JOURNAL OF CLINICAL MICROBIOLOGY, May 1989, p. 915–921
0095-1137/89/050915-07$02.00/0

Vol. 27, No. 5

Bacteriological and Electron Microscopy Examination of Brown Pigment Stones

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Received 31 August 1988/Accepted 13 January 1989

Bacteriological and morphological studies of 38 brown pigment common duct stones were performed. Stone cultures were positive for bacteria in 80.5% of those studied. Enterococci were the most common organisms that were isolated. Scanning electron microscopy showed the presence of bacteria in 84.2% of the stones. The bacteria were seen embedded within an amorphous matrix in alternating layers of flakelike crystals. Transmission electron microscopy showed the presence of gram-positive and gram-negative bacteria surrounded by a ruthenium red-stained exopolysaccharide material. Results of the bacteriological and morphological studies confirmed the close relationship between the presence of bacteria and the development of brown pigment stones.

Calcium bilirubinate stones are classified by their biochemical composition and morphological appearance into laminated brown stones and amorphous black stones (17). Brown pigment stones are an important cause of acute cholangitis in Oriental populations (5). Bacterial infection and the deconjugation of bile by bacterial β-glucuronidase have been implicated as etiological factors in the formation of brown pigment stones (3, 6), but controversy exists concerning their primary importance and causal relationship (17). A much more definite causal relationship has been established (2, 8) between the presence of bacteria, and the activity of bacterial urease, in the development of struvite stones in the infected kidney. In order to determine the relationship between bacteria and brown pigment stone formation, we performed similar morphological studies of 38 brown pigment stones removed endoscopically from the common bile ducts of patients with cholangitis. These stones were examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In addition, bacteriological examinations of the stones and bile specimens obtained from the respective patients were undertaken.

MATERIALS AND METHODS

Stones. Thirty-eight biliary stones were obtained from patients suffering from cholangitis. The stones were removed from the common bile duct or intrahepatic ducts after endoscopic sphincterotomy and were retrieved with a basket through the mouth. Bile specimens were obtained via an indwelling nasobiliary catheter. The stones were immediately sent to the laboratory for processing. Gross inspection of the stones was performed, and the stones were sectioned into eight portions and processed for subsequent analyses, which included biochemical assays for P_i and lipid contents. Sections of the stones were processed for SEM and TEM. In addition, a portion of the stones and bile collected was submitted for bacteriological culture by standard techniques.

Chemical analyses of stones. (i) Determination of P_i content. A portion of the stones was weighted, ground into powder form, and made into an ash by heating the powder at 500°C for 24 h to remove all the organic components. The samples were then dissolved in nitric acid (1.58 M) to extract the P_i. The phosphate content was determined with a P-kit (bioMerieux Laboratory Reagents and Products, Charbonnières les Bains, France).

(ii) Lipid assay. Fatty acids were extracted from the stones with ether. The lipid and cholesterol contents were assayed by gas chromatography with a gas chromatograph (HP5890; Hewlett-Packard Co., Palo Alto, Calif.) and a fused-silica capillary column cross-linked with 5% phenylmethyl silicose (HP-5 19091J-202; Hewlett-Packard). The chromatograph settings were as follows: oven temperature, 160°C; injector temperature, 160°C; detector temperature, 260°C; running

TABLE 1. Stone culture results

Organism (n = 42)	n	%
Enterococci	17	40.5
Escherichia coli	7	16.7
Klebsiella sp.	4	9.5
Pseudomonas aeruginosa	3	7.1
Bacteroides sp.	3	7.1
Citrobacter sp.	3	7.1
Acinetobacter sp.	2	4.8
Proteus mirabilis	1	2.4
Candida sp.	1	2.4
Diphtheroids	1	2.4

TABLE 2. Bile culture results

Organism (n = 69)	n	%
Escherichia coli	18	26.1
Enterococci	16	23.2
Klebsiella sp.	9	13.1
Acinetobacter sp.	5	7.2
Pseudomonas aeruginosa	4	5.8
Diphtheroids	4	5.8
Citrobacter sp.	3	4.3
Bacteroides sp.	3	4.3
Candida sp.	3	4.3
Proteus mirabilis	2	2.9
Streptococcus group D	1	1.5
Aeromonas sp.	1	1.5

