pigment stones and never in cholesterol or black pigment stones ($p < 0.001$).

Bile culture data are presented in Table 2 and Figure 3. Bacteria grew from the gallbladder or common duct bile of all five patients with brown pigment stones. The organisms most frequently isolated were *Escherichia coli* and enterococcus (Table 2). Bile cultures were positive in only 13% and 14% of patients with cholesterol and black pigment gallbladder stones, respectively. The organisms that were isolated from these patients were similar to those cultured from patients with brown pigment stones (Table 2). However, the incidence of positive bile cultures was significantly higher ($p < 0.001$) among the five patients with brown pigment stones.

Gallstone Composition

The per cent of cholesterol by dry weight in the three gallstone groups is presented in Figure 4. The mean cholesterol content in the 46 patients with cholesterol stones was 85.6% ± 2.4%. In comparison, patients with black pigment stones had 1.6% ± 0.8% cholesterol, and patients with brown pigment stones had 13.9% ± 1.7% cholesterol. Both pigment groups had significantly less ($p < 0.001$) cholesterol in their stones than did cholesterol stone patients. In addition, brown pigment stones had significantly more ($p < 0.001$) cholesterol than did black pigment stones.

Black and brown pigment stones were further differentiated by the individual calcium salts and cholesterol



FIGS. 2A–D. (A) A low magnification SEI of bacterial casts within a brown pigment gallstone. (B) Higher magnification showing longitudinal fracture plane through bacterial casts. (C) High magnification of a bacterial rod from a brown pigment common bile duct stone. (D) Windowless EDX spectrum identifying bacterial cast matrix as calcium bilirubinate.[21]

observed by SEM and windowless EDX (Fig. 5). Calcium bilirubinate was seen in almost all patients in both pigment stone groups. However, calcium phosphate ($p = 0.06$) and calcium carbonate were only observed in black pigment stones. Moreover, calcium palmitate ($p < 0.005$) and cholesterol crystals ($p < 0.001$) were observed significantly more often in brown than in black pigment stones.

Discussion

Gallbladder and common bile duct stones as well as bile for culture were obtained from 67 consecutive patients undergoing cholecystectomy and/or common bile duct exploration. All stones were assayed for cholesterol content and studied with scanning electron microscopy equipped with windowless energy-dispersive x-ray microanalysis. Gallbladder stones from 65 patients were classified as cholesterol in 71%, black pigment in 26%, and brown pigment in 3%. Common duct stones from 10 patients were classified as cholesterol in 40%, black pigment in 20%, and brown pigment in 40%. Bacteria were found only in the calcium bilirubinate-protein matrix of brown pigment stones. No bacteria were present in cholesterol or black pigment stones, despite the presence of calcium bilirubinate in the majority of these stones. Moreover, patient characteristics and stone composition differed between patients with black and brown pigment stones.

The observation in this study that bacteria are found only within brown pigment stones is similar to the findings of Cetta et al.[26–28] These investigators found infected bile in 40% of patients with secondary common bile duct stones but in 100% of those with recurrent common bile duct stones.[26] In the present study these figures were 50% and 100%, respectively. In comparing black and brown pigment stones Cetta et al.[27] found positive bile cultures in 25% of patients with black stones and in 100% of patients with brown pigment stones. In the present study these figures were 14% and 100%, respectively. Moreover in 1984, Cetta et al.[28] reported "large amounts of bacteria" by scanning electron microscopy in five patients with recurrent common bile duct stones.

Scanning electron microscopic studies of gallbladder and common bile duct stones have also been reported by Malet et al.,[14] by Wosiewitz,[29,30] and by Potamitis.[31] In

TABLE 2. *Bacteriology by Scanning Electron Microscopy (SEM) and Culture*

	Galbladder Stones				Common Bile Duct Stones			
		Pigment				Pigment		
	Cholesterol	Black	Brown	All	Cholesterol	Black	Brown	All
n	46	17	2	65	4	2	4	10
Bacteria (% patients)								
SEM	0	0	100*	3	0	0	100*†	40
Culture	13	14	100*	16	50	50	100*	70
Bacteriology (# patients)								
E. coli	0	1	2	3	0	0	2	2
Enterococcus	1	0	0	1	2	0	2	4
Enterobacter	2	0	0	2	0	0	1	1
Strep viridans	1	1	0	2	0	1	0	1
Pseudomonas	1	0	0	1	0	0	1	1
Klebsiella	0	0	1	1	0	0	1	1

* $p < 0.001$ all brown stones *versus* cholesterol and black pigment gallbladder stones.

