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Comparison of 16S Ribosomal RNA Targeted Sequencing and Culture for Bacterial Identification in Normally Sterile Body Fluid Samples: Report of a 10-Year Clinical Laboratory Review

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As 16S ribosomal RNA (rRNA)-targeted sequencing can detect DNA from non-viable bacteria, it can be used to identify pathogens from clinical samples even in patients pretreated with antibiotics. We compared the results of 16S rRNA-targeted sequencing and culture for identifying bacterial species in normally sterile body fluid (NSBF): cerebrospinal, pericardial, peritoneal and pleural fluids. Over a 10-year period, a total of 312 NSBF samples were evaluated simultaneously using 16S rRNA-targeted sequencing and culture. Results were concordant in 287/312 (92.0%) samples, including 277 (88.8%) negative and 10 (3.2%) positive samples. Of the 16 sequencing-positive, culture-negative samples, eight showed clinically relevant isolates that included *Fusobacterium nucleatum* subsp. *nucleatum, Streptococcus pneumoniae*, and *Staphylococcus* spp. All these samples were obtained from the patients pretreated with antibiotics. The diagnostic yield of 16S rRNA-targeted sequencing combined with culture was 11.2%, while that of culture alone was 6.1%. 16S rRNA-targeted sequencing in conjunction with culture could be useful for identifying bacteria in NSBF samples, especially when patients have been pretreated with antibiotics and when anaerobic infection is suspected.

Key Words: 16S ribosomal RNA, Sequencing, Culture, Diagnostic yield, Normally sterile body fluid

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The identification of pathogens by culture of normally sterile body fluid (NSBF) is crucial for accurate diagnosis of invasive infections, including meningitis, pericarditis, peritonitis, and empyema [1]. However, culture frequently fails to detect clinically important pathogens owing to stringent growth requirements or prior empirical antibiotic treatment [2]. In recent years, broad-

range PCR has been used in clinical laboratories to identify clinical pathogens from normally sterile sites, especially in cases of fastidiously slow growing bacteria and biochemically unidentifiable bacteria [3]. Although 16S ribosomal RNA (rRNA) PCR and targeted sequencing can produce false-positive results because of contamination, they remain useful for detecting bacterial infection [4]. In our clinical laboratory, 16S rRNA-targeted sequencing has been applied to tissue and fluid samples and has assisted in diagnosing culture-negative cases since September 2009. We retrospectively evaluated the clinical utility of 16S rRNA-targeted sequencing in comparison with culture for identifying bacterial species in NSBF samples. Although, the results of our study are consistent with those of a previous study [2], to our knowledge, this is the largest single center study that summarizes the results of 16S rRNA-targeted sequencing in NSBF samples. We reviewed the records for a decade to emphasize the clinical utility of 16S rRNA targeted sequencing.

A total of 312 NSBF samples from 248 patients collected from Mar 2009 to May 2018 were evaluated. Samples were submitted to the laboratory of Samsung Medical Center, Seoul, Korea for 16S rRNA-targeted sequencing in addition to culture. We analyzed 154 cerebrospinal fluid (CSF), 29 pericardial fluid, 24 peritoneal fluid, and 105 pleural fluid samples. Clinical data were reviewed retrospectively to assess the likelihood of infection, including clinical signs and symptoms, laboratory findings, radiological findings, and patient antibiotic therapy response. This study was approved by the Institutional Review Board (IRB) of Samsung Medical Center, Seoul, Korea (IRB No. 2018-12-012), and informed consent requirements were waived.

Samples were Gram-stained and cultured following standard laboratory procedures [5]. Four drops of each sample were directly inoculated on 5% sheep blood agar, chocolate agar, MacConkey agar, and Brucella agar (except for CSF) and incubated at 35°C in a 5% CO_2 incubator for 18–48 hours. For enrichment, residual samples were inoculated into thioglycolate broth and incubated for seven days. Some samples were also inoculated into BacT/ALERT blood culture bottles (Organon Teknika Corporation, Durham, NC). Microorganisms were further identified using the VITEK 2 system (bioMérieux, Marcy-l'Étoile, France) or by matrix-assisted laser desorption ionization time-of-flight mass spectrometry using the VITEK MS system (bioMérieux).

