Evaluation of direct 16S rRNA PCR from clinical samples for bacterial detection in normally sterile body sites

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Abstract
Introduction: In addition to antibiotic treatment, slow-growing and non-cultivable bacteria can lead to false-negative results for sterile body site infections. In this study, we investigated the efficacy of 16S rRNA polymerase chain reaction (PCR) for such infections.

Methodology: Following routine culture procedures, 16S ribosomal RNA (16S rRNA) PCR was performed for samples collected from sterile body sites between July 2017 and September 2018. The samples were separated into two groups for likely (group 1) and unlikely infections (group 2) based on clinical and laboratory findings, as well as clinician opinion. Sequence analysis was performed for PCR-positive samples using 16S rRNA primers. Mixed chromatograms were analyzed with the RipSeq Mixed program, and Stata 15.1 was used for statistical analysis.

Results: Eighty-seven of 139 samples collected from 116 patients were placed in group 1, and 52 were placed in group 2. Compared with culture as the reference method, the sensitivity, specificity, positive predictive value, and negative predictive value for 16S rRNA PCR were 89.8%, 85.6%, 77.2%, and 93.9%, respectively. 16S rRNA PCR identified infections in 13 culture-negative samples. Among these, three had Bartonella quintana, Mycoplasma salivarium, and Mycobacterium avium complex infections, which cannot be detected with commercial multiplex PCR kits.

Conclusions: Our study demonstrates that 16S rRNA PCR is effective for the diagnosis of sterile body site infections, especially for cases of meningitis and infective endocarditis where routine cultures fail.

Key words: Bacterial infections; 16S rRNA; polymerase chain reaction; culture; body fluids, tissue.


Introduction
Infections at sterile body sites have life-threatening consequences, making fast and accurate diagnosis critical [1]. Uncultivable bacteria, a low number of microorganisms, or the use of antimicrobial drugs can cause difficulties in the isolation of microorganisms [2]. In recent years, molecular detection methods have become very popular, as they are fast and highly sensitive [3-5]. However, commercial molecular detection assays can only identify specific targets and ignore other microorganisms, limiting their diagnostic capability [6].

The detection of 16S ribosomal RNA (16S rRNA) genes through polymerase chain reaction (PCR) amplification is a widely accepted method for the identification of bacteria [7-8]. As this gene encodes the RNA component of the 30S subunit of the bacterial ribosome and is present in all bacteria, sequence analysis of 16S rRNA can be used for taxonomic studies and bacterial species identification [9-12].

In this study, we aimed to evaluate the performance of 16S rRNA PCR by comparing the results with those obtained from culture.

Methodology
Sample analysis and ethics
This prospective study was performed using samples from sterile body sites collected from patients at all Marmara University Hospital clinical departments and sent to the microbiology laboratory between July 2017 and September 2018. The peritoneal, pleural, pericardial, cerebrospinal, synovial, and vitreous fluids and tissue, biopsy, and abscess samples were taken from sterile sites, immediately placed in a sterile container, and transported to the microbiology laboratory. The collected samples were primarily used for routine culture analysis and were only included in this study if sufficient sample remained for 16S rRNA PCR. Samples from drains, chest tubes, and shunts and samples that were insufficient for PCR were excluded.
Since the samples included in the study consisted of tissue remaining after routine culture analysis, informed consent was not obtained from the patients. Clinical manifestations, radiological findings, biochemical parameters (white blood cell count, C-reactive protein, procalcitonin, etc.), microbiological findings (presence of leukocytes and bacteria in gram examination, serology parameters, previous culture results), and antibiotic usage were evaluated. Samples judged by physicians to have a high likelihood of bacterial infection, according to these parameters, were placed in the likely infection group. The sensitivity and specificity of PCR were calculated with bacterial culture as the reference. The samples were separated into two aliquots for culture and PCR in class 2 biosafety cabinets, and the culture and pretreatment steps for PCR were performed in these cabinets to prevent contamination.