† $p < 0.001$ *versus* other common bile duct stones.

none of these series, however, were bacteria or bacterial casts observed within gallstones. In comparison, in two recent studies by Stewart et al.[17] and by Smith et al.[18] from the United States, bacteria were found in the majority of black as well as brown pigment stones. As a result, these authors concluded (1) that bacterial infection is a primary factor in both black and brown pigment stone pathogenesis and (2) that "the distinction between black and brown stones has been somewhat overstated in recent literature."[17]

The different conclusions in the present study compared to those by Stewart and associates[17] and by Smith et al.[18] may be explained, in part, by different patient populations. Stewart et al.[17] examined the gallstones from patients in San Francisco and in Essex, England. Only 41% of their 85 patients were female, and 18% were Oriental. In the present study the patient population was a more typical Western population with 64% women and no Oriental patients. Moreover, 54% of the stones examined by Stewart et al.[17] were common bile duct stones compared to only 15% in the present study. Similarly, 42 of the 69 patients (61%) reported by Smith et al.[18] had undergone common duct procedures. In these two studies, "composite" stones accounted for 21% and 20% of the stones, respectively. In the present study, "composite" stones have been classified as either cholesterol or brown pigment stones, depending on their predominant visual appearance and percentage of cholesterol by weight.

A recent SEM report by Speer et al.[20] from Australia confirmed the presence of bacteria in four patients with brown pigment stones. These authors also noted that bacteria adhered to the surface of cholesterol gallstones in a biofilm.[19] In the present study, the core and periphery, but not the surface, of gallstones was examined, whereas

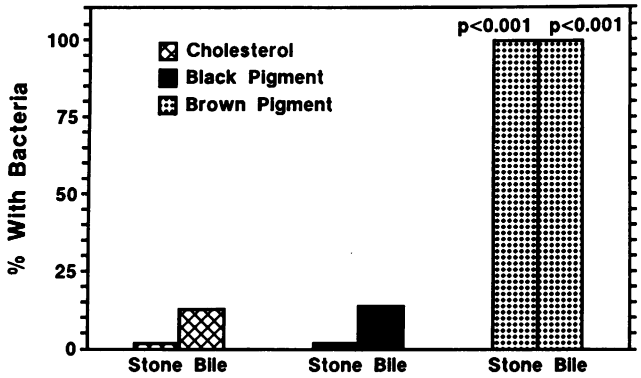


FIG. 3. The percentage of bacteria seen within stones by SEM or cultured from gallbladder or common duct bile. Statistical analysis compares brown pigment stones and bile with both cholesterol and black pigment stones and bile, respectively.

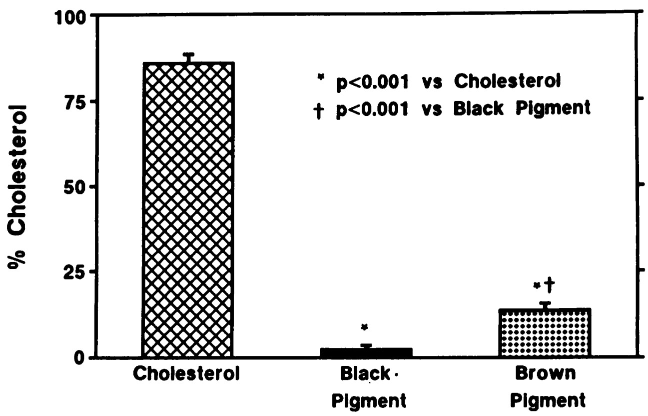


FIG. 4. Per cent of cholesterol by dry weight in the cholesterol (n = 46), black pigment (n = 17), and brown pigment (n = 5) stone groups.

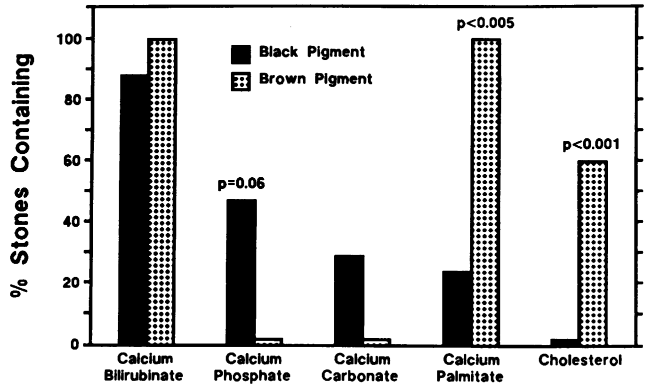


FIG. 5. Per cent of black (n = 17) and brown (n = 5) stones containing individual calcium salts and cholesterol. Statistical analysis compares black and brown pigment stone groups.

Speer et al.[19] focused their observations on the surface of cholesterol stones. Surface characteristics may reflect late, but not the early, stages in gallstone formation. Moreover, the overinterpretation of calcium phosphate crystals as bacterial cocci may also explain some of the differences among various studies. The identification of calcium phosphate by windowless energy-dispersive x-ray microanalysis in the present study prevented this potential problem.

The relative incidence of cholesterol, black pigment, and brown pigment stones observed in the gallbladder and common bile duct in this series are similar to those previously reported in consecutive series by Trotman et al.[11] and by Malet and his colleagues[32] In the present study 71% of gallbladder stones were cholesterol and 29% were pigment. Trotman and his associates from Philadelphia[11] reported almost identical values of 73% and 27%, respectively. In the present study only 3% of gallbladder stones were brown pigment stones containing bacteria. Similarly low percentages of brown pigment gallbladder stones have also been reported in a number of series[11,30,32,34] from Western countries.

