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Identification of *Staphylococcus aureus* and Determination of Methicillin Resistance Directly from Positive Blood Cultures by Isothermal Amplification and a Disposable Detection Device▽

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A simple, rapid, and user-friendly procedure has been developed to identify *Staphylococcus aureus* and determine its methicillin resistance directly from gram-positive cocci in cluster-containing blood culture medium. The specimens were diluted and heated prior to amplification of the *nuc* and *mecA* genes with isothermal helicase-dependent amplification. Amplicons were detected using a disposable detection device. The analytical sensitivity of the assays was 50 CFU per reaction, and the clinical sensitivity and specificity were both 100% for *S. aureus* detection and 100% and 98% for methicillin resistance determination, respectively.

Staphylococcus aureus, including methicillin-resistant *S. aureus* (MRSA), is the most common antibiotic-resistant species identified in bacteremia and is associated with a high mortality rate (5). Coagulase-negative staphylococci (CoNS) such as *Staphylococcus epidermidis* are usually normal flora of skin and mucous membranes and are common contaminants in blood cultures (2). Timely identification and differentiation of pathogenic from contaminant staphylococcal isolates from the patient's blood have great therapeutic, prognostic, and economic significance. Currently, definitive identification and antimicrobial susceptibility testing of gram-positive cocci in clusters (GPCC) are time-consuming, which may lead to the unnecessary use of antimicrobial agents while awaiting results. To take advantage of the rapid growth in enriched media used for automated blood culture instruments, several procedures have recently been reported to rapidly identify organisms directly from clinically positive blood cultures that contain GPCC (3, 4, 6, 9–14).

We are reporting the development and validation of the IsoAmp RapidStaph Detection kit (BioHelix Corporation, Beverly, MA), which uses helicase-dependent amplification (HDA) coupled with a self-contained disposable amplicon detection device, the BioHelix Express strip (BESt) cassette for the rapid detection of *S. aureus* and determination of its methicillin resistance directly from blood culture medium specimens containing GPCC. HDA is a unique isothermal DNA amplification technique that has been developed by employing a DNA helicase enzyme to unwind double-stranded DNA and RNA-DNA hybrids (1, 7). The BESt cassette is a disposable device designed for instrument-free, cross-contamination-proof detection of amplicons derived from HDA and other nucleic acid amplification reactions.

The IsoAmp RapidStaph Detection kit contains two separate amplification reaction mixtures and detection cassettes for detecting the *S. aureus*-specific *nuc* gene and the methicillin resistance-related *mecA* gene, respectively. Aliquots from the blood cultures were diluted 1:100 with distilled water, and 25 μl of diluted sample was heated at 95°C for 5 min. The 25-μl processed sample was then directly mixed with 21.5 μl IsoAmp Staph reaction mix and 3.5 μl IsoAmp enzyme mix. The HDA mix contained 2× annealing buffer (20 mM KCl and 40 mM Tris-Cl [pH 8.8] at 25°C), 7.0 mM $MgSO_4$, 80 mM NaCl, 800 μM deoxynucleoside triphosphates, 6 mM dATP, 400 nM biotin-labeled forward primer, 200 nM reverse primer, and 100 nM fluorescein isothiocyanate (FITC)-labeled probe. The primers and probes used by the kit are 5′-biotin-CAA AGA ACT GAT AAA TAT GGA CGT GGC TTA (forward), 5′-TAG CCA AGC CTT GAC GAA CTA AAG C (reverse), and 5′-GTT TAC CAT TTT TCC ATC AGC AT-FITC (probe) for *nuc* and 5′-biotin-GAA AAA TGA TTA TGG CTC AGG TAC TGC (forward), 5′-TGG ATA GAC GTC ATA TGA AGG TGT GCT (reverse), and 5′-TAA TAA TTC ACC TGT TTG AGG GT-FITC (probe) for *mecA*.

