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Species-Level Identification of Staphylococcal Isolates by Real-Time PCR and Melt Curve Analysis

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A real-time PCR assay was developed to identify common staphylococcal species. A single set of consensus primers was designed to amplify a portion of the 16S rRNA gene, and a pair of fluorescence resonance energy transfer probes was used to identify species based on the unique melt properties of the probes resulting from sequence variations in the amplicons from each species. Nine common staphylococcal strains (*S. aureus*, *S. capitis*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. schleiferi*, *S. simulans*, and *S. warneri*) were used for assay development. The species-specific melting profiles were validated by correctly identifying 36 of 37 coagulase-negative staphylococcal (CoNS) isolates identified by ribotyping. In a study of clinical isolates, the PCR/melt curve approach correctly identified 56/56 *S. aureus* isolates identified by coagulase/protein A latex agglutination. Fifty-four CoNS clinical isolates characterized using the API Staph assay were studied, with the PCR/melt curve approach yielding matching identifications for 32/54 (59%). The API Staph assay was unable to identify 18 CoNS isolates, and differing results were obtained for 4 isolates. Sequencing of the 22 discrepant or unidentified CoNS samples revealed that the PCR/melt curve results were correct for all but one isolate. Thus, PCR/melt curve analysis achieved a nearly 100% accuracy and performed better than biochemical testing. Performance of the PCR/melt curve approach requires less than 2 h after colony selection. This method thus provides a rapid and accurate approach to the identification of staphylococcal species in the clinical laboratory.

Staphylococci cause a variety of serious infections and are threatening public health worldwide. The bacteria are prevalent in hospitals, where they pose a serious health risk to immunocompromised patients (11, 18). Accounting for almost 30% of all nosocomial infections and 50% of nosocomial septicemia, staphylococci are the most commonly isolated organisms in clinical laboratories (6, 19). The high occurrence of staphylococcal infections is directly correlated to the abundance of the bacteria on the skin, their minimal nutritional requirements, and the increasing use of implanted medical devices (3, 6, 14, 18).

Although there are over 32 staphylococcal species, 95% of bloodstream infections are caused by the coagulase-positive *Staphylococcus aureus* or one of three coagulase-negative staphylococcal (CoNS) species: *S. epidermidis*, *S. haemolyticus*, and *S. hominis* (16, 17). While once considered benign clinical contaminants, CoNS are now widely accepted as clinically relevant pathogenic bacteria (4, 5, 8, 9, 10, 15, 21, 22, 23). However, not all CoNS are clinically important; therefore, it is important to distinguish relevant CoNS isolates in a rapid and effective manner.

Establishing the clinical significance of usual human pathogens may require repeated isolation of the same species from the same specimen source. In fact, algorithms proposed for the workup of CoNS from multiple blood cultures include timely identification of the species involved (12). Additionally, complete species identification is suggested for CoNS isolates from other normally sterile sites, such as joint or cerebral fluid, when these infections are considered clinically significant (1). Conventional, phenotypic identification, using physical properties and substrate degradation, or commercial kit identification systems and automated instruments have an accuracy of 70 to 90%, with most requiring overnight incubation (13). The expense, time, and low accuracy of identification have resulted in policies for many laboratories that do not included identification of CoNS, even when they are considered clinically significant (24). A simple and accurate assay for the identification of selected strains of CoNS would improve the clinical usefulness of microbiology reports.

The aim of this study was to develop a rapid-cycle real-time PCR assay that would distinguish various clinically relevant strains of *Staphylococcus* via unique melting curve profiles of each species. The assay was based upon the ability of this approach to detect sequence variation present within the 16S rRNA amplicons of each species. After development and validation using a panel of staphylococcal isolates identified by ribotyping, a larger series of 110 clinical isolates was examined.