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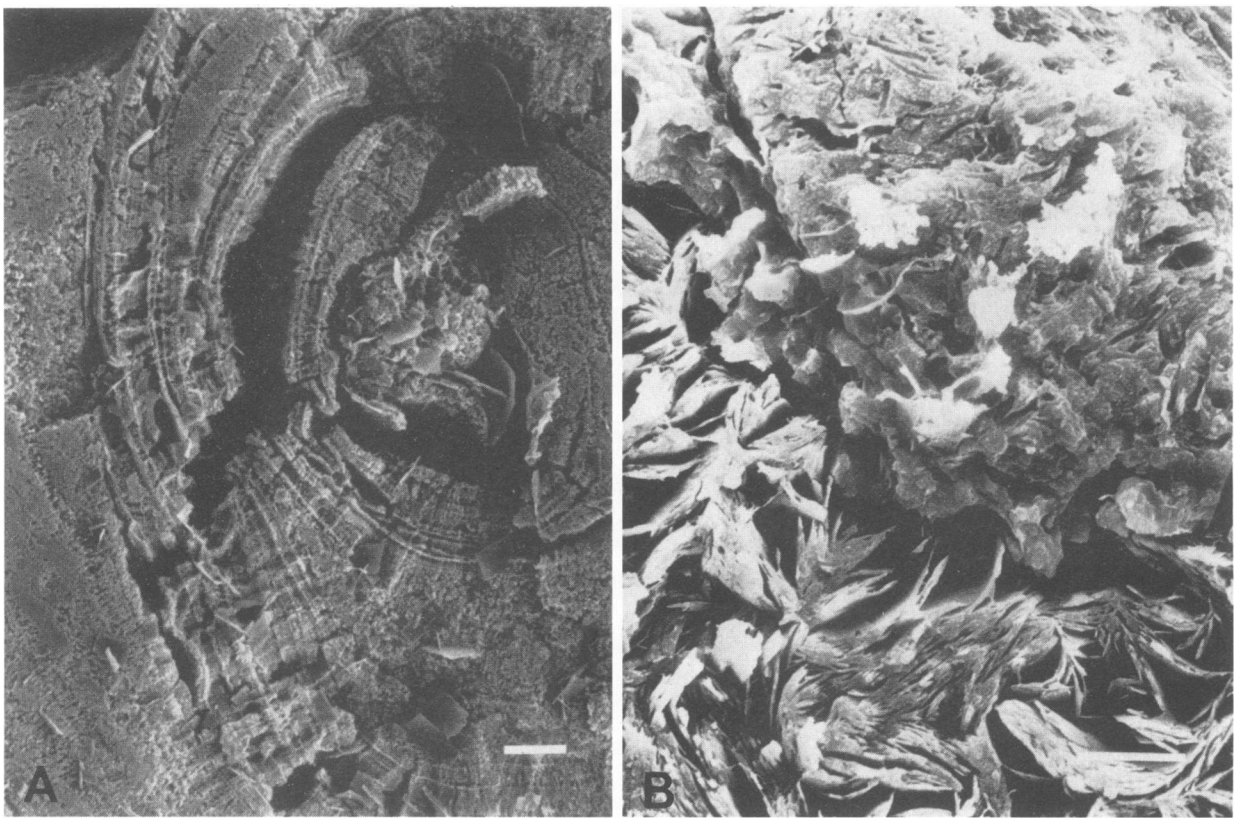

FIG. 1. Scanning electron micrograph of brown pigment stones showing concentric layering structure of the stone (bar, = 100 μm) (A) and an area of flakelike plates alternating with amorphous material (bar, 10 μm) (B).

FIG. 2. (A) Microcolonies of cocci embedded in amorphous material. Bacterial imprints were seen on the cross-sectional surface of the stone. (B) Abundant cocci and bacilli seen mixed with the amorphous material, but less so in the area with flakelike crystals. Bars, 10 μm.

