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Cholesterol gallstones and bile host diverse bacterial communities with potential to promote the formation of gallstones

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ABSTRACT

The prevalence of cholesterol gallstones has increased in recent years. Bacterial infection correlates with the formation of gallstones. We studied the composition and function of bacterial communities in cholesterol gallstones and bile from 22 cholesterol gallstone patients using culture-dependent and culture-independent methods. Altogether fourteen and eight bacterial genera were detected in cholesterol gallstones and bile, respectively. *Pseudomonas* spp. were the dominant bacteria in both cholesterol gallstones and bile. As judged by diversity indices, hierarchical clustering and principal component analysis, the bacterial communities in gallstones were different from those in bile. The gallstone microbiome was considered more stable than that of bile. The different microbial communities may be partially explained by differences in their habitats. We found that 30% of the culturable strains from cholesterol gallstones secreted β-glucuronidase and phospholipase A2. *Pseudomonas aeruginosa* strains showed the highest β-glucuronidase activity and produced the highest concentration of phospholipase A2, indicating that *Ps. aeruginosa* may be a major agent in the formation of cholesterol gallstones.

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1. Introduction

The prevalence of cholesterol gallstones, a common disease including gallstones, common bile duct gallstones, and intrahepatic bile duct gallstones, has increased in recent years, especially in the Western world [1]. In China, along with the population aging, obesity, hyperlipidemia and diabetes, the incidence of the cholesterol gallstones has risen to 10% [2–4]. The secretion of cholesterol in bile and abnormal metabolism are considered as the primary pathophysiological causes in the formation of cholesterol gallstones. Factors that are thought to or known to promote the formation of cholesterol gallstones include estrogen and progestogens, cholesterol-lowering medications, obesity and rapid weight loss [5,6]. The consistency and relevance of symptomatic gallbladder stones in identical twins is significantly higher than in double egg twins, indicating that both genetic and environmental factors are involved [7]. The frequent Cyp7a1 polymorphism in A allele in Chinese patients with cholesterol gallstone implies that gallstone formation is related to the expression of genes [8]. Other potential factors associated with the formation of cholesterol gallstones include mucoprotein, aminopeptidase N and vesicular protein [9,10].

Bacterial colonization correlate with the formation of gallstones. Bacterial infection has been proposed as a key factor in the pathogenesis of pigment gallstones [11]. Some study confirmed that nanobacteria may use its self-propagation to cause the formation of black pigment gallstones in rabbits [12,13]. Meanwhile the role of bacteria in cholesterol gallstones formation has aroused increasingly more attention. Swidsinski et al. [14] used RT-PCR (reverse transcription – polymerase chain reaction) to release an 80% detection rate of bacterial DNA in cholesterol gallstones. Chen et al. [15] showed that the detection rate of bacterial DNA in bile, gallstone and biliary tract mucous membrane was 77%, 84% and 62%, respectively. Bacteria may play a critical role in the formation of cholesterol crystals [16]. Alterations in the gastrointestinal microbiome may change aspects of

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cholesterol gallstone pathogenesis and then these changes may impact cholelithogenesis [17]. *Pseudomonas aeruginosa* and *Enterococcus faecalis* shortened the cholesterol crystallization time in the model bile indicating that these species may be crucial in the formation of the cholesterol gallstones [18]. These findings indicated that bacterial community composition might influence the formation of cholesterol gallstones. Thus, it is crucial to understand the structure of bacterial community and its potential connection in the pathogenesis of cholesterol gallstones.

Bacteria speed up the gallstone formation by secrefing crystal formation factors. β-glucuronidase and phospholipase A2 have been identified as the major factors leading to the formation of gallstones. The role of β-glucuronidase in gallstone formation process has been fully recognized [19]. The hydrolysis of phospholipids by phospholipase A2 consequently caused the crystallization of cholesterol and finally led to the formation of gallstones [20]. Furthermore, multidrug-resistance efflux pump proteins expressed by bacteria may help bacteria to survive in bile [21–25].

Traditionally characterizing bacteria has relied on cultivation. However, most of the bacteria in are non-cultivable by conventional methods. Therefore, culture-independent methods like polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) have been widely used to study microbial communities present in biological samples [26–28]. To get a more wide view on the microbial communities in gallstones and bile, we used both cultivation and the culture-independent PCR-DGGE method. In addition, we tested the β-G activity and the PLA2 production of the isolated bacteria to better understand the bacterial community in the cholesterol gallstones and to assess which bacteria have potential to promote the formation of cholesterol gallstones.