MATERIALS AND METHODS

Bacterial strains and clinical samples. Nine methicillin-susceptible American Type Culture Collection (ATCC) (Manassas, VA) reference staphylococcal strains were used for assay development and subsequently as positive controls. Organisms were grown on blood agar plates overnight at 37°C and subcultured three times to eliminate contamination and ensure colony purity. Additionally, 37 isolates identified by ribotyping were obtained from the University of Iowa and were used to assess the accuracy of the species identification test. Lastly, 111

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TABLE 1. Nucleotide sequences and positions of primers and probes

Oligonucleotide[a]	Sequence	Position[b]	Product length (bp)
16S3 up	5′-ATGCAAGTCGAGCGAAC-3′	28–44	282
16S3 down	5′-TGTCTCAGTTCCAGTGTGGC-3′	309–290	
16S FITC probe	5′-CACTTATAGATGGATCCGCGCCGCATTAGCTAGT-FITC-3′	199–232	
16S Red probe	5′-Red-GTAAGGTAACGGCTTACCAAGGCAACGAT-Phos-3′	235–263	

[a] FITC, fluorescein isothiocyanate; Red, LightCycler-Red 640.
[b] Position of the nucleotide sequence in the *S. epidermidis* 16S rRNA gene (accession number L37605).

clinical isolates from the Microbiology Laboratory at Evanston Northwestern Healthcare were used to clinically validate the assay.

Staphylococcus aureus isolates were identified using colony morphology, catalase, Gram stain morphology, and coagulase/protein A testing via latex agglutination (Staphaurex; Murex Diagnostics Inc., Norcross, GA). CoNS were identified using API Staph (bioMerieux Vitek, Inc., Hazelwood, MO) and supplementary tests as suggested by the kit instructions.

DNA extraction from strains. A rapid boiling procedure was used to prepare template DNA (20). Two 10-μl loops of bacteria taken from the blood agar plate were collected and suspended in 200 μl of a lysis buffer comprised of 1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. After boiling for 10 min, the suspension was centrifuged for 2 min to sediment bacterial debris. The supernatant was removed, and 2 μl was used directly for PCR amplification. The residual sample was stored at −20°C.

Real-time PCR primers and probes. The sequences and positions of the amplification primers and the detection probes are shown in Table 1. The oligonucleotides were designed based on 16S rRNA gene sequence data obtained from GenBank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD). Primers were chosen to amplify a variable region of the 16S rRNA gene. Probe sequences were chosen by aligning the 16S rRNA amplicon across nine species (*S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. capitis*, *S. simulans*, *S. schleiferi*, and *S. warneri*) and selecting the region with suitable variability between strains (Fig. 1). The TM Utility software supplied by Idaho Technologies (Salt Lake City, UT) was used to predict theoretical melting temperatures (T_ms) for each species. Probe sequences were chosen that were predicted to result in seven distinct melt profiles with T_m peak temperatures separated by at least 1°C for each of the staphylococcal species. Both probes were fluorescently labeled; the upstream probe was 3′ labeled with fluorescein and the other recognized adjacent sequences in the amplicon and was 5′ labeled with LightCycler-Red 640 and phosphorylated on the 3′ end. The probe sequences were homologous to *S. epidermidis* and varied by a maximum of six nucleotides from the amplicon sequences of the other species. IT BioChem (Salt Lake City, Utah) synthesized the primers and the probes.

Real-time PCR assay conditions. Amplification and melt curve analysis were performed using a LightCycler (Roche Diagnostics, Indianapolis, IN). Reactions were carried out in a total volume of 20 μl. PCR mixes contained primers and probes at final concentrations of 0.5 μM and 0.2 μM, respectively, 2 μl of 10× LightCycler DNA FastStart master hybridization probe kit mix (10 mM $MgCl_2$, *Taq* DNA polymerase, deoxynucleoside triphosphates and reaction buffer) (Roche Diagnostics, Indianapolis, IN), 2 μl of bacterial DNA, and $MgCl_2$ at a final concentration of 3.5 mM. Cycling conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 57°C for 10 s, and 73°C for 10 s. Melt curves were generated by holding the capillaries at 95°C for 10 s, followed by 40°C for 15 s. The temperature was then gradually increased at 0.2°C/s to 95°C with step fluorescence acquisition. Melt curve profiles were assessed and analyzed using LightCycler software.

