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Profiling of the Bacteria Responsible for Pyogenic Liver Abscess by 16S rRNA Gene Pyrosequencing

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Pyogenic liver abscess (PLA) is a severe disease with considerable mortality and is often polymicrobial. Understanding the pathogens that cause PLA is the basis for PLA treatment. Here, we profiled the bacterial composition in PLA fluid by pyrosequencing the 16S ribosomal RNA (rRNA) gene based on next-generation sequencing (NGS) technology to identify etiological agents of PLA and to provide information of their 16S rRNA sequences for application to DNA-based techniques in the hospital. Twenty patients with PLA who underwent percutaneous catheter drainage, abscess culture, and blood culture for isolates were included. Genomic DNAs from abscess fluids were subjected to polymerase chain reaction and pyrosequencing of the 16S rRNA gene with a 454 GS Junior System. The abscess and blood cultures were positive in nine (45%) and four (20%) patients, respectively. Pyrosequencing of 16S rRNA gene showed that 90% of the PLA fluid samples contained single or multiple genera of known bacteria such as *Klebsiella*, *Fusobacterium*, *Streptococcus*, *Bacteroides*, *Prevotella*, *Peptostreptococcus*, unassigned *Enterobacteriaceae*, and *Dialister*. *Klebsiella* was predominantly found in the PLA fluid samples. All samples that carried unassigned bacteria had 26.8% reads on average. We demonstrated that the occurrence of PLA was associated with eight known bacterial genera as well as unassigned bacteria and that 16S rRNA gene sequencing was more useful than conventional culture methods for accurate identification of bacterial pathogens from PLA.

Keywords: pyogenic liver abscess, abscess culture, metagenomics, 16S rRNA gene sequencing, next generation sequencing

Introduction

Pyogenic liver abscess (PLA) remains a severe disease with considerable mortality (Lee *et al.*, 2001; Yu *et al.*, 2004; Cerwenka, 2010) and is often polymicrobial. Advanced imaging tools such as high resolution computed tomography (CT) and ultrasound have improved early diagnosis of PLA (Malik *et al.*, 2010). The trend regarding the initial treatment choice for PLA has changed to antibiotics and radiological intervention (Cerwenka, 2010; Malik *et al.*, 2010). Therefore, antibiotic treatment and accurate identification of bacteria is the basis for PLA treatment.

Bacteria are traditionally identified using phenotypic tests, including Gram smears, biochemical testing, and blood or abscess cultures. However, some limitations of these methods are rare bacteria, slow-growing bacteria, uncultivable bacteria, and culture-negative infections. Thus, clinicians may have difficulty choosing the correct antibiotic treatment.

Molecular-based tools such as polymerase chain reaction (PCR) and DNA sequencing have been employed in the diagnostic field. The most commonly used target for bacterial identification in clinical laboratories is the 16S ribosomal RNA (rRNA) gene (Fredericks and Relman, 1996; Kolbert and Persing, 1999; Kiratisin *et al.*, 2003; Woo *et al.*, 2008). The 16S rRNA gene is considered the gold standard for phylogenetic studies of microbial communities and for assigning taxonomic names to bacteria (Huse *et al.*, 2012). However, this gene sequencing method is not routinely used in hospitals. Profiling of the bacteria responsible for infectious lesions such as PLA is necessary to develop a molecular-based diagnosis as well as to understand the disease etiology.

Pyrosequencing of 16S rRNA provides immense information regarding microbial communities in healthy or diseased humans and animals (Petrosino *et al.*, 2009; Hiyari and Bennett, 2011; Costa *et al.*, 2012; Hooda *et al.*, 2012). Therefore, the bacteria in PLA fluid was investigated in the present study by pyrosequencing the 16S rRNA gene based on next-generation sequencing technology. We provide a bacterial profile for PLA and information on the 16S rRNA sequences to apply molecular-based techniques in the hospital. Additionally, 16S rRNA gene sequencing resulted in identifying significantly more bacteria than those of the conventional cultured method.

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Materials and Methods

Patient population

This prospective study was reviewed and approved by the Institutional Review Board (2013-SCMC-035-00), and written informed consent was obtained from all participants. Twenty-two patients (10 men and 12 women) at the Samsung ChangWon Hospital were initially considered for the study between January 2012 and December 2013. Abscesses that were not large enough to be considered for percutaneous drainage were excluded. Thus, two patients with hepatic abscess were excluded.

