Rapid Detection and Identification of Clinically Important Bacteria by High-Resolution Melting Analysis after Broad-Range Ribosomal RNA Real-Time PCR

Ju-Chien Cheng, Chien-Ling Huang, Chung-Ching Lin, Chi-Ching Chen, Yi-Chih Chang, Shy-Shin Chang, and Ching-Ping Tseng

Background: Broad-range PCR provides valuable information for detecting bacterial infections. This study assesses the combined use of broad-range real-time PCR and high-resolution melting analysis for rapid detection and identification of clinically important bacteria.

Methods: We subjected 46 bacterial culture colonies representing 25 clinically important bacterial species to LightCycler real-time PCR amplification of the 16S rRNA gene in the presence of LCGreen I fluorescent dye. We performed high-resolution melting analysis of the PCR products with the HR-1 instrument and used melting profiles as molecular fingerprints for bacterial species identification. We validated this method via assessment of 54 consecutive bacteria culture colonies obtained from a clinical microbiology laboratory.

Results: The 16S rRNA gene of all 25 bacterial species was amplifiable by this method, with PCR product lengths of 216 or 217 bp. Of the 25 bacterial species, we identified 11 via a 1-step post-PCR high-resolution melting analysis. The remaining bacterial species were identified via the high-resolution melting plots obtained by heteroduplex formation between the PCR products of the tested and reference bacterial species or by a 2nd real-time PCR targeting a different region of the 16S rRNA gene. A high-resolution melting database and a working protocol were established for identifying these 25 bacterial species. In the validation assay, a 94% accuracy rate was achieved when the bacterial species were in the high-resolution melting database.

Conclusions: This assay requires no multiplexing or hybridization probes and provides a new approach for bacterial species identification in a molecular diagnostic laboratory.

Improved schemes for rapid diagnosis of bacterial infections are critical to patient management and antibiotic therapy of bacteremia-induced severe sepsis, a primary cause of morbidity and mortality in hospitalized patients worldwide (1). Two PCR-based strategies have been developed for nonculture diagnosis of bacteremia. The 1st approach targets species-specific genes for amplification (2), and the 2nd uses broad-range PCR amplification of conserved bacterial DNA sequences, such as the 16S rRNA, 23S rRNA, and 16S–23S rRNA interspace regions (3–5). Numerous studies have demonstrated that broad-range PCR generates valuable information that complements results of time-consuming and subjective phenotypic tests for detecting bacterial infections (6) and can be used to differentiate bacterial from viral and other infections (7, 8).

In clinical applications, real-time PCR for broad-range amplification of bacterial DNA offers additional benefits...
including minimal labor, rapid turnaround time, and a decreased risk of PCR carryover contamination. For rapid detection of bacteria, we used the primer pair p201 and p1370 for real-time amplification of 16S rRNA gene, with PCR product lengths of 216 or 217 bp (9). The 176-bp interprimer region likely contains information for at least partial phylogenetic characterization for subclassifying clinically important bacterial species (10). Although probe-based assays and DNA sequencing of highly variable regions within the universal PCR amplicon have been used for phylogenetic analysis that in most cases leads to species-level identification, they are generally time-consuming and relatively expensive (11, 12). Recently, a high-resolution melting analysis incorporating the fluorescent dye LCGreen I has been used for detecting heterozygous and homozygous sequence variants for genotyping and variation scanning (13–15). This approach is a closed-tube technique that does not require fluorescently labeled probes or separation steps (16). In contrast to traditional melting-curve analysis, high-resolution melting with the HR-1 instrument reliably detects single-base differences in homozygous and heterozygous sequences (17). This technique is cost-effective, has high sensitivity and specificity, and can be completed in <2 min after PCR.

In this study, we report a novel and powerful scheme combining LightCycler broad-range real-time PCR and high-resolution melting analysis for rapid species identification of clinical bacteria culture colonies. Without multiplexing or hybridization probes, 25 bacterial species can be identified by this method with ≤2 PCRs within 1.5 h.

