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Development of a rapid diagnostic assay for methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative *Staphylococcus*

Wesley C. Lindsey[a,1], Evelyn S. Woodruff[a,*], Diane Weed[b], David C. Ward[c], Robert D. Jenison[a,2]

[a]*Inverness Medical Innovations, Inc., Louisville, CO 80027, USA*
[b]*Microbiology Department, Denver Health Medical Center, Denver, CO 80204, USA*
[c]*Nevada Cancer Institute, Las Vegas, NV 89135, USA*

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Abstract

We describe here a rapid assay for the detection of the *tuf* gene for the identification of *Staphylococcus* genus, the *femB* gene for the identification of *Staphylococcus aureus* species, and the *mecA* gene for the identification of methicillin resistance directly from BACTEC blood culture bottles showing Gram-positive cocci in clusters. The test, configured on a thin-film biosensor platform, allows for detection of genomic DNA from blood culture samples without the need for nucleic acid amplification. In an initial study to validate the technology, 107 consecutive positive blood cultures were tested on the thin-film biosensor, and the assay exhibited 100% concordance in comparison with standard microbiological methods for identifying methicillin-susceptible and methicillin-resistant *S. aureus* and for identifying methicillin-susceptible and methicillin-resistant coagulase-negative *Staphylococcus*. Results were obtained within 90 min directly from signal positive bottles with no instrumentation required.

Keywords: MRSA; MSSA; Thin-film biosensor chip; *mecA*; *femB*; CoNS

1. Introduction

The prevalence of antibiotic resistance in *Staphylococcus* strains has dramatically increased over the past decade (Centers for Disease Control and Prevention [CDC], 2001; Diekema et al., 2004). If not diagnosed early and treated appropriately, these human pathogens can cause a range of life-threatening illnesses, including septicemia and toxic shock syndrome (Diekema et al., 2001). Although methicillin-resistant *Staphylococcus aureus* (MRSA) has garnered much of the attention as the leading "superbug" in nosocomial and community-acquired infections, bloodstream infections with methicillin-resistant coagulase-negative staphylococci (MRCoNS) have also been implicated with an increased risk of morbidity and mortality for infected patients (Gastmeier et al., 2003; Refsahl and Andersen, 1992; Silva et al., 2000). MRCoNS are now regarded as major human pathogens responsible for life-threatening bloodstream infections (Karchmer, 2000; Kloos and Bannerman, 1994).

Appropriate early treatment of nosocomial infections has been associated with improved outcomes in patients. Clinical outcome studies have shown that reducing the time to diagnosis decreases the patient's length of stay and morbidity and mortality rate (Beekmann et al., 2003; McClelland et al., 1999; Minino and Smith, 2001; Pittet et

* Corresponding author. Tel.: +1-303-530-3888x681; fax: +1-303-581-6405.
E-mail addresses: evelynwoodruff@hotmail.com (E.S. Woodruff), rob.jenison@gmail.com (R.D. Jenison).
[1] Current address: SomaLogic, Inc., 1775 38th St., Boulder, CO 80301, USA.
[2] Current address: Great Basin Scientific, 2400 Trade Center Ave., Longmont, CO 80503, USA.

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al., 1994; Rello, 1999). Rapid detection of MRSA and MRCoNS has a significant positive impact on preventative infection control strategies, leading to cost savings for the hospital (Abramson and Sexton, 1999; DiGiovine et al., 1999; Orsi et al., 2002).

The standard microbiology methodologies for identifying MRSA and MRCoNS from a patient's blood culture are time consuming, often requiring 24 to 72 h after microbial activity has been detected by an automated continuous-monitoring blood culture system (Bannerman, 2003). Recent polymerase chain reaction (PCR)-based assays for MRSA identification have significantly reduced the amount of time to reach a correct diagnosis for the patient (Costa et al., 2005; Fang and Hedin, 2003; Francois et al., 2003; Grisold et al., 2002; Huletsky et al., 2004; Reischl et al., 2000; Tan et al., 2001). Instead of conventional testing of phenotypic characteristics, these methodologies amplify and detect conserved DNA sequences within the bacterial genome. However, such PCR-based formats have certain disadvantages in the clinical laboratory setting, including the requirement for expensive equipment and specialized technical training.