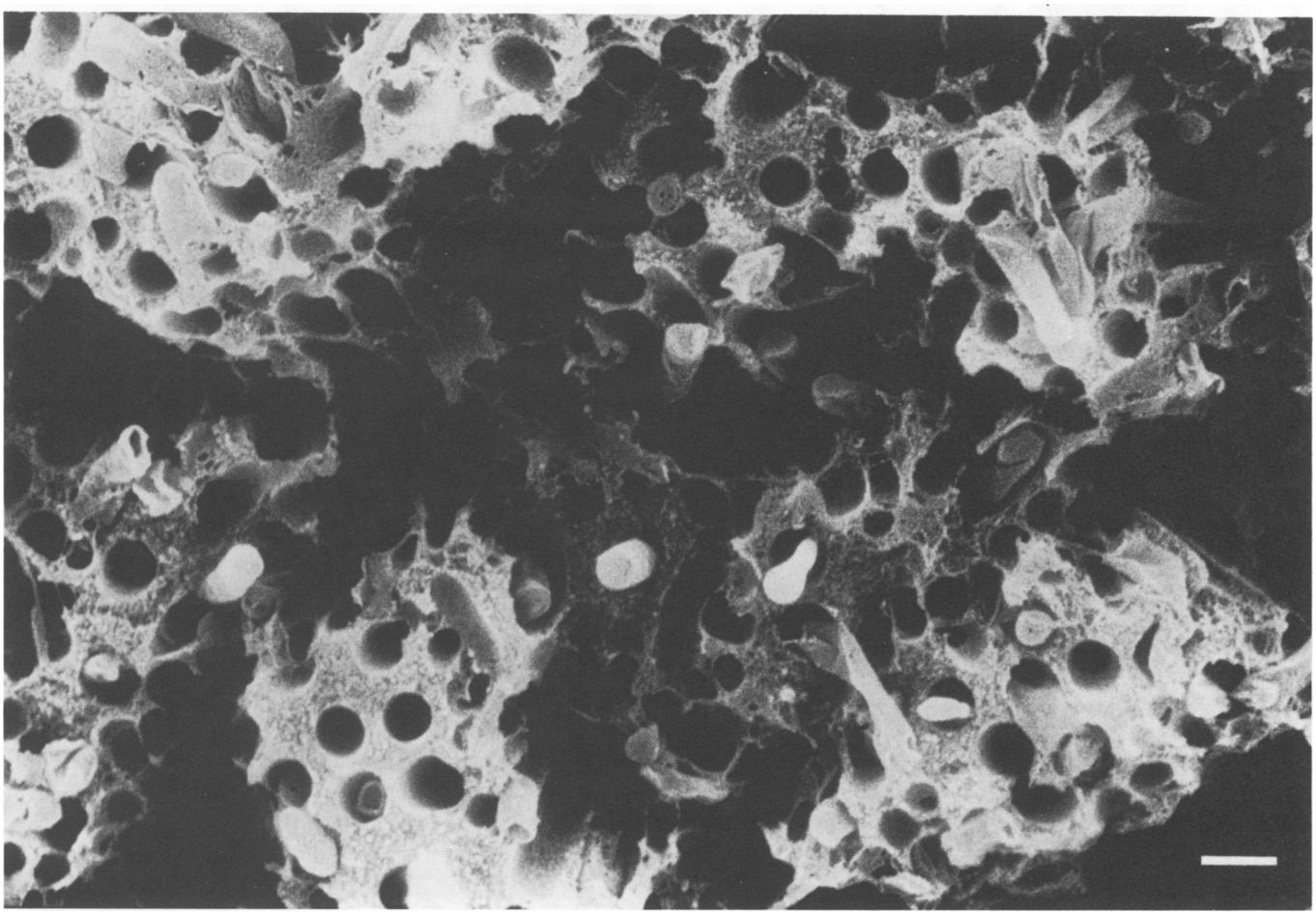

FIG. 3. Scanning electron micrograph showing microcolonies of bacteria embedded in amorphous material. The honeycomb appearance was due to the negative imprints in the amorphous material from which the bacteria were removed. Bar, 1 μm.

time, 8 min; head column pressure, 70 kPa. Helium was used as the carrier gas. The individual lipids were expressed as a percentage of the total lipid content.