Nucleic acids were extracted from 200 µL of fresh samples using a MagNA Pure LC instrument (Roche Diagnostics, Mannheim, Germany) or MagNA Pure 96 instrument (Roche Diagnostics), according to the manufacturer's protocol. PCR was performed in a 20-µL sample containing DNA template, specific primers, and the AccuPower ProFi Taq PCR PreMix (Bioneer, Daejeon, Korea) after decontaminating the PCR master mix solutions including primers with a PCR decontamination kit (ArcticZymes, Tromsø, Norway). 16S rRNA was amplified using semi-nested PCR with two primer sets. The external primers were 4F (5′-TTGGAGAGTTTGATCCTGGCTC-3′) and 1,492R (5′-GGTTACCTTGTTACGACTT-3′), and the internal primers were 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 801R (5′-GGCGTGGACTTCCAGGGTATCT-3′) [6]. If indicated, alternative target genes, for instance, *tuf* and *gyrB*, were used to obtain species-level identification [7, 8]. Amplification conditions for the first and second rounds consisted of denaturation at 94°C for five minutes; 32 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for seven minutes. Sequencing was performed on an automated ABI Prism 3730 instrument using the BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). The 16S rRNA sequences were compared with those of reference strains in the NCBI GenBank database and the EzTaxon database (http: //www.eztaxon.org/). Sequencing results were interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI) MM18-A guidelines [6]. The 16S rRNA-targeted sequencing and culture results were compared, and the concordance rate was determined. Clinically relevant isolates were defined as bacteria identified by either 16S rRNA-targeted sequencing or culture when the patient with identified bacteria exhibited clinical manifestations, laboratory findings and/or radiological evidence of infection, and clinical improvement in response to antibiotic treatment [9, 10]. Clinically relevant isolates were categorized by two doctors based on the clinical information of each patient.

Of the 312 samples, 26 (8.3%) and 19 (6.1%) were positive for bacteria identification by 16S rRNA-targeted sequencing and culture, respectively; 10 (3.2%) were positive by both methods, 16 (5.1%) were positive by 16S rRNA targeted sequencing only, nine (2.9%) were positive by culture only, and the remaining 277 (88.8%) were negative for bacteria identification by both methods. The concordance rate between methods was 92.0% (287/312). Of the 25 discordant samples, nine sequencing-negative, culture-positive samples showed three coagulase-negative staphylococcal, two streptococcal, one *Enterococcus faecium*, and three *Enterobacteriaceae* isolates (Fig. 1). Of these, six were clinically relevant isolates (see Supplemental Data Table S1). Of the 16 sequencing-positive, culture-negative samples, eight showed clinically relevant isolates (Fig. 1). All these samples were obtained from the patients pretreated with empirical antibiotics

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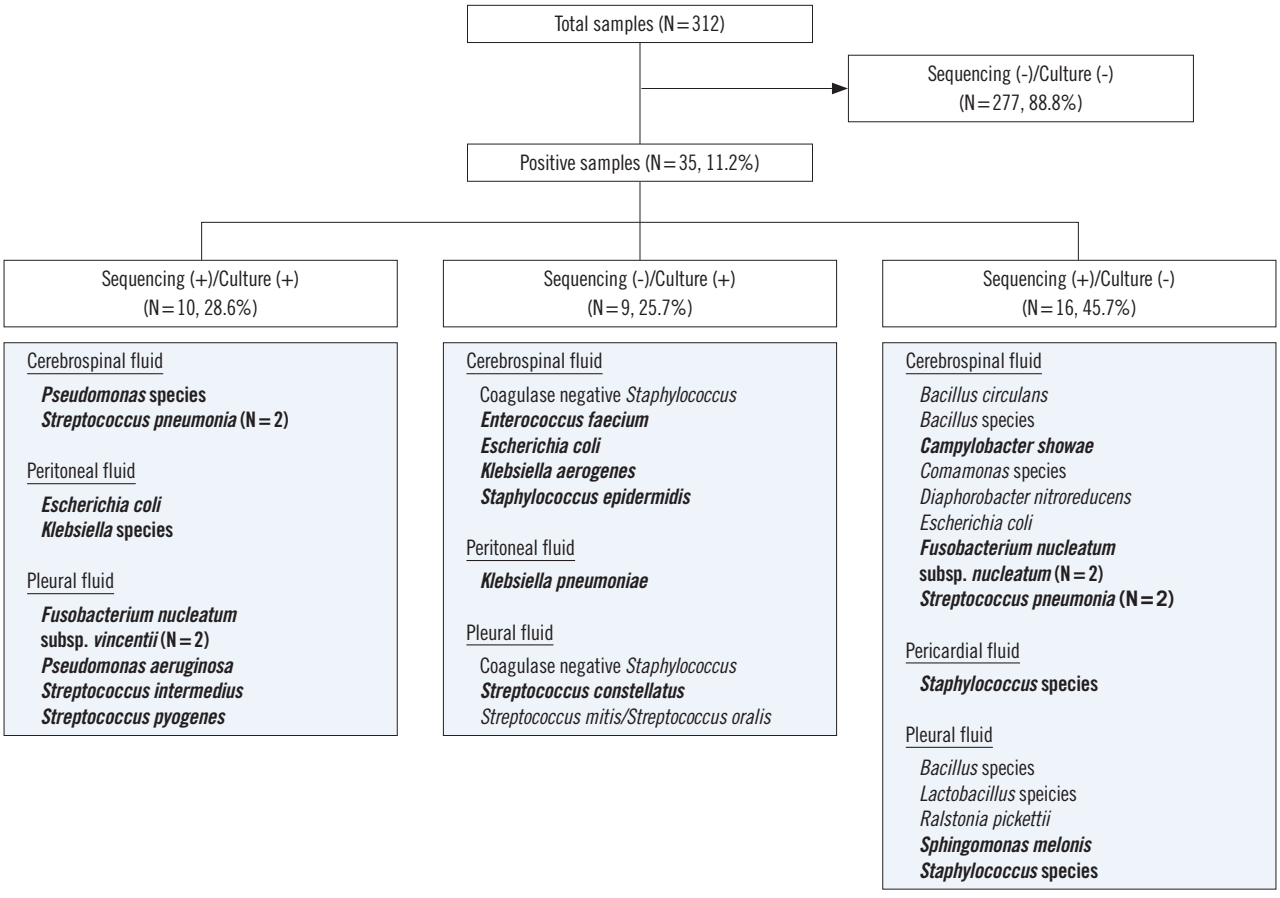