Sequencing of discrepant specimens. Cycle sequencing using the Cy5/Cy5.5 Dye Primer cycle sequencing kit (Visible Genetics, Toronto, Ontario, Canada) was performed utilizing a 22-μl master mix containing 2 to 4 μl of an amplification reaction mixture without fluorescently labeled probes (approximately 100 ng of amplified products), 2.5 μl of the 10× sequencing buffer, 100 pmol of each fluorescently labeled primer (5′ Cy5.5 forward primer and 5′ Cy5 reverse primer), 14% dimethyl sulfoxide and 0.3 unit of ThermoSequenase enzyme. Five microliters of this master mix was added to 3 μl of nucleotide termination mixes in separate PCR tubes for amplification. The cycling parameters for amplification in a Perkin-Elmer 2400 thermal cycler were as follows: an initial 5 min at 94°C; 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min; and a final extension step at 72°C for 7 min. Six microliters of MGB loading dye (Visible Genetics) was added to each nucleotide termination mix, followed by sample denaturation at 95°C for 3 min and rapid chilling on ice immediately prior to gel loading. The sequencing reaction products were analyzed utilizing 6% polyacrylamide gels in 1× Tris-borate-EDTA buffer in a MicroGene Clipper automated fluorescent DNA sequencer and a two-color detection system (Visible Genetics).

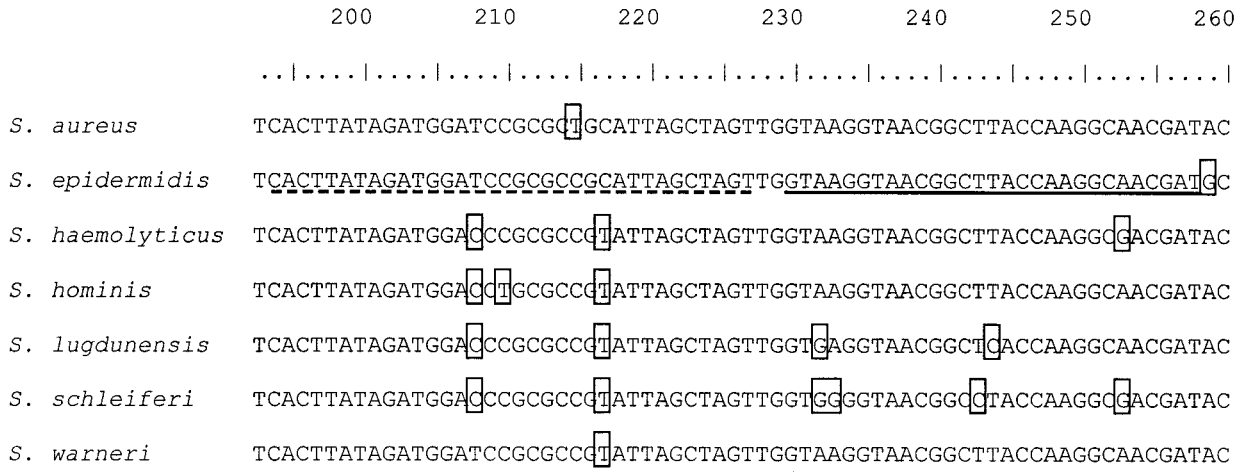

FIG. 1. Locations of fluorescently labeled hybridization probes within the 16S rRNA gene. The fluorescein isothiocyanate (dashed underline)- and LightCycler-Red 640 (solid underline)-labeled probes extend from position 199 to 232 and 235 to 263, respectively. Probe sequences are homologous to *S. epidermidis*. Boxed bases indicate differences from the probe sequence. Probes were selected to maximize these differences.