2. Material and methods

2.1. Patients and sample collection

The 22 cholesterol gallstone samples (S1–S22) and 12 bile samples (B1–B12) from 22 gallbladder-stone patients were provided by Chengdu Military General Hospital. The 12 male and 10 female provided a signed informed consent to partake in the study. The average age of the patients was 45.1 ± 14.2 (SD). The patients did not receive any antibiotics or probiotics before the study. Gallstones were removed aseptically from the gallbladder of each patient. At the same time, gallbladder bile was aspirated from indwelling catheters into 10 ml sterile tubes from 12 patients. All samples were stored in their original tubes at −40 °C until further analyses. The gallstones were classified as cholesterol gallstones with above 70% cholesterol content by Fourier transform infrared spectroscopy as described [29,30].

2.2. Total DNA extraction

Cholesterol gallstones (180–200 mg) were homogenized by grinding with a sterile mortar and then by mechanical disruption with a bead beater. Total DNA from homogenized gallstones and bile samples was extracted by DNA Stool Kit (OMEGA) following the manufacturer's instructions.

2.3. PCR amplification of V3 region of 16S rDNA

PCR amplification was performed in S1000™ Thermal Cycler (BIO-RAD) with reagents obtained from TaKaRa (Japan). In the first step of a nested PCR method, 16S rRNA gene fragments were amplified using 20 pmol of each primer 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGC TAC CTT GTT ACG ACT T-3′) in 50 μl of amplification buffer (250 μM of each dNTP, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl and 2.5 U Taq DNA polymerase) with 5 μl sample DNA as the template. The amplification was performed with the following temperature profile: an initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min; and a final extension at 72 °C for 10 min. The amplification products were subjected to electrophoresis in 1% (w/v) agarose gel and extracted by Gel Extraction Kit (OMEGA) according the manufacturer's instructions.

The extracted products were used as template for amplification of V3 region of 16S rDNA. The V3 region was amplified using primers 338F (5′-ACT CCT ACG GGA GGC AGC AG-3′) with a GC-clamp (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3′) at the 5′ end and 518R (5′-GTA TTA CCG CGG CTG CTG GCA-3′) in 50 μl of amplification buffer as described above with 5 μl extract as the template. The amplification was performed with the following temperature profile: 5 min at 95 °C; 10 cycles of denaturation at 94 °C for 30 s, annealing for 30 s with decreasing the temperature by 0.5 °C per cycle from 60 to 55 °C, extension at 72 °C for 35 s; 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s; and a 7 min final extension at 72 °C.

The amplification products were subjected to electrophoresis in 1% agarose gels (Invitrogen) using a D2000 DNA Marker (TIANGEN BIOTECH, Beijing, China) as a molecular weight standard. The gels were run at 120 V for 20 min in 1 × TAE, stained with 0.1 μl ml^{-1} (v/v) Green View and evaluated as described [31,32].

2.4. DGGE analysis

DGGE was performed with the D-Code™ universal mutation detection system (BioRad, Lab., USA). The amplification products from cholesterol gallstone samples were separated in 8% (w/v) polyacrylamide gels in a denaturing gradient of 40%–65% (100% denaturant defined as 7 M urea and 40% formamide in 1 × TAE buffer, pH 7.4) at 110 V and 60 °C for 6.5 h. The amplification products from bile samples were separated in a denaturing gradient of 45%–55% under conditions described above for separating preferably bands. Gels were stained with SYBR Green (Invitrogen) for 45 min, visualized with a UV transilluminator (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed with Quantity One software (Bio-Rad, USA).

2.5. Amplification and sequencing of excised DGGE bands

Selected bands were excised from the DGGE gel, resuspended in sterile water and kept at 4 °C for 24 h before amplification of the V3 region as described above. The amplification products were sequenced at Invitrogen (Shanghai, China). The sequences were compared with NCBI database to determine the closest match sequence.

2.6. Isolation and identification of culturable bacteria

Cholesterol gallstone and bile samples were inoculated onto modified tryptic soy broth (TSB). The plates were incubated at 37 °C under aerobic conditions (CO_2 concentration 5%). DNA was extracted from bacterial mass taken from single colonies by Bacterial DNA Extraction kit (TIANGEN, Beijing, China) while the rest of the colony was preserved for further testing. The 16S rDNA was amplified using primers 27F and 1492R as described above and sequenced at Invitrogen (Shanghai, China). The sequences were compared with NCBI database to determine the closest match sequence.

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