JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2009, p. 1082–1086 Vol. 47, No. 4
0095-1137/09/$08.00+0 doi:10.1128/JCM.01850-08


# Identification of *Staphylococcus* Species Directly from Positive Blood Culture Broth by Use of Molecular and Conventional Methods▽

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Received 24 September 2008/Returned for modification 14 November 2008/Accepted 5 February 2009

**We compared two real-time PCR assays (both by the use of melting curve analysis) for their ability to identify *Staphylococcus* species directly from 200 positive blood culture bottles. The PCR assays correctly identified 83% to 94% of the *Staphylococcus* isolates to species clusters. Molecular testing significantly outperformed commercially available latex tests (sensitivity for both latex tests, <15%) when it was used directly with broth from signal-positive blood cultures.**

*Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) are among the bacteria most commonly isolated from clinical blood culture specimens (15, 17). While the isolation of *S. aureus* from blood cultures is considered to represent a true infection, the isolation of CoNS more typically reflects contamination (1) and hence is reported as CoNS without further identification. At this time, there is only one report of a commercial assay that successfully differentiates sensitive and resistant strains of *S. aureus*, and it does not identify CoNS strains to the species level (22). However, imprecise species identification may foster this practice and may impair the ability of clinicians and researchers to associate certain species with a predilection to cause disease. Species-level identification may be necessary when isolates are recovered from multiple blood culture bottles (7), immunocompromised patients (6, 7, 13), or patients with indwelling devices (4, 8, 13). Although *S. epidermidis* is the most commonly isolated CoNS (19), other CoNS species have been shown to cause nosocomial infections, including bacteremia (3, 5, 10, 18), making fast and accurate species determination useful for the identification and management of patients infected with such strains.

Standard methods for the identification of *Staphylococcus* species from positive blood culture broths include the performance of a Gram stain and subculture to an appropriate solid medium to obtain colonies for further testing. Several commercial assays can differentiate colonies of *S. aureus* from CoNS and further differentiate CoNS into species (20), but phenotypic methods for the identification of different CoNS species often suffer because of a lack of accuracy (9). We have previously described a real-time PCR assay that can differentiate *S. aureus* from CoNS by the use of an aliquot directly from the blood culture broth (14); however, no available tests currently give an accurate identification of species of other staphylococci directly from blood cultures.

Our goal for this project was to compare two real-time PCR assays, an assay developed in-house (20) and a laboratory-optimized assay that used analyte-specific reagents (ASRs; Roche Diagnostics Corp., Indianapolis, IN) for their abilities to correctly identify *Staphylococcus* species directly from positive blood culture bottles. We also evaluated how well two commercially available latex agglutination tests, the Staphaurex Plus test and the PBP2′ test (Remel, Inc., Lenexa, KS), performed when they were directly inoculated with positive blood culture broths.

## MATERIALS AND METHODS

**Samples for study.** All blood culture samples came from the patient population at NorthShore University HealthSystem (formerly Evanston Northwestern Healthcare). Blood samples for culture were collected by standard techniques. Aerobic bottles (BD Bactec Plus and Aerobic/F bottles) and anaerobic bottles (BD Bactec standard Anaerobic/F bottles) were incubated in Bactec 9240 instruments (BD Diagnostics, Sparks, MD) at 35°C for up to 5 days. Broth from bottles that gave a positive signal were Gram stained, and those that showed gram-positive cocci in clusters were included in the study.

