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ORIGINAL ARTICLE

Application of next-generation sequencing to study ascitic microbiome in cirrhotic patients with or without spontaneous bacterial peritonitis

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Background: Spontaneous bacterial peritonitis is an important cause of morbidity and mortality in cirrhotic patients with ascites. The key step in the pathogenesis of spontaneous bacterial peritonitis is bacterial translocation from intestinal lumen to mesenteric lymph nodes, and from there to the systemic circulation and ascitic fluid. We aimed to study the ascitic microbiota of cirrhotic patients with or without spontaneous bacterial peritonitis.
Methods: Both the 16S polymerase chain reaction approach and the whole genome shotgun approach were adopted for the next-generation sequencing technology. We compared the results derived from the two methods.

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Results: The bacterial culture-negative ascites in cirrhotic patients, which even failed for amplification of 16S ribosomal DNA, were found to contain much less bacterial DNA than the culture-positive ones, indicating that the paucity of bacteria, instead of the difficulty of bacterial culture, was possibly the main reason for negative culture result of the ascites. *Escherichia coli* was the predominant species in all samples, and the bacteria of low abundance were also identified by the next-generation sequencing technology.
Conclusion: Whole genome shotgun-based next-generation sequencing is an appropriate method for depicting the microbiome of ascites or of other specimens with a low abundance of bacterial DNA.

Introduction

Spontaneous bacterial peritonitis (SBP) is a frequent infectious complication of cirrhosis with ascites.[1,2] The key step in the pathogenesis of SBP is bacterial translocation, defined as the migration of viable bacteria or bacterial products of enteric origin from intestinal lumen to mesenteric lymph nodes, and from there to the systemic circulation and ascitic fluid. The prevalence of SBP in hospitalized patients ranges between 10% and 30%.[3]

An accurate diagnostic method for identifying the causative organism responsible for SBP is particularly useful in acute care settings in that a timely and appropriate antibiotic treatment can improve the clinical outcome significantly. Traditionally, identification of bacteria that cause SBP depends on culturing, but it normally takes several days to detect the pathogens in the ascitic fluid. Furthermore, the rate of negative ascitic fluid cultures is usually 10—60% among patients with the clinical manifestations of SBP, with the supposed reason that many fastidious organisms in the ascites cannot be cultured by the general medium.[4] Current laboratory diagnosis of SBP often uses the count of polymorphonuclear neutrophils (PMNs) in the ascitic fluid as an indicator of inflammation in ascites. A PMN count in the ascitic fluid of $\geq$250/dL is defined as SBP, but lysis of PMNs during transport to the laboratory may lead to false-negative results.[5] In that case, lactoferrin can be used as a biomarker for SBP.[6]

In the past decade, amplification of 16S ribosomal DNA (rDNA) has come to the forefront of infectious disease diagnostic development, including the researches on ascites. Following this method, 16S rDNA within ascites was directly extracted and amplified, and then the polymerase chain reaction (PCR) amplicon was cloned and sequenced by the Sanger method.[7—10] Culture growth independence allows the 16S PCR method to achieve a substantial increase in the detection rate of ascitic bacteria compared to the conventional culture methods. Recently, Appenrodt et al[7] coupled the 16S PCR method with high-resolution melt analysis. As high-resolution melt analysis offers a simple and low-cost approach to amplicon analysis, the capacity of the 16S PCR method has been enhanced further. However, both Sanger sequencing and high-resolution melt analysis cannot resolve the mixed infection caused by multiple agents, not to mention depicting the whole ascitic microbiome.

The next-generation sequencing (NGS) technology has achieved a rapid progress and has been widely applied for characterizing the human microbiome from multiple body sites.[11] The main advantage of NGS is its ultrahigh throughput, by which even trace amounts of bacterial DNA (bactDNA) can be detected. Most microbiomic studies were based on by the 16S PCR approach, in which the 16S rDNA amplicons of the samples were sequenced by NGS. However, the whole genome shotgun (WGS) approach has also been developed,[12] to directly sequence the entire genomic DNA by NGS. In this study, we adopted both approaches to delineate the microbial profiles in cirrhotic patients with or without SBP.