Stones and bile culture. The portion of the stones submitted for bacterial cultures was washed with normal sterile saline to remove surface contaminants. The stones were then crushed and inoculated onto a blood agar plate (containing 5% horse blood) and a MacConkey agar plate (containing peptone water, agar, bile salt, lactose, and neutral red). The bile samples were inoculated onto similar media. These plates were then incubated aerobically for 24 h, and the bacterial colonies were identified by standard bacteriological methods.

SEM. A portion of the stones was placed immediately into 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.2) with 0.15% ruthenium red and kept at 4°C for 4 h. The fixed specimens were then washed five times with 0.1 M cacodylate buffer containing 0.05% ruthenium red, postfixed with osmium tetroxide, and dehydrated in a series of ethanol gradients before they were dried to the critical point with Freon 13 (E. I. de Pont de Nemours & Co., Inc., Wilmington, Del.). The specimens were then sputter coated with gold by using a coater (S150B; Edwards) and examined with a scanning electron microscope (JSM-35-CF; JEOL) at an accelerating voltage of 15 kV.

TEM. A portion of the stones adjacent to the area taken for SEM were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.2) with 0.15% ruthenium red at 4°C for 24 h. The specimens were then washed again with buffer containing 0.05% ruthenium red, postfixed with 2% osmium tetroxide, and washed again before dehydration in a series of ethanol gradients. Further dehydration was performed with propylene oxide. The specimens were then infiltrated and embedded in Spurr resin, sectioned, and stained with uranyl acetate and lead citrate (11). Examination was performed with a transmission electron microscope (JEM-100-CX2; JEOL) at an accelerating voltage of 80 kV.

RESULTS

Gross appearance and biochemical composition. The stones were brownish yellow and soft. A cross-section of the stones showed a laminated appearance. All the stones contained a low P_i content that ranged from 0.05 to 0.98% of the total dry weight and a high palmitate level that ranged from 16.5 to 85.9% of the total lipid content. Thirty-four stones contained no cholesterol, but another four stones contained between 11.4 and 29.9% cholesterol and were classified as mixed stones.

Stone and bile cultures. Positive bacteriological cultures were obtained from 29 of the 36 (80.6%) stone cultures studied. A total of 42 organisms were isolated from the stones; stones from eight patients had mixed isolates. Enterococci were the most common organisms isolated, fol-