A representative schematic and result readout are shown in Fig. 1. By using a biotin-labeled primer and a 3′ FITC-labeled oligonucleotide probe that can bind to the biotin labeled DNA strand generated by the HDA reaction, amplicon-probe complexes were detected via a sandwich format (Fig. 1A). Amplification is asymmetric because the biotin-labeled primer is present in excess such that more biotin-labeled strands are produced than unlabeled complementary strands, and thus, no denaturation is required to make the biotin strand available for binding by the FITC-labeled probe. After a 60-min incubation at 65°C in a water bath, or a heat block, the reaction tube was placed directly into the BESt cassette (BioHelix Corporation, Beverly, MA) for amplicon detection (Fig. 1B). The device was

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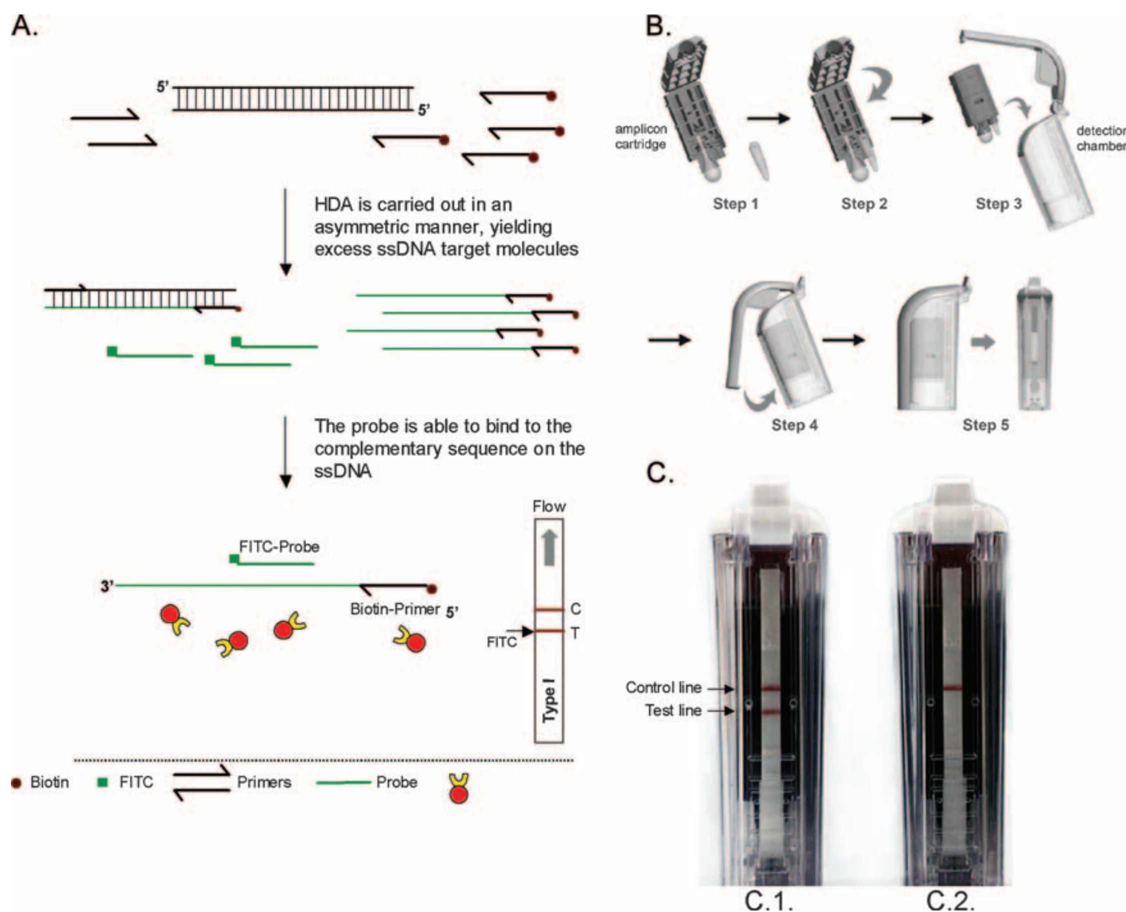

FIG. 1. (A) The steps involved in the IsoAmp RapidStaph assay begin with asymmetric HDA generating single-stranded DNA (ssDNA) capable of binding the specific probe. The probe is bound by the cassette insert, and the biotin moiety binds the chromogen, resulting in the red test line. (B) The amplification reaction vessel is placed into an amplicon cartridge (step 1), the cartridge is closed to immobilize the reaction vessel (step 2), the amplicon cartridge is inserted into the detection chamber (step 3), the handle of the detection chamber is closed to seal the vessel into the chamber and to cut open the running buffer reservoir and the reaction vessel (step 4), and after 10 min, the detection window of the chamber is read by eye to score the assay result (step 5). (C) Readout of the type 1 BESt cassette, C.1. is a positive reaction, and C.2. is a negative reaction.

closed to trigger the release of the amplicons by cutting the closed tube inside of the self-contained device (with amplicons and the running buffer mix at the bottom of the cassette). The mixture then flowed vertically up the strip, and the test results were shown as a visible colored line (Fig. 1C). The entire procedure, starting from specimen processing and ending at result readout, can be completed in less than 1.5 h.