In this study, we describe a rapid assay that can detect and identify MRSA or MRCoNS within 90 min directly from a positive blood culture and that requires neither nucleic acid amplification nor expensive instrumentation. This easy-to-perform test detects hybridization events between staphylococcal genomic DNA and complementary nucleic acid probes immobilized on a thin-film biosensor surface (Jenison et al., 2001; Sandstrom et al., 1985). Because of specific optical principles on the thin-film chip surface, intermolecular interactions trigger optical thickness changes that result in a visible color change that is easily visualized (Jenison et al., 2006). The method is sensitive to thickness changes in the angstrom range, allowing for visual detection of attomole quantities of nucleic acids (Jenison et al., 2001). Furthermore, the test is in an easy-to-use single device format that is practical for the typical hospital clinical laboratory.

2. Materials and methods

2.1. Oligonucleotide probes

The design of the capture and detector probes was performed using Vector NTI® software (Invitrogen, Carlsbad, CA). Homology was analyzed for highly conserved sequences of *Staphylococcus* species, including multiple *S. aureus* strains, for the following genes: *mecA*, *femB*, and *tuf*. BLAST analysis was performed on all of the candidate probes to eliminate cross-reactivity. The *mecA* gene, unique to methicillin-resistant staphylococci, encodes for penicillin-binding protein 2a (PBP2a), allowing for methicillin resistance (Chambers, 1997; Marshall et al., 1998). The *femB* and *tuf* probes are specific for *S. aureus* and the *Staphylococcus* genus, respectively (Grunberg-Manago, 1996; Hurlimann-Dalel et al., 1992). The candidate probes had similar melting temperatures and were optimized for performance. This combination of probes was chosen to provide an ability to discriminate *S. aureus* from other *Staphylococcus* species and to determine methicillin resistance. A 4th probe pair was designed to function as an assay chemistry control. The probes were synthesized and high-performance liquid chromatography purified by Integrated DNA Technologies (Skokie, IL).

2.2. Clinical samples and ATCC strains

One hundred seven blood culture samples containing Gram-positive cocci in clusters (GPCC) were collected from the Microbiology Department of the Denver Health Medical Center (Denver, CO). These were a set of consecutive routine clinical patient blood cultures that were flagged as positive for microbial growth during incubation in a Becton Dickinson (BD) BACTEC 9240 (Becton Dickinson, Franklin Lakes, NJ) continuous-monitoring system. Microbial strains used for seeding blood culture bottles were obtained from the American Type Culture Collection (ATCC, Manassas, VA). For the analytical strain panel, approximately 5 to 7 mL of human blood were injected into aerobic BD BACTEC^{+} blood culture bottles. The bottles then were seeded with an ATCC strain of interest, initially grown on 5% sheep blood agar, and placed onto a BD BACTEC automated continuous-monitoring blood culture system until microbial activity was detected.

2.3. Microbiological testing

Standard microbiology methodologies were performed in parallel on the clinical samples in the microbiology department at Denver Health Medical Center to validate culture results. Those bottles positive for microbial growth and containing GPCC in the Gram stain were subcultured on 5% sheep blood agar and CDC anaerobic blood agar (Remel, Lenexa, KS). Plates were incubated overnight at 35 °C in 5% to 10% CO_2 and anaerobic atmosphere, respectively. Staphylococcal isolates were identified morphologically and biochemically. Latex agglutination (BactiStaph; Remel) was performed for identification of *S. aureus* and coagulase-negative staphylococci (CoNS). Oxacillin susceptibilities (MicroScan Walkaway 96 SI; Dade Behring, West Sacramento, CA) were determined for all isolates according to Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards, 2000) methods. Results of the tests were blinded until after the results of the biosensor chip assays were completed.