The PLA diagnosis was made based on ultrasound or computed tomography imaging findings. We routinely perform percutaneous drainage and antibiotic treatment in patients with PLA at our hospital. All patients were started on antibiotics prior to percutaneous abscess drainage. A blood sample was taken and sent for culture before administration of antibiotics. The time interval between antibiotic administration and drainage was < 8 h in all cases.

Finally, 20 patients (nine males and 11 females; mean age, 61.84 ± 13.21 years; range, 33–83 years) were enrolled in this study.

Intervention

All percutaneous procedures were performed under ultrasound (Acuson x300, Sequoia 512, Siemens, USA) guidance. Eighteen gauge P.T.C Chiba needles (Unimed, Switzerland) of varying lengths were used to puncture the abscesses. The aspirated isolates including cultures were sent for microbiological analysis and 16S rRNA gene sequencing. Under fluoroscopic guidance, 2–4 ml of undiluted contrast media was instilled into the abscess cavity through the 18G needle and then a 0.035-inch wire (Terumo, Japan) was inserted into the abscess cavity. After serial dilatation, an 8.5F pigtail catheter (Cook Medical, USA) was inserted into the abscess. Follow-up ultrasonography of the liver was performed 1–2 weeks later. The drainage catheter was removed when the abscess cavity collapsed on follow-up ultrasonography and when catheter output decreased to < 10 ml daily.

Preparation of genomic DNAs from abscess effusions

Genomic DNAs from the abscesses were prepared by a method described previously with a modification (Holder *et al.*, 2012). A 500 µl aliquot of abscess effusion was mixed with an equal volume of TE buffer (pH 7.0). The abscess diluent was mixed with 15 U mutalysin (Sigma, USA) and 600 µg lysozyme (Genery, China) and incubated at 37°C for 1 h. The mixture was treated with 10 µl 10% SDS solution and 20 µg RNase (RBC, Taiwan) at 37°C for 1 h and then with 120 µg Protease K (GeNet Bio, Korea) at 37°C for 1 h. The samples were mixed with 1/10 volume of 5% cetyltrimethylammonium bromide (BDH Chemicals Ltd., England)-0.5 M NaCl solution and allowed to stand at 37°C for 1 h. The same volume of phenol: chloroform: isoamyl alcohol (25:24:1) solution was added and vortexed vigorously. The mixtures were centrifuged for 10 min at 12,000 rpm and the aqueous layer was subjected to chloroform extraction. After centrifugation, the aqueous phase was collected and mixed with 1/10 volume 3 M sodium acetate (pH 5.2) and two volumes of ethanol. The sample tubes were stored at -20°C for 20 min and then centrifuged at 12,000 rpm for 10 min. The pellets were washed with 1 ml 70% ethanol, dried completely, and dissolved in 30 µl TE buffer. The purified genomic DNA, the PCR primers and the PCR products were deposited to the *Helicobacter pylori* Korean Type Culture Collection (HpKTCC, http://hpktcc.knrrc.or.kr).

PCR

Extracted DNA (60 ng) was subjected to PCR amplification of the 16S rRNA gene V1–V3 region using AccuPower PCR PreMix (Cat. No K-2016, Bioneer, Korea) containing 1 unit of Top DNA polymerase, 1 mM dNTPs, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, and 1.5 mM $MgCl_2$ with the 27F primer (5′-GAGTTTGATCCTGGCTCAG-3′) and the 518R primer (5′-ATTACCGCGGCTGCTGG-3′). The 5′ ends of the forward primers were subsequently attached with an adaptor 1 sequence (5′-CCATCTCTCCCTGCGTGTCTCC GAC-3′), a key sequence (TCAG), and sample-specific multiplex identifier sequences. The reverse primer was added with an adaptor 2 sequence (5′-CCTATCCCCTGTGTGC CTTGGCAGTC-3′) and a key sequence (TCAG). The PCR reactions were carried out by a pre-denaturation of 4 min at 94°C and 20 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C, followed by a final extension of 7 min at 72°C. Amplified DNAs were separated on a 1% agarose gel and purified with GeneALL® ExpinTM PCR SV (Geneall Biotechnology Co. Ltd., Korea).