This study was approved by the Marmara University Clinical Research Ethics Committee (Decision No: 09.2017.312).

**Microbiological methods**

**Culture**

Gram-stained slides were examined under a light microscope to identify the presence of leukocytes and microorganisms. Four solid agar plates (5% sheep blood agar (bioMérieux, Marcy l’Etoile, France), Mac Conkey agar (bioMérieux, Marcy l’Etoile, France), chocolate agar (bioMérieux, Marcy l’Etoile, France) and brucella blood agar (Difco, Detroit, Michigan, USA) supplemented with hemin and vitamin K) were used, with thioglycollate broth as the enriching liquid medium. All agar plates were incubated for five days at 37°C. The 5% sheep blood agar, Mac Conkey agar, and chocolate agar samples were incubated in an aerobic environment while the brucella blood agar samples were incubated in an anaerobic environment (anaerobic jar or anaerobe cabinet). Subcultures with the liquid media were performed for samples that showed growth in the liquid media but no growth in the solid media. Culture-positive samples were identified using conventional methods and MALDI-TOF MS (bioMérieux, Marcy l’Etoile, France). After the culture process, all samples were stored at −80°C until 16S rRNA PCR.

**16S rRNA PCR and sequence analysis**

Genomic DNA was extracted from all samples using the DNeasy Blood and Tissue kit (Qiagen, Valencia, California). Different pretreatment and lysis steps were performed for different sample types. Bone biopsies, tissue, and viscous samples were prepared, with modifications made according to the manufacturer’s protocol. These samples were homogenized using MagNA Lyser (Roche, Mannheim, Germany) and MagNA Lyser green bead tubes. Each sample was transferred to a MagNA Lyser green bead tube, and 350 µL bacterial lysis buffer was added. After homogenization at 7,000 rpm for 60 seconds, cooling was performed (2°C–8°C for 90 seconds). The steps were repeated three times, and centrifugation was performed at 14000 × g for 1–3 minutes [13]. Afterward, 20 µL proteinase K was added to the samples.

For sterile body fluids, 500 µL of the sample was centrifuged for 10 minutes at 5000 × g (7500 rpm) [14]. After centrifugation, 180 µL buffer ATL and 20 µL proteinase K were added to the bacterial pellet.

Pre-prepared specimens were incubated at 56°C for 3 hours. Subsequently, DNA extraction was performed using a 50 µL elution volume according to the manufacturer’s protocol. The DNA region encoding the 16S rRNA gene of the isolates was amplified by PCR using universal 8UA and 907B primers. The amplification products were visualized after electrophoresis on a 1% agarose gel, and the products with the correct band size were sent to the identification laboratory (GATC Biotech/Germany) for sequence analysis (Figure 1).

Positive and negative controls were added to each PCR cycle. Sterile water was used for the negative control, and ATCC 25922 *Escherichia coli* DNA was used as the positive control. Amplification of the gene for human glyceraldehyde-3-phosphate dehydrogenase was performed on negative samples for DNA isolation and PCR inhibition. Healthy human blood was added to serial dilutions of ATCC 25922 *E. coli* and ATCC
**Staphylococcus aureus** to determine the detection limit for 16S rRNA PCR.

The positive PCR products were sequenced with the ABI prims 3730XL genetic analyzer (Eurofins Genomics / GATC Biotech, Konstanz, Germany) and the sequences were evaluated using data from GenBank (www.ncbi.nlm.nih.gov). The data were compared between the 16S rRNA gene sequences in the GenBank database, and ≥ 98% similarity was used for species level identification [15]. The RipSeq Mixed web application (Pathogenomix, Santa Cruz, California, USA) was used to analyze mixed chromatograms. This program makes it possible to analyze up to three different bacteria at the species level based on the 16S rRNA gene profile [16].

**Statistical analysis**

Statistical analysis of the data was performed using the Stata 15.1 program. A value of \( p < 0.05 \) was considered statistically significant.