2.4. Deoxyribonucleic acid extraction

Positive blood culture samples (0.5 mL) were mixed with 150 μL of 1 N NaOH, vortexed, and centrifuged at 3000 × *g* for 1 min. The supernatant was discarded, and the pellet was washed with 250 μL of 10 mmol/L Tris, pH 7.0. After a 2nd centrifugation, the pellet was resuspended in 100 μL of an achromopeptidase-based enzyme extraction solution (Staph-Lyser; Inverness Medical-BioStar, Louisville, CO), mixed,

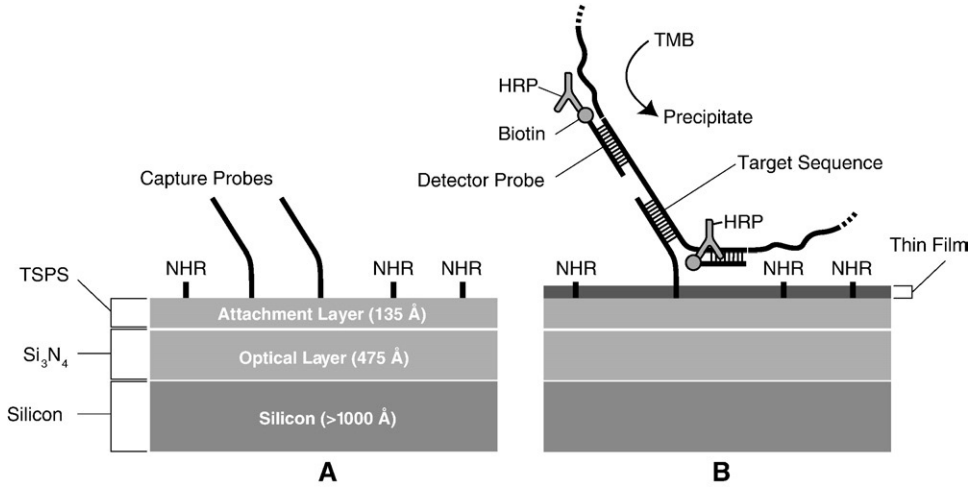

Fig. 1. Schematic representation of the thin-film biosensor. (A) Unreacted thin-film biosensor surface with covalently attached capture probes. The surface coating, Si_3N_4, creates a gold colored surface. (B) Surface reacting with target sequence to create thin film. Target immobilization triggers reactions that enzymatically transduce the formation of hybrids on the surface into a molecular thin film causing a surface color change from gold to blue.

and incubated for 10 min at room temperature and then treated with 5 μL of stop solution.

2.5. Hybridization and thin-film biosensor detection

To detect staphylococcal sequences, we configured a sandwich hybridization assay on the thin-film biosensor surface (Fig. 1). Capture probes were covalently attached on the chip surface as previously described (Zhong et al., 2003). One hundred twenty-five microliters of hybridization buffer (5× saline sodium citrate [SSC], 0.1% sodium dodecyl sulfate [SDS], 0.5% BlockAid®; Inverness Medical-BioStar) containing biotin-labeled detector probes (20 nmol/L each) was added to the chip surface and preheated to 53 °C for 10 min. The entire DNA extraction mix (125 μL) was added to the chip and incubated at 53 °C for 45 min. Subsequently, the chips were washed with wash A (0.1× SSC, 0.1% SDS) and wash B (0.1× SSC), and an antibiotin antibody/horseradish peroxidase (HRP) conjugate solution was added. After room temperature incubation for 10 min, the chips were washed with wash B, and an HRP-precipitating substrate solution, tetramethyl benzidine (BioFx Laboratories, Owings Mills, MD), was added. After a final incubation for 10 min at room temperature, the chips were washed with water, then 95% ethanol, and allowed to dry.

3. Results

3.1. Assay principle and design

The conserved genomic regions of 3 specific genes, *mecA*, *femB*, and *tuf*, were targeted for analysis in this study. The *mecA* gene is part of the staphylococcal cassette chromosome *mec* (SCC*mec*), a mobile genetic element found in all MRSA strains. It encodes for an altered cell wall enzyme, PBP2a, that, unlike the wild-type protein, has a low affinity for all β-lactam antibiotics, including methicillin. This low affinity accounts for the methicillin resistance of MRSA strains. The *femB* gene is part of the *femAB* operon, a housekeeping gene found in all *S. aureus* strains and involved in peptidoglycan biosynthesis. The *tuf* gene, which encodes for the peptide elongation factor Tu in bacteria, is conserved in all members of the *Staphylococcus* genus.

