Detection of Bacteremia in Emergency Department Patients at Risk for Infective Endocarditis Using Universal 16S rRNA Primers in a Decontaminated Polymerase Chain Reaction Assay

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Prompt definitive diagnosis of acute bacterial endocarditis in febrile injection drug users (IDUs) remains problematic because of delays associated with blood culture. Rapid detection of bacteremia by polymerase chain reaction (PCR) by use of “universal” primers has been hampered by background bacterial contamination. Broad-range eubacterial primers selected from the 16S rRNA gene were used in a PCR assay coupled with a simple pre-PCR decontamination step. All PCR reagents were pretreated with the restriction enzyme AluI, which has multiple digestion sites in the amplicon but none in the primer sets. When 4 different bacterial species were spiked into healthy human blood specimens, the assay identified each pathogen with an analytic sensitivity of 5 bacteria/PCR reaction. A clinical trial with 51 febrile IDUs revealed that PCR had a sensitivity and specificity of 86.7% and 86.9%, respectively, versus blood culture. Importantly, all (8/8) patients with blood culture–positive infective endocarditis were determined to be positive by PCR. This assay provides a promising diagnostic for rapid identification of bacteremia, particularly valuable in acute care settings.

Infective endocarditis (IE) is one of the most serious medical complications associated with febrile injection drug users (IDUs), with significant morbidity and mortality occurring in unrecognized cases. Presenting clinical features associated with IE are usually nonspecific. Since fever occurs in ~98% of IDUs with IE, current practice is to hospitalize all febrile IDUs who present to hospital emergency departments for further inpatient evaluation [1].

A pivotal diagnostic feature of IE is accurate identification of bacteremia. Blood culture, the current “gold standard” test, has several inherent limitations, including lengthy time delays to results (typically at least 24–48 h), relatively low sensitivity (30%–50%) among patients who meet criteria for sepsis syndrome, and diminished sensitivity in patients already on antibiotics [2].

Polymerase chain reaction (PCR) is a technique that allows for rapid nucleic acid amplification and detection of small amounts of target pathogens (e.g., bacterial RNA or DNA). Molecular diagnostics for clinical use have shown promise when primers for a specific pathogen are used in selected clinical settings [3]. Numerous investigations also have been carried out by use of universal primers from the highly conserved regions of the 16S rRNA gene, which allows for amplification of all bacterial species. Several recent studies indicate that PCR may be useful for detection of bacteria in highly infected tissue specimens (e.g., resected heart valves or skin abscesses) [4, 5]. Less-compelling findings have been described with universal screening of blood samples, most commonly due to contaminant bacterial DNA [6–8].

In this study, we describe refinement and application of a method for removing background bacterial DNA from a universal PCR assay, which uses primers from a highly conserved region of the 16S rRNA gene. The decontamination procedure was first developed in an in vitro system, with spiked bacterial DNA and human whole blood. Results from a prospective clinical study that used this assay on human whole blood specimens from febrile IDUs in an urban emergency department are reported.
Materials and Methods:

Study design. This was a prospective identity-unlinked investigation. Excess blood was retained from patients aged <18 years who presented to the Johns Hopkins Hospital Emergency Department and had blood samples drawn for culture. Enrolled patients were assigned a unique study number to code excess blood, laboratory, and descriptive data. After coding, all patient identifiers were deleted.

Patients and sample collection. From 25 December 1999 to 8 July 2000, febrile IDUs had a 3 mL aliquot of excess whole blood collected simultaneously with the standard 2 sets of blood cultures, set aside for PCR assay. Samples were collected in a sterile fashion. Blood samples obtained for culture were inoculated into aerobic and anaerobic bottles (BACTEC; Beckton Dickinson) and were processed in the clinical microbiology laboratory. The 3-mL whole blood aliquots were inoculated into Na2-EDTA tubes and were stored at 4°C for batched, identity-unlinked PCR analysis. For assay refinement and control purposes, 3 mL of blood was collected from healthy volunteers by use of the same standard sterile techniques.

DNA extraction. Bacterial colonies from Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, and Enterococcus faecalis were obtained from clinical isolates and were resuspended in diethyl pyrocarbonate (DEPC) water. Each of the 4 bacterial samples was spiked into whole blood specimens taken from healthy volunteers, to optimize the DNA extraction and the PCR assay. Bacterial colonies from representative pathogens of the HACEK group (Hae-mophilus aphrophilus, Cardiobacterium hominis, Eikenella corrodens, and Kingella kingae) acquired from clinical isolates also were spiked into whole blood specimens to demonstrate the potential use of the PCR assay for early detection of the slow-growing pathogens that cause IE.