Materials and methods

Patients

The study was approved by the Institutional Review Board of Chang Gung Memorial Hospital, Taoyuan, Taiwan. Ascitic samples were collected from three groups of patients: Group 1, uninfected, asymptomatic patients with negative bacterial cultures; Group 2, infected patients with negative bacterial cultures; and Group 3, patients with culture-confirmed SBP. All the ascitic samples were examined for the white blood cell count, percentage of PMNs, bacterial culture, and lactoferrin concentration. SBP was defined as an increased ascitic white blood cell count with PMN predominance and a positive culture. Symptomatic patients with an increased white blood cell count but without positive culture results were deemed infected and allocated to Group 2 if the test results showed PMN predominance or increased lactoferrin. In this group of patients, bacterial cultures were performed at least twice. Asymptomatic patients without evidence of infection were included in Group 1. Lactoferrin concentrations were determined using a polyclonal antibody-based enzyme-linked immunosorbent assay (Bethyl Laboratories, Montgomery, TX, USA). Data on these patients and samples are summarized in Table 1.

Ascites and preparation of genomic DNA

Genomic DNA was extracted immediately after the ascites were collected using a kit according to the manufacturer's instructions. Ascites were centrifuged at 1200 $\times$ *g* at 4°C for

Table 1 Information of the ascitic fluid from patients

Group	Age (y)/sex	Cytology		Lactoferrin (ng/mL)	Gram stain	Culture	
		WBC	PMN (%)			Aerobic	Anaerobic
1	63/F	26	5	31.43	N	N	N
	43/F	41	1	12.41	N	N	N
	40/M	13	28	7.19	N	N	N
2	70/M	136	61	35.8	N	N	N
	65/M	4250	97	157.97	N	N	N
	72/F	540	92	465.39	N	N	N
3	73/F	231	82	82.90	Gram-negative bacilli	*E. coli*	N

N = negative; PMN = polymorphonuclear neutrophil; WBC = white blood cells.

10 minutes, and the pellets were treated with lysozyme (4 mg/mL; Sigma, St. Louis, MO, USA) and lysostaphin (20 mg/mL; Sigma), and then incubated at 37°C for 30 minutes. Genomic DNA from ascites was extracted using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). For Groups 1 and 2, the amount of DNA from one patient was not enough for the following experiments, so equal amounts of DNA were pooled together from three patients.

Microbiota analysis by the 16S PCR approach

The 16S rDNA sequences from the genus *Acinetobacter*, *Bacteroides*, *Clostridium*, *Enterococcus*, *Escherichia*, *Helicobacter*, *Klebsiella*, *Propionibacterium*, *Ralstonia*, *Ruminococcus*, *Pseudomonas*, and *Staphylococcus* were aligned, and the conserved regions were chosen as the primer candidates. The following primers were finally used to amplify the V3 region of bacterial 16S rDNA: 16sV3F 5′-ACTCC TACGGGAGGCAGCAGT-3′ and 16sV3R 5′-ACCGCGGCTGCTG GCAC-3′. The primer sequences do not match with the 18S rDNA of *Homo sapiens*. The expected size of the PCR amplicon was 200 bp. The protocol for PCR amplification was as follows. Each 20 μL reaction mix contained 10 ng ascitic DNA from each group as the template. The PCR condition was as follows: 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 10 minutes. The PCR amplicon, if available, was sequenced by an Illumina Miseq sequencer (San Diego, CA, USA). The paired-end 2 × 150 bp protocol was adopted. The software FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) was used for trimming the low-quality ends of reads. The program Stitch (https://github.com/audy/stitch) was used for assembling the overlapping paired-end reads into a single contig for each pair. The preformatted 16S microbial database was downloaded from the National Center for Biotechnology Information (NCBI)/Basic Local Alignment Search Tool (BLAST) database. Merged reads were searched against this database using the NCBI blastn program. If the DNA identity of the best hit was >98.5%, the read was then assigned to the genus of the best hit.