Fig. 1. Direct 16S ribosomal RNA sequencing versus culture for identifying bacteria in normally sterile body fluid samples (N=312). Clinically relevant isolates are indicated in bold.

Table 1. Clinically relevant isolates identified by only 16S ribosomal RNA-targeted sequencing in NSBF samples (N=8)

Sample	Bacteria identified by 16S ribosomal RNA gene PCR	% Identity	Antibiotic treatment prior to sampling	Treatment change after reporting	Final diagnosis
Cerebrospinal fluid	*Campylobacter showae*	569/569 (100%)	Ceftriaxone+Vancomycin	Ceftriaxone	Intraventricular abscess
	Fusobacterium nucleatum subsp. *nucleatum*	678/678 (100%)	Ampicillin+Ceftriaxone+Vancomycin	Ampicillin+Ceftriaxone	Meningitis
	Fusobacterium nucleatum subsp. *nucleatum*	445/445 (100%)	Cefepime+Vancomycin	Cefepime	Brain abscess with meningoencephalitis
	Streptococcus pneumoniae	724/728 (99.5%)	Ampicillin+Cefepime+Vancomycin	Ceftriaxone+Vancomycin	Pneumococcal meningoencephalitis
	Streptococcus pneumoniae	704/704 (100%)	Ceftriaxone+Vancomycin	Ampicillin+Ceftriaxone	Bacterial meningoencephalopathy
Pericardial fluid	*Staphylococcus* species	728/730 (99.7%)	Ceftriaxone+Gentamicin+ Rifampin+Vancomycin	Gentamicin+Nafcillin+ Rifampin	Pericarditis
Pleural fluid	*Sphingomonas melonis*	584/584 (100%)	Meropenem+Azithromycin	Meropenem+TMP/SMX	Bronchopneumonia with pleural effusion
	Staphylococcus species	477/489 (97.5%)	Azithromycin+Cefotaxime	TMP/SMX	Pleural effusion

Abbreviations: NSBF, normally sterile body fluid; TMP/SMX, trimethoprim/sulfamethoxazole.

(Table 1).

The distribution of clinical samples and identified species from the 35 samples positive by either 16S rRNA-targeted sequencing or culture or both is shown in Fig. 1. Most bacterial species were identified in CSF (N=18), of which *S. pneumoniae* was the clinically relevant species most frequently detected (N=4) by 16S rRNA-targeted sequencing. The second highest number of bacterial species were identified in pleural fluid (N=13); *F. nucleatum* subsp. *vincentii* was detected in these samples by both 16S rRNA-targeted sequencing and culture. All species isolated from pericardial (N=1) and peritoneal fluid samples (N=3) were clinically relevant.

Diagnostic yield increased from 6.1% (19/312) with culture to 11.2% (35/312) with the addition of 16S rRNA-targeted sequencing. Direct amplification and sequencing in clinical samples are especially useful for patients pretreated with antibiotics [11]. Consistent with this observation, all sequencing-positive, culture-negative samples were obtained from the patients with prior antibiotic treatment. Specifically, two of the four *S. pneumoniae* isolates (the most common bacterial meningitis pathogen [12]) were detected only by 16S rRNA-targeted sequencing. A retrospective review of the effects of parenteral antibiotic pretreatment in suspected *S. pneumoniae* meningitis suggested that CSF sterilization occurs only four hours after initiation of parenteral antibiotics [13]. Therefore, identifying pathogen DNA by 16S rRNA-targeted sequencing could be advantageous, especially in CSF samples when antibiotic pretreatment could affect CSF culture yield.