Materials and Methods

We purchased the LCGreen I reagent set and HR-1 instrument from Idaho Technology, the Taq DNA polymerase, and PCR reagents from Invitrogen, and LightCycler capillaries from Roche Applied Science. Bacteria strains were clinical isolates identified by the China Medical University Hospital (Taichung, Taiwan, Republic of China) and Li Shin Hospital (Taoyuan, Taiwan, Republic of China).

Isolation of Bacterial Genomic DNA

Bacteria were cultured overnight, and then 1.5 mL was centrifuged at 4500g for 10 min, and the pellet was resuspended in 400 μL of P1 buffer (50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L EDTA, 100 mg/L RNase A). The bacterial suspension was incubated with mutanolysin (6.25 × 10^4 U/L) and lysostaphin (60 mg/L) at 37 °C for 30 min when augmented digestion of the streptococcal and staphylococcal cell wall was needed. Proteinase K (200 mg/L) and 1% SDS with incubation at 56 °C for 30 min were then used to lyse bacteria. After phenol/chloroform extraction, supernatant was transferred to a clean microcentrifuge tube, and the DNA was precipitated with 400 mL/L isopropanol. After 2 washes with 750 mL/L ethanol, DNA pellets were resuspended in water for quantification by spectrophotometry.

LightCycler Broad-Range Real-Time PCR for Amplification of 16S rRNA Gene

Real-time PCR amplification of the 16S rRNA gene was performed with the LightCycler thermal cycler with the primer pairs p201 (5′-GAGGAAGGGIGGIAAGCT-3′) and p1370 (5′-AGICCGGGAACGTATTCAC-3′) (9) or E721F (5′-AAGACTGACGCTCAGGTGCGA-3′) and E1035R (5′-GCCATGCAGCACCTGTCTCAC-3′). Briefly, bacterial genomic DNA (200 pg) or bacterial suspension (1 μL) was added to a reaction mixture containing 0.5 μmol/L of each primer, 2 mmol/L MgCl_2, 50 mmol/L Tris-HCl (pH 8.3), 0.2 mmol/L of each dNTP, 250 mg/L bovine serum albumin, 1× LCGreen I dye, and 2.5 U of Taq DNA polymerase in 10 μL. Cycling conditions were 1 cycle of 94 °C for 2 min, 40 cycles of 94 °C for 15 s, and 60 °C for 1 min at a transition rate of 20 °C/s. In this study, most of the PCR amplifications were performed with bacterial suspensions. Purified bacterial genomic DNA was used only for comparison.

High-Resolution Melting-Curve Acquisition and Analysis

Glass capillaries containing amplification products were transferred directly from the LightCycler to the HR-1 high-resolution melting instrument. Fragments were melted at 60–95 °C at a rate of 0.3 °C/s. Melting profiles were assessed with HR-1 software with fluorescence normalization and temperature overlay to superimpose the curves at 20%–60% fluorescence to obtain minimal interrun variability.

Heteroduplex Formation

Heteroduplex formation was achieved by mixing equal amounts of PCR product from the tested and reference bacteria. The value of fluorescence intensity after final amplification of PCR was used as a basis for calculating the required volume of PCR products for mixing. To induce heteroduplex formation, the DNA mixtures were subjected to heating at 95 °C for 1 s and cooling to 40 °C for 10 s at a rate of 20 °C/s.

Results

To assess the combined use of broad-range real-time PCR and high-resolution melting analysis for rapid detection and identification of clinically relevant bacterial species, 10-fold serially diluted *Escherichia coli* genomic DNAs, 100 ng to 100 fg, were PCR amplified by the primer pair p201 and p1370 in the presence of LCGreen I (see Fig. 1A in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue11). As little as 100 fg of bacterial DNA could be detected with a dynamic linear range for quantification across at least 6 logs of DNA concentration. The detection limit was increased to 1 pg when temperature holds were
decreased to 94 °C for 5 s and 60 °C for 15 s to increase the speed of PCR amplification (data not shown). The crossing points for the broad-range real-time PCR ranged from \[9.52 (0.34), n / \text{H11005} 4, \text{to } 27.88 (0.99), n / \text{H11005} 4\] for 100 ng to 100 fg of \textit{E. coli} genomic DNA. For the no-template control (NTC) reaction, the crossing point was at the cycle number of \[28.20 (0.85), n / \text{H11005} 4\].