**Conventional phenotypic testing.** A slight modification of the method used by Rappaport et al. (16) was used to process the positive blood culture broths before the agglutination tests were performed. Eight milliliters of blood from positive culture bottles was transferred into a conical tube, and the tube was centrifuged at 110 × *g* for 10 min to sediment the blood cells in the sample. The supernatant was then transferred into another conical tube, and the tube was centrifuged at 3,000 × *g* for 10 min to sediment the bacterial cells. The bacterial cell pellet was then washed once by resuspending it in 8 ml of deionized (DI) water and recentrifuged at 3,000 × *g* for 10 min. The supernatant water and cell debris were discarded, and the bacterial cell pellet was resuspended in 2 ml of DI water. This suspension was used to perform the Staphaurex Plus (Murex Biotech Ltd., Dartford, Kent, United Kingdom) and the PBP2′ (Oxoid Ltd., Basingtoke, Hants, United Kingdom) latex agglutination tests. For the Staphaurex Plus test, 2 drops of the bacterial suspension was added to the test latex and the control latex, and the test card was rotated for 1 min, which was longer than the manufacturer's recommended 20 s so that positive samples with low bacterial cell counts could be captured. For the PBP2′ test, 12 to 15 drops of the bacterial suspension was added to reagent 1 (provided with the test kit), and the test was completed by the manufacturer's protocol. Any agglutination seen in either latex test was considered positive, and no agglutination was reported as a negative result.

For conventional culture, a small aliquot of every positive blood culture was also plated onto a blood agar plate and a chocolate agar plate (Remel, Inc.), and the plates were incubated at 35°C for 24 to 48 h. After incubation, representative colonies resembling staphylococci were tested by the Staphaurex latex agglutination test (Murex Biotech Ltd.), and all positive colonies were identified as *S. aureus*. Testing with an API Staph test strip (bioMerieux, Durham, NC) was done according to the manufacturer's protocol with all isolates not identified as *S. aureus*. API Staph strips were held for a full 24 h before the results were read. The test was repeated for any isolate for which genus and species identifications

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▽ Published ahead of print on 11 February 2009.

TABLE 1. $T_m$s for species clusters for the in-house real-time PCR assay

| *Staphylococcus* species | $T_m$(°C) | Organism cluster | SD | 95% CI |
|---|---|---|---|---|
| *S. epidermidis* | 70.26 | 1 | 0.41 | 70.74–70.9 |
| *S. capitis* | 70.12 | 1 | | |
| *S. lugdunensis* | 64.76 | 2 | 0.36 | 64.92–65.62 |
| *S. haemolyticus* | 64.90 | 2 | | |
| *S. saprophyticus* | 65.32 | 2 | | |
| *S. simulans* | 59.75 | 3 | 0.58 | 61.35–61.83 |
| *S. schleiferi* | 58.57 | 3 | | |
| *S. hominis* | 60.8/62.00[a] | 3 | | |
| *S. aureus* | 67.13 | 4 | 0.40 | 67.47–67.65 |
| *S. warneri* | 66.57 | 4 | | |

[a] *S. hominis* demonstrated two distinct melting curves with separate $T_m$ values.

were not provided by the API Staph system the first time. If no identifications were provided the second time, it was marked as "no species identified" and was considered to have a discrepant result by use of the API Staph system.

**Real-time PCR assays.** A total of 200 μl of broth (not manipulated in any fashion) from the same positive blood culture bottle used for latex agglutination testing and subculture was directly placed onto a Generation capture column (Gentra, Minneapolis, MN) and washed according to the manufacturer's instructions (14). DNA was recovered in 100 μl of elution buffer and was used for real-time PCR. For the in-house PCR for *Staphylococcus* species identification described previously, 2 μl was used (20), and 5 μl of DNA was used for the Roche ASR laboratory-optimized assay. The in-house PCR did not have an internal control, and hence, there was no monitor for inhibition. However, no inhibition was observed since the amplification of all samples occurred. The Roche ASR test contained an internal control so that inhibition could be monitored if no amplification for any *Staphylococcus* species occurred. Specimens inhibited on the initial run of the Roche ASR assay were retested. If they were still inhibited, the DNA was diluted 1:10 with sterile DI water and the test was repeated again.