Microbiota analysis by the WGS approach

Ten microgram ascitic DNA from each group was fragmented into approximately 300 bp and sequenced by Illumina Hiseq with one lane of paired-end run (2 × 100 bp). The reads were first mapped against human genome to filter those of a human origin. The human genome hg19 was downloaded from the University of California Santa Cruz (UCSC) database, and the program Bowtie2 was used for alignment.[13] Unaligned reads were then searched against the NCBI nonredundant nucleotide sequence database using the NCBI mega-blast program. If the DNA identity of the best hit was >90%, the read was then assigned to the species of the best hit.

Calculation of Simpson's Index of Diversity

The Simpson's Index (D) is calculated using the following formula:

$$D = \sum n(n-1)/N(N-1), \quad [1]$$

where n is the total number of organisms of a particular species and N is the total number of organisms of all species.[14]

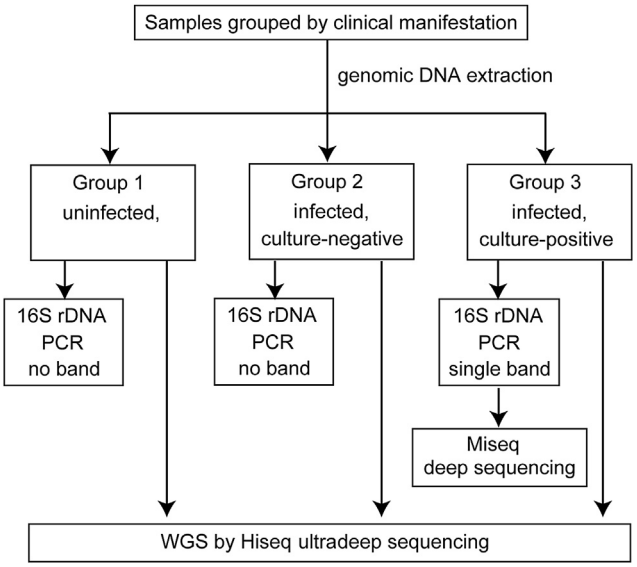

Figure 1. Flowchart of the experimental design. PCR = polymerase chain reaction; WGS = whole genome shotgun.

The Simpson's Index of Diversity is (1 − *D*), the value of which ranges between 0 and 1. The greater the value, the greater the sample diversity.

Results

Microbiome identified by the 16S PCR approach

A flowchart of the experimental design is provided in Fig. 1. First, we adopted the 16S PCR approach to explore the ascitic microbiome. The V3 region of 16S rDNA gene was amplified. As a result, no bands appeared in Groups 1 and 2, whereas Group 3 had a single band, with an approximate size of 200 bp. The PCR amplicon from Group 3 was sequenced by Illumina Miseq and eventually 429,400 read pairs were obtained. The bacterial profile of Group 3 is shown in Fig. 2A. Half of the 16S reads belonged to the genus *Escherichia*, and one-quarter to the genus *Sphingomonas*. The Simpson's Index of Diversity of this microbiota was 0.66.

Microbiome identified by the WGS approach

We also applied the WGS approach to explore the ascitic microbiome. Ascitic DNA from each group was sequenced by Illumina Hiseq, with approximately 20 Gbp of throughput. Due to the presence of human cells, over 99.9% of reads were derived from human source. However, the WGS approach can still detect the trace amount of bactDNA from Groups 1 and 2: they contained 642 and 100 bacterial reads, respectively. By contrast, the number of bacterial reads from Group 3 was larger by two orders of magnitude (25,346 reads).