We then used the HR-1 instrument to perform high-resolution melting analysis. Derivative plots revealed melting peaks corresponding to single PCR product (see Fig. 1B in the online Data Supplement). Because of the presence of contaminated bacterial DNA in the PCR reagents \([9, 18, 19]\), a small melting peak was observed for NTC. Low concentrations of initial template DNA usually led to a primer dimer with a melting peak at \(74 \ ^\circ \text{C}\). After fluorescence normalization and temperature overlay, the melting plots for the broad-range real-time PCR products were similar over a million-fold range of concentration (see Fig. 1C in the online Data Supplement). The melting plot for the NTC reaction was eliminated automatically by HR-1 analytical software when the option “Delete some bad samples” was selected during fluorescence normalization.

To simplify and increase the pace of high-resolution melting analysis in bacterial detection, we determined whether direct amplification of bacterial suspensions could be used without DNA purification. Bacterial suspensions from single colonies of \textit{Staphylococcus aureus} or \textit{E. coli} subjected to PCR amplification followed by high-resolution melting revealed similar curves to those obtained from purified genomic DNA (see Fig. 1D in the online Data Supplement). Serial dilution analysis indicated that the melting plot was not altered with as little as 1 bacterium for PCR amplification (data not shown). These data indicate that direct PCR amplification of bacterial suspension can be used for high-resolution melting analysis. Therefore, we used this method as a broad-range real-time PCR protocol to facilitate bacterial detection.

Broad-range real-time PCR and high-resolution melting analysis were performed with 46 clinical isolates belonging to 25 clinically important bacterial species (Table 1). These bacterial species accounted for \(>80\%\) of gram-positive and gram-negative bacteria identified from blood cultures in our hospital during the past few years. The 16S rRNA genes of all these isolates were amplifiable.

### Table 1. High-resolution melting profiles for clinically important bacterial species disclosed by broad-range 16S rRNA real-time PCR.

