Evaluation of the LightCycler *Staphylococcus* M^{GRADE} Kits on Positive Blood Cultures That Contained Gram-Positive Cocci in Clusters

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We evaluated the Roche LightCycler *Staphylococcus* M^{GRADE} kits to differentiate between *Staphylococcus aureus* and coagulase-negative staphylococci in blood cultures growing clusters of gram-positive cocci. Testing 100 bottles (36 containing *S. aureus*), the assay was 100% sensitive and 98.44% specific for *S. aureus* and 100% sensitive and specific for coagulase-negative staphylococci.

Gram-positive cocci in clusters (GPCC) noted on Gram staining of blood cultures are usually either *Staphylococcus aureus* or coagulase-negative staphylococci (CoNS). While the latter may be considered contaminants, the former is usually considered a true pathogen. It takes an additional day and supplementary testing to differentiate these two organisms by conventional methods. Multiple studies have reported rapid and accurate detection of *S. aureus* in blood culture bottles growing GPCC, which speak to the feasibility and interest in this approach (1–3, 5, 6, 9, 10). Accurate detection of the presence of all staphylococci in blood culture bottles and simultaneous identification of *S. aureus* among them using real-time PCR have also been demonstrated (8). This approach identifies CoNS by actually detecting its presence rather than by assuming its presence in the absence of *S. aureus* in cultures growing GPCC. A limitation to the use of real-time PCR for detection of staphylococci in a clinical laboratory is the expertise necessary to conduct real-time PCR and control for all the variables that might give rise to inconsistencies in the reaction over a period of time. The availability of a standardized kit would ease the process to the point where staphylococcal detection in blood cultures by real-time PCR may be seriously considered for practical application in clinical microbiology laboratories. The purpose of this study was to evaluate the LightCycler *Staphylococcus* M^{GRADE} kits for the detection and identification of *S. aureus* and CoNS in blood culture bottles growing GPCC.

Aerobic and anaerobic FAN-containing blood culture bottles that gave a positive signal in the BacT/ALERT blood culture system (bioMérieux, Inc., Durham, NC) and revealed the presence of GPCC were studied. A 0.5-ml aliquot of fluid was immediately removed for PCR from the positive blood culture bottle after brief manual agitation. Identification of the bacterium proceeded according to the standard laboratory protocol that included inoculation onto 5% sheep blood agar, incubation at 37°C, and identification based on colony mor-

phology and coagulase test results. The aliquot removed for PCR was processed to remove the charcoal and DNA was prepared by a differential centrifugation and lysis buffer treatment as previously described (9). Charcoal was removed by centrifuging the aliquot at 850 × g for 2 min and discarding the pellet. The supernatant was centrifuged at 11,500 × g for 5 min. The resulting pellet was resuspended in 200 µl of a lysis buffer (7) containing 1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA and incubated in a screw-cap reaction tube at 100°C for 10 min. The mixture was again centrifuged at 850 × g for 2 min, and the supernatant was removed and stored at –20°C for future testing.

Real-time PCR was performed in the LightCycler instrument (Roche Diagnostics, Indianapolis, IN) using the following LightCycler reagents (Roche): FastStart DNA master hybridization probes, *Staphylococcus* primer/hybridization probes, and the *Staphylococcus* template set. With these kits, a portion of the ITS region (internal transcribed spacer between the 16S and 23S genes) of *Staphylococcus* spp. are amplified and *S. aureus* and CoNS are detected and differentiated by melting curve analysis using fluorescence resonance energy transfer probes. In addition, PCR inhibition is monitored by the inclusion of a probe for the recovery template.

Each 20-µl PCR mixture consisted of 8.4 µl of PCR H₂O, 1.6 µl of 25 mM MgCl₂ (final concentration of 3 mM), 2.0 µl of primer/hybridization probe mix, 1.0 µl of the recovery template diluted 1:10, 2.0 µl of Fast Start mix and 5 µl of DNA target. The LightCycler reaction protocol was as follows: denaturation and *Taq* polymerase activation (95°C for 10 min), 45 cycles of PCR (1 cycle consists of 95°C for 10 s, 50°C for 15 s, and 72°C for 10 s), a melting phase (95°C for 60 s, and 40°C to 80°C in 60 s), and a cooling phase at 40° for 30 s. Positive amplification curves indicated the presence of *Staphylococcus* spp. Melting curve analysis was performed with channel F2 (LightCycler Red 640 probe) for differentiating between *S. aureus* and CoNS and with channel F3 (LightCycler Red 705 probe) for detecting the recovery template (internal control for PCR inhibition). Interpretation of melting temperatures was done according to the package insert for the *Staphylococcus* primer/hybridization probe M^{GRADE} kit.

The specimens were also analyzed by another real-time PCR

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FIG. 1. Box plot comparing crossing thresholds for *S. aureus* (SA) and CoNS. *, data point that lies more than 1.5 times the interquartile range from the end of the box.

FIG. 2. Box plot comparing melting temperatures for *S. aureus* (SA) and CoNS. *, data point that lies more than 1.5 times the interquartile range from the end of the box.

assay that had been previously described, targeting the *sa442* DNA fragment of *S. aureus*, to serve as an independent confirmation of the presence of *S. aureus* (9).