In this study, *F. nucleatum* subsp. *nucleatum* and *C. showae* were identified in CSF samples using 16S rRNA-targeted sequencing, but not using culture, because we do not routinely perform anaerobic culturing with CSF. *F. nucleatum* subsp. *nucleatum* causing several systemic infections and *C. showae* with unknown significance of pathogenicity are rarely isolated anaerobic gram-negative rods that are primarily involved in periodontal diseases [14, 15]. Similar to a previous study, we found that 16S rRNA-targeted sequencing is particularly valuable for identifying anaerobic pathogens that are difficult to culture [16].

For sequencing-positive, culture-negative samples, we also considered the possibility of false-positive 16S rRNA-targeted sequencing results due to contamination in the DNA extraction kit, PCR reagents, or samples [17]. Based on a thorough review of the 16 sequencing-positive, culture-negative samples, eight were inconsistent with the clinical context, suggesting contamination. Of these, *Ralstonia pickettii* is a common contaminant in DNA extraction kits [18], and it was isolated from the pleural fluid of a patient with invasive pulmonary aspergillosis. The false-positive results can be derived from contaminants or nonviable bacteria and seem to be an inherent feature of PCR. Therefore, clinical correlation would be needed when a false-positive result is suspected.

Out of the nine sequencing-negative, culture-positive samples, six clinically relevant isolates were recovered from patients with bacterial meningitis, liver abscess, or pneumonia with combined empyema. Of these, two isolates, *Klebsiella pneumoniae* and *S. constellatus*, were recovered from a blood culture bottle, and one *E. faecium* isolate was recovered from an enrichment culture with thioglycolate broth. The false-negative sequencing results could be due to low microbial concentration and/or presence of PCR inhibitory substances in the samples subjected to 16S rRNA-targeted sequencing. Inhibitory substances may be present in the original sample and also may be unintentionally added as a result of the sample processing and DNA extraction from reagent [19].

This study has several potential limitations. First, the positive rates for culture were relatively low compared with those in a previous report, in which culture recovered 78.8% and 84.6% of significant isolates from peritoneal and pleural fluids [20]. The positive rates from culture in our study were 5.2% (8/154), 12.5% (3/24), and 7.6% (8/105) for CSF, peritoneal fluid, and pleural fluid samples, respectively. The main reason for low positive rates is that we included not only samples from the initial work-up but also follow-up samples obtained during empirical antibiotic treatment. In addition, many samples were collected from patients with a low probability of infection. Second, owing to the retrospective design of this study, we determined the clinical relevance of the isolates based solely on recorded, clinically important characteristics.

Despite these limitations, to our knowledge, this is the largest-scale single center study that summarizes the results of 16S rRNA-targeted sequencing in NSBF samples. We demonstrated that 16S rRNA-targeted sequencing in conjunction with culture can be useful for identifying the etiological agent in NSBF samples, especially when patients have been pretreated with antibiotics and when anaerobic infection is suspected.

Author Contributions

All authors have accepted their responsibility for the entire content of this manuscript and approved submission.

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Conflicts of Interest

None declared.

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Supplemental Data Table S1. Clinically relevant isolates identified only by culture in normally sterile body fluid samples (N=6)

Body fluid	Gram staining result	Bacteria identified by culture	Antibiotic treatment prior to sampling	Treatment change after reporting	Final diagnosis
Cerebrospinal fluid	No microorganisms observed	*Klebsiella aerogenes*	Ceftazidime+Vancomycin	Meropenem	Bacterial meningitis
	No microorganisms observed	*Enterococcus faecium*	Meropenem+Vancomycin	Vancomycin	Bacterial meningitis
	Gram-negative bacilli	*Escherichia coli*	Meropenem	Meropenem	Relapsed meningitis with *E. coli*
	No microorganisms observed	*Staphylococcus epidermidis*	Cefotaxime+Vancomycin	Cefotaxime+Vancomycin	Bacterial meningitis after craniotomy and tumor removal
Peritoneal fluid	Gram-negative bacilli	*Klebsiella pneumoniae**	Cetriaxone	Tazoferan	Liver abscess
Pleural fluid	Gram-positive cocci	*Streptococcus constellatus**	Cefotaxime+Vancomycin	Ampicillin/sulbactam	Pneumonia with combined empyema

*These were obtained from a blood culture bottle.

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