


With the ability to exponentially amplify regions of DNA, two different PCR-based strategies have been developed for noncultivation diagnosis of bacteremia. The first approach targets the species-specific genes for amplification (1), whereas the second approach involves universal PCR amplification of conserved bacterial DNA sequences, such as the 16S rRNA, the 23S rRNA, and the 16S-23S rRNA interspace regions (2–4). Although universal PCR is not able to distinguish bacteria to the species level, numerous studies have shown that this method provides valuable information complementary to the results of time-consuming and subjective phenotypic tests used in detection of bacterial infection (5) and can be used to differentiate bacterial from viral or other infections (6). In clinical applications, real-time PCR for broad-range amplification of bacterial DNA sequences could offer additional benefits: it is less labor-intensive, less time-consuming, and reduces the risk of PCR carryover contamination.

A major obstacle for broad-range PCR amplification is the presence of bacterial DNA in the Taq DNA polymerase and real-time PCR master mixture (6–10). The contaminating DNA is effectively amplified, giving rise to false-positive results. Efforts have been taken to eliminate contaminating DNA, including the use of Sau3AI restriction endonuclease (11), DNase I (12,13), and ultraviolet irradiation (7,8). Although these strategies may be effective for conventional PCR, the study by Corless et al. (14) indicated that the contamination issue could not be avoided without affecting sensitivity when TaqMan probe-based real-time PCR is performed. Therefore, an
appropriate method for removal of contaminating bacterial DNA and subsequent real-time amplification is required. In this study, we address this issue and report optimized conditions for broad-range amplification of the bacterial 16S rRNA gene by real-time PCR using SYBR Green I dye (15) as the fluorescent signal.

Bacterial genomic DNA was isolated by use of the MasterPure DNA purification reagent set (Epicentre Technologies). Briefly, pellets from bacterial cultures were resuspended in 300 μL of Tissue and Cell Lysis Solution containing proteinase K (160 mg/L) and incubated at 65 °C for 15 min. After the lysis process, RNase A (160 mg/L) was added for 1 h at 37 °C. We then added 150 μL of MPC Protein Precipitation Reagent and centrifuged the mixture at 20 000g for 10 min. The supernatant was transferred to a clean microcentrifuge tube, and the DNA was precipitated with isopropanol. After two washes with 750 mL/L ethanol, the DNA pellet was resuspended in water for real-time PCR analysis.

The broad-range real-time PCR was first set up to amplify the *Escherichia coli* 16S rRNA gene fragment with the universal primers p1370/p201 (13). The template DNA was added into the reaction mixture containing 25 μL of 2× SYBR Green PCR master mixture (Applied Biosystems), 1 μL of p201 (5 × 10⁻⁶ M; 5'-GAGGAAGGIGIGGAIGACGT-3'), and 1 μL of p1370 (5 × 10⁻⁶ M; 5'-AGICCCGIGAACGTATTCAC-3') in a final volume of 50 μL. PCR was performed with the GeneAmp 5700 Sequence Detection System (Applied Biosystems). After initial activation of AmpliTaq Gold DNA polymerase at 95 °C for 10 min, 40 PCR cycles of 95 °C for 15 s and 60 °C for 1 min were performed. Immediately after PCR amplification, we performed melting curve analysis (16) by cooling the reaction to 60 °C and then heating it to 95 °C in an ~20-min period. The SYBR Green I fluorescence (F) was measured continuously during the heating period, and the signal was plotted against temperature (T) to produce a melting curve for each sample. The melting peak was then generated by plotting the negative derivative of the fluorescence with respect to temperature against temperature (−dF/dT vs T). The melting temperature (Tm) was considered the peak data point in the melting curve analysis.

The amplification plot (Fig. 1) shows a threshold cycle value (C_T) of 20.79 cycles for 200 pg of *E. coli* genomic DNA. Melting curve analysis revealed a melting peak with a Tm at 83.3 °C. The melting peak corresponded to a single PCR product with an expected size of 216 bp in agarose gel electrophoresis. Sequencing analysis confirmed that the PCR product was the *E. coli* 16S rRNA gene fragment (data not shown). In the no-template control (NTC) reaction, we detected a small but distinct single melting peak with a Tm of 84.3 °C, indicating that the PCR master mixture contained amplifiable bacterial DNA.

To remove the contaminating bacterial DNA, we treated 2× SYBR Green PCR master mixture with DNase I (10⁻³ U/μL; Invitrogen) at 37 °C for 60 min followed by heat inactivation at 90 °C for 30 min before running real-time PCR. The melting peak was eliminated in the NTC reaction, indicating that DNase I effectively reduced the contaminating bacterial DNA (Fig. 1). Notably, a typical amplification plot with a C_T of 21.94 cycles and a
melting peak with a $T_m$ of 83.3°C was still generated when we used *E. coli* genomic DNA as the template.