<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>
<th>Pathogen with identical PCR amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. epidermidis} (1)</td>
<td>85.2 (0.6)</td>
<td>47.2</td>
<td>L37605</td>
<td>\textit{Staphylococcus xylosus, Staphylococcus cohnii}</td>
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<tr>
<td>\textit{S. aureus} (2)</td>
<td>85.8 (1.0)</td>
<td>47.7</td>
<td>Y15856</td>
<td></td>
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<tr>
<td>\textit{S. saprophyticus} (2)</td>
<td>85.8 (0.2)</td>
<td>47.7</td>
<td>L37596</td>
<td></td>
</tr>
<tr>
<td>\textit{H. influenzae} (1)</td>
<td>86.5 (0.3)</td>
<td>52.8</td>
<td>Z22806</td>
<td></td>
</tr>
<tr>
<td>\textit{S. marcescens} (2)</td>
<td>86.7 (0.2)</td>
<td>50.2</td>
<td>M59160</td>
<td>\textit{Enterobacter aerogenes}</td>
</tr>
<tr>
<td>\textit{P. mirabilis} (2)</td>
<td>86.7 (0.4)</td>
<td>51.9</td>
<td>AF008582</td>
<td>\textit{Proteus vulgaris}</td>
</tr>
<tr>
<td>\textit{C. freundii} (1)</td>
<td>87.1 (0.5)</td>
<td>52.3</td>
<td>CFR23340B</td>
<td>\textit{Klebsiella oxytoca}</td>
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<tr>
<td>\textit{K. pneumoniae} (3)</td>
<td>87.1 (0.7)</td>
<td>51.9</td>
<td>AF511429</td>
<td></td>
</tr>
<tr>
<td>\textit{S. bovis} (1)</td>
<td>87.3 (0.1)</td>
<td>53.7</td>
<td>M58835</td>
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<td>\textit{M. morganii} (1)</td>
<td>87.4 (0.2)</td>
<td>52.0</td>
<td>AJ301681</td>
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<td>\textit{A. baumannii} (2)</td>
<td>87.6 (0.3)</td>
<td>54.2</td>
<td>AW738399</td>
<td></td>
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<td>\textit{Bacillus spp.} (1)</td>
<td>87.8 (0.2)</td>
<td>53.2</td>
<td>DQ232746</td>
<td></td>
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<tr>
<td>\textit{S. pyogenes} (1)</td>
<td>88.0 (0.3)</td>
<td>53.7</td>
<td>AB023573</td>
<td></td>
</tr>
<tr>
<td>&lt;tt&gt;\textit{E. cloacae} (1)&lt;/tt&gt;</td>
<td>88.1 (0.3)</td>
<td>53.7</td>
<td>DQ089673</td>
<td></td>
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<td>\textit{S. agalactiae} (1)</td>
<td>88.2 (0.3)</td>
<td>53.7</td>
<td>AB002479</td>
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<td>\textit{E. faecalis} (3)</td>
<td>88.5 (0.6)</td>
<td>54.6</td>
<td>AB036835</td>
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<tr>
<td>\textit{E. faecium} (1)</td>
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<td>54.2</td>
<td>AJ420800</td>
<td>\textit{Streptococcus dysgalactiae}</td>
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<td>\textit{S. flexneri} (1)</td>
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<td>54.6</td>
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<td>\textit{S. pneumoniae} (3)</td>
<td>88.5 (0.3)</td>
<td>54.6</td>
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<td>\textit{S. typhimurium} (3)</td>
<td>88.6 (0.4)</td>
<td>54.6</td>
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<td></td>
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<td>\textit{S. enteritidis} (1)</td>
<td>88.6 (0.4)</td>
<td>54.6</td>
<td>U90318</td>
<td></td>
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<tr>
<td>\textit{E. coli} (4)</td>
<td>88.6 (0.2)</td>
<td>54.6</td>
<td>AF511430</td>
<td></td>
</tr>
<tr>
<td>\textit{P. aeruginosa} (4)</td>
<td>88.7 (0.4)</td>
<td>54.6</td>
<td>Z76672</td>
<td></td>
</tr>
<tr>
<td>\textit{Micrococcus spp.} (1)</td>
<td>89.2 (0.8)</td>
<td>54.8</td>
<td>AJ296288</td>
<td></td>
</tr>
<tr>
<td>\textit{B. fragilis} (3)</td>
<td>89.2 (0.9)</td>
<td>56.9</td>
<td>X83943</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The variance of 3–6 measurements with the indicated number of isolates.

\(^b\) The sequences of these PCR amplicons are identical in these 4 bacterial species.
in the presence of LC Green I. The melting temperature (T_m) for the PCR product was recorded with the HR-1 instrument without temperature shifting (Table 1). With the melting profile of *Citrobacter freundii* as a reference, we generated difference plots of these 25 bacterial species after fluorescence normalization and temperature overlay to superimpose the curves at 20%–60% fluorescence. Each bacterial species had a characteristic difference plot with minimal inter- and intrarun variability (Fig. 1; see Fig. 2 in the online Data Supplement). The 9 bacterial species that could be identified directly via their difference plots were *C. freundii*, *S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Morganella morganii*, *Micrococcus* spp., and *Bacteroides fragilis* (Fig. 1A). To avoid misidentification as a result of closely overlapping difference plots, the remaining 16 bacterial species were classified into 4 melting groups to facilitate species identification (see Fig. 2 in the online Data Supplement). According to the patterns of difference plots, group 1 included *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Serratia marcescens*; group 2 included *Haemophilus influenzae* and *Acinetobacter baumannii*; group 3 included *Staphylococcus saprophyticus*, *Streptococcus bovis*, *Bacillus* spp., *Enterococcus faecalis*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*; and group 4 included *E. coli*, *Salmonella enteritidis*, *Salmonella typhimurium*, and *Shigella flexneri*. Of the group 3 bacteria, *S. saprophyticus* was identified by its relatively low T_m.