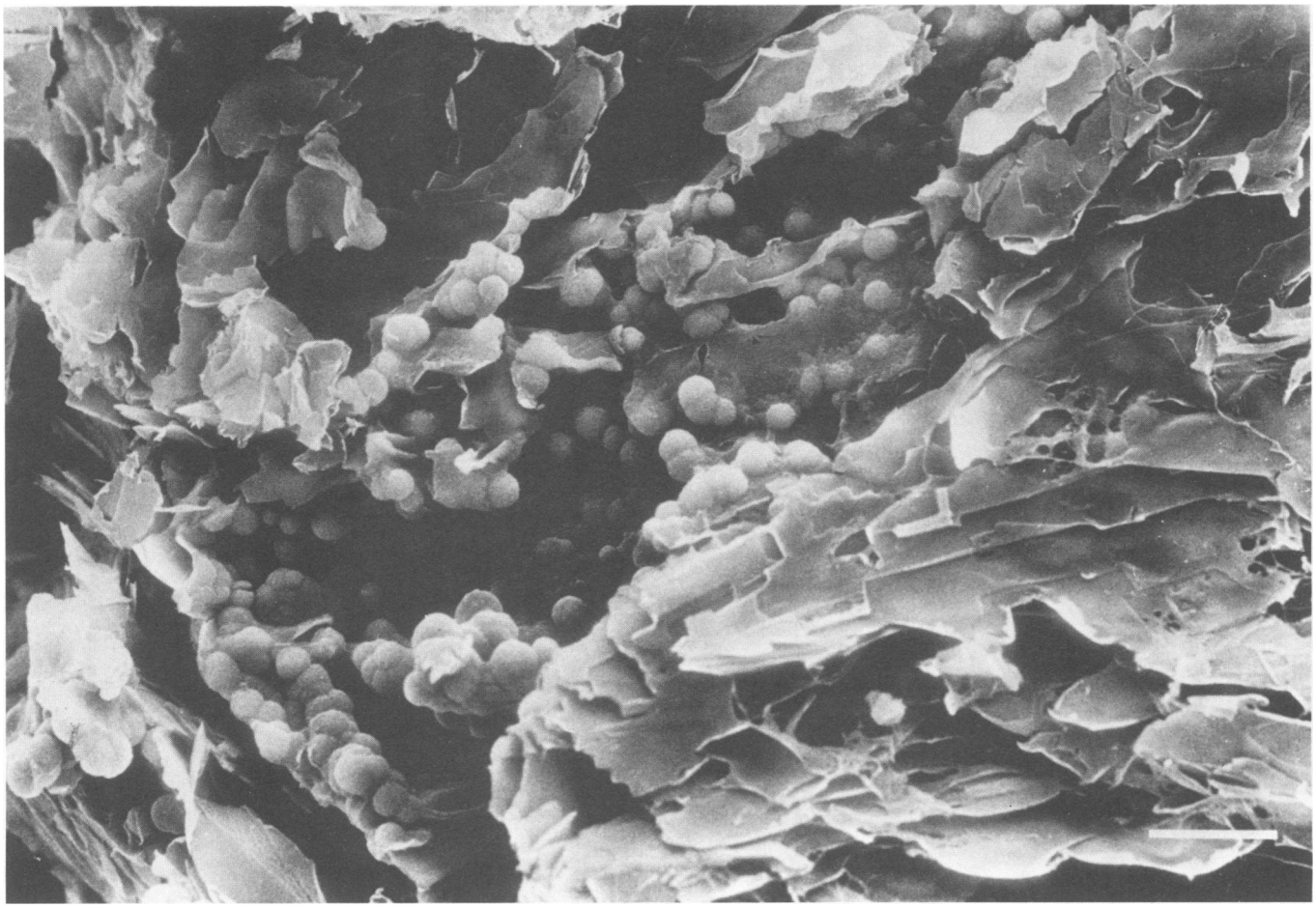

FIG. 4. Scanning electron micrograph showing microcolonies of cocci embedded in palmitate crystals. Bacterial division can be seen in some areas. Bar, 1 μm.

lowed by *Escherichia coli*, *Klebsiella* sp., *Pseudomonas aeruginosa*, *Bacteriodes* sp., *Acinetobacter* sp., *Citrobacter* sp., and *Proteus mirabilis* (Table 1).

Bile cultures were positive in 33 of 36 (91.7%) patients. A total of 69 organisms were isolated; 25 patients had bile with a mixed infection. The organisms were similar to that found in the stones, but *E. coli* was the most common organism isolated from the bile (Table 2).

SEM. Low-magnification examination of a cross section of brown stones showed concentric layers (Fig. 1). Higher magnification revealed the presence of alternating layers of flakelike projecting plates arranged in rosettes (resembling palmitate crystals) (19) mixed with areas of amorphous material containing bacterial imprints and microcolonies of cocci and bacilli embedded in the amorphous material (Fig. 2). Further close-up examination of this area showed the presence of a granular substance molded into an amorphous matrix mixed with microcolonies of bacteria and a honeycomb appearance representing footprints of bacteria that were washed off during the processing (Fig. 3). In other areas, microcolonies of cocci were seen mixed with the palmitate crystals (Fig. 4). SEM showed the presence of bacteria in 32 of 38 (84.2%) stones studied. The bacteria were seen in both the superficial and inner layers of the stones. They were mixed with the palmitate crystals and calcium bilirubinate but were more abundantly mixed with the latter. Bacteria undergoing binary fission were observed in some cases. The number and distribution of the bacteria observed by SEM varied in different areas of the stones that were studied.

TEM. TEM showed the presence of mixed microcolonies of gram-positive and gram-negative bacteria embedded in an amorphous anionic matrix. Some bacteria encased in the dense matrix were lysed, giving the appearance of ghost cells (Fig. 5). In addition, a layer of ruthenium red-stained, condensed, amorphous material was seen surrounding the bacteria (Fig. 6).