Pyrosequencing and data anlaysis

Barcoded amplified DNAs of the 20 samples were mixed with the same concentrations and applied to 454 pyrosequencing. Pyrosequencing was performed unidirectionally from the 27F primer end and on a 454 GS Junior System platform in a single full-plate run. These SFF-files of primary sequencing reads were then de-multiplexed based on the MIDs. Subsequently, MIDs, U-linkers, and primers were trimmed away and the sequences were quality filtered. Sequences not passing the FLX quality controls were discarded, the 454-specific portions of the primers were trimmed, the raw sequences were sorted according to tag sequences, and reads

Table 1. Culture results

Organism	Number (%)
Blood (n=20)	
Klebsiella pneumonia	2 (10.0%)
Staphylococcus caprae	1 (5.0%)
E. coli	1 (5.0%)
Non	16 (80.0%)
Abscess (n=20)	
Klebsiella pneumonia	5 (40.0%)
Streptococcus spp./*Klebsiella pneumonia*[a]	3 (15.0%)
E. coli /*Staphylococcus epidermidise*[b]	1 (5.0%)
Non	11 (55.0%)
Total	20 (100%)

[a] *Streptococcus* spp. /*Klebsiella pneumonia* = *Streptococcus* spp. is predominant, [b] *E. coli* /*Staphylococcus epidermidise* = *E. coli* is predominant, Non = no growth.

with low quality scores (quality scores below 30) and short lengths (< 80 bp) were removed. For 16S rRNA sequences, de-multiplexing, trimming, and quality filtering were done using AmpliconNoise (Quince *et al.*, 2011). Trimmed 16S rRNA sequences were identified using the Basic Local Alignment Search Tool (BLASTBN) algorithm and the National Center for Biotechnology Information (NCBI) non-redundant (NT) sequence database with an E-value cutoff of 10^{-5} based on total reads. The species richness analysis was performed by using MEGAN base on total sequencing reads. The MEGAN platform uses the lowest common ancestor (LCA) algorithm to classify reads to certain taxa based on their best-scoring matches in the BLAST result. When processing the BLAST files by MEGAN we used parameter settings of Min Score 35, Top Percent 10 and Min Support 5 for read sequences. The results of the total read classification were constructed into a rooted taxonomic tree where each clad represents a taxon. Some reads which do not have any match to the respective database are placed under "No hit" node, and some reads that are originally assigned to a taxon that did not meet our selected threshold criterion are placed under "Not assigned" category. Genus counts for each subject were extracted from MEGAN. Clustering and diversity analysis of the sequence data were performed using the CD-HIT (Li and Godzik, 2006) software package.

Statistical analysis

Results are presented as arithmetic means (ranges) and associated standard deviations. Categorical data are expressed as the number of subjects or as clinical variables with a corresponding percentage.

Results

Blood and abscess cultures were processed for all 20 patients. Abscess cultures were positive in nine (45%) patients; five (55.6%) were monomicrobial and four (44.4%) were polymicrobial (≥2 organisms isolated). *Klebsiella pneumoniae* was the most commonly isolated bacteria from the abscess cultures. No culture was obtained in 11 patients. Blood cultures were positive in four (20%) patients, and 50% of these had *K. pneumoniae*.

Table 2. Pyrosequencing of the 16S rRNA genes in the genomic DNAs extracted from liver abscesses on the 454 GS Junior System and classification and species richness of the bacteria in liver abscesses

