Characterization of The Microbiome In Bile From Normal Human Gallbladders

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Abstract

Background: Scientists previously believed that bacteria could not grow in bile, which is rich in bacteriostatic compounds such as bile acids. Therefore, the biliary tract was considered to be a sterile environment. However, high-throughput (i.e., amplicon) sequencing analysis methods have recently been used to discover the bacterial flora in gallbladder bile in brain-dead patients with normal gallbladders. In a bacterial flora analysis targeting the 16S ribosomal gene, a specific flora was present in the bile of normal gallbladders. However, these results were not obtained from truly healthy individuals. Therefore, the aim of this study was to analyze the microbial flora of bile collected from pathologically normal gallbladders that were surgically removed from patients with hepatobiliary pancreatic diseases who had normal liver function.

Results: All 12 bile samples obtained from the gallbladders had negative culture results, although a bacterial flora was detected in all samples via 16S ribosome gene analysis. The composition of the bacterial flora was very simple, and the *Firmicutes*, *Proteobacteria*, and *Actinobacteria* phyla were identified in all samples. Based on 16S rRNA gene profile analysis, the composition ratio accounted for more than 80% of the total number of reads. The *Anaerobacillus*, *Delftia*, *Bacillus*, *Ralstonia*, *Ochrobactrum*, *Acidovorax*, and *Curvibacter* genera were detected in all 12 samples. Based on 16S rRNA gene profile analysis, at the genus level, *Anaerobacillus* and *Delftia* accounted for 58.62%–87.63% of the identified bacteria in each sample.

Conclusions: In this study, the bacterial flora in the gallbladder bile was not diverse. Contrary to previous reports, few bacteria belonging to the *Bacteroidetes* phylum were detected. The functional significance of the gallbladder bacterial flora requires further investigation.

Background

Newly developed high-efficient gene analysis technology has allowed advances in bacterial microbiome research targeting the intestinal bacteria, its relationship with various diseases, and the etiology of such diseases [1, 2]. Determining the functional role of bacteria using 16S rRNA gene analysis alone is difficult; however, with this new technology, identifying bacterial species that are difficult to detect using normal culture techniques is possible because of the small number of bacteria.

The bacterial flora within an organ may be involved in the pathology of certain diseases, as previously suggested [3, 4]. The bile of the gallbladder was previously considered to be sterile in healthy patients because bile does not provide a suitable environment for bacteria to survive [5]. However, a recent study reported a diverse bacterial flora in the bile of the gallbladder of seemingly healthy patients [6]. A hypothesis is that the presence of a small amount of a special bacterial flora in the bile that is difficult to culture with normal methods may inhibit the invasion of other pathogenic bacteria.

In nonhuman studies, a recent genetic analysis of a healthy porcine gallbladder bile revealed that specific bacteria are present within the gallbladder bile [7]. In addition, age-related changes in the bile

microbiome of rabbits have been reported [8]. However, to sample the bile of healthy humans is ethically impossible. Owing to the high detection sensitivity of genetic analysis, DNA contamination always exists. Furthermore, the test procedure may be a risk factor for biliary tract infection, even when bacterial flora analysis is conducted via endoscopic retrograde cholangiopancreatography (ERCP), during which bile collection is possible [9–11]. Until recently, the bacterial flora of bile in healthy gallbladders has not been analyzed for ethical reasons. Molinero et al. [12] aseptically collected bile from the gallbladder of brain-dead patients who had abnormalities in the biliary system. They reported that some bacteria existed at the genetic level. However, these results were determined using bile from patients in a special circumstance who were likely to have an underlying disease; therefore, the continual presence of certain bacteria in the normal gallbladder of healthy individuals cannot be assumed. In this study, bile was collected from the gallbladders of relatively healthy patients to clarify the presence of a microbiome using 16S ribosomal gene analyses.

Methods

Patients

Bile samples were collected from the gallbladders of patients who were undergoing hepatobiliary and pancreatic surgery on account of malignant or nonmalignant causes and required a simultaneous cholecystectomy for procedural reasons at Juntendo University Hospital (Tokyo, Japan) from August 2018 to May 2019. The gallbladders included in this study were free of inflammation and infection, and the bile samples were collected aseptically during surgery by the hepatobiliary and pancreatic surgeon. The bile was withdrawn from the fundus of the gallbladder by using an 18 G needle. The collected specimens were immediately divided into 2-cc sterile testing tubes and refrigerated at -80°C until they were transported for analysis. All patients received a 2-gram cefmetazole infusion 1–2 hours before the cholecystectomy.

Patients who had no gallstones, a pathologically normal gallbladder, no hepatobiliary enzyme abnormalities, and no inflammatory markers, and who had not undergone ERCP or biliary drainage or had not received antibiotics 3 months before the study were considered to have a healthy gallbladder and were included in the study.

All patients provided informed consent before the procedure. This study was approved by the Juntendo University Hospital Ethics Committee (approval number: JHS18-0309) and was conducted according to the principles of the Declaration of Helsinki.