To evaluate the limit of the decontamination procedure without the interference of contaminating DNA, we added to the master mixture various amounts of *Staphylococcus aureus* genomic DNA (from 1 pg to 20 fg) and treated it according to the DNase I procedure. We then performed real-time PCR and melting curve analysis with the *S. aureus*-specific primers Sa224-F (5'-TCGGTACAC-GATATTCTTCAC-3') and Sa224-R (5'-ACTCTCGTAT-GACCAGCTTC-3') to detect the residual undigested genomic DNA (17). The DNase I pretreatment procedure eliminated up to 500 fg of genomic DNA. These results suggest that optimizing pretreatment of the master mixture with DNase I reduces contaminating bacterial DNA and is crucial for successful development of broad-range rRNA real-time PCR.

To assess the reproducibility and reliability of the assay, we repeated the real-time PCR three times with 20 pg of *E. coli* genomic DNA under identical conditions. The $C_T$s were 25.75, 26.16, and 26.73 cycles, respectively, with the mean ± SD equivalent to 26.21 ± 0.49 cycles. The detection limit was determined with 10-fold serial dilutions of *E. coli* genomic DNA, ranging from 200 pg to 20 fg. As little as 20 fg of DNA could be detected with a dynamic linear range for quantification across at least four logs of DNA concentration (correlation coefficient = -0.9959). Assuming that the *E. coli* genome is ~4.6 Mbp (18) and that each genome contains seven copies of the 16S rRNA gene (19), the detection limit was equivalent to four copies of 16S rRNA gene or genomic DNA from 0.5 bacterium.

The feasibility of this technique for use in detecting DNA from other bacteria was determined with genomic DNA from 39 clinically important isolates representing 9 gram-positive and 9 gram-negative bacteria species (Table 1). These bacterial species account for >80% of the gram-positive and -negative bacteria identified from blood cultures in our hospital during the past few years. The 16S rRNA genes of all these isolates were amplifiable by the broad-range real-time PCR with a detection limit for the isolates of *Salmonella* group D and *Pseudomonas aeruginosa* equivalent to the limit reported for *E. coli* (data not shown). Melting curve analysis revealed distinct melting profiles, with the $T_m$s for different isolates of the same species within a limited range of variation (Table 1). The 18 species we analyzed could be classified into four subgroups with mean $T_m$s of 80.3 ± 0.3 (n = 5), 82.0 ± 0.0 (n = 6), 82.8 ± 0.2 (n = 6), and 83.5 ± 0.2°C (n = 22), respectively. Except for the subgroup with a $T_m$ of 83.5 ± 0.2°C, the other three subgroups were either gram-positive ($T_m$s of 80.3 ± 0.3 and 82.8 ± 0.2°C) or gram-negative ($T_m$ of 82.0 ± 0.0°C) bacteria only.

The length and GC content for the expected PCR product were determined from the corresponding bacterial 16S rRNA sequence deposited in the GenBank database (Table 1). All of the species had an expected 216-bp PCR product, with the exception of the PCR product of *P. fluorescens*, which had an expected length of 217 bp. Within the four subgroups, bacteria had distinct GC contents (Table 1).

Real-time PCR has been useful in various applications, including pathogen detection, allelic discrimination, and gene expression (20–23). Our approach to simultaneous detection and classification of bacteria required careful consideration of several universal primer pairs (2, 13, 24, 25). The primer pair p201 and p1370 appeared to have advantages over others. For example, the amplicon was relatively small (216 or 217 bp) and was suitable for efficient amplification by real-time PCR. The p201 and p1370 primers could amplify at least 96% of bacterial DNA (13, 26). In contrast, the 176-bp interprimer region

**Table 1. Melting peak profiles for clinically important bacterial strains disclosed by broad-range, real-time PCR of the 16S rRNA gene.**