The reaction mixture and conditions for the in-house PCR assay were identical to those described by Skow and colleagues (20). *S. aureus* was used as a positive control, and sterile water was used as the blank control. The reaction mixture used for the Roche ASR assay consisted of 5 μl extracted DNA, 2 μl of LightCycler FastStart DNA master hybridization probes $M^{GRADE}$ mix, 2 μl of LightCycler *Staphylococcus* primer-hybridization probes $M^{GRADE}$, 1 μl of a 1:10 dilution of LightCycler *Staphylococcus* recovery template $M^{GRADE}$, and 10 μl of sterile water. LightCycler *Staphylococcus* template set $M^{GRADE}$ was used as a positive control, and sterile water was used as the blank control. All the reagents were obtained from Roche Diagnostics. The cycling conditions that we used were 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 50°C for 15 s, and 72°C for 10 s, with fluorescence acquisition occurring at the end of each annealing. Melting curves were generated by holding the capillaries at 95°C for 60 s and then at 40°C for 60 s. The temperature was then gradually increased by 0.1°C/s to 80°C with continuous fluorescence acquisition.

The sequences and the positions of the primers and detection probes for the in-house PCR assay were those described by Skow and colleagues (20). The Roche ASR is proprietary and was not disclosed to us. The melting temperatures ($T_m$s) for the in-house assay and the ASR (Roche) protocol depicting the organism clusters are listed in Tables 1 and 2, respectively. Amplification and melting curve analysis for both real-time PCR tests were performed on a Roche LightCycler instrument. An in-house PCR assay for the detection of *mecA* (14) and a laboratory-optimized Roche ASR test for *mecA* were also performed with the extracted DNA.

**Determination of results and discrepant analysis for method comparison.** If the result of each PCR for the detection of *mecA* matched, it was considered the true result and the "gold standard" for the determination of methicillin resistance and calculation of the sensitivity and the specificity of the PBP2′ latex agglutination test. Microbiologic culture results were considered the gold standard for calculation of the sensitivity and the specificity of the Staphaurex Plus test performed with the blood culture broth specimen.

Both real-time PCR assays differentiated staphylococcal species into clusters on the basis of melting curve analysis. Clusters represented more than one species with the same melting temperature (Tables 1 and 2). For each specimen, we matched the melting temperature to the clusters from the in-house PCR assay and the Roche ASR PCR assay; when the results for one species common to the clusters from both assays agreed, then that was considered the final identification for both PCR assays. If two or more staphylococcal species belonged to the same cluster in both PCR assays, then the API Staph test strip results were matched. If the species identified by each PCR assay and the API Staph identification method matched, the identification was considered the true gold standard identification. If there was no species agreement between the PCR assays and the API Staph test, then the identification was resolved by 16S rRNA sequencing (20). If sequencing did not resolve the discrepancy, then both PCR assays were repeated with isolated colonies grown from the blood culture (20) and consensus among the colony-based test and sequencing established the final species identification. The results obtained by each PCR assay and the Staph API assay were compared to the final resolved species identification, and the percent agreement was calculated.

## RESULTS

**Conventional phenotypic testing.** From a total of 200 unique blood culture broth specimens tested by the Staphaurex Plus test, one specimen gave an invalid result and was excluded from the data analysis. For the remaining 199 specimens, the Staphaurex Plus test had a sensitivity of 10.9% (95% confidence interval [CI], 4.9 to 21.8%), a specificity of 99.3% (95% CI, 95.3 to 100%), a positive predictive value 87.5% (95% CI, 46.7 to 99.3%), and a negative predictive value of 70.2% (95% CI, 63.1 to 76.4%) with a sample prevalence for *S. aureus* of 31%. For all 200 specimens, the PBP2′ test had a sensitivity of 13.2% (95% CI, 7.8 to 21.1%), a specificity of 96.5% (95% CI, 89.4 to 99.1%), a positive predictive value of 83.3% (95% CI, 57.7 to 95.6%), and a negative predictive value of 45.6% (95% CI, 38.3 to 53.1%) with a sample prevalence for methicillin resistance of 57%.

**Real-time PCR assays.** The *mecA* PCR results were identical for both the in-house PCR assay and the laboratory-optimized Roche ASR assay, similar to our prior experience (12). Of the 199 specimens tested, 85 (42.7%) were negative and 114 specimens (57.3%) were positive by both assays.

