·临床研究　Clinical research·

16S rRNA 基因测序技术在肝脓疡细菌鉴定中的作用

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【摘要】 **目的**　评价 16S 核糖体 RNA（rRNA）基因测序在肝脓疡中细菌鉴定中的应用价值。**方法** 2012 年 1 月—2013 年 12 月间共 20 例肝脓疡行经皮置管引流的患者，分别行脓液培养，血培养和 16S rRNA 基因测序。利用 454 GS Junior System 对脓液基因组 DNA 行 PCR 和 16S rRNA 基因测序。脓液培养，血液培养和 16S rRNA 基因测序结果进行分别评价。**结果**　脓液和血液培养阳性的患者分别是 9 例（45%）和 4 例（20%）。16S rRNA 基因测序细菌鉴定率为 90%，明显高于传统的培养方法。**结论**　16S rRNA 基因测序方法较传统的培养方法能更准确和有效对肝脓疡进行细菌鉴定。

【关键词】 肝脓疡；脓肿培养；宏基因组学；16S rRNA 基因测序

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Usefulness of 16S rRNA Gene Sequencing for Identification of Bacteria from Pyogenic Liver Abscess

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【**Abstract**】 **Background/Aims**　To evaluate the usefulness of 16S ribosomal RNA (rRNA) gene sequencing for an accurate and better identification of bacteria from pyogenic liver abscess (PLA). **Methodology**　20 patients with PLA were included who underwent percutaneous catheter drainage, abscess culture, blood culture and 16S rRNA gene sequencing for isolates from January 2012 to December 2013. Genomic DNAs of abscess fluids were subjected to PCR and sequencing of 16S rRNA gene by on a 454 GS Junior System. The results were evaluated between abscess cultures, blood and 16S rRNA gene sequencing for isolates. **Results**　Abscess and blood cultures were positive in 9 (45%) and 4 (20%) patients, respectively. The 16S rRNA gene sequencing showed with 90% identification of bacteria a significantly greater identification than conventional cultured methods. **Conclusion**　This study showed a greater usefulness of 16S rRNA gene sequencing than conventional cultured methods for accurate and better identification of bacteria from PLA. (J Intervent Radiol, 2014, 23: 906-912)

【**Key words**】 Pyogenic liver abscess; Abscess culture; Metagenomics; 16S rRNA gene sequencing

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INTRODUCTION

A liver abscess is still a severe disease with considerable mortality[1-3]. It is often polymicro-bial (PLA). Advanced imaging tools such as high resolution computed tomography (CT) and ultrasound device have improved a possible early diagnosis for PLA [4]. Actually, the treatment regimen is changing. Recently, the trend regarding initial treatment choice for PLA has changed into antibiotics and radiological intervention[3-4]. Therefore, an antibiotic treatment with an accurate identification of bacteria is the basis for PLA treatment.

Traditionally, the identification of bacteria was performed using phenotypic tests, including gram smear and biochemical tests as well as blood or abscess cultures. However, there are some limitations such as rare bacteria, slow-growing bacteria, uncultivable bacteria and culture-negative infection in those traditional identification methods. So the clinicians may have problems in the choice and duration of antibiotic treatments.

In the last decades, molecular-based tools with invention of PCR and DNA sequencing have been employed in the diagnostic fields. Nowadays, the most commonly used and commercially available method for the bacterial identification in clinical laboratories is 16S ribosomal RNA (rRNA) gene sequencing [5-8]. It has provided immense information regarding to microbial communities in human and in variety of animals with healthy or pathological conditions. However, the gene sequencing method is not routinely used in hospitals.

Therefore, the purpose of our study was to prospectively evaluate the usefulness of 16S rRNA gene sequencing for more accurate and better identification of the causative aetiology of PLA.