One hundred positive bottles analyzed in this manner contained 36 *S. aureus* isolates, 63 CoNS isolates, and 1 *Micrococcus* species. Positive and negative controls reacted appropriately. The LightCycler *Staphylococcus* M^{GRADE} kits detected the presence of staphylococci in all 99 specimens that contained the microorganism. The specimen containing *Micrococcus* returned a negative result. All bottles containing *S. aureus* were correctly identified. The LightCycler *Staphylococcus* M^{GRADE} kits revealed the presence of CoNS in all of the 63 positive blood culture bottles that contained this organism. However, one of these bottles that grew only CoNS also tested positive for the presence of *S. aureus* by the LightCycler *Staphylococcus* M^{GRADE} kits by postamplification melting curve analysis. The result of the *S. aureus*-specific *sa442* assay on this specimen was negative. Interestingly, this specimen came from a patient on hemodialysis; patients requiring hemodialysis are known to be at risk for *S. aureus* infection. The possibility that the LightCycler *Staphylococcus* M^{GRADE} kit PCR detected DNA from nonviable *S. aureus* and that the discrepancy between the two PCR assays represents differences in sensitivity remains. Although the *sa442* assay was previously shown to have a sensitivity of 100% (9), it may really be less sensitive than the *Staphylococcus* M^{GRADE} kit PCR when examining small bacterial loads because the *sa442* DNA fragment exists as a single copy and the ITS region is present in multiple copies, but this remains speculative. This result was categorized as a false positive for the LightCycler *Staphylococcus* M^{GRADE} kit. The mean crossing thresholds (standard deviations shown in parentheses) were 30.13 (3.58) and 29.07 (4.84) cycles for *S. aureus* and CoNS, respectively; Fig. 1 shows their distributions graphically. The mean melting temperatures (standard deviations shown in parentheses) were 60.68°C (0.34) and 51.41°C (1.16), respectively, with Fig. 2 demonstrating that the two were clearly distinguishable. Therefore, in this analysis, we found the LightCycler *Staphylococcus* M^{GRADE} kit to be 100% sensitive and specific for the detection of the

Staphylococcus genus, 100% sensitive and specific for the detection of the presence of CoNS, and 100% sensitive and 98.44% specific for the detection of *S. aureus* in positive blood culture bottles that contained GPCC.

It would be ideal if we could use PCR to identify microorganisms in blood culture bottles as soon as they give a positive signal. A limitation of many real-time PCR assays is that they allow for accurate identification of a selected species or genus of microorganism. Multiplex PCR using multiple-species-specific probes are one approach for the simultaneous detection of multiple microorganisms (13). However, the complexities of these assays, which arise mainly from the interactions between the various oligonucleotide primers and probes make this approach less practical in many laboratories. Other approaches to rapid identification of bacteria in blood cultures have been reported (11). Although PCR–single-strand conformation polymorphism (PCR–SSCP) analysis has been shown to be able to detect a wide variety of microorganisms, multiple organisms may have the same SSCP pattern, and mixtures of organisms will confuse the interpretation of results. A more practical approach would be to identify specific important microorganisms by targeted real-time PCR in positive blood culture bottles. As a genus, staphylococci are the commonest cause of positive blood cultures, and of these staphylococci, *S. aureus* is almost always a significant pathogen (12). Thus, laboratories may be interested in targeting this genus, and specifically *S. aureus*, for rapid identification in blood culture systems, and the LightCycler *Staphylococcus* M^{GRADE} kit is one commercially available system that is available to laboratories that contemplate doing so, especially those that already have a LightCycler in place. The question that arises is if there is any utility in knowing the identity of the microorganism 1 day in advance while susceptibility test results are still pending. The assay could make a difference where single cultures are positive. If the microorganism were identified as *S. aureus*, the culture result would be taken seriously with further workup initiated immediately, rather than hesitating to proceed with immediate further workup because of the possibility that a single CoNS may be a contaminant. In our laboratory we are

currently using fluorescence in situ hybridization with peptide nucleic acid probes for the identification of *S. aureus* as soon as blood culture bottles turn positive (4). By this strategy we identify gram-positive cocci in blood culture bottles as being *S. aureus* 1 day earlier than would have been possible by conventional methods. The use of the LightCycler *Staphylococcus* M^{GRADE} kits would be an equally effective alternative for achieving the same end. In order to provide same-day results, this would need planning on batching of specimens with cutoff times specified in a manner that would suit the workflow of the laboratory planning to use the test. It would be more effective if combined with real-time PCR detection of methicillin resistance. The cost of the assay, excluding capital costs, using the kits described in this study would be about \$25 per sample, of which the three reagent kits account for \$16.43, assuming a batch size of 20 with a positive and negative control in every batch and a 15% repeat rate. Institutions will have to decide if the added cost is worth the knowledge of the identity of the staphylococcus 1 day earlier than would be available by conventional testing. Real-time PCR identification of *S. aureus* and methicillin resistance in signal-positive blood culture bottles targeting the *femA* and *mecA* genes, respectively, has been implemented in the routine work of a lab (6). This signifies an interest in early identification of *S. aureus* and the presence of methicillin resistance in positive blood cultures. We conclude that *S. aureus* and CoNS can be accurately detected and identified using the LightCycler *Staphylococcus* M^{GRADE} kits in positive blood cultures that contain GPCC. The availability of such kits makes the application of real-time PCR for detection of staphylococci in positive blood culture bottles practical. The use of PCR assays augments our diagnostic capabilities when used in conjunction with automated blood culture systems.

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