Barcode primers	Total reads	Reads after processing	Assigned		Not assigned		No hit		*Klebsiella*		*Bacteroides*		*Fuso-bacterium*		*Prevotella*		*Streptococcus*		*Pepto-streptococcus*		*Dialister*		*Entero-bacteriaceae*[a]		Assigned but not genus[b]	
			Reads (%)[c]		Reads (%)[c]		Reads (%)[c]		Reads (%)[d]		Reads (%)[d]		Reads (%)[d]		Reads (%)[d]		Reads (%)[d]		Reads (%)[d]		Reads (%)[d]		Reads (%)[d]		Reads (%)[d]	
01F	1558	1263	965	(76.4)	295	(23.4)	3	(0.2)	959	(99.4)															6	(0.6)
02F	2017	1641	1292	(78.7)	349	(21.3)			1276	(98.8)															16	(1.2)
03F	4481	2974	1515	(50.9)	1459	(49.1)									610	(40.3)	352	(23.2)	60	(4.0)	51	(3.4)	53	(3.5)	389	(25.7)
04F	3153	2434	1617	(66.4)	817	(33.6)			123	(7.6)	137	(8.5)	715	(44.2)			375	(23.2)							267	(16.5)
05F	3143	2744	2005	(73.1)	738	(26.9)	1	(0.0)			757	(37.8)					1218	(60.7)							30	(1.5)
06F	3928	3286	2928	(89.1)	358	(10.9)			2892	(98.8)															36	(1.2)
07F	3953	3122	2809	(90.0)	312	(10.0)	1	(0.0)	2722	(96.9)															87	(3.1)
08F	254	241	162	(67.2)	79	(32.8)			160	(98.8)															2	(1.2)
09F	4462	3409	2949	(86.5)	460	(13.5)							2943	(99.8)											6	(0.2)
10F	4363	3600	3172	(88.1)	428	(11.9)			3142	(99.1)															30	(0.9)
11F	3265	3004	650	(21.6)	2354	(78.4)							505	(77.7)	69	(10.6)									76	(11.7)
12F	158	158	0	(0.0)	158	(100.0)																			0	
13F	3519	3372	3265	(96.8)	107	(3.2)			3215	(98.5)															50	(1.5)
14F	4779	3800	2955	(77.8)	844	(22.2)	1	(0.0)	2904	(98.3)															51	(1.7)
15F	8914	8216	3295	(40.1)	4921	(59.9)			3257	(98.8)															38	(1.2)
16F	862	540	0	(0.0)	540	(100.0)																			0	
17F	6971	6365	5556	(87.3)	809	(12.7)			5445	(98.0)															111	(2.0)
18F	9598	7222	5685	(78.7)	1537	(21.3)			5607	(98.6)															78	(1.4)
19F	4022	2884	2530	(87.7)	354	(12.3)											2338	(92.4)							24	(0.9)
20F	2969	2930	2912	(99.4)	18	(0.6)			2871	(98.6)															41	(1.4)
Un-identified	1480												168	(6.6)												
Total	77849	63205	46262	(73.2)	16937	(26.8)	6	(0.0)	34573	(74.7)	894	(1.9)	4331	(9.4)	679	(1.5)	4283	(9.3)	60	(0.1)	51	(0.1)	53	(0.1)	1338	(2.9)

[a] Reads finally assigned to the family as *Enterobacteriaceae* in BLASTBN. [b] Reads classified into the orders and families of assigned genera in the rooted taxonomic tree but finally not assigned to the genus.
[c] A percentage against reads after processing. [d] A percentage against assigned reads.

Two patients had concordant blood and abscess culture results and two had positive blood cultures with negative abscess cultures (Table 1).

16S rRNA gene was PCR-amplified from genomic DNA extracted from purulent fluids abscess of abscesses and read by pyrosequencing. Of the 63,205 reads remaining after processing, 46,262 reads (73.2%) were assigned to the existing 16S rRNA database. The other 16,937 reads (26.8%) were not assigned and could not be classified into known bacteria. Therefore, they might represent novel lineages. Of 46,261 assigned reads, *Klebsiella* was the most abundant genus (74.7%) and the remaining reads were classified as *Fusobacterium* (9.4%), *Streptococcus* (9.3%), *Bacteroides* (1.9%), *Prevotella* (1.5%), *Peptostreptococcus* (0.1%), unassigned *Enterobacteriaceae* (0.1%), and *Dialister* (0.1%).

Of the 20 abscess samples from patients with liver abscesses, 12 (01F, 02F, 06F, 07F, 08F, 10F, 13F, 14F, 15F, 17F, 18F, and 20F) exclusively contained *Klebsiella* species (>96% of the reads) and one sample (09F) exclusively contained *Fusobacterium*, except the unassigned and no-hit reads. Sample 03F was composed of *Prevotella* (40.3%), *Streptococcus* (23.2%), *Peptostreptococcus* (4.0%), *Dialister* (3.4%), and *Enterobacteriaceae* (3.5%). Sample 04F was composed of *Klebsiella* (7.6%), *Bacteroides* (8.5%), *Fusobacterium* (44.2%), and *Streptococcus* (23.2%). Sample 05F was composed of *Bacteroides* (37.8%) and *Streptococcus* (60.7%). Sample 11F was composed of *Fusobacterium* (77.7%) and *Prevotella* (10.6%), and sample 19F was composed of *Fusobacterium* (6.6%) and *Streptococcus* (92.4%). Two samples (12F and 16F) did not contain any assigned bacteria (Table 2).

Discussion

Recent advances have been made in interventional radiology, intensive care unit care, antibiotics, culture technique, and imaging devices for the diagnosis and treatment of PLA. However, PLA remains a life-threatening disease (Lee *et al.*, 2001; Yu *et al.*, 2004; Cerwenka, 2010; Malik *et al.*, 2010).