METHODOLOGY

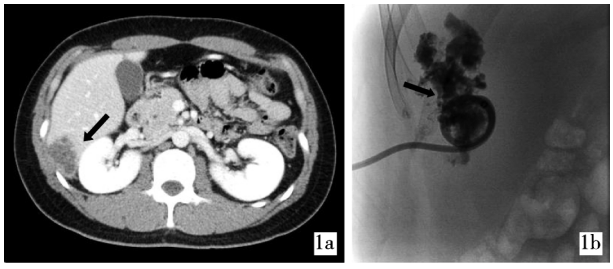
Patients

This prospective study was reviewed and approved by our Institutional Review Board 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. Two patients with hepatic abscess were excluded because the abscess was not large enough to be considered for percutaneous drainage. Finally, 20 patients were enrolled in this study. There were 9 male (45%) and 11 female (55%) patients. The mean age(± standard deviation) at the time of examination was 61.84 ± 13.21 years (age range 33 – 83 years).

The diagnosis of PLA was made on the basis of clinical and imaging findings with ultrasound or CT. We performed percutaneous drainage and antibiotic treatment in patients with pyogenic liver abscess in our hospital. All patients started with antibiotics prior to percutaneous abscess drainage. A blood sample was taken and sent to culture before the administration of antibiotics. In all cases, the time interval between antibiotic administration and drainage was less than 8 hours.

Intervention

All percutaneous procedures were performed under ultrasound (Acuson × 300, Sequoia 512, Siemens, USA) guidance. 18 G P.T.C Chiba needle (Unimed, Lausanne, Switzerland) with varying lengths was used for puncturing the abscess. 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 18 G needle and then a 0.035-inch wire (Terumo, Tokyo, Japan) was inserted into the abscess cavity. After serial dilatation, an 8.5 Frenchpigtail catheter (CooK, Bloomington, In, USA) was inserted into the abscess (Fig. 1). An ultrasonography of the liver performed 1 – 2 weeks after the insertion of a percutaneous drainage catheter. The drain catheter was removed when the abscess cavity had collapsed on follow-up ultrasonography and catheter output had decreased < 10 ml daily.



1a Contrast enhanced computed tomographic scan of the liver shows hypodense pyogenic liver abscess in segment VI (arrow) **1b** Spot image shows pyogenic liver abscess (arrow) with insertion of percutaneous drainage catheter

Fig. 1 43-year-old male with pyogenic liver abscess treated by insertion of percutaneous drainage catheter

Preparation of Genomic DNAs from Abscess effusions

Genomic DNAs from abscesses were prepared by the modification method described previously [9]. The abscess effusions of 500 ul were mixed by the same volume of TE buffer (pH 7.0). The abscess diluents were added with 15 U mutalysin (Sigma, St. Louis, MO) and 600 ug lysozyme(Genery, Shanghai, China) and then incubated for 1 hour at 37℃. The mixtures were treated with 10 ul 10% SDS solution and 20 ug RNase (RBC, Taipei, Taiwan) for 1 hour at 37℃ and then with 120 ug protease K (GeNet Bio, Daejeon, Korea) for 1 hour at 37℃. Thereafter, the mixtures were added with 1/10 volume of 5% cetyltrimethylammonium bromide (BDH Chemicals Ltd., Poole, England) - 0.5 M NaCl solution and allowed to stand for 1 hour at 37℃. The mixtures were added with the same volume of phenol-chloroform-isoamyl alcohol (25∶24∶1) solution and vigorously vortexed.

The mixtures were centrifuged for 10 minutes at 12 000 rpm and aqueous layers were subjected to choloroform extraction. After centrifugation the aqueous phase was collected and added with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes ethanol. The sample tubes were stored at −20℃ for 20 minutes and centrifuged for 10 minutes at 12 000 rpm. The pellets were washed with 1 ml of 70% ethanol, completely dried and dissolved with 30 ul of TE buffer.