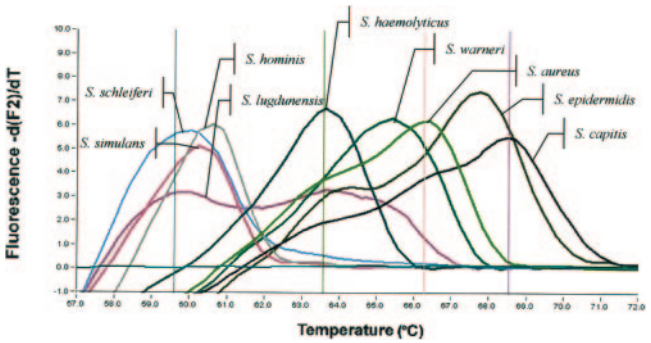

FIG. 2. Characteristic melt curves generated by differential binding of template DNA to hybridization probes.

The fluorescence data collected were automatically analyzed by the GeneObjects software, and the sequences were then compared to published sequences available in GenBank.

RESULTS

PCR amplification and species determination. The 16S rRNA gene primers and probes chosen successfully amplified and detected all of the staphylococcal species chosen for study (data not shown).

The ability of the probes to distinguish between different staphylococcal species was assessed by testing the melt curve assay on the nine control strains obtained from the ATCC. The assay produced distinct melting profiles (Fig. 2). Overall, this melt curve analysis yielded T_m values varying from 60 to 70°C, with reproducible results for each species. Each species had a characteristically shaped melt profile; therefore, not only were the peak T_ms important for species identification, but the shapes of the curves were useful as well. For example, while *S. epidermidis* and *S. capitis* resulted in prominent peaks with similar T_m values, a shoulder or small secondary peak was present for *S. epidermidis* isolates. The presence of a shoulder or a second peak can be explained in part by the presence of mismatched bases in both probe regions. In three instances, melt curve analysis yielded peaks that were so close in T_m that the individual species could not be accurately differentiated by this approach; *S. lugdunensis* and *S. haemolyticus* were thus considered together in subsequent analysis, as were *S. hominis*, *S. schleiferi*, and *S. simulans*. *S. capitis* and *S. epidermidis* could generally be distinguished by a shoulder, despite similar T_m peak values. *S. aureus* and *S. warneri* yielded unique T_m patterns.

The melt curve analysis proved to be very reproducible in replicate studies, as shown in Table 2. In studies comparing different bacterial colonies isolated from the same sample and different batches of master mix, essentially no variation in the T_m values was seen. However, slight T_m variations did exist between different LightCycler instruments, leading us to utilize a single instrument for the studies reported in this paper.

Additionally, melt curve results were reproducible when different isolates of the same species were used, indicating that little sequence heterogeneity exists in the region targeted for this assay. This is further supported by the results of validation and clinical studies using this assay, as described below.

TABLE 2. Average T_m values of nine ATCC reference strains

Species	Strain	T_m (°C)[a]	
		Avg	SD
S. aureus	ATCC 29213	67.68, 63.08	1.11, 0.834
S. epidermidis	ATCC 14990	68.14	1.08
S. capitis	ATCC 27840	69.26	1.66
S. warneri	ATCC 27836	66.22	1.49
S. haemolyticus	ATCC 29970	64.25, 58.49	2.02, 1.20
S. lugdunensis	ATCC 43808	64.57, 58.87	1.31, 1.56
S. hominis	ATCC 27844	61.26	1.23
S. schleiferi	ATCC 43809	59.37	1.34
S. simulans	ATCC 27848	59.83	1.34

[a] T_m values and standard deviations were generated using data from six replicate runs. Two T_ms indicate two distinct peaks.