<table>
<thead>
<tr>
<th>Bacterial species (no. of isolates)</th>
<th>$T_m$ ± SD, °C</th>
<th>GC content, %</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. epidermidis</em> (1)</td>
<td>80.1 ± 0.5</td>
<td>47.2</td>
<td>L37605</td>
</tr>
<tr>
<td><em>S. aureus</em> (2)</td>
<td>80.4 ± 0.3</td>
<td>47.7</td>
<td>Y15856</td>
</tr>
<tr>
<td><em>S. saprophyticus</em> (2)</td>
<td>80.4 ± 0.1</td>
<td>51.8</td>
<td>L37596</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (2)</td>
<td>82.0 ± 0.0</td>
<td>51.8</td>
<td>AF511429</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> (3)</td>
<td>82.0 ± 0.1</td>
<td>51.8</td>
<td>AF008582</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em> (1)</td>
<td>82.0 ± 0.0</td>
<td>52.3</td>
<td>CFR233408</td>
</tr>
<tr>
<td><em>Streptococcus group A</em> (2)</td>
<td>82.8 ± 0.0</td>
<td>53.7</td>
<td>AB023575</td>
</tr>
<tr>
<td><em>Streptococcus group B</em> (2)</td>
<td>82.9 ± 0.1</td>
<td>53.7</td>
<td>AB002479</td>
</tr>
<tr>
<td><em>Streptococcus group D</em> (2)</td>
<td>82.7 ± 0.4</td>
<td>53.7</td>
<td>M58835</td>
</tr>
<tr>
<td><em>Enterococcus spp.</em> (3)</td>
<td>83.4 ± 0.1</td>
<td>54.6</td>
<td>AB036835</td>
</tr>
<tr>
<td><em>E. coli</em> (3)</td>
<td>83.4 ± 0.3</td>
<td>54.6</td>
<td>AF511430</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (3)</td>
<td>83.6 ± 0.1</td>
<td>54.6</td>
<td>Z70672</td>
</tr>
<tr>
<td><em>Salmonella</em> group B (2)</td>
<td>83.4 ± 0.1</td>
<td>54.6</td>
<td>X09681</td>
</tr>
<tr>
<td><em>Salmonella</em> group D (3)</td>
<td>83.4 ± 0.1</td>
<td>54.6</td>
<td>U90318</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> (3)</td>
<td>83.6 ± 0.2</td>
<td>55.1</td>
<td>Z70672</td>
</tr>
<tr>
<td><em>S. viridans</em> (1)</td>
<td>83.6 ± 0.4</td>
<td>56.2</td>
<td>AB002522</td>
</tr>
<tr>
<td><em>P. fluorescens</em> (1)</td>
<td>83.7 ± 0.2</td>
<td>56.9</td>
<td>AF145244</td>
</tr>
<tr>
<td><em>Bacteroides fragilis</em> (3)</td>
<td>83.7 ± 0.2</td>
<td>56.9</td>
<td>X83943</td>
</tr>
</tbody>
</table>
was expected to contain information for at least partial phylogenetic characterization that was useful to subclassify clinically important bacterial species (13). The variability of this interprimer region was not revealed clearly in conventional PCR because of the low-resolution capability of agarose gel electrophoresis. However, the intrinsic variability of real-time PCR products could be disclosed by high-resolution melting curve analysis, where $T_m$ could serve as a measure of DNA length and nucleotide composition (Table 1).

We identified four subgroups of bacteria each with distinct $T_m$s in a limited range. Similar to any broad-range PCR detection of bacterial DNA, the assay could not determine the viability of the organism, particularly during the treatment with antibiotics, or identify the bacterial species. However, because it detects a broad range of bacteria usually present in clinical specimens, this assay may complement the time-consuming blood culture test and supply timely information needed by physicians to determine whether bacterial infection has occurred and to plan treatment regimens. The unique feature of the method presented in this study (the classification information disclosed by the melting peak profiles) could be used in the design of multiplex PCR to confirm the identity of infectious bacterial species in a clinical or laboratory setting.

In summary, the decontamination procedure and the broad-range real-time PCR method allow rapid detection, quantification, and classification of several clinically important bacteria and may facilitate rapid detection of local and systemic infection.

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References


Detection of Microchimeric Cells in the Peripheral Blood of Nonpregnant Women Is Enhanced by Magnetic Cell Sorting before PCR, Lori A. Cox, Ronald C. Ramos, Tara N. Dennis, Sergio A. Jimenez, J. Bruce Smith, and Carol M. Artlett* (Thomas Jefferson University, Department of Medicine, Division of Rheumatology, Philadelphia, PA 19107; * address correspondence to this author at: Thomas Jefferson University, Department of Medicine, Division of Rheumatology, Room 509, Bluemle Life Sciences Bldg., 233 South 10th St., Philadelphia, PA 19107-5541, e-mail Carol.Artlett@mail.tju.edu)

Our laboratory and others have demonstrated the presence of microchimeric cells in the peripheral blood of nonpregnant patients with systemic sclerosis (SSc) (1–4). In addition, we have demonstrated the presence of ma-