For each genomic region targeted, a surface-bound capture probe and a pair of detector probes were designed for optimal specificity and sensitivity. The thin-film biosensor described here was configured on a multilayered optically coated silicon surface that was highly reflective with a gold appearance under white light (Fig. 1A). The gold color was created by controlled deposition of Si_3N_4 and is a consequence of destructive interference of specific wavelengths of light reflected from the surface (Jenison et al., 2001; Sandstrom et al., 1985). Although Si_3N_4 is sufficient for optical performance criteria, an attachment layer is required for immobilization of biological capture molecules. Capture probes were covalently attached to the surface as described (Zhong et al., 2003). Staphylococcal cells, present in positive blood cultures, were lysed, and the extracted genomic DNA was combined with biotinylated detector probes. The genomic DNA/biotinylated detector probe complex was annealed to specific capture probes on the chip surface. The resulting surface-immobilized hybrids were then detected with antibiotin antibody/HRP conjugates. In the presence of a precipitable substrate for HRP, a molecular thin film was deposited on the surface (Fig. 1B). The additional mass on the surface altered the path length of light reflected from the optical surface layers and attenuated specific wavelengths of light through destructive interference. The result was an apparent surface color change from gold to purple.

The oligonucleotide capture probes were printed onto the thin-film biosensor surface in a pattern as depicted in Fig. 2

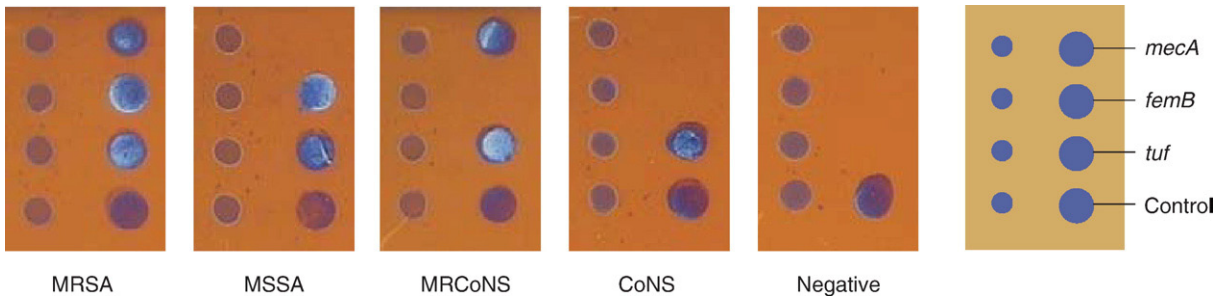

Fig. 2. Sample results for the detection of *Staphylococcus* species directly from blood culture. Probe map is shown on the right panel. The left column of the chip contains the 4 fiducial markers (200 nL of carboxylated polystyrene microspheres dried onto surface). The right column contains the test probe spots.

(far right panel). The left column contains 4 fiducial markers (carboxylated polystyrene microspheres) necessary for orientation of the chip and indicates the positions of the printed oligonucleotide capture probes. In the right column, probes that recognize sequences in the *mecA*, *femA*, and *tuf* genes were printed as shown. A 4th probe that serves as a chemistry procedural control was printed. The control probe functions to ensure that the hybridization reaction and thin-film color development were performed properly by annealing to a complementary biotin-labeled detector probe added to the hybridization buffer. The *tuf* gene marker further serves as a sample sufficiency and DNA extraction control because this probe should signal in the presence of all *Staphylococcus* species. Fig. 2 illustrates the 5 possible outcomes for a successful assay: MRSA, methicillin-sensitive *S. aureus* (MSSA), MRCoNS, or methicillin-sensitive CoNS, as well as a negative result.