DNA isolation

Bacterial DNA was extracted from the bile samples using DNeasy PowerSoil Kits (QIAGEN, Venlo, the Netherlands). All specimens were stored under the same conditions and were processed by skilled staff using identical protocols.

Preparation of the 16S ribosomal DNA sequencing library

A library was created for each specimen by using a primer set (27Fmod: 5' AGR GTT TGA TCM TGG CTC AG 3' and 338R: 5' TGC TGC CTC CCG TAG GAG T 3') targeting the V1-V2 region of the 16S ribosomal gene, based on the guidelines of the *Illumina 16S Metagenomic Sequencing Library Preparation Guide* [13, 14]. The 251-base pair (bp) pair-end sequencing of this amplicon was conducted using MiSeq (Illumina, Inc., San Diego, CA, USA).

Sequence processing

The 251 bp paired-end sequence obtained from each specimen was processed using QIIME2 (version 2021.4) [15]. First, the Illumina MiSeq pair-end sequence (Fastq format) was demultiplexed (demux) to QIIME2. The DADA2 plugin was then used to trim the ends so that the average quality score was 37 or higher, and the low-quality sequences and chimeric sequences were removed [16]. Therefore, the sequences obtained from each specimen had a minimum number of reads of 68,185, a maximum number of reads of 165,362, and an average number of reads of 102,623. Each sequence then underwent the MAFFT multiple sequence alignment. Positional conservation and gap filtering were conducted using the mask plugin.

Sequence analysis

A phylogenetic tree was created using the fasttree plugin and the midpoint-root plugin. Silva-138-99 sequences were then obtained from the QIIME 2 website (<https://docs.qiime2.org/2021.4/data-resources/#silva-16s-18s-rna>), and a dataset of sequence data spliced from the region bracketed by the 27Fmod and 338R primers and operational taxonomic units were created [13]. Class names in this dataset were extrapolated from sequence data obtained from samples, as classifiers in the QIIME2 q2-feature-classifier plugin (Additional Table 1).

Results

Twelve patients (9 men and 3 women; age range, 43–89 years) were included in this study (Additional Table 2). No bacterial growth in any of the 12 bile specimens was detected in aerobic cultures with blood agar/bromothymol blue agar medium or in anaerobic cultures with phenylethyl alcohol/*Bacteroides* bile esculin agar medium. However, three groups of the *Firmicutes*, *Proteobacteria*, and *Actinobacteria* phyla were found in all specimens during the 16S ribosomal gene analysis. These three groups accounted for more than 80% of the detected flora (Figure 1). The phylum *Fusobacteria* was found in 3/12 specimens, but there was no disease with characteristics.-

Within the *Firmicutes* phylum, the genera *Anaerobacillus* (33.41% $\pm$ 7.0441% of the detected flora) and *Bacillus* (6.623% $\pm$ 3.8955% of the detected flora) were detected in all specimens, based on the 16S rRNA gene profile analysis. All bile samples contained the following genera in the *Proteobacteria* phylum: *Delftia* (18.68% $\pm$ 4.3935% of the detected flora), *Ralstonia* 4.14% $\pm$ 6.1401% of the detected flora),

Ochrobactrum (4.518% $\pm$ 2.3837% of the detected flora), *Acidovorax* (3.016% $\pm$ 1.943% of the detected flora), and *Curvibacter* (1.561% $\pm$ 1.0242% of the detected flora) (Figure 2). No bacteria of the phylum *Actinobacteria* were detected in any sample. Based on the 16S rRNA gene profile analysis, bacteria in the *Anaerobacillus* genus and the *Delftia* genus accounted for 42.53%–64.63% of the detected flora in each specimen.

Discussion

This study demonstrated that the bacterial flora in gallbladder bile is simple. The functional significance of the bacterial flora identified in this study remains unclear.

Bile acids possess antibacterial qualities that can dissolve bacterial membranes and damage bacterial DNA; therefore, bile in the biliary tract was believed to be sterile in healthy individuals [17]. However, some bacteria such as *Salmonella* spp. and *Listeria monocytogenes* may survive in the gallbladder and be associated with infections and the development of gallstones [18, 19].

Bacterial flora analysis, based on gene sequencing using a high-efficiency sequencer, has recently provided a new perspective in the field of microbiology. Flora that is not able to be identified using conventional morphological cultures can be detected using gene sequencing, thereby allowing for flora classification. Recent studies have reported the presence of bacteria in gallbladder bile of relatively healthy individuals. Gene sequencing has been used to detect bacteria in the bile of the gallbladder of healthy swine and rabbits. However, the analysis of bile in healthy humans is not ethical, and the collection of bile using an endoscope is limited by contamination. Furthermore, the gallbladder of a healthy human cannot be excised for pathological examination. To overcome these limitations, Molinero et al. [12] performed gene sequencing on bile from the gallbladders of brain-dead patients and identified a bacterial flora that cannot be detected using conventional bacterial cultures. The Molinero study also analyzed the bacterial flora of the bile of patients with cholelithiasis, and reported that it differed from the bacterial flora in patients with healthy gallbladders. However, the medical histories of brain-dead patients were diverse, and a pathological examination of the gallbladder and detailed liver function tests are typically not conducted in these patients. In the current study, the bile samples were collected from patients with a normal liver function and a pathologically normal gallbladder who did not receive any antibiotics or tests that would have affected the biliary system before the study. However, most patients included in this study had cancer, which may have affected the results of this study. These patients had pancreatobiliary diseases, although they had no medical history suggestive of inflammation and no bias toward a specific disease. Therefore, we determined that the likelihood of infected bile in the gallbladder was low, based on laboratory data. In this study, a microbiome was identified in all bile samples using 16S ribosomal DNA sequencing.