The analytical sensitivity of the IsoAmp RapidStaph Detection kit was evaluated by spiking live MRSA cells (ATCC 33591) into a pooled negative blood culture medium mixture specimen. Serial dilutions containing different CFU of *S. aureus* cells from correlated measurements of the optical density at 600 nm and quantitative culture plate counts were mixed with 5 μl of the pooled negative specimen, limiting variance among cultures as a source of error. Each dilution was performed in triplicate. The analytical sensitivity of the kit was 50 CFU/reaction for both *nuc* and *mecA* detection, which is equivalent to 2×10^4 *S. aureus* cells per ml of blood culture medium specimen. The analytical specificity of the IsoAmp RapidStaph assay was evaluated by testing a panel of six bacterial species commonly encountered in blood culture. The test was negative for both *nuc* and *mecA* for *Escherichia coli* (ATCC 25922), *Staphylococcus saprophyticus* (Vanderbilt University Medical Center [VUMC]), *Pseudomonas aeruginosa* (ATCC 27583),

TABLE 1. Sensitivity and specificity of the IsoAmp RapidStaph assay for rapid detection of MRSA infections directly from positive blood cultures

Organism	No. of isolates with result:				Sensitivity (%)	Specificity (%)
	Reference$^+$/test$^+$	Reference$^-$/test$^+$	Reference$^+$/test$^-$	Reference$^-$/test$^-$		
S. aureus	39	0	0	80	100	100
MRSA	19	2	0	98	100	98

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Klebsiella pneumoniae (VUMC), and *Streptococcus pneumoniae* (ATCC 49619). A methicillin-resistant CoNS isolate recovered in VUMC was positive for *mecA* but negative for *nuc* by the IsoAmp RapidStaph assay. Methicillin-sensitive (ATCC 25923) and -resistant (ATCC 33591) strains of *S. aureus* were used extensively during the design of the detection kit and were used as controls for *nuc* and *mecA* detection.

The IsoAmp RapidStaph Detection kit was validated using 119 frozen GPCC-containing blood culture medium specimens, which were collected from a Bactec 9240 blood culture system (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD) in the Clinical Microbiology Laboratory at VUMC. Subculturing was performed, and bacterial isolates were further characterized by biochemical and genotypic methods (14). They were identified as 74 CoNS, 18 methicillin-sensitive *S. aureus* (MSSA) isolates, 18 MRSA isolates, 6 negative/nonstaphylococcus isolates, 2 mixtures of MSSA and CoNS, and 1 mixture of MRSA and CoNS, respectively. The IsoAmp RapidStaph assay detected the *nuc* gene in 39 of the 39 specimens that previously tested positive for *S. aureus* and in none of the CoNS samples, giving 100% sensitivity and specificity. The assay detected *mecA* in all 19 blood specimens containing MRSA and in two specimens in which both MSSA and methicillin-resistant CoNS were recovered, giving 100% sensitivity and 98% specificity (Table 1).

The IsoAmp RapidStaph assay combines simple sample processing, isothermal amplification technology, and a self-contained disposable amplicon detection device for the rapid detection of *S. aureus* and identification of MRSA directly from positive blood cultures. As it does not require any special instrumentation or complicated specimen handling, the assay provides a rapid tool for the diagnosis of bloodstream MRSA infections in a routine clinical microbiology laboratory. The *nuc* and *mecA* tests can be run either sequentially or simultaneously (in this study) depending on the clinical circumstance and time constraints of the user. At VUMC, for example, blood culture specimens are not routinely collected by phlebotomists, and the rate of recovery of contaminant CoNS from blood culture could be very high. The use of a sequential procedure, i.e., running the *nuc* test first, may significantly save reagents and decrease costs. The development of a duplex IsoAmp MRSA assay that couples the multiplex amplification of the *nuc* and *mecA* genes in one tube with a single BESt cassette for the detection of both amplicons is under way.

While CoNS are usually negative for the *nuc* gene, they carry the *mecA* gene in high frequencies. It was noticed that when both MSSA and methicillin-resistant CoNS exist in one specimen, the IsoAmp RapidStaph assay generated false-positive results by calling MRSA. However, such mixed cultures are rarely detected in positive blood cultures (8, 14), and their incidence can be reduced further by implementing strict site sterilization procedures prior to collecting the patient's blood.

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