Polymerase chain reaction

Extracted DNA of 60 ng was subjected to PCR amplification of the V1-V3 region of the 16S rRNA gene using AccuPower PCR PreMix (Cat. No K-2016, BiONEER, Daejon, Korea) containing 1 unit of Top DNA polymerase, 1 mM dNTPs, 10 mM Tris-HCl [pH9.0], 30 mM KCl, and 1.5 mM $MgCl_2$ with 27 F primer (5′-GAGTTTGATCCTGGCTCAG-3′) and 518R primer (5′-ATTACCGCGGCTGCTGG-3′). The 5'ends of forward primers were subsequently attached with an adaptor 1 sequence (5′-CCATCTCTCCCTGC-GTGTCTCCGAC-3′), a key sequence (TCAG), and sample-specific multiplex identifiers sequences. Reverse primer was added with an adaptor 2 sequence (5′-CCTATCCCCTGTGTGCCTTGGCAGTC-3′) and a key sequence (TCAG). The PCR reactions were carried out by a pre-denaturation for 4 seconds at 94℃ and 20 cycles of 30 seconds at 94℃, 30 seconds at 55℃ and 30 seconds at 72℃ and then followed by a final extension of 7 minutes at 72℃. Amplified DNAs were separated with 1% agarose gel and purified with GeneALL® ExpinTM PCR SV (Geneall Biotechnology Co. Ltd., Seoul, Korea).

Pyrosequencing and Data Analysis

The barcoded amplified DNAs of 20 samples were mixed with the same concentrations and applied to 454 pyrosequencing. Pyrosequencing was unidirectionally performed from the 27 F primer end 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 sequence quality was filtered. For 16S rRNA sequences, de-multiplexing, trimming and quality filtering was done using AmpliconNoise[10]. 16sRNA 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. The alignment results were further processed by the Metagenome Analyzer (MEGAN) program to statistically analyze the abundance of microorganisms

in each sample.

Statistical analysis

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

RESULTS

Blood and abscess cultures were processed for all 20 patients. Abscess cultures were positive in 9 (45%) patients; in which 5 (55.6%) were monomicrobial and 4(44.4%) were polymicrobial (≥ 2 organisms isolated). *Klebsiella pneumonia* was most commonly isolated from the abscess cultures. No culture was obtained in 11 patients. Blood cultures were positive in 4 (20%) patients and 50% of these presented as *Klebsiella pneumonia*. Two patients had concordant blood and abscess culture results and two had positive blood cultures with negative cultures in the abscess (Table 1).

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)

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

Genomic DNA was extracted from purulent fluids of abscesses. Of 63 207 reads remained after the processing, 46 261 reads (73.2%) were assigned in the existing 16S rRNA database. Other 16 937 reads (26.8%) were not assigned and couldn't be classified into known bacteria. Therefore they might represent novel lineages. Of a total of 46,261 assigned reads was Klebsiella the most abundant genus (74.7%) and the remained reads were classified to the genus *Fusobacterium*(9.3%), *Streptococcus*(5.0%), *Bacteroides*(1.9%), *Prevotella*(1.5%), *Peptostreptococcus* (0.1%), unassigned *Enterobacteriaceae* (0.1%) and *Dialister*(0.1%), respectively.

Of 20 abscess samples from patients with liver abscess, 12 samples (01 F, 02 F, 06 F, 07 F, 08 F, 10 F, 13 F, 14 F, 15 F, 17 F, 18 F, and 20 F) were exclusively occupied with *Klebsialla* species (more than 96% of reads) and 1 sample (09 F) was exclusively contained with *Fusobacterium* when excepted the unassigned and no hit reads. The sample 03F was composed of *Prevotella* (40.3%), *Streptococcus* (23.2%), *Peptostreptococcus* (4.0%), *Dialister* (3.4%) and *Enterobacteriaceae* (3.5%). The sample 04 F was composed of *Klebsiella*(7.6%), *Bacteroides* (8.5%), *Fusobacterium* (44.2%) and *Streptococcus* (23.2%). The sample 05 F was composed of *Bacteroides*(37.8%) and *Streptococcus* (60.7%). The sample 11 F was composed of *Fusobacterium* (77.7%) and *Prevotella* (10.6%) and sample 19 F was composed of *Fusobacterium*(5.1%) and *Streptococcus* (14.0%). Two samples (12 F and 16 F) did not contain any assigned bacteria (Table 2).