The composition of the bile bacterial flora identified in this study was significantly less diverse than that reported by Molinero et al. [12]. More than 80% of the detected bacteria belonged to the *Firmicutes* and *Proteobacteria* phyla. The *Anaerobacillus* and *Delftia* genera accounted for most bacteria in each

specimen. Molinero et al. reported that that bacteria from the genera *Lactococcus*, *Propionibacterium*, *Bifidobacterium*, and *Bradyrhizobium* were predominant, which is inconsistent with the results of this study. These differences may be because of the fact that the QIIME system was used in the Molinero study and the QIIME2 system was used in this study. When the previous study's reported sequence data were analyzed using QIIME2, the results differed from those reported in that study (data not shown).

Bacteria may have entered the gallbladder in a retrograde fashion from the duodenum or from the portal vein. When the duodenal papilla function normally, the duodenal fluid (including bacteria) does not enter the bile duct. Investigators have reported that a distal cholangiocarcinoma that may result in dysfunction of the duodenal papilla can lead to an overgrowth of bile in the bile duct [19]. Papillary dysfunction was possible among the patients included in this study, although most patients were determined to have normal papillary dysfunction. Bile is constantly produced and is excreted in the duodenum when food is eaten. Therefore, duodenal bacteria would only be expected to be in the bile of the gallbladder under pathological conditions such as biliary obstruction. The differences between the results of this study and those of Molinero et al. [12] are consistent with the findings of another study reporting that the intestinal flora of Japanese patients is unique and differs from that of Chinese and Western patients [20].

Conclusion

Previous studies have reported the presence of a diverse spectrum of bacteria in the gallbladder bile of brain-dead patients. However, the results of this study demonstrated that the bacterial flora in gallbladder bile is simple. The functional significance of the bacterial flora identified in this study remains unclear. Further studies are needed that include patients in whom the duodenal papilla is less likely to be dysfunctional. A functional and quantitative evaluation of the bacterial flora present in the gallbladder is also needed. The role of these flora in preventing inflammation of the gallbladder from pathogenic flora requires further investigation.

Abbreviations

ERCP: endoscopic retrograde cholangiopancreatography

Declarations

Ethics approval and consent to participate

All experimental procedures used in the study complied with the ethical standards of the national guidelines of the Japanese government on human experimentation and the Declaration of Helsinki. The study was approved by the institutional review board of Juntendo University (approval number: JHS 18-0309), and written informed consent was obtained from all patients.

Consent for publication

All patients agreed to the publication of the data.

Availability of data and material

All sequence data and metadata have been deposited in the DDBJ Sequence Read Archive (<https://www.ddbj.nig.ac.jp/dra/index-e.html>) under the accession number DRA012314.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

SW and TS conceived and designed the study. MM and AS explained the study to the patients, obtained consent forms, and collected and processed the biological samples. DM and SN conducted the staging, processing, and the MiSeq analyses. SW, TS, MT, and TK performed the data management. SW drafted the manuscript. SW, TS, MT, and TK corrected and reviewed the manuscript. All authors read and approved the content of the manuscript, including the accuracy of the data, ethical legitimacy, and validity of the results.

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Figures

Figure 1

Phylum-level composition of the bacterial flora in the bile from a normal gallbladder 16S ribosomal gene amplicon sequencing was used to identify the bacterial flora of the bile from a normal gallbladder. The composition ratios are shown at the phylum level, when possible; otherwise, the closest taxonomic rank is provided, preceded by unknown member (U. m.). The Firmicutes, Proteobacteria, and Actinobacteria phyla exist in all 12 specimens and account for more than 80% of the detected bacterial flora.

Figure 2

Genus-level composition of the bacterial flora in bile from a normal gallbladder 16S Ribosomal gene amplicon sequencing was used to identify the bacterial flora of the bile from a normal gallbladder. The composition ratios are shown at the genus level, when possible; otherwise, the closest taxonomic rank is provided, preceded by the unknown member (U. m.). In the Firmicutes phylum, the genera Anaerobacillus and Bacillus exist in all 12 specimens. In the Proteobacteria phylum, the genera Delftia, Ralstonia, Ochrobactrum, Acidovorax, and Curvibacter exist in all 12 specimens.

Supplementary Files

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