DISCUSSION

It is generally accepted that pigment stones can be differentiated from cholesterol stones by their gross appearance (9, 16). Based on their chemical compositions, pigment stones can be further subdivided into laminated brown stones and amorphous black stones (17). P_i is present only in very small amounts in brown stones, and calcium palmitate is rarely found in black stones (12). Using these two criteria, we classified the stones analyzed in this study as brown pigment stones ($n = 34$) or mixed brown pigment-cholesterol stones ($n = 4$).

The role of bacterial infection in the pathogenesis of common bile duct stones remains controversial. Maki (6) has

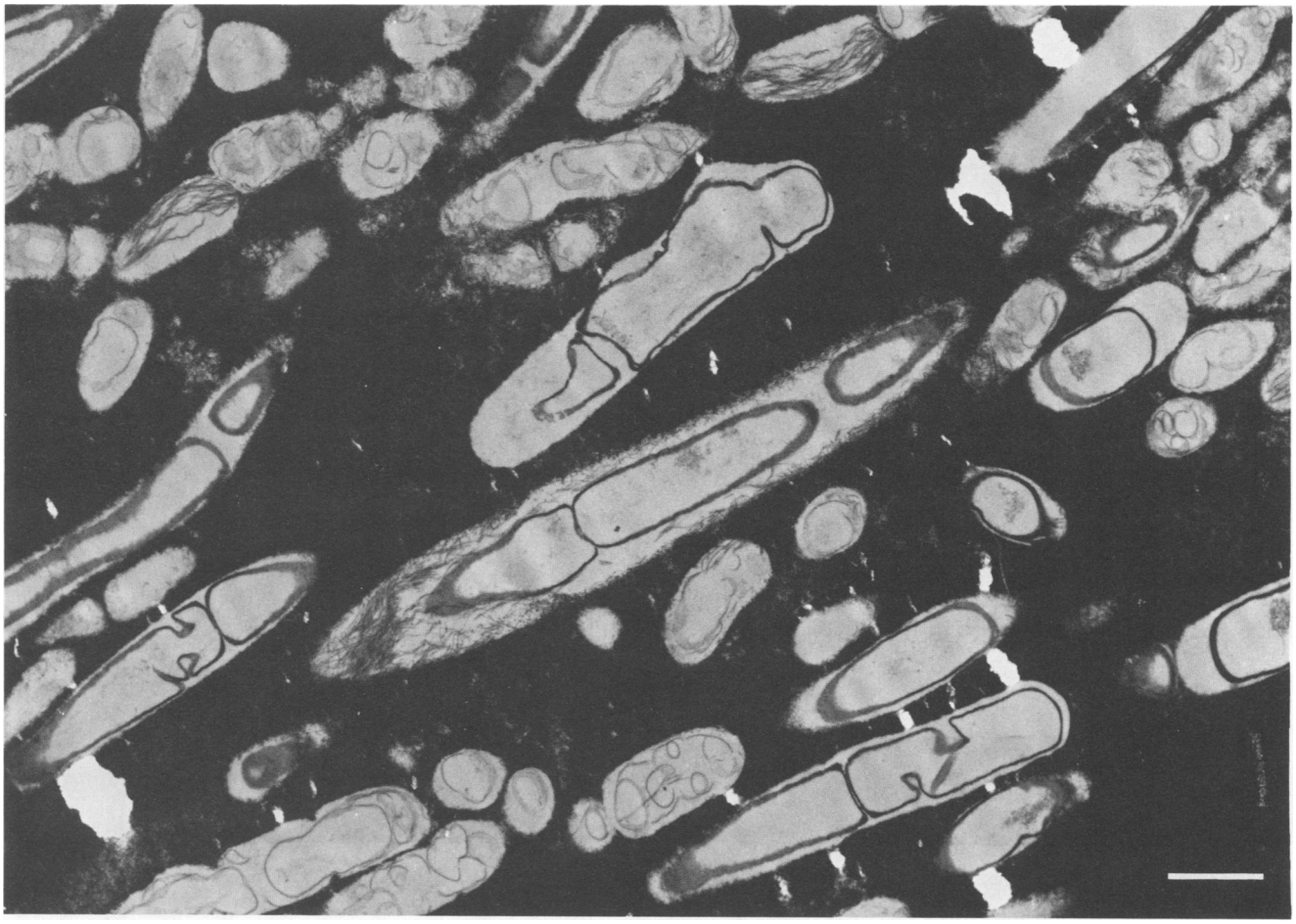

FIG. 5. Transmission electron micrograph of brown pigment stone showing bacteria encased in a dense matrix; some bacteria were lysed, giving the appearance of ghost cells. Bar, 1 μm.