Fig. 1. High-resolution difference plots as the molecular fingerprints of 25 common and clinically important bacterial species.

(A), difference plots for the indicated bacterial species were obtained by high-resolution melting analysis of the broad-range real-time PCR products. Fluorescence normalization and temperature overlay were performed with the HR-1 software. *C. freundii* was selected as a reference species for plotting the graph. Bacterial species that can be identified directly via their distinct and recognizable patterns of difference plots are presented. The remaining bacterial species were classified into 4 melting groups (see Fig. 2 in the online Data Supplement). For each bacterial species, the data represent 3–6 measurements with the clinical isolates as described in Table 1. (B), bacterial species with similar shapes of difference plots were plotted together to reveal the differences among melting groups and the similarity within each melting group. To decrease graph complexity, only 1 representative difference plot is presented for each bacterial species. The difference plots for *S. aureus*, *S. epidermidis*, and *E. faecium* are not shown.
compared with those of S. bovis, Bacillus spp., E. faecalis, S. pyogenes, S. agalactiae, and S. pneumoniae (Table 1; Fig. 2). Moreover, S. bovis was identified by its broad melting pattern in derivative plot (Fig. 2) or its unique difference plot (see Fig. 2 in the online Data Supplement) compared with those of Bacillus spp., E. faecalis, S. pyogenes, S. agalactiae, and S. pneumoniae. In all, we identified 11 bacterial species directly via 1-step post-PCR high-resolution melting analysis.

We used different strategies for identification of bacterial species within each melting group. Sequence analysis of the bacterial 16S rRNA genes indicated that sequence variations of the PCR amplicons within each melting group were 0–31 bp (see Fig. 3A in the online Data Supplement). No sequence variation existed in the amplicons of E. coli, S. typhimurium, S. enteritidis, or S. flexneri (data not shown). We determined whether heteroduplex formation between the PCR products of the tested and reference bacterial species produced unique high-resolution melting plots suitable for species identification. By heteroduplexing with the PCR amplicon of P. mirabilis, we identified the group 1 bacteria P. mirabilis, K. pneumoniae, and S. marcescens by their unique high-resolution melting plots (Fig. 3A). Similarly, by heteroduplexing with the PCR amplicon of A. baumannii, we identified the group 2 bacteria A. baumannii and H. influenzae (see Fig. 4A in the online Data Supplement). By heteroduplexing with the PCR amplicon of S. pneumoniae, we identified the group 3 bacteria S. pneumoniae, E. faecalis, Bacillus spp., and S. pyogenes/S. agalactiae (see Fig. 4B in the online Data Supplement). S. pyogenes and S. agalactiae were indistinguishable by high-resolution melting analysis because only a 1-bp variation exists between these 2 species (see Fig. 3A in the online Data Supplement).

Because of the lack of sequence variation in the PCR amplicon of E. coli, S. typhimurium, S. enteritidis, and S. flexneri, we designed a 2nd real-time PCR to distinguish these bacterial species by use of primer pair E721F and E1035R that amplified nucleotides (nt) 721-1035 of the 16S rRNA gene (GenBank accession no. AF511430). The amplicons of these 4 species had sequence variants of 3–18 bp (see Fig. 3B in the online Data Supplement). High-resolution melting differentiated these 4 Enterobacteriaceae species without post-PCR handling (Fig. 3B). Overall, these results demonstrated that our HR-1 melting-based protocol can be performed without multiplexing or hy-

![Fig. 2](image1.png)

**Fig. 2.** Derivative plots for S. saprophyticus and S. bovis. After broad-range real-time PCR and high-resolution melting analysis, derivative plots for the indicated bacterial species were generated. Note the distinct Tm, for S. saprophyticus and the flat and broad shape melting peak of S. bovis.