Table 1
Staphylococcus analytical panel data

Organism	ATCC no.	Positive (+) or negative (−) result for the following gene[a]		
		tuf	*femB*	*mecA*
MSSA	6538P	+	+	−
MSSA	13150	+	+	−
MSSA	25923	+	+	−
MSSA	11632	+	+	−
MSSA	14776	+	+	−
MSSA	14458	+	+	−
MSSA	14993	+	+	−
MSSA	14775	+	+	−
MRSA[b]	33591	+	+	+
MRSA[b]	33592	+	+	+
MRSA[b]	43300	+	+	+
Staphylococcus auricularis	33753	+	−	−
Staphylococcus capitis	35661	+	−	−
S. capitis	49326	+	−	−
Staphylococcus caprae	51548	+	−	−
Staphylococcus epidermidis[b]	12228	+	−	+
S. epidermidis	700562	+	−	−
S. felis	49168	+	−	−
Staphylococcus haemolyticus	29968	+	−	−
Staphylococcus hominis	27844	+	−	−
Staphylococcus intermedius	29663	+	−	−
Staphylococcus lugdunensis	49576	+	−	−
Staphylococcus pasteuri	51129	+	−	−
Staphylococcus pulvereri	51698	+	−	−
Staphylococcus saprophyticus	15305	+	−	−
Staphylococcus schleiferi	43808	+	−	−
Staphylococcus sciuri	29060	+	−	−
Staphylococcus simulans[b]	700576	+	−	+
Staphylococcus warneri	27836	+	−	−
Staphylococcus xylosus	29971	+	−	−

[a] *Tuf*, *Staphylococcus* genus; *femB*, *S. aureus* specific; *mecA*, determinant of methicillin resistance.
[b] Methicillin-resistant strain.

3.2. Analytical panel

To verify specificity and sensitivity of the assay, a sample set of spiked blood cultures was tested (Tables 1 and 2). These samples contained various strains of bacteria obtained

Table 2
Negative panel data

Organism	ATCC no.	Positive (+) or negative (−) result for the following gene[a]		
		tuf	*femB*	*mecA*
Gram positives				
Bacillus cereus	14579	−	−	−
B. subtilis	6051	+	−	−
Clostridium butyricum	19398	−	−	−
Enterococcus gallinarum	49573	−	−	−
Micrococcus luteus	4698	−	−	−
M. luteus	27141	−	−	−
Streptococcus[b]—group A		−	−	−
Gram negatives				
A. calcoaceticus	23055	+	−	−
Enterobacter aerogenes	15038	−	−	−
Enterobacter cloacae	13047	−	−	−
Escherichia coli[b]		−	−	−
Haemophilus parainfluenzae	33392	−	−	−
P. vulgaris	29905	+	−	−
Pseudomonas aeruginosa	10145	−	−	−
Salmonella enterica	13311	−	−	−
S. enterica	43973	−	−	−
Serratia marcescens	13880	−	−	−
Shigella flexneri	25929	−	−	−
Shigella sonnei	29930	−	−	−

[a] *Tuf*, *Staphylococcus* genus; *femB*, *S. aureus* specific; *mecA*, determinant of methicillin resistance.

from the ATCC that had been incubated on agar plates and then seeded into BD BACTEC^{+} blood culture bottles containing 7 to 10 mL of human blood. The blood culture bottles were then incubated in the automated continuous-monitoring blood culture system until microbial activity was detected. Table 1 shows the results of 30 unique *Staphylococcus* strains. Analysis indicates that, as expected, all of the tested *Staphylococcus* strains reacted with the *tuf* probe (Table 1). Testing of 11 *S. aureus* strains and 5 methicillin-resistant strains all resulted in positive results with the *femB* and *mecA* probes, respectively. Table 2 shows the results from the negative analytical panel, a set of Gram-positive and Gram-negative non-*Staphylococcus* bacteria. As expected, none of the nonstaphylococcal bacterial strains cross-reacted with the *femB* or *mecA* probes. However, the *tuf* probe cross-reacted with the following species: *Acinetobacter calcoaceticus*, *Bacillus subtilis*, and *Proteus vulgaris*. Sequence alignment of the *tuf* probe in the BLAST database predicts this cross-reactivity with *B. subtilis* (data not shown), but sequence data for *A. calcoaceticus* and *P. vulgaris* were incomplete for the *tuf* gene and, therefore, not analyzed. However, it should be noted that the cross-reactivity of the *tuf* gene probe with the other microbial species does not affect the specificity or sensitivity in detecting MRSA or MRCoNS species.