DISCUSSION

Recently there have been advances in interventional radiology, ICU care, antibiotics, culture technique and imaging devices in the diagnosis and treatment of PLA. However, it is still a life-threatening disease[1-4].

The most frequently isolated microorganisms were the polymicrobial *E. coli* and *Streptococcus spp.* and were polymicrobial[11-12]. Although the cause is mostly a cryptogenic abscess, a highly virulent *K. pneumoniae* had emerged as a predominant cause of PLA in Asian countries and areas recently[13-16]. Also, *K. pneumoniae* was the predominant cause of PLA in our study.

In our study, blood cultures were positive in 20% and pus cultures were positive in 45% of patients. Several studies revealed before positive blood cultures 10.2% – 55% and pus cultures in 48% – 85%(4,17,18). Usually, the positive rate of abscess cultures is higher than that of blood cultures[3]. The

Table 2 Pyrosequencing of the 16S rRNA genes in 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*	*Fusobacterium*	*Prevotella*	*Streptococcus*	*Peptostreptococcus*	*Dialister*	*Enterobacteriaceae*
			Reads/%[a]	Reads/%[a]	Reads/%[a]	Reads/%[b]	Reads/%[b]	Reads/%[b]	Reads/%[b]	Reads/%[b]	Reads/%[b]	Reads/%[b]	Reads/%[b]
01 F	1 558	1 263	965(76.4)	295(23.4)	3(0.2)	959(99.4)							
02 F	2 017	1 641	1 292(78.7)	349(21.3)		1 276(98.8)							
03 F	4 481	2 974	1 515(50.9)	1 459(49.1)					610(40.3)	352(23.2)	60(4.0)	51(3.4)	53(3.5)
04 F	3 153	2 443	1 617(66.4)	817(33.6)		123(7.6)	137(8.5)	715(44.2)		375(23.2)			
05 F	3 143	2 744	2 005(73.1)	738(26.9)	1(0.0)		757(37.8)			1 218(60.7)			
06 F	3 928	3 286	2 928(89.1)	358(10.9)		2 892(98.8)							
07 F	3 953	3 122	2 809(90.0)	312(10.0)	1(0.0)	2 722(96.9)							
08 F	254	241	162(67.2)	79(32.8)		160(98.8)							
09 F	4 462	3 409	2 949(86.5)	460(13.5)				2 943(99.8)					
10 F	4 363	3 600	3 172(88.1)	428(11.9)		3 142(99.1)							
11 F	3 265	3 004	650(21.6)	2 354(78.4)				505(77.7)	69(10.6)				
12 F	158	158	0(0.0)	158(100.0)									
13 F	3 519	3 372	3 265(96.8)	107(3.2)		3 215(98.5)							
14 F	4 779	3 800	2 955(77.8)	844(22.2)	1(0.0)	2 904(98.3)							
15 F	8 914	8 216	3 295(40.1)	4 921(59.9)		3 257(98.8)							
16 F	862	540	0(0.0)	540(100.0)									
17 F	6 971	6 365	5 556(87.3)	809(12.7)		5 445(98.0)							
18 F	9 598	7 224	5 685(78.7)	1 537(21.3)		5 607(98.6)							
19 F	4 022	2 884	2 530(87.7)	354(12.3)				129(5.1)		354(14)			
20 F	2 969	2 930	2 912(99.4)	18(0.6)		2 871(98.6)							
Unidentified	1 480												
Total	77 849	63 216	46 261(73.2)	16 937(26.8)	6(0.0)	34 573(74.7)	894(1.9)	4 292(9.3)	679(1.5)	2 299(5.0)	60(0.1)	51(0.1)	53(0.1)

Note: [a]a percentage against reads after processing, [b]a percentage against assigned reads

causative bacterial identification was not completely obtained in the blood and pus cultures in the present study. We believe such results may occur due to the previous use of antibiotics and pure pus (neutrophils) collected abscesses. PLA remains as a serious illness with a considerable morbidity and mortality until today[4,19]. In our study, the hospital mortality rate was zero. However, in previous studies, the PLA mortality rate remained high at 9% – 25 %[11,17]. Therefore, an accurate and rapid identification of bacterial isolates and targeted antibiotics are needed to control PLA.