![Fig. 3](image2.png)

**Fig. 3.** High-resolution melting plots for the indicated bacterial species. (A), high-resolution melting plots were obtained by heteroduplex formation (HD) between the PCR products of the indicated bacterial species and P. mirabilis. (B), high-resolution melting plots for E. coli, S. typhimurium, S. enteritidis, and S. flexneri were obtained after a 2nd real-time PCR targeting nt 721-1035 of the 16S rRNA gene.
bridization probes to identify 25 common and clinically important bacterial species, with the exception of *S. pyogenes*/*S. agalactiae*, which have closely similar melting plots (Fig. 4). We also generated a high-resolution melting database for rapid identification of bacterial species.

We validated this novel approach in bacterial species identification by performing a blind test with 54 consecutive bacteria culture colonies from the clinical microbiology laboratory at Li Shin Hospital. Bacterial suspensions were subjected to broad-range real-time PCR followed by high-resolution melting analysis. In this study, the difference plots of 46 samples matched those of the high-resolution melting database and had analytical results comparable to those obtained from bacterial cul-

ture (see Table 1 in the online Data Supplement). Of the 8 unmatched samples, 5 were not in our high-resolution melting database [*Aeromonas hydrophila* (n = 1), *Burkholderia cepacia* (n = 1), *Providencia alcalifaciens* (n = 1), *Stenotrophomonas maltophilia* (n = 1), and *Yersinia enterocolitica* (n = 1)]. The remaining 3 samples that were not correctly identified were *E. coli* (n = 2) and *S. marcescens* (n = 1). Sequencing of the PCR amplicons showed that base variants of C/T occurred at nt 1238 of the 2 *E. coli* strains (GenBank accession no. AF511430) and G/A at nt 1245 of the 1 *S. marcescens* strain (GenBank accession no. M59160). Consequently, the heterogeneity of PCR ampli-
cons altered the high-resolution melting patterns and led to mistyping of these 3 samples. Excluding the 5 samples...

![Fig. 4. Work flow for rapid detection and identification of 25 clinically important bacterial species.](image)

An unknown bacterial isolate was subjected to broad-range real-time PCR and high-resolution melting analysis. According to the Tm, difference plot and derivative plot, 11 of the 25 bacterial species can be identified directly via 1-step post-PCR analysis. The remaining species can be classified into 4 melting groups. These bacterial species were identified via the unique high-resolution melting plots obtained by heteroduplex formation between the PCR products of the tested and reference bacterial species (asterisk) or via high-resolution melting analysis of a 2nd real-time PCR targeting a different region of the 16S rRNA gene.
for which the bacterial species were not in the database, we achieved a 94% (46 of 49) overall accuracy rate for bacterial species identification.

**Discussion**

A principal task in clinical microbiology is naming the bacterial species of microbial isolates to provide insight into the agent causing an infectious disease. A new standard based on the phylogenetic relationships of bacteria has evolved for identifying bacteria by comparing a stable section of the genetic code. In clinical microbiology laboratories, this task is typically performed via DNA sequencing that conclusively identifies the bacterial species (11). Recent trends in molecular diagnostic techniques offer alternatives to sequencing. For example, denaturing HPLC has been used for rapid detection and identification of various bacterial species (20). SYBR Green I–based broad-range real-time PCR followed by melting curve analysis has also been used to enhance the speed of bacterial detection (9). A recent study elucidated the application of high-resolution melting analysis of heat shock protein 65 PCR products for rapid species identification within the *Mycobacterium chelonae–abscessus* group (21). In this study, we demonstrate that clinically important bacterial species can be identified simply through high-resolution melting analysis of broad-range real-time PCR products. This novel approach is based on the same concept used for single-base variation identification and genetic defect scanning in thalassemia, acute myeloid leukemia, and various genetic diseases (13–15, 22, 23).