We determined the analytical limit of detection using a dilution series of the positive blood cultures. Dilutions were tested on the thin-film biosensor chip and plated onto blood agar. The thin-film biosensor could detect 5×10^7 CFU/mL (data not shown). We found the typical positive blood culture bottle to contain at least 1×10^9 CFU/mL (data not shown).

3.3. Clinical samples

For the clinical sample set, we tested 107 consecutive routine patient blood cultures obtained from the Denver Health Medical Center, which were identified as having GPCC. These sample sets were tested by the thin-film chip rapid assay as well as by using conventional microbiology methods in a double-blind study (Table 3). All of the 107 clinical samples were positive for the *tuf* marker, indicating that these were of the *Staphylococcus* genus. This was 100% in correlation with the conventional microbiological results. Of the 107 samples, 62 were positive for the *femB* marker, indicating *S. aureus*, and 15 of the 62 were also positive for the *mecA* marker. This indicated that of these 62 samples, 15 were MRSA and 47 were MSSA. This also was 100% in agreement with the microbiological results. The remaining 45 of the 107 clinical samples were negative for the *femB* marker and, therefore, CoNS. Of these 45 CoNS, 32 were also positive for the *mecA* marker and, therefore, MRCoNS, with 13 methicillin-sensitive CoNS. Compared with the standard microbiology testing, the thin-film test for the *mecA* and *femB* probes was 100% sensitive and specific in this study.

4. Discussion

Our results demonstrate that when the rapid thin-film assay was used to test blood cultures positive for GPCC, it accurately identified *S. aureus* and CoNS in analytical testing and an initial patient sample clinical study; methicillin resistance was measured with 100% concordance compared with standard microbiological approaches. Although the performance of the thin-film biosensor in this study appears rather promising, more clinical samples need to be tested with genetically diverse strains of *Staphylococcus*. This will further validate the performance of the gene probe sequences chosen.

The components of the thin-film biosensor surface were selected by chemical and optical criteria such that hybridization-dependent thickness increases on the order of 20 Å were sufficient to generate a visually detectable color change (Jenison et al., 2006). This creates a surface with excellent sensitivity as evidenced by previously observed detection limits of less than 100 000 copies, or 10 fmol/L concentration, of a DNA target in a model system (Jenison et al., 2006). Furthermore, the surface configuration is designed to be chemically inert and molecularly flat, thereby limiting nonspecific interactions. These characteristics of the

Table 3
Results of testing 107 clinical samples positive for GPCC

Organism	*n*	No. of isolates	No. of positive results for the following gene[a]			Correlation with microbiologic results (%)
			tuf	*femB*	*mecA*	
All staphylococci	107					
Methicillin resistant		47	47	15	47	100
Methicillin susceptible		60	60	47	0	100
S. aureus	62					
Methicillin resistant		15	15	15	15	100
Methicillin susceptible		47	47	47	0	100
CoNS	45					
Methicillin resistant		32	32	0	32	100
Methicillin susceptible		13	13	0	0	100

[a] *Tuf*, *Staphylococcus* genus; *femB*, *S. aureus* specific; *mecA*, determinant of MET resistance.

platform have allowed for testing in the presence of complicated clinical matrices including urine, feces, blood, mucus, and saliva with excellent clinical specificity and sensitivity when applied to numerous pathogens including *Clostridium difficile*, group B *Streptococcus*, group A *Streptococcus*, and respiratory viruses such as influenza and respiratory syncytial virus.