Nowadays, conventional culture-based methods are used for the identification of bacteria in most hospitals. The methods are relatively inexpensive, but they have some limitations regarding rare, slow-growing and uncultivable bacterial identifications. Also, an identification of some particular bacteria, such as anaerobes and mycobacteria, would require additional equipment and expertise that are not available in most of hospitals. Therefore the clinicians have problems in the choice and duration of antibiotic treatments and the appropriate infection control procedures. Non-culture-based techniques (metagenomics) are required. There already exist identification methods by hybridization and sequencing. The hybridization method will be a valuable tool for bacterial identification, but the default of the method is to identify what an unknown isolate is. Sequencing is a more powerful molecular identification method with the increasing availability of DNA sequencing and PCR[20-24].

Today, 16S rRNA gene sequencing is used in clinical laboratories for routine identifications, especially for slow-growing, unusual or fastidious bacteria and also poorly differentiated bacteria by conventional methods. In our study, the 16S rRNA gene sequencing showed 90% identification of bacteria with unusual bacteria such as *Dialister*. However, the conventional methods showed positive in 20% and 45%, respectively. So this method can provide more definite taxonomic classification than culture-based approaches for many organisms[25-28].

The 16S rRNA gene exits universally among bacteria and includes regions with species specific variability. Also the 16S rRNA function has

constantly remained over a long period. Therefore, 16S rRNA gene sequencing makes it possible to identify bacteria to its genus or species level by comparison with databases in the public domain [20,25,29,30]. Also, previous authors have reported its use as a tool for bacterial identification[27,31-37]. This approach has been demonstrated to improve a more rapid recognition of novel isolates and the accuracy of organism identification compared to non-molecular testing[20,33,38].

In our study, there was a number of unassigned reads with an average of 26.8% per sample. This unassigned reads were not identified because these sequences were missing from the database. It suggests that novel lineages of pathogens might be one of the pathogenic members responsible for provoking and maintaining the polymicrobial environment contributing to an arising liver abscess. *Staphylococcus caprae* and *staphylococcus epidermidis* were cultured in blood and abscess isolates. We do not think that they were causative agents, but rather were a result of a contamination during aspiration procedure. A great number of bacterial species were inhabited in the superior and inferior surfaces of the human body[39]. However, limited bacterial species are found in a liver abscess only. It demonstrated that rare species of bacteria hold pathogenic mechanisms for evading or breaking host defense barriers to form an abscess in deep tissues like in the liver. So, the profiling of pathogenic species responsible for forming various tissue abscesses is prerequisite to understand etiologies and to provide new therapeutic approaches against abscesses.

There are many requirements for this 16S rRNA gene sequence method; i.e. reagent, instrumentation for amplification and sequencing, a database of known sequences and software for sequence editing and database comparison. However, commercial reagents are available, laboratory-developed assays for amplification and sequencing have been reported and there is an increasing number of commercial and public databases[20].

Our study had several limitations. First, 16S rRNA gene sequencing provides no information about antibiotic resistance. Second, gene sequencing is a relatively expensive method of identification. However, an introduction of more automated methods will decrease the costs of the sequencing method. Third, this method requires accurate and complete genetic databases.

In conclusion, this study showed the greater usefulness of 16S rRNA sequencing than of conventional cultured methods for more accurate and better identification of bacteria in patients with pyogenic liver abscess.

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