**Results**

Our study included 139 samples from 116 patients. Of these samples, 87 (63%) were classified as likely infections, and 52 (37%) were classified as unlikely infections, according to clinical, radiological, and laboratory findings. The sample types, PCR, and culture results are given in Table 1. The detection limit of PCR was \( 10^3 \) cfu/ml for standard strains (Figure 2). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 16S rRNA PCR were 89.8% (with 95% confidence interval (CI) 77.8–96.6%), 85.6% (with 95% CI 76.6–92.1%), 77.2% (with 95% CI 64.2–87.3%), and 93.9% (with 95% CI 86.3–98%), respectively, compared with culture, which was the reference method.

**Analysis of PCR-positive, culture-negative results**

16S rRNA PCR identified bacterial infection in 13 samples that were culture-negative (Table 2). As shown in the table, six patients (patients 14, 21, 56, 57, 71, and 79) were referred to our emergency department with meningitis findings. Antibiotic treatment was initiated before lumbar punctures were performed for all but two patients (patients 14 and 56).

Three patients (patients 13, 42, and 116) underwent surgery for infective endocarditis, and the heart valves were sent for microbiological analysis. *S. aureus* was isolated from the blood cultures of two of these patients before surgery. All heart valve cultures were negative, but *S. aureus* was detected with PCR in the heart valves of the two patients mentioned above. Pre-operative blood culture from patient 116 was negative, and the patient was treated empirically with ampicillin sulbactam. Despite antimicrobial therapy, the size of the vegetation continued to increase. Hence, surgical treatment was performed, and the heart valve was sent to our laboratory. Heart valve culture was also negative, but *Bartonella quintana* was detected with 16S rRNA PCR.

One patient (patient 26) diagnosed with osteomyelitis had been treated with cefazolin at a pediatric clinic because of previous *S. aureus* isolation

### Table 1. PCR and culture results for samples in likely and unlikely infection groups.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Group 1a</th>
<th>Group 2b</th>
<th>Group 1c</th>
<th>Group 2c</th>
<th>Group 1d</th>
<th>Group 2d</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF (n)</td>
<td>9</td>
<td>-</td>
<td>8</td>
<td>1</td>
<td>20</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Other body fluids (n)</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Tissue-biopsy (n)</td>
<td>6</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Abscess (n)</td>
<td>3</td>
<td>4</td>
<td>10</td>
<td>27</td>
<td>15</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>4</td>
<td>20</td>
<td>29</td>
<td>42</td>
<td>15</td>
<td>7</td>
</tr>
</tbody>
</table>

C: Culture, CSF: Cerebrospinal fluid, (+): Positive, (-): Negative; aSamples in likely infection group; bSamples in unlikely infection group; cPeritoneal, pleural, pericardial, synovial and vitreous fluids.
in tissue cultures. Since there was no regression with treatment, the patient underwent surgery, and tissue samples were taken from three different sites in the affected bone and sent to our laboratory. *S. aureus* was isolated from only one of the three samples in the first part of the inoculated medium. However, using 16S rRNA PCR *S. aureus* was detected in all three samples using 16S rRNA PCR. Antibiotic treatment for the patient was modified in light of these findings.

Patient 72, diagnosed previously with AIDS, was admitted to the emergency room with fever and shivering, and a bone marrow biopsy was sent to our laboratory. *Mycobacterium avium complex* was detected with 16S rRNA PCR.

The pleural fluid from patient 95, who was diagnosed with empyema, was one of the culture-negative PCR-positive samples. Gram staining of the sample showed many polymorphonuclear leukocytes with no microorganisms. Although culture was negative on conventional media, *Mycoplasma salivarium* was detected by PCR.

**Analysis of PCR-negative, culture-positive results**

In the likely infection group, three samples were culture-positive only. These samples were from synovial fluid, mediastinal tissue, and brain tissue, and *Staphylococcus epidermidis* was isolated in all three. Two of these were isolated from solid and liquid media (only in the first part of the solid media), and one was isolated from only liquid media.