Using the WGS approach, we found that *Escherichia coli* accounted for more than half the abundance in all groups (Fig. 2B–D). *Propionibacterium*, *Cupriavidus*, and *Pseudomonas* accounted for a considerable proportion in both Groups 1 and 2, but the detail bacterial compositions of the two groups differed a lot. Values of the Simpson's Index of Diversity of the two microbiota were 0.58 and 0.63, respectively. In Group 3, *Helicobacter pylori* was the second dominant bacterium, followed by two Firmicute genera: *Enterococcus* and *Lactococcus*. This profile was entirely different from that revealed by the 16S PCR approach, in which *Sphingomonas*, *Tetragenococcus*, and *Thermus* accounted for a large proportion. The Simpson's Index of Diversity calculated from the two methodologies was also different. The value for the WGS approach was 0.73, 7% higher than that of the 16S PCR approach. In other words, the WGS approach was better at identifying microbes with a low abundance.

Discussion

To our knowledge, this is the first study that applied NGS for exploring the ascitic microbiome. We found that the culture-negative ascitic samples contained much less bactDNA than the culture-positive ascitic samples. The result suggests that the paucity of bacteria, instead of the difficulty in culturing the fastidious organisms, is the main reason leading to the low rate of bacterial detection in SBP. It is conceivable that a small number (less than a required minimum) of bacteria fail to grow into a colony in the

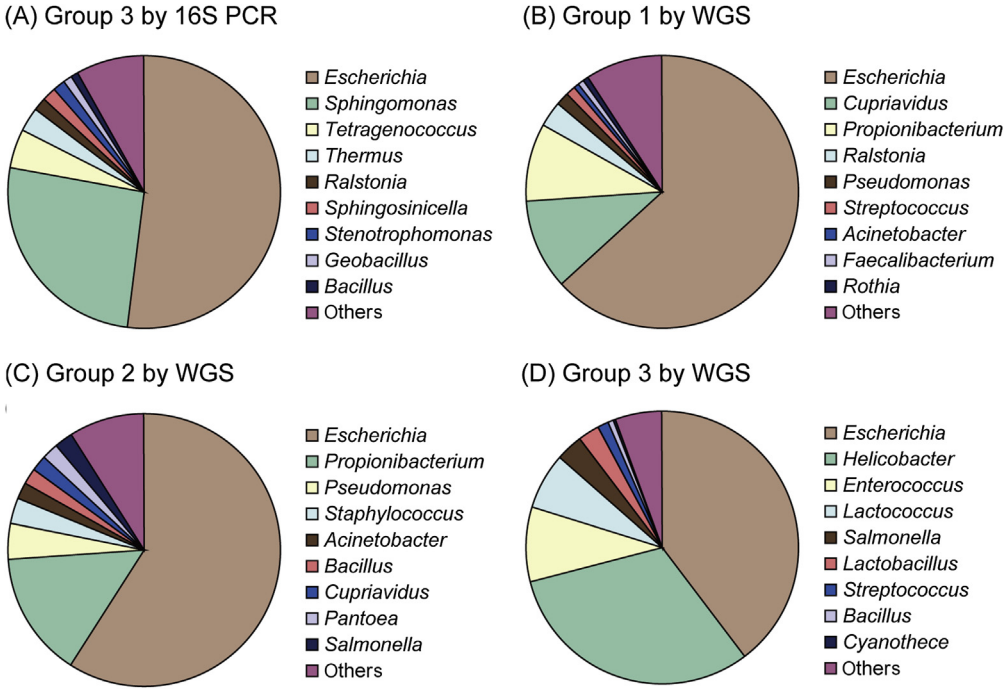

Figure 2. Ascitic bacterial profiles revealed by the NGS technology. (A) Bacterial profile of ascites revealed by the 16S PCR approach. (B–D) Bacterial profile revealed by the WGS approach. NGS = next-generation sequencing; PCR = polymerase chain reaction; WGS = whole genome shotgun.

laboratory. However, such few bacteria have already been able to elicit a disease, manifested by the patients of Group 2.