DNA was extracted from whole blood samples by use of a phenol-chloroform-isoamyl alcohol procedure. Then, 200 µL of whole blood was diluted in 450 µL of DEPC water and incubated at room temperature for 10 min, and 40 µL of proteinase K and 15 µL of 5.0 mM NaCl were added, followed by 30 min incubation at 55°C. Then, 1 mL phenol-chloroform-isoamyl alcohol (25:24:1) was added, followed by centrifugation at 12,000 g for 15 min. Three hundred microliters of the aqueous layer then was aliquoted into 1 mL of 200-proof ethanol. Samples were subsequently centrifuged at 12,000 g for 15 min, washed with 1 mL 70% ethanol, and air-dried. The DNA pellet was resuspended in 100 µL of DEPC water.

Primer selection. PCR primers were designed to detect a broad range of bacteria by targeting conserved regions of the 16S rRNA gene. Primer set PEU7/PEU8 was tested against a broad range of eubacterial DNA. Strains were obtained from the clinical microbiology laboratory at Johns Hopkins Hospital (Baltimore, MD) and the American Type Culture Collection (Manassas, VA). Figure 1A shows the primer pairs, sequences, and size of the amplified PCR product. Figure 1B shows alignment of the target sequences of the primer set with the 16S rRNA gene of several common bacterial pathogens.

Restriction endonuclease digestion. Before amplification of the desired target DNA, all PCR reagents were treated with the restriction endonuclease, AluI (New England Biolabs). The targeted sequence contains 3 sites for AluI, which is extremely specific for the nucleotide sequence AG–CT. No sites for AluI exist in the PCR primers themselves. Predigestion thus destroys all contaminating DNA targets but leaves the primer set intact. The 90 µL PCR reaction cocktail contained 2.5 U of HotStar Taq DNA polymerase (Qiagen), 25 pmol of each primer, 200 µM of each nucleotide, 4 mM MgCl2, and 10X PCR buffer (Qiagen). Then, 10.0 U of AluI was added to each 90 µL PCR reaction and was incubated at 37°C for 1.5 h, followed by inactivation of restriction enzyme by heating to 65°C for 30 min before the addition of target DNA for PCR amplification.

PCR amplification and detection of products. PCR was performed by use of 10 µL target DNA in 90 µL of processed PCR cocktail. The reaction consisted of a heat-mediated enzyme activation at 95°C for 10 min and 35 cycles of amplification in a Perkin-Elmer Gene-Amp 4800 Thermal Cycler, with the following conditions for each cycle: denaturation at 95°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 90 s, followed by an elongation step at 72°C for 7 min. Amplified products were detected by gel electrophoresis on a 3% agarose gel stained with ethidium bromide.

Sequencing. Amplified products were sequenced by use of the automated fluorescent DNA Sequencer (Perkin-Elmer), to confirm the identity of the PCR product from spiked pathogens.

Blood culture results and clinical data. Blood culture findings, and final discharge diagnosis were retrieved from the electronic patient record (EPR) system at the Johns Hopkins Hospital. Diagnosis of IE was based on the Duke Criteria [9]. Patients with single isolates of coagulase-negative staphylococci were excluded from analysis, since this is the most common contaminant in blood cultures and is, for all practical purposes, not associated with bacterial endocarditis [9]. Furthermore, the identity-unlinked protocol rendered it impossible to determine which of the 2 or 3 blood samples for cultures obtained in the emergency department was used for PCR analysis.

Statistical analysis. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of PCR versus blood culture were calculated.

Results

The PCR amplification assay was first tested in a mock sample containing water with no added bacteria, to determine whether the reagents themselves contained contaminating sources of bacterial DNA that might lead to false-positive results. PCR amplification that used the 16S rRNA primers described in figure 1A gave a product of the expected size, which indicates that contaminant bacterial DNA was amplified. Predigestion with AluI added to all components of the PCR cocktail eliminated this background signal. A titration profile for AluI and time identified 10 U/PCR reaction for 1.5 h to be the optimal enzyme concentration and time for digestion.