Of a total of 200 unique blood culture specimens tested, 3 were excluded from the data set because single isolates from 2 cultures did not yield a final identification and 1 was contaminated during laboratory processing. Of the remaining 197 specimens, 189 specimens had a single staphylococcal isolate and 8 had two distinct staphylococcal isolates. The in-house PCR correctly identified 184 of the 189 (97.3%) single isolates, while the Roche ASR assay correctly identified 164 (86.7%) isolates directly from blood culture broths ($P < 0.001$). The phenotypic identification by the Staphaurex Plus latex agglutination test combined with the API Staph test was correct for 171 (90.4%) strains when isolated colonies were tested ($P < 0.01$ compared to the results of the in-house PCR) (Table 3). When the performance of the assays for individual species is considered, the only significant difference between the identification methods was for *S. hominis* (Table 3), for which the in-house PCR outperformed both of the other two approaches and the phenotypic identification performed better than the Roche ASR assay.

Of the eight specimens that had two staphylococcal isolates, the in-house PCR demonstrated two melting curve peaks indicating two different species identifications for one of eight specimens, and these results were in agreement with the results

TABLE 2. $T_m$s for the species clusters for the laboratory-optimized Roche ASR assay

| Cluster | *Staphylococcus* species | $T_m$ (°C) | SD | 95% CI | $T_m$ range (°C) for cluster |
|---|---|---|---|---|---|
| None detected | *Staphylococcus hominis* subsp. *novobiosepticus* | | | | |
| | *Staphylococcus intermedius* | | | | |
| | *Staphylococcus lentus* | | | | |
| | *Staphylococcus schleiferi* subsp. *coagulans* | | | | |
| | *Staphylococcus schleiferi* subsp. *schleiferi* | | | | |
| | *Staphylococcus sciuri* subsp. *camaticus* | | | | |
| | *Staphylococcus sciuri* subsp. *rodentium* | | | | |
| | *Staphylococcus sciuri* subsp. *sciuri* | | | | |
| | *Staphylococcus simulans* | | | | |
| | *Staphylococcus vitulinus* | | | | |
| 1 | *Staphylococcus arlettae* | 44.1 | | | 44–45 |
| | *Staphylococcus auricularis* | 44.9 | | | |
| | *Staphylococcus kloosii*[a] | 48.7 | | | |
| 2 | *Staphylococcus equorum* | 49.9 | 0.31 | 51.28–51.40 | 50–51 |
| | *Staphylococcus saprophyticus* subsp. *saprophyticus* | 50.0 | | | |
| | *Staphylococcus xylosus* | 50.0 | | | |
| | *Staphylococccus saprophyticus* subsp. *bovis* | 50.1 | | | |
| | *Staphylococcus epidermidis* | 50.8 | | | |
| | *Staphylococcus haemolyticus* | 51.4 | | | |
| | *Staphylococcus caprae*[a] | 52.6 | | | |
| 3 | *Staphylococcus gallinarum* | 53.4 | 0.26 | 54.27–54.67 | 53–54 |
| | *Staphylococcus cohnii* subsp. *cohnii* | 53.9 | | | |
| | *Staphylococcus capitis* subsp. *capitis* | 54.1 | | | |
| | *Staphylococcus capitis* subsp. *ureolyticus* | 54.1 | | | |
| | *Staphylococcus hominis* subsp. *hominis* | 54.3 | | | |
| | *Staphylococcus cohnii* subsp. *urealyticum*[a] | 54.6 | | | |
| 4 | *Staphylococcus lugdunensis* | 56.0 | 0.55 | 56.47–57.19 | 56–57 |
| | *Staphylococcus pasteuri* | 56.5 | | | |
| | *Staphylococcus warneri* | 56.7 | | | |
| 5 | *Staphylococcus aureus* | 61.0 | 0.34 | 61.3–61.46 | 61 |

[a] This species exhibits its own $T_m$ and does not fall within any of the defined clusters.

of the other tests. Five of the eight specimens had a mixture of two *Staphylococcus* species that had the same $T_m$, making differentiation between the two species by PCR impossible. The remaining two specimens demonstrated only one melting peak. When the isolates were tested from colonies, the in-house PCR correctly identified all 16 isolates. The Roche ASR method detected only one of the two isolates in all eight broth specimens with more than one species. When the individual colonies from those mixed specimens were tested, the Roche ASR assay correctly identified 15 of the 16 isolates. The missed isolate was an *S. hominis* isolate that the Roche ASR assay does not detect.