**Polymicrobial infections**

In three cases, the 16S rRNA PCR results contained mixed chromatograms, which were resolved using the RipSeq Mixed program. In the first case, four bacteria (*Bacteroides fragilis*, *Fusobacterium necrophorum*, *Slackia spp.*, and *Bacteroides spp.*) were isolated from cerebrospinal fluid (CSF) culture, but the molecular detection method only identified two of them (*Bacteroides spp.* and *Fusobacterium spp.*). In the second case, *Klebsiella pneumoniae* was only isolated from brain abscess culture, whereas 16S rRNA PCR showed the presence of polymicrobial infection (*K. pneumoniae* and *E. coli*). The third case was that of a patient with liver abscess from which *Streptococcus intermedius* was isolated in culture, whereas 16S rRNA PCR detected both *S. intermedius* and *Fusobacterium nucleatum*.

**Discussion**

Infections at sterile body sites should be diagnosed and treated immediately due to the risk of severe morbidity and resulting into mortality [17]. Broad-range 16S rRNA PCR performed with clinical specimens may identify bacteria that cannot be grown in culture [18]. In our study, the sensitivity and specificity of PCR were found to be 89.8% and 85.6%, respectively, compared with culture. In the literature, studies investigating the efficacy of 16S rRNA PCR for the diagnosis of sterile body site infections. However, the sensitivity and specificity of this approach differ between the studies. In a more recent study with a sample size of 32, the sensitivity, specificity, PPV, and NPV of 16S rRNA PCR were reported to be 58%, 85%, 70%, 77.2%, respectively, in comparison to blood culture [19]. Grif *et al.* investigated the use of 16S rRNA PCR for the detection of bacterial pathogens in normally sterile body sites and reported the diagnostic sensitivity and specificity of the PCR as 88.5% and 83.5%, respectively, similar to our study [3]. Wellinghausen *et al.* also compared 16S rRNA PCR with blood culture for the diagnosis of bloodstream infections and reported a sensitivity of 87% with 342 blood samples [20].

**Table 2. 16S rRNA PCR-positive, culture-negative samples.**

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Diagnosis</th>
<th>Sample</th>
<th>Culture</th>
<th>16S rRNA PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>Meningitis</td>
<td>CSF</td>
<td>-</td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>21</td>
<td>Meningitis</td>
<td>CSF</td>
<td>-</td>
<td><em>Neisseria meningitidis</em></td>
</tr>
<tr>
<td>56</td>
<td>Meningitis</td>
<td>CSF</td>
<td>-</td>
<td><em>S. pneumoniae</em></td>
</tr>
<tr>
<td>57</td>
<td>Meningitis</td>
<td>CSF</td>
<td>-</td>
<td><em>S. pneumoniae</em></td>
</tr>
<tr>
<td>71</td>
<td>Meningitis</td>
<td>CSF</td>
<td>-</td>
<td><em>N. meningitidis</em></td>
</tr>
<tr>
<td>79</td>
<td>Meningitis</td>
<td>CSF</td>
<td>-</td>
<td><em>S. pneumoniae</em></td>
</tr>
<tr>
<td>13B</td>
<td>Infective endocarditis</td>
<td>Heart valve</td>
<td>-</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>42</td>
<td>Infective endocarditis</td>
<td>Heart valve</td>
<td>-</td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td>116</td>
<td>Infective endocarditis</td>
<td>Heart valve</td>
<td>-</td>
<td><em>Bartonella quintana</em></td>
</tr>
<tr>
<td>26A</td>
<td>Osteomyelitis</td>
<td>Bone abscess</td>
<td>-</td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td>26C</td>
<td>Osteomyelitis</td>
<td>Tissue</td>
<td>-</td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td>72</td>
<td>AIDS</td>
<td>Bone marrow biopsy</td>
<td>-</td>
<td><em>Mycobacterium avium complex</em></td>
</tr>
<tr>
<td>95</td>
<td>Empyema</td>
<td>Pleural fluid</td>
<td>-</td>
<td><em>Mycoplasma salivarium</em></td>
</tr>
</tbody>
</table>

