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

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-
Targeted sequencing vs culture in body fluids

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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.
**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 meningocerephalitis   |
|                 | *Streptococcus pneumoniae*                      | 724/728 (99.5%) | Ampicillin+Cefepime+Vancomycin       | Ceftriaxone+Vancomycin           | Pneumococcal meningocerephalitis        |
|                 | *Streptococcus pneumoniae*                      | 704/704 (100%) | Ceftriaxone+Vancomycin                | Ampicillin+Ceftriaxone           | Bacterial meningocerephalopathy         |
| 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*                        | 472/489 (97.5%) | Azithromycin+Cefotaxime                | TMP/SMX                         | Pleural effusion                         |

Abbreviations: NSBF, normally sterile body fluid; TMP/SMX, trimethoprim/sulfamethoxazole.
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 picketti 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.
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 |
|                  |                      | Enterococcus faecium | Meropenem+Vancomycin | Vancomycin | Bacterial meningitis |
|                  | Gram-negative bacilli | Escherichia coli | Meropenem | Meropenem | Relapsed meningitis with E. coli |
| Pleural fluid    | Gram-positive cocci  | Streptococcus constellatus* | Cefotaxime+Vancomycin | Ampicillin/sulbactam | Pneumonia with combined empyema |

*These were obtained from a blood culture bottle.