Previous studies, which were based on conventional methods, showed that Enterobacteriaceae were the major organisms responsible for SBP, although there has been a trend toward an increase in infections caused by Gram-positive bacteria, particularly *Staphylococcus*, *Streptococcus*, and *Enterococcus*.[7–10,15–18] In our study, *E. coli* was the predominant bacteria in all the three microbiota as well as in the result of culture-confirmed microbiota (data not shown). Although Gram-positive bacteria were also present in all samples, they accounted for a small proportion. Note that the real causative agents are not necessarily the most abundant bacterial species because a tiny amount of bacteria are also able to elicit the disease. Therefore, we cannot neglect the pathogenic role of these Gram-negative bacteria. Likewise, *H. pylori* was found to exist in culture-confirmed SBP cases, but it has never been documented earlier that *H. pylori* can participate in the pathogenesis of SBP. Dore et al[18] even suggested that infections caused by *H. pylori* and *Helicobacter* spp. were unlikely to be associated with SBP. Although *H. pylori* colonizes in the upper gastrointestinal tract, it is not surprising for them to translocate into the abdominal cavity.

Because ascitic bacteria were thought to come from the gut, we compared our ascitic samples with gut microbiota and found that they showed entirely different bacterial profiles. In the gut, which is actually represented by stool samples, *Bacteroidetes* and *Firmicutes* are most abundant, whereas Enterobacteriaceae are under-represented.[12] It is known that stools reflect the bacterial community from the colon, but ascitic bacteria may stem from other parts of the intestine, such as the small bowel.[19] In addition, bacteria in the gut may have unequal ability to translocate across the intestinal wall and survive within the abdomen. This factor also contributes to the difference between the bacterial profiles of stools and ascites.

In this study, we employed both 16S PCR and WGS approaches to explore the ascitic microbiome. The WGS approach has at least three advantages over the 16S PCR approach, which are likely to be the reasons why results varied significantly between the two approaches. First, because the purified ascitic DNA is mixed with a large amount of human DNA, the amount of ascitic bactDNA become relatively small. In addition, the larger amount of DNA used (10 μg) for the WGS approach may be the reason for its higher sensitivity for microbiota detection compared to the PCR-mediated 16S rDNA assay (which used 10 ng DNA). Thus, little or lack of bactDNA remaining in the purified ascitic DNA would not be a guarantee for the 16S rDNA PCR assay to produce a positive amplicon. Under such circumstances, the WGS approach is the only feasible way for depicting the ascitic microbiome. Second, given the tiny amount of ascitic bactDNA, PCR amplification of the 16S rDNA may require a larger number of cycles, which may introduce a significant bias to the final bacterial composition.

Third, the powerful discriminating ability of 16S rDNA typing is based on the high conservation of 16S rDNA sequences among the entire bacteria kingdom. However, 16S rDNA sequences of the species of the same genus are too similar to each other and their 16S rDNA typing is no longer appropriate to make the interspecies typing. By contrast, whole genome sequence is highly variable between species. For example, the DNA identity of full-length 16S rDNA is up to 99.2% between *Escherichia fergusonii* (represented by str. ATCC 35469) and *E. coli* (represented by str. K-12 substr. MG1655), but the average DNA identity of their shared 2859 orthologous protein-coding genes is 93.5%, with the remaining genes being species specific. Therefore, the WGS approach can assign the NGS reads to the level of species, whereas the 16S PCR approach can assign them to the level of genus only.

The WGS approach also has its drawbacks. First, it needs a database of bacterial genome, against which the NGS reads are searched. If a certain bacterium species does not have its whole genome sequence deposited in the database, it cannot be identified even if it is abundant in the sample. Second, the WGS approach still costs much more than the 16S PCR approach. Consequently, one lane of Hiseq sequencing can be used for one or two ascitic samples only, whereas the same throughput can resolve at least 100 samples by the 16S PCR approach. As a methodological attempt, this study revealed three microbiota only. We could not observe any statistically significant difference between the profiles of the three groups. Future studies accommodating more samples can provide a comprehensive view of the ascitic microbiome, based on which the association between disease states and specific microbiota can be determined.

Conflicts of interest

All authors report no conflicts of interest relevant to this article.

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