The characteristics of the thin-film surface facilitate the application of a crude lysate from a blood culture to detect genomic DNA targets directly without any culture step or target amplification. The advantages of this approach are simplicity and rapidity in testing. No complicated sample preparation is required to purify nucleic acids. Furthermore, because nucleic acid amplification is not necessary, the presence of any amplification inhibitors in the blood culture media will not compromise this assay. Also, the risks associated with "carryover" contamination from previously amplified samples are eliminated. A further advantage of the described test is that the signal is visually detectable and permanent. Therefore, no specialized instrument is required for interpretation of a test result. In addition, this technology provides clinicians answers for methicillin resistance/susceptibility, genus and species in 1 test.

The limitations of this assay are similar to other genotypic tests in that it is unable to distinguish between MRSA and a mixed culture of MSSA and MRCoNS within the same culture bottle (Shrestha et al., 2002). This problem could be mitigated by the addition of probe sets that recognize SCC*mec* DNA containing *mecA* in the *S. aureus* chromosome (Huletsky et al., 2004). SCC*mec* is a mobile genetic element that contains the *mecA* gene and *orfX*, a highly conserved open reading frame in *S. aureus* that is the site of SCC*mec* integration into the genome. Therefore, SCC*mec* is a marker specific for MRSA. If SCC*mec* probes were added to the described panel, the presence of a mixed culture of MSSA and MRCoNS would yield a positive signal on the *tuf*, *femB*, and *mecA* test probes and negative for the SCC*mec* probe sets. One advantage of this probe set over other reported PCR detection methods using the SCC*mec* as the sole marker for detection (Desjardins et al., 2006; Huletsky et al., 2004) is the ability to identify CoNS and its resistance profile by a positive, not a negative, result. A further advantage of this probe set should be improved specificity for MRSA identification compared with real-time PCR tests. Huletsky et al. (2004) describe that the SCC*mec* marker incorrectly called 4.6% of tested MSSA strains as MRSA. Testing with both the SCC*mec* and *mecA* gene markers would provide a secondary check for specificity on these SCC*mec*-positive MSSA strains. Having a result of *mecA* gene marker signal negative with positive or negative signal for SCC*mec* and a positive signal for *femB* and *tuf* markers would indicate the presence of MSSA.

Based on the testing parameters used, the *tuf* gene marker proved to be useful and adequate for the purposes of this study. However, cross-reactivity of the *tuf* marker to *A. calcoaceticus*, *B. subtilis*, and *P. vulgaris* was observed in analytical testing. Because *A. calcoaceticus* and *P. vulgaris* are Gram-negative bacteria, and we propose that the "gate" for the use of this chip is a Gram stain result of GPCC, these bacteria would not likely be tested by this assay. In addition, no cross-reactivity was observed for bacterial species that are phenotypically similar to staphylococci such as strains of *Micrococcus*, *Streptococcus*, or *Enterococcus*. Therefore, even with the observed cross-reactivity, the specificity of this probe may be appropriate for making a determination of CoNS if the *tuf* probe is the only one to generate signal on the chip (besides the procedural control). However, any candidate *tuf* gene probes will need to undergo extensive analytical testing with other bacteria that can be confused with staphylococci by Gram stain assessment, such as short-chain–forming *Streptococcus* strains, *Enterococcus faecalis*, *Enterococcus faecium*, *Acinetobacter baumannii*, and numerous others. We are currently evaluating other promising sequences in the *tuf* gene that may improve its specificity as a marker for *Staphylococcus* genus identification.

Studies to improve the limit of detection are also planned for this test. Although we observed the analytical limit of sensitivity to be adequate for all studies performed here and to detect the numbers of organisms present in positive blood cultures, improved clinical sensitivity may be a benefit to the end user.

With a short assay time and simplified visual detection method, this test is currently practical for a clinical laboratory setup and would fit in the workflow pattern of most clinical laboratories. Because the results from the thin-film test are visualized within 2 h of a blood culture flagging positive, this would help clinicians identify *Staphylococcus* bacteremia 24 to 48 h sooner than conventional techniques that involve subculture and phenotypic analysis. Such an improvement over the current methods could have an impact on patient mortality, costs to the hospital, and effective infection control management.

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