## DISCUSSION

We observed several interesting findings in this investigation. One important observation was the unsatisfactory performance of latex agglutination tests for the detection of *S. aureus* and methicillin resistance when they were done directly with specimens from blood culture broth, even though such tests might be used by clinical laboratories as a rapid means of identification of gram-positive cocci found in positive blood culture bottles. However, our findings are not new. McDonald and Chapin (11) performed a study using seeded blood culture bottles and clinical blood culture broths and observed that the sensitivity of the Staphaurex Plus latex agglutination test was as low as 8% to 13% and that its specificity was 96% to 100%, which were similar to our findings. Speers and colleges used the Staphaurex Plus test, the same test that we used in the present study, and showed that the sensitivity was 23% (21); they also found that the test was not acceptable for clinical use. The single study showing a positive utility of this type of direct, rapid test was by Rappaport and colleagues, who found that the test had a better performance with a sensitivity of 77.3% and a specificity of 100% (16); they concluded that the Staphaurex Plus test is the preferred method for the identifi-

TABLE 3. Comparison of results between in-house PCR, laboratory-optimized Roche ASR assay, and Staphaurex Plus and API Staph tests

| Final species identification (no. of isolates) | Species (no. of isolates) identified by: | | |
|---|---|---|---|
| | In-house PCR[a] | Roche PCR[a] | Staphaurex Plus and API Staph tests[b] |
| *S. aureus* (62) | *S. aureus* (62) | *S. aureus* (62) | *S. aureus* (62) |
| *S. epidermidis* (79) | *S. epidermidis* (79) | *S. epidermidis* (79) | *S. epidermidis* (78), *S. hominis* (1) |
| *S. hominis* (20) | *S. hominis* (19),[c,d] *S. epidermidis* (1) | *S. hominis* (1),[c,e] *S. epidermidis* (2), no identification (2), no amplification (15) | *S. hominis* (13),[d,e] *S. epidermidis* (3), *S. lugdunensis* (2), *S. saprophiticus* (1), *S. aureus* (1) |
| *S. warneri* (8) | *S. warneri* (8) | *S. warneri* (8) | *S. warneri* (5), *S. saprophiticus* (2), *S. haemolyticus* (1) |
| *S. capitis* (7) | *S. capitis* (7) | *S. capitis* (5), *S. epidermidis* (1), no amplification (1) | *S. capitis* (6), *S. epidermidis* (1) |
| *S. haemolyticus* (5) | *S. haemolyticus* (3), *S. warneri* (1), no identification (1) | *S. haemolyticus* (5) | *S. haemolyticus* (3), *S. hominis* (1), *S. warneri* (1) |
| *S. simulans* (3) | *S. simulans* (3) | No amplification (3) | *S. simulans* (1), *S. sapriphyticus* (1), no identification (1) |
| *S. pettenkoferi* (2) | *S. warneri* (2) | No identification (1), no amplification (1) | *S. capitis* (1), no identification (1) |
| *S. lugdunensis* (1) | *S. lugdenensis* (1) | *S. lugdunensis* (1) | *S. lugdunensis* (1) |
| Not *Staphylococcus* sp. (2) | No amplification (2) | No amplification (2) | No identification (2) |

[a] Results directly from blood culture broth.
[b] Results from colonies recovered from positive blood culture broth.
[c] Difference between in-house and Roche ASR PCRs significant at $P < 0.0001$.
[d] Difference between in-house PCR and API Staph test identification significant at $P < 0.05$.
[e] Difference between API Staph test identification and Roche ASR PCR significant at $P < 0.0001$.

cation of S. *aureus* directly from blood culture bottles. Chapin and Musgnug evaluated the same PBP2a latex agglutination test that we used directly with specimens from positive blood culture broths and showed a sensitivity of 18% (2), findings again similar to our own. Taken all together, these latex agglutination tests should not be performed directly with specimens from positive blood culture broths in any clinical setting for the identification of *S. aureus* and methicillin-resistant *S. aureus*.