shown that bacterial β-glucuronidase produced by *E. coli* is an important enzyme which deconjugates bilirubin diglucuronide, resulting in the release of free bilirubin and glucuronic acid (6). The former precipitates with calcium ion to form calcium bilirubinate, which is the major component of brown pigment stones. Wong et al. (18) have also found a strong association between the presence of bacteria and the occurrence of ductal stones in patients with recurrent pyogenic cholangitis. In the present study, bacterial cultures were positive in 80.6% of the stones that were analyzed and were positive in 91.7% of the bile specimens that were analyzed. Mixed growth of bacteria was obtained from the bile cultures in over 75% of the patients. *E. coli* was the most common organism isolated from bile cultures, followed by enterococci and *Klebsiella* sp. Enterococci appeared to be more commonly isolated from the stone cultures. However, in some cases in which only enterococci were cultured, bacilli were seen on microscopic examination of these stones, suggesting their presence, despite a negative culture.

In the literature, previous demonstration of the association between bacteria and brown pigment stones has relied solely on bacteriological culture (18). Electron microscopy (SEM and TEM) has made a major impact in the morphological study of the association between bacteria and infection-induced struvite stones in the urinary system (8). The microstructure of cholesterol gall stones has also been described (1, 7, 10). In this study, SEM demonstrated that the brown pigment stones are composed of alternating layers of calcium bilirubinate and calcium palmitate crystals (Fig. 1). Bacteria were seen in the matrix of the stones in up to 80.6% of the patients (Fig. 2). This was in contrast to cholesterol stones and black pigment stones, in which bacteria are not commonly found (15). A honeycomb appearance representing the negative imprints or fossilized appearance of the bacteria indicated that the bacteria were present within the stone and were not just surface contaminants (14). The bacteria were seen throughout the stones in all layers, suggesting that they were involved in the early stages of stone formation. The bacteria were found in abundance in the layers of calcium bilirubinate (Fig. 2), supporting the hypothesis of bacterial deconjugation and precipitation of calcium bilirubinate. The TEM study of sections of the stones showed that the bacteria were covered with ruthenium red-stained material (Fig. 6) which was condensed on the cell surface as a result of fixation and dehydration during preparation for SEM and TEM (2). This polyanionic material surrounding the bacteria, presumably the bacterial glycocalyx, formed a protective cover for the bacteria and allowed them to multiply within the hostile environment of the biliary system. We postulate that this, in turn, leads to the formation of a bacterial biofilm and to the trapping of more bacteria, with further deconjugation and precipitation of