Regardless of the haploid nature of bacterial chromosomes, 44% (11 of 25) of the bacterial species can be identified directly via a 1-step post-PCR high-resolution melting analysis. The remaining species can be differentiated via the shift of melting plot during heteroduplex formation with a PCR amplicon of a reference bacterial species or via melting analysis of a 2nd PCR amplicon. This approach greatly enhances the application of broad-range real-time PCR and high-resolution melting analysis to identify bacteria at the species level.

High-resolution melting analysis of broad-range PCR products offers several advantages and can supply timely information to physicians in a clinical laboratory setting. The time required to perform 16S rRNA gene sequence analysis in a routine clinical microbiology laboratory is roughly 60 samples in 40 h, whereas the material cost per isolate—not including instrument purchase and labor costs—is approximately US$44 (11). Although a traditional culture-based assay is relatively cost-effective, it usually requires at least 24 h to identify the bacteria via biochemical and phenotypic analysis. When combined with rapid-cycle PCR, high-resolution melting analysis requires minimal time, and the material cost is usually <US$2. The time required for bacteria identification is considerably shorter when PCR is performed directly on clinical specimens, as mentioned in several reports (7, 8).

In some situations, this method can lead to misidentification or nontyping of bacterial species. First, different bacterial species can have the same DNA sequences within the PCR amplicon. A National Center for Biotechnology Information BLAST search (http://www.ncbi.nlm.nih.gov) indicates that most of the clinically relevant bacterial species (16 of 25) enrolled in this study have a unique PCR amplicon (Table 1). In addition to the 4 common and clinically important Enterobacteriaceae species (*E. coli*, *S. flexneri*, *S. typhimurium*, and *S. enteritidis*), the other 5 bacterial species have identical PCR amplicons with relatively rare pathogens and can cause misidentification. This potential problem can be practically overcome by designing a 2nd PCR that targets another fragment of 16S rRNA gene. We have demonstrated the feasibility of this strategy in differentiating *E. coli*, *S. flexneri*, *S. typhimurium*, and *S. enteritidis*. This scheme can be used to distinguish between bacterial species with identical PCR amplicons.

The 2nd scenario that can lead to misidentification is an uncharacterized bacterial species with a melting profile similar to another species in the database. This potential mistyping can be resolved by the use of a confirmation test of heteroduplex formation between the PCR products of the test sample and a standard strain of the putative bacterial species. When a test sample differs from the putative bacterial species, the melting plot should be changed after heteroduplex formation. No change to the melting plot occurs when a test sample matches the bacterial species of the standard strain. Alternatively, increasing the size of the high-resolution melting database by inclusion of a larger panel of pathogenic and non-pathogenic bacteria should help to rule out potential misidentification and cross-reactions with contaminants.

A 3rd issue is the heterogeneity of 16S rRNA gene within a bacterial species, which we encountered in 3 clinical samples—1 *S. marcescens* strain and 2 *E. coli* strains—during our validation study. Sequence variations between different members of the 16S rRNA gene within a bacterial isolate have been reported previously in *Mycoplasma mycoides*, *Mycoplasma spp.*, and *E. coli* (24–27). Consequently, the heterogeneity of PCR amplicons altered the high-resolution melting patterns and resulted in mistyping of these 3 samples. Because the reported primary variable sequences of the 16S rRNA gene (26) are outside the PCR amplicon used in this method, misidentification arising from sequence heterogeneity was minimal and should not have a notable effect on applying high-resolution melting analysis to bacterial species identification.

Finally, polymicrobial bacteremia was identified in 5%–22% of cases of bacteremia (28–30). In addition, *S. epidermidis*, *Micrococcus*, and *Propionibacterium acnes* are recognized common contaminants, with an expected contamination rate of 2%–3% (30). These clinical complexities may not be crucial when bacterial isolates are used for broad-range PCR amplification. Similar to other sequencing and broad-range amplification techniques, species
identification by high-resolution melting analysis may be compromised by sample contamination or multiple infections when PCR is performed directly on clinical specimens. Whether high-resolution melting profiles, such as the presence of multiple melting peaks in the derivative plot, provide additional information for differentiating single vs. multiple infections in clinical specimens is worthy of further investigation.

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