Another interesting observation from our study was the performance of the API Staph test with isolated colonies. The Staphaurex Plus latex agglutination test together with the API Staph test identified all staphylococci with an accuracy of 90.4%. If we evaluate the performance of the API Staph test for the identification of CoNS, *S. epidermidis* was the sole species that was reliably identified (Table 3). For all other species, confirmatory testing would be essential for final species determination.

A third interesting observation was the performance of the two real-time PCR assays. The in-house PCR test incorrectly identified one *S. hominis* isolate, two *S. haemolyticus* isolates, and two *S. pettenkoferi* isolates (Table 3). When colony PCR was performed with these five strains, one *S. hominis* strain and one of the *S. haemolyticus* strains were correctly identified. This is noteworthy, as the original test was developed to rapidly identify staphylococci from colonies and not blood culture broth. Despite this finding, the assay that we describe for blood culture broth specimens performed well, even though the growth medium for the broth specimens had characteristics very different from those of the specimens used for the original colony assay. *S. pettenkoferi* was not included in the PCR assay design, so it is not surprising that these strains were not correctly identified. It is important to note that *S. pettenkoferi* had the same $T_m$ as the *S. aureus*-*S. warneri* cluster, and so further testing is needed to separate these species.

The real-time PCR Roche ASR test that we describe failed to amplify or misidentified 25 of 189 isolates, as shown in Table 3. Among those isolates, 18 were *S. hominis* and *S. simulans*, species that the Roche ASR test does not amplify, and 2 were *S. pettenkoferi*, which was not included in the Roche ASR assay design. When only those species claimed to be identified by the Roche ASR method are considered, 96.3% were correctly characterized.

One limitation of both PCR assays was the inability to differentiate between species within a cluster that had the same $T_m$; and therefore, supplemental biochemical testing of specimens from colonies was required to separate the species once a cluster was identified. The most important differentiation issue for the in-house PCR was differentiation between *S. aureus* and *S. warneri*, which would require a confirmation test for *S. aureus* by using a colony at 24 h after the blood culture bottle was sampled and subcultured. While the clustering is of unknown consequence, further identification of this or other species would add to the time required to obtain a final result. The two PCR assays that we used can be completed in less than 3 h, including the times for broth preparation, PCR amplification, and melting curve analysis. It should be noted that both PCR assays outperformed conventional biochemical testing when they were performed directly with specimens from blood culture bottles with positive signals. Another potential limitation is the limited number of species tested that might be affected by regional variations in genomes that would cause different results to occur. Since the commercial primers are proprietary and undisclosed to us, it is impossible to know whether any genetic variability in the primer regions may affect

a $T_m$ analysis. This is an important area for future investigation.

The real-time PCR tests performed very well in identifying *Staphylococcus* species by the use of specimens directly from positive blood culture broths and showed results equivalent to or better than those obtained by conventional biochemical tests done with bacterial colonies recovered from the same positive blood culture broth. The results of new molecular diagnostic assays like these can be a key factor for medical decision making, including antimicrobial management for patients with bacteremia. This work demonstrates that new identification approaches by the use of molecular tests not only perform comparably to conventional tests but also have the added advantage of providing results more quickly by eliminating the time needed to recover and grow colonies on solid agar media.

## ACKNOWLEDGMENTS

This work was supported by a research grant from Roche Diagnostics Corp. to Lance R. Peterson. There are no other conflicts of interest for any of the other authors.

We thank Mohammed Tajuddin for sequencing all discrepant specimens for this project.

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