Of the 10 common duct stones in this series, cholesterol, black pigment, and brown pigment stones were found in 40%, 20%, and 40%, respectively. In comparison, Malet et al.[32] from the University of Pennsylvania found that these percentages were 43%, 18%, and 39% among 56 consecutive patients with choledocholithiasis. Slightly lower percentages (25% to 27%) of brown pigment common bile duct stones have been reported by Bernhoft et al.[33] from San Francisco and London, by Wosiewitz[30] from West Germany, and by Whiting and Watts[34] from South Australia. In the Orient brown pigment stones account for the vast majority of stones found in the intra- and extrahepatic biliary tree.[2-8] Interestingly, Yamashito et al.[8] have reported that intrahepatic brown pigment stones contained significantly less bilirubin and more cholesterol than brown pigment stones found in the extrahepatic ducts. These authors did not report data on bile cultures from the two subgroups but they suggested that the site of formation may modify stone pathogenesis.

The black and brown pigment stones in the present series differed in cholesterol content and in individual calcium salts. Brown stones were more likely to contain cholesterol or calcium palmitate but did not contain calcium phosphate or carbonate. In 1984 Cetta and his Italian colleagues[27] made these same observations. In that series all 19 patients with brown pigment stones had cholesterol and calcium palmitate detected by x-ray diffractometry and infrared spectroscopy. In comparison, cholesterol was found in only 5 of 20 patients and calcium palmitate was not detected in any of the 20 patients with black pigment stones. These same trends have also been observed recently by Soloway et al.[16] and by Malet and his associates.[32] These observations, as well as the differences in clinical characteristics, further support different pathogenic mechanisms for black and brown pigment stones.

These data suggest that biliary infection is more likely to result in the precipitation of calcium bilirubinate and calcium salts of fatty acids (calcium palmitate) and is less likely to cause the precipitation of calcium phosphate or carbonate. Maki[1] was the first to suggest that β-glucuronidase of bacterial origin resulted in precipitation of calcium bilirubinate. Glucuronidases are also present in the gallbladder and liver[35,36] so that conjugated bilirubin may be hydrolysed by enzymes from these tissues as well as from bacterial sources. Similarly, both bacteria and hepatobiliary tissues contain phospholipases.[37] As a result, the palmitate present in brown, but not black, pigment stones could be the product of either bacterial or tissue hydrolysis of biliary phospholipids.[38,39] Cetta[40] has recently documented in two patients that bacterial infection actually precedes brown pigment stone formation. A bacterial source for these enzymes, therefore, is supported by the findings of this study and those of Cetta et al.[26,27] Thus, we believe that bacterial infection is an initiating and/or promoting factor in brown pigment stones but is not associated with black pigment or cholesterol gallstone formation.

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Discussion

DR. ROBERT E. HERMANN (Cleveland, Ohio): Dr. Berry, Dr. Jones, Members, and Guests: I enjoyed this interesting paper by Dr. Pitt and his colleagues very much and I appreciate the opportunity to review the manuscript. They have focused in this study on the role of bacteria on the formation of gallstones and bile-duct stones.

I believe most of the evidence to date accumulated in the many institutions that are studying the lithogenicity of bile, in other words, the formation of stones, have shown that there are probably several factors that contribute to the development of stones in the biliary system. Among these are first and foremost, the degree of saturation or supersaturation of cholesterol for primary gallstones, the role of stasis or inadequate emptying for both gallstones and bile-duct stones, and the role of infection, which Dr. Pitt and his colleagues have focused on today.

It is always difficult, it seems to me, to determine whether the infection is primary, an initiating factor, or if it is a secondary factor.

Dr. Pitt, I wonder if you could speak to this a bit further. Do you feel stasis comes first or does the infection comes first? Because in your studies most of the stones, the brown stones or stasis-type stones, in the bile duct are those that are associated with bacteria, how much of a role do you believe stasis plays? Second, once the bile duct has bacteria in it and you correct the obstruction by connecting it to the intestinal tract for better drainage and bile flow, does the bile duct or the bile ever become sterile again? In other words, I am concerned about the role of stasis and whether, once infection is there, can you ever overcome this problem?

DR. R. SCOTT JONES (Charlottesville, Virginia): I wanted to ask a question and make a comment.

The first question concerns the bacteria. In the beginning of Dr. Pitt's presentation, he mentioned the Maki hypothesis for the formation of pigment stones and the basis for that is that certain bacteria produce an enzyme, beta-glucuronidase, that hydrolizes conjugated bilirubin to produce the deconjugated form. The deconjugated form is much less soluble in water than is the conjugated form, and my question, therefore, is: In the bacteria that you recovered from the bile of the patients or from the stones, did you test these to determine if they were the species of bacteria that produced beta-glucuronidase, certain strains of E. coli, or did you, in fact, measure for the enzyme in the bacterial isolates?

The comment that I wanted to make was concerning the finding of ileal disease in patients with pigment stones. We usually think that ileal disease participates in causing gallstones by producing abnormal bile