Assay validation. In order to more thoroughly assess the accuracy of the species identification assay, 37 blinded CoNS isolates were obtained from the Molecular Epidemiology and Fungus Testing Laboratory at the University of Iowa College of Medicine and were tested. The species identities of these isolates were definitively determined via ribotyping methods at the University of Iowa (2). Melting profiles generated by the species identification assay correctly identified 36 of the 37 isolates, as shown in Table 3. One isolate of *S. capitis* was misidentified as *S. epidermidis*.

Analysis of clinical isolates. One hundred ten clinical isolates from the Microbiology Laboratory at Evanston Northwestern Healthcare were tested to fully validate the assay in the clinical setting. The samples were supplied in a blinded fashion and included 56 *S. aureus* and 54 CoNS isolates. The PCR/melt curve method matched the API Staph assay for all 56 of the *S. aureus* specimens (100%) and for 32 of the 54 CoNS isolates (59%), as shown in Table 4. Melt curves were generally easily identified and closely matched those for all other isolates of the same species; this high degree of reproducibility indicates the minimal sequence variation occurring with in the portion of the 16S rRNA gene chosen for study.

Consensus identifications by the PCR/melt curve and API Staph assays were considered definitive identifications. However, the API Staph assay could not provide any identification for 18 (33%) of the 54 isolates tested; all of these were identified by PCR melt curve analysis. The API Staph assay was most effective in the identification of *S epidermidis*, but it performed less well for other species of CoNS, as summarized in

TABLE 3. Molecular identification of 37 ribotyped CoNS isolates by PCR/melt curve analysis

Species identified by ribotyping	No. of isolates identified by PCR/melt curve analysis/total
S. capitis	0/1[a]
S. epidermidis	11/11
S. haemolyticus/*S. lugdunensis*	13/13
S. hominis/*S. simulans*/*S. schleiferi*	7/7
S. warneri	5/5

[a] One isolate of *S. capitis* was called *S. epidermidis* as a result of the similar T_m values for these species.

TABLE 4. Identification of 110 staphylococcal blood culture isolates by molecular and conventional methods

Consensus result[a] or result confirmed by gene sequencing (*n*)	PCR melt curve identification (*n*)	Conventional identification (coagulase and API Staph) (*n*)
S. aureus (56)	*S. aureus* (56)	*S. aureus* (56)
S. epidermidis (34)	*S. epidermidis* (33), *S. hominus* (1)	*S. epidermidis* (30), *S. lugdunensis* (1), no identification (3)
S. capitis (2)	*S. capitis*/*S. epidermidis* (2)	*S. capitis* (1), no identification (1)
S. hominis (11)	*S. hominis*/*S. schleiferi*/*S. simulans* (11)	*S. lugdunensis* (1), *S. epidermidis* (1), no identification (9)
S. haemolyticus (3)	*S. haemolyticus* (3)	No identification (3)
S. warneri (2)	*S. warneri* (2)	*S. hominus* (1), no identification (1)
S. simulans (2)	*S. simulans*/*S. schleiferi*/*S. hominis* (2)	*S. simulans* (1), no identification (1)

[a] Consensus of results obtained from PCR/melt curve and API Staph analyses.

Table 4. Some species, such as *S. hominis*, were never correctly identified by the API Staph phenotypic assay.

Discrepant analysis. Twenty-two of the CoNS isolates for which either API Staph yielded no definitive result or discrepant molecular and phenotypic results were obtained were subjected to 16S rRNA gene sequencing to determine the correct species. Twenty-one of 22 sequences matched the species categorization by the PCR/melt curve method as opposed to the API Staph assay, bringing the accuracy rate for the PCR/melt curve species identification method to 53/54, or over 98%, for the identification of CoNS. A single isolate of *S. epidermidis* was called *S. hominis* (or *S. schleiferi* or *S. simulans*) by the PCR melt curve approach and was classified as *S. lugdunensis* by the API Staph phenotypic assay.