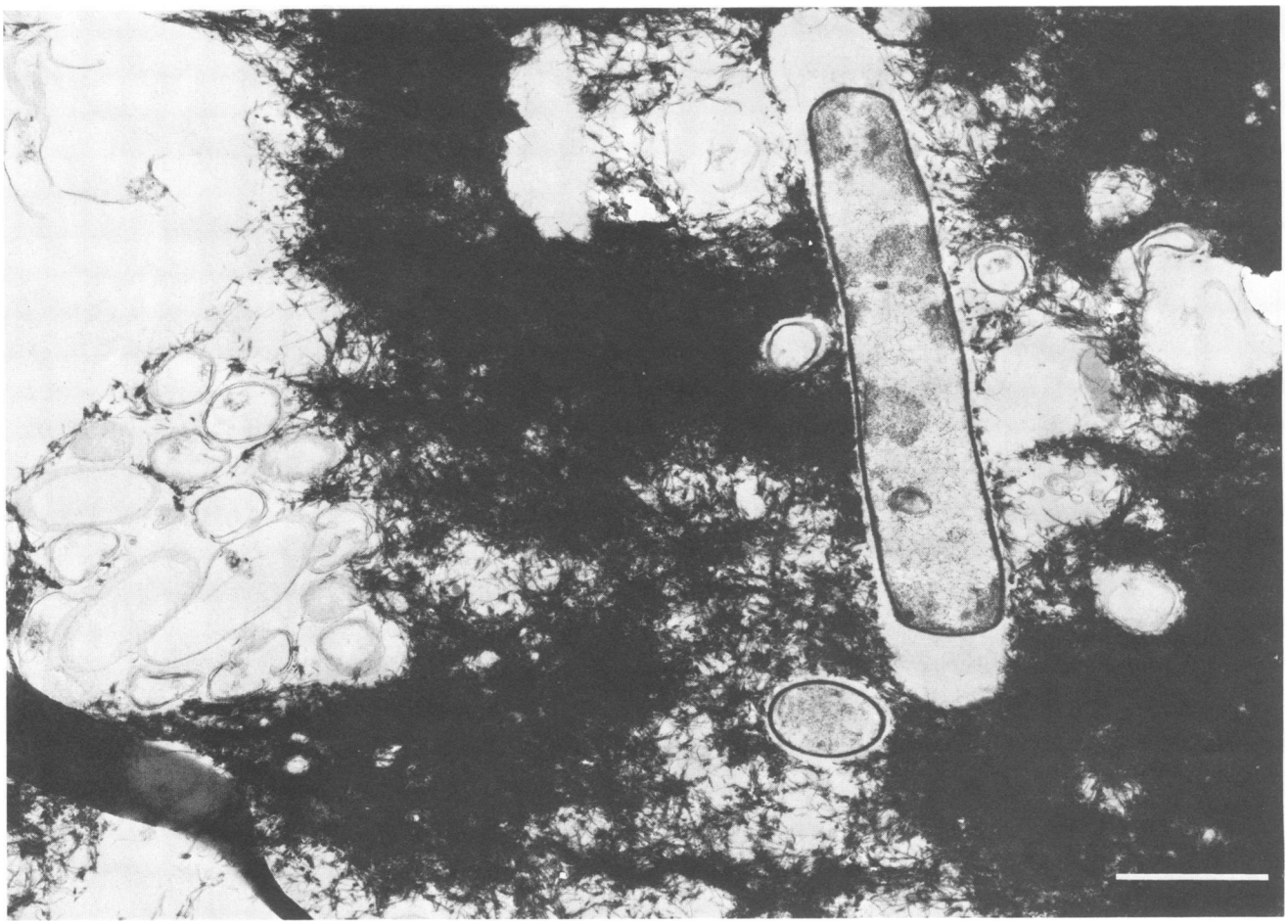

FIG. 6. Transmission electron micrograph showing the condensed glycocalyx, which appears as a ruthenium red-stained material surrounding gram-positive and gram-negative bacteria. Bar, 1 μm.

calcium bilirubinate resulting ultimately in stone formation. The appearance of these stones in many ways is similar to that of urinary struvite stones, in which bacteria play an important etiological role (8). In a study of the relationship between bacteria and biliary sludge formation, Leung and colleagues (4; J. W. C. Leung, Gut **27**:A602, 1986 [abstract]) and Speer et al. (13) have previously shown that live bacteria play an important role in the formation of the biliary sludge that is responsible for blocking biliary stents. The biliary sludge had a chemical composition similar to that of brown pigment stones (U. Wosiewitz, B. Schrameyer, and L. Safrany, Gastroenterology **88**:1706, 1985 [abstract]). SEM studies showed that the sludge consisted of microcolonies of bacteria and granular amorphous material (2; Leung, Gut **27**:A602, 1986) which had a morphological appearance similar to that of brown pigment stones.

In conclusion, results of the bacteriological examination of bile and stones are in good correlation with the results of morphological studies. Electron microscopy, however, undoubtedly proved that bacteria are present at all levels within the stones and that they are closely associated with the amorphous matrix. It is possible that brown pigment stones may represent the end result of bacterial colonization and biofilm formation within the biliary system. More work is needed to assess the detailed mechanism of stone formation. The presence of bacteria within brown pigment stones may serve as a continuing bacterial nidus and may account for the repeated cholangitis in patients with Oriental cholangitis.

ACKNOWLEDGMENT

We acknowledge Jean Kung, Department of Anatomy, Chinese University of Hong Kong, for helping with the SEM and TEM studies.

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