Healthy human whole blood then was spiked with 1 of 4 bacterial isolates, after which DNA was extracted. PCR amplification reactions were subsequently carried out with inclusion of the decontamination step described above. The amplified product of 418 bp was detected for each of the spiked reactions but not for the negative control (healthy human whole blood spiked

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with water). DNA sequencing correctly confirmed the identity of each spiked pathogen. Similar experiments with the 4 representative HACEK pathogens yielded a product of the expected size. A titration profile found that 5 bacteria/PCR reaction, calculated from exact quantification of bacteria per milliliter taken from plated colonies, was the lower limit of detection of the assay.

DNA was next isolated from whole blood specimens from 60 febrile IDUs with suspected bacteremia. Valid data for complete analysis were available for 51 patients. As per protocol, 7 were excluded because of a single isolate of coagulase-negative staphylococci; 2 were excluded because of missing blood culture reports. There were 13 samples which were positive by both PCR and blood culture.

Compared with that of blood culture, PCR had a sensitivity of 86.7%, specificity of 86.9%, PPV of 76.5%, and NPV of 94.1%. Discrepancies between blood culture findings and PCR results occurred in 6 cases (4 false positives and 2 false negatives). The 8 cases of blood culture positive for IE were PCR positive.

Discussion

PCR has long been recognized as a potential alternative to blood culture as a screening tool for rapid universal bacterial identification in the acute care setting, which is critical for triage decisions regarding treatment and disposition. Published clinical trials with such universal PCR assays have included early detection of bacteremia in neutropenic patients with cancer [6], as well as in critically ill patients in the intensive care unit [7]. However, concerns related to contamination have prevented general acceptance of these assays for routine use [8].

We describe a modification of a universal PCR assay that allows for removal of background DNA contamination, thereby improving upon existing methodologies for identification of the presence of bacterial organisms in the blood. There are 3 essential features of our broad-range assay, which uses a primer pair from the highly conserved 16S rRNA sequence: (1) presence of a restriction site in the amplicon that is not encoded in the PCR primer set, allowing for digestion or decontamination of all com-
Components of the PCR cocktail, including the primers before PCR amplification; (2) presence of multiple copies of this restriction site within the amplified product, which increases the probability of cleaving contaminating DNA into “nonamplifiable” product; and (3) use of HotStart Taq DNA polymerase, which is only activated at high temperatures (95°C), which prevents DNA polymerization from occurring during the decontamination step, which is performed at 37°C.

Restriction endonuclease digestion has been described as a method for elimination of false positives, as described elsewhere [10]. Importantly, the amplification target was not the 16S rRNA gene, where contamination is particularly problematic because of the highly conserved nature of this gene. More recently, decontamination studies have focused on the removal of bacterial DNA from Taq DNA polymerase by restriction endonuclease digestion of the polymerase before PCR amplification [11]. Although promising, that methodology did not permit decontamination of all components of the PCR cocktail. Of interest, a recent report describing restriction endonuclease digestion for elimination of false positives in a real-time universal PCR reaction failed to show consistent decontamination [12]. Possible explanations include presence of contaminating DNA that was not digested by the restriction enzyme chosen and relative inhibition of Taq DNA polymerase during inactivation of the restriction enzyme. Other decontamination methodologies have been described, but remain under investigation [13].

The 2 cases of positive blood culture but negative PCR were from gram-positive organisms (one was Corynebacterium species and the other contained mixed organisms, including group G streptococci, Propionibacterium species and coagulase-negative staphylococci). The patient with the blood culture positive for Corynebacterium species was treated for a localized cellulitis. Organisms grew from the aerobic bottle in only 1 of 3 sets of cultures and were believed by the treating physician to be a contaminant. Thus, it is likely that this patient was not bacteremic. The other blood culture–positive, PCR-negative patient had septic arthritis of the knee and developed pneumonia later during the hospitalization. Group G streptococci organisms grew in 1 anaerobic bottle at 2 days, and Propionibacterium species grew in a different anaerobic bottle after 7 days. This probably was a case of early bacteremia that was missed by PCR. A possible explanation for the missed cases include inadequate lysis of the bacterial cell wall, a phenomenon described elsewhere [14].

Specimen handling and processing might be an alternative explanation. Single collection of several blood samples, which could be used for both culture and PCR testing, may help to resolve such discrepancies in future studies; additionally, specimen handling errors could be limited by restricting storage time of specimens and DNA quantification after extraction, before running the PCR assay.