Previous reports shows that the most frequently isolated microorganisms are the polymicrobial *E. coli* and *Streptococcus* spp. (Corbella *et al.*, 1995; Lopez-Cano Gomez *et al.*, 2012). Although the cause is mostly a cryptogenic abscess, a highly virulent *K. pneumoniae* has emerged as a predominant cause of PLA in Asian countries (Li *et al.*, 2010; Siu *et al.*, 2011; Chung *et al.*, 2012; Liu *et al.*, 2013). *K. pneumoniae* was also the predominant cause of PLA in our study.

In our study, blood cultures were positive in 20% of patients and pus cultures were positive in 45% of patients. Several studies have revealed positive blood cultures in 10.2–55%, and positive pus cultures in 48–85% of patients (Zibari *et al.*, 2000; Malik *et al.*, 2010; Ali *et al.*, 2013). The positive rate of abscess cultures is typically higher than that of blood cultures (Cerwenka, 2010). The causative bacteria were not completely identified in the blood and pus cultures in the present study. We believe such results may have occurred due to the previous use of antibiotics and the pure pus (neutrophils) abscesses. PLA remains a serious illness with considerable morbidity and mortality (Ochsner *et al.*, 1938; Malik *et al.*, 2010). In our study, the hospital mortality rate was zero. However, in previous studies, the PLA mortality rate was 9–25% (Zibari *et al.*, 2000; Lopez-Cano Gomez *et al.*, 2012). Therefore, accurate and rapid identification of bacterial isolates and targeted antibiotics are needed to control PLA.

Conventional culture-based methods are used to identify bacteria in most hospitals. However, culture-based identification of bacteria is often unsuccessful because of slow-growing or unculturable bacteria present in the samples. Therefore, non-culture-based techniques (metagenomics) are required. Sequencing of conserved essential genes such as 16S rRNA is a powerful molecular identification method with the increasing availability of DNA sequencing and PCR (Denys and Carey, 1992; Lebrun *et al.*, 1992; Padhye *et al.*, 1992; Bourbeau *et al.*, 1997; Patel, 2001).

In our study, 16S rRNA gene sequencing had a 90% rate for identifying bacteria including unusual bacteria such as *Dialister*. However, conventional methods showed 20% and 45% positive rates respectively. Thus, 16S rRNA gene sequencing provides a more definite taxonomic classification for many organisms (Clarridge, 2004; Petti *et al.*, 2005; Mignard and Flandrois, 2006; Salipante *et al.*, 2013).

A number of unassigned reads (average, 26.8% per sample were revealed in our study. This result suggests that novel pathogen lineages may have been responsible for provoking and maintaining the polymicrobial environment contributing to liver abscess. *Staphylococcus caprae* and *Staphylococcus epidermidis* were cultured in blood and abscess isolates. We do not think that they were causative agents, but were a result of contamination during the aspiration procedure. *Peptostreptococcus*, *Dialister*, and genus-unassigned *Enterobacteriacea* of 03F sample couldn't be ruled out as the contaminants because of their relatively low percentage against assigned reads compared to others. However, these bacteria might be associated with occurrence of PLA because several researchers have isolated *Peptostreptococcus* and *Dialister* in body abscesses (Rousée *et al.*, 2002; Stein *et al.*, 2011). A great number of bacterial species inhabit superior and inferior surfaces of the human body (Huse *et al.*, 2012). However, limited bacterial species are found in a liver abscess. Our results demonstrated that rare species of bacteria can evade host defense barriers to form an abscess in deep tissues such as the liver. Thus, profiling of pathogenic species responsible for forming various tissue abscesses is prerequisite to understand the etiology and provide new therapeutic approaches against abscesses.

Our study had several limitations. First, 16S rRNA gene sequencing provides no information about antibiotic resistance. Second, gene sequencing is a relatively expensive identification method. Third, this method requires accurate and complete genetic databases.

We revealed bacterial communities responsible for producing PLA, which consisted of well-known genera and unknown bacteria. The known bacteria included *Klebsiella*, *Fusobacterium*, *Streptococcus*, *Bacteroides*, *Prevotella*, *Peptostreptococcus*, genus-unassigned *Enterobacteriaceae*, and *Dialister*. In addition, all PLA harbored large parts of sequences of unknown bacteria which could be novel lineages of bacterial pathogens related to PLA. Such new pathogen lineages must be characterized to identify the causative

agents of PLA.

In conclusion, our results provide a bacterial profile of PLA and information about the 16S rRNA sequences, which could be the basis for developing a molecular-based method to identify the etiological agent responsible for PLA and overcome above-mentioned obstacles in the hospital. In addition, we showed the superior utility of 16S rRNA sequencing compared to that of conventional cultured methods for an accurate identification of bacteria in patients with PLA.

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