CSF: Cerebrospinal fluid, AIDS: Acquired Immune Deficiency Syndrome.
We performed culture and 16S rRNA PCR for 139 samples taken from sterile body sites. All clinical, laboratory, and radiological findings were gathered, and the samples were classified under likely and unlikely infections. Culture was used as the gold standard for this study. Thirteen culture-negative PCR-positive samples were found in the likely infection group. Six of these samples were from the CSF, and according to the clinical data, four of the patients were given antibiotics before the lumbar puncture was performed. Previous studies have indicated that the CSF is free of microorganisms within 2–6 hours after the administration of antibiotics; thus, false-negative results can occur in subsequent culture [21]. According to Brouwer et al. [22], CSF culture was positive for only one in 10 patients previously treated with antibiotics in developing countries. Afifi et al. also reported that the rate of culture-positive CSF samples was low (8%) for suspected cases of bacterial meningitis [23].

Another problematic area for clinical microbiology laboratories is blood and heart valve cultures from infective endocarditis cases. Up to 30% of samples from these patients are culture-negative, mostly due to the presence of atypical microorganisms and/or antimicrobial use [19]. Peeters et al. found that the sensitivity of 16S rRNA PCR (87%) was much higher than that of valve culture (26%) and suggested that the molecular approach was particularly useful for patients with culture-negative infective endocarditis [24]. In our study group, especially for patient 116, 16S rRNA PCR was the most important diagnostic tool for B. quintana, an agent of culture-negative infective endocarditis. Treatment for this patient was changed to doxycycline. Similarly, in a recent study, the treatment regimen for three of eight patients with infective endocarditis was modified based on the 16S rRNA PCR results [19].

Another prominent sample with PCR-positive culture-negative results was the pleural fluid, in which M. salivarium was detected by PCR. Since this bacterium has no cell wall, it was not detected in gram staining or isolated in routine culture media. Only one case of M. salivarium in the pleural fluid with empyema has been reported in the literature [25]. The second case was found in our study, diagnosed by means of molecular detection.

Three samples categorized under likely infection were PCR-negative but culture-positive. In these samples, bacteria were isolated only in a liquid medium or only in the first part of the inoculated solid medium. This result can be attributed to the detection limit of PCR, which was 10³ cfu/ml in our study. Xu et al. performed 16S rRNA PCR with the QIAamp Blood kit (Qiagen) for acute meningitis cases and reported a similar detection limit of this kit for S. aureus and E. coli [26].

We detected polymicrobial infections in three samples using either culture or PCR in our study group. For two of these samples, the cultures were positive only for one type of bacteria. The other two types of bacteria were detected with PCR with the aid of the RipSeq Mixed web program, which can analyze mixed chromatograms.

For sterile body sites, 16S rRNA PCR can provide a positive or negative result within 24 hours with a PPV of 77.2% and NPV of 93.9%. However, a positive result can take approximately one week for identification at the species level using sequence analysis. This is one limitation of our study. Additionally, PCR can detect the remains of dead bacteria after antibiotics intake, leading to false-positive results [27]. In our study, some patients were treated with antibiotics before sample collection. Therefore, we cannot rule out the possibility that PCR may have detected dead bacteria. This is another limitation of our study.

**Conclusion**

Microorganisms, such as Bartonella spp., Mycoplasma spp., and atypical mycobacteria, which are difficult to grow in routine culture, can be detected with 16S rRNA PCR. Broad-range 16S rRNA PCR appears to be highly effective, especially for patients with meningitis or infective endocarditis who previously underwent antibiotic therapy. We recommend the use of 16S rRNA PCR for samples taken from sterile body sites, as this method appears to be beneficial for patients with a high clinical suspicion of infection and negative culture results. However, culture remains the gold standard, and PCR should always be performed together with culture because of its inability to differentiate between dead and live bacteria and the possibility of false-positive results.

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