All 4 PCR-positive, but culture-negative cases had clinically identifiable infections: 2 urinary tract infections (1 with possible pyelonephritis), 1 cellulitis/abscess, and 1 pneumonia (see table 1). All of them received antimicrobial therapy for at least 7 days on the basis of clinical appearance. Possible explanations for the discordant findings in these cases include false-positive PCR results (which we believe unlikely) or improved sensitivity of the PCR assay over standard blood culture for detection of early bacteremia. Several recent reports describe the existence of culture negative, PCR positive IE [4, 15].

Several limitations of the PCR assay remain. The assay only allows for identification of presence or absence of bacterial organisms, and not for speciation. Design of species-specific primers, which could be used in conjunction with the universal primers might offer a solution, are already under investigation. Another concerns the methodology of the clinical specimen collection and our a priori decision to exclude patients with single isolates of coagulase-negative staphylococci, which reduced our total sample size. All 7 cases in which single isolates of coagulase-negative staphylococci were found were considered to be “blood culture contaminants” by the clinicians who were unaware of our results. However, it is impossible to determine whether these represent blood culture contaminants or low levels of bacteremia. One other limitation concerns confirmatory sequence identity of the PCR products from our clinical study. For definitive correlation, sequencing of amplicons from patient samples probably will require cloning and sequencing of the PCR product obtained, with comparison to the primary blood culture pathogen. Furthermore, although successful sequencing of the PCR amplicon after spiking bacteria in the blood demonstrates the integrity of the assay, this does not guarantee reproducible amplification of the pathogen DNA in true bacteremia, in which the number of organisms tends to be low. In this regard, although our titration profile indicates the ability to detect as little as 5 bacteria/PCR reaction, determination of whether this is adequate for consistently identifying bacteremia in patients presenting to acute care.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Blood culture</th>
<th>Discharge diagnosis (grouped) and corresponding organisms isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>+</td>
<td>IE 8 (Staphylococcus aureus, group A streptococci, 2; S. aureus + viridans group streptococci, 1; viridans group streptococci, 1; urosepsis 1 (Enterobacteriaceae + Actinobacteriaceae); cellulitis 1 (group A streptococci); pyelonephritis, 1 (Escherichia coli + Corynebacterium); fever unknown origin (left against medical advice), 1 (viridans group streptococci); and drug fever, 1 (Propionibacterium species)</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>UTI 1, UTI with possible pyelonephritis, 1, cellulitis/abscess, 1, pneumonia, 1</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>Septic arthritis and pneumonia, 1 (group G streptococci + Propionibacterium); cellulitis 1 (Corynebacterium sp.)</td>
</tr>
<tr>
<td>32</td>
<td>–</td>
<td>Cellulitis/abscess, 7; pneumonia, 7, drug fever, 6, bronchitis, 3, cellulitis, 2; UTI 2, leg ulcer, 2; sinusitis, 1, neck abscess, 1; PID, 1</td>
</tr>
</tbody>
</table>

NOTE. IE, infective endocarditis; PID, pelvic inflammatory disease; UTI, urinary tract infection; +, positive; –, negative.
settings awaits validation. A larger clinical trial underway, which will be described in a follow-up study to this pilot investigation, will address these issues.

In conclusion, we have refined a PCR assay for detection of bacterial species in the blood of febrile patients. Testing of our assay with spiked bacterial pathogens in whole blood showed that the decontamination step was highly effective in eliminating background DNA contamination, while preserving the sensitivity of the assay. Findings from the pilot clinical trial conducted in an acute care setting suggest that this tool may ultimately have important practical use in triage and hospitalization decisions for patients in whom recognition of bacteremia is of critical importance, such as febrile IDUs known to be at increased risk for IE [1]. Of note, mean time from sample collection to reporting of positive results was 6.5 h for the PCR assay, which is significantly shorter than the mean of 22.7 h (range, 14–99 h) known to be associated with reporting of first positive blood culture in IDUs with IE at our institution (R.E.R., author’s unpublished data). Another valuable application of this assay indicated by our experiments includes earlier detection of the slow-growing HACEK organisms associated with initially culture-negative cases of IE. Further potential usefulness, currently under investigation is improved detection of culture-negative IE associated with blood samples obtained from patients already on antibiotics.

Before the assay can be recommended for treatment or disposition decision-making, additional assay refinement, and more extensive clinical trials will be required.

References