DISCUSSION

We successfully developed a staphylococcal species identification assay based on differential melting of fluorescently labeled hybridization probes from 16S rRNA gene amplicons. This assay can be completed in under 2 h, including DNA preparation from a bacterial colony, PCR amplification, and melt curve analysis. Development was based on melt curve analysis of nine ATCC control strains selected because they represent likely pathogenic or contaminating bloodstream isolates in clinical microbiology laboratories. Melt curve analysis was validated, in a blinded experiment, by correctly identifying 36 of 37 CoNS isolates previously identified by ribotyping. PCR melt curve identification misidentified one *S. capitis* isolate as *S. epidermidis*. Finally, melt curve analysis was used in a blinded clinical evaluation to identify 56 of 56 *S. aureus* and 53 of 54 CoNS strains detected in patient blood cultures. In the clinical evaluation, PCR correctly identified over 98% of the staphylococcal strains, while conventional identification using latex agglutination (coagulase and protein A detection) and API Staph correctly identified only 79% of the 110 strains. The one PCR misidentification was an *S. epidermidis* strain identified by melt curve analysis as *S. hominis*. Species identities were confirmed by gene sequencing when PCR and conventional identifications did not agree.

The 16S rRNA gene primers and probes chosen successfully amplified and detected all staphylococci but were unable to reliably differentiate some species. *S. lugdunensis* and *S. haemolyticus* had similar T_m values and could not be separated, and *S. hominis*, *S. schleiferi*, and *S. simulans* could not be differentiated for the same reason. Although this represents a shortcoming for the melt curve analysis approach, *S. lugdunensis* can be differentiated rapidly from *S. haemolyticus* by performing a slide coagulase test (*S. lugdunensis* is positive) (1). *S. schleiferi* can be separated from *S. hominis* and *S. simulans* by using slide coagulase and staphylococcal coagulase tests (*S. schleiferi* subspecies are positive with at least one of these two tests) (1). *S. hominis* and *S. simulans* can be differentiated using anaerobic growth in thioglycolate medium (*S. hominis* shows heavier growth in the top of the tube only, while *S. simulans* grows well throughout the aerobic and anaerobic portions of the medium) (1). In practice, it may not be necessary to routinely differentiate the coagulase-negative species *S. hominis* and *S simulans* in most clinical situations. It is important to note that the coagulase/clumping factor-positive species, including *S. aureus*, *S. schleiferi*, and *S. lugdunensis*, can be readily differentiated by PCR and melt curve analysis.

Characteristic melt curves were reproducible consistently from run to run, using replicate studies and different master mix lots. Equally important, reproducibility was maintained across different strains representing the same species. Slight variations did exist between different LightCycler instruments, prompting us to use a single instrument for all comparisons.

Another real-time PCR approach has been developed to identify species of CoNS (7). Edwards and colleagues used melt curves generated by the combination of a fluorescent intercalating probe along with a labeled hybridization probe to discriminate 15 staphylococcal species. However, in contrast to our approach, three separate PCR amplifications and multiple sets of probes were required (7). Either of these PCR/melt curve approaches offers certain advantages over another molecular approach, the sequencing of 16S rRNA gene sequences. Real-time PCR/melt curve analysis is far less costly and time-consuming than sequencing, requiring only the time, expense, expertise, and equipment needed for real-time PCR. 16S rRNA analysis requires automated sequencing equipment and considerably more expertise and time, and at present it is practical in fewer laboratories. On the other hand, sequencing will likely provide a more definitive identification of the organism and can be applied to a wider spectrum of unknown organisms.

In summary, the combination of real-time PCR and melt curve analysis is a rapid and accurate method for the identification of staphylococci grown from clinical specimens, including blood and other normally sterile body fluids and tissues. The entire specimen preparation and assay can be finished within 2 h, and the assay is more accurate than conventional

identification methods. Real-time PCR instruments, such as the LightCycler, offer molecular technology to laboratories that were unable to develop gel-based PCR methods. Studies are currently under way in our laboratory to adopt this assay for direct identification of staphylococci in blood culture broths and other clinical specimens.

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