A Multiplex Molecular Diagnostic Platform and Test for Identification of Staphylococcus species and Detection of the *mecA* gene Directly from Positive Blood Cultures

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Abstract

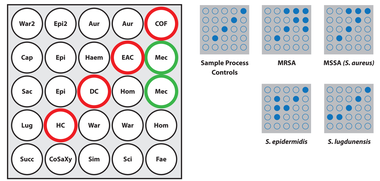
Background: Staphylococcal blood stream infections are increasingly prevalent and cause high morbidity and mortality. An automated molecular diagnostic platform, Portrait Dx, consists of a disposable cartridge run by a small instrument. The system combines multiplex amplification with multiplex chip-based detection. Here we describe development of the Staph ID/R assay for simultaneous identification of 8 *Staphylococcal spp* and the *mecA* gene. A blinded study of clinical blood culture samples compares Staph ID/R to an automated biochemical detection system.

Methods: Gene sequences were aligned and primers were identified that use Helicase-Dependent Amplification (HDA) to amplify conserved regions of the *tuf* and *mecA* genes. An array of hybridization probes was immobilized onto a silicon surface. For blood culture testing, a 50 uL blood culture aliquot was extracted enzymatically for 10 min, amplified for 30 min, and hybridized for 5 minutes producing produces eye-visible signals. Phenotypic determination of species results and methicillin-resistance was performed on the MicroScan. For discrepant results, definitive species identification was achieved by sequencing of *rpoB* and/or 16S rDNA genes, and definitive methicillin resistance was determined by growth on oxacillin-containing agar.

Results: The limit of detection for *S. aureus* was 3e3 CFU/mL, or 1 copy of input DNA, placing sensitivity on par with real-time PCR. 8 Staph spp, were identified and resolved with limits of detection ranging from 1-250 CFU/reaction. In a blinded study using clinical blood culture samples, the Microscan biochemical system correctly identified 93/98 samples that were mono-microbial infections for species and methicillin-resistance results, while Staph ID/R correctly identified 98/98 for bacterial species and methicillin-resistance. In 5 poly-microbial cultures Microscan was correct in 0/5 samples while Staph ID/R identified 4/5 samples correctly as defined by detection of all *Staphylococcal* species present in each sample.

Conclusion: Staph ID/R shows high sensitivity and specificity, equivalent to the gold standard for Staphyloccocal species identification, *rpoB* gene sequencing. Staph ID/R represents an effective, low-cost, user-friendly approach to blood culture detection of *S aureus* and additional spp along with methicillin resistance. HDA and eye-visible detection together permit low-cost instrumentation. Clinical evaluations of the Portrait Dx platform are in progress at several sites.

Bacterial Identification Array



Control Features *MecA* Gene

Feature	Staph species
Succ	succinus
CoSaXy	cohnii, saprophyticus, xylosus
Sim	simulans
Sci	sciuri
Fae	Entercoccus faecalis
Lug	lugdunensis
HC	Hyb. Control
War	warneri
War	warneri (replicate)
Hom	hominis
Sac	saccharolyticus
Epi	epidermidis
DC	Detect Control

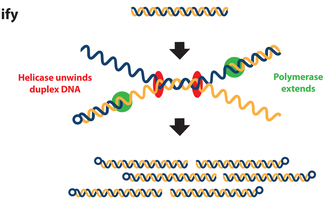
Feature	Staph species
Hom	hominis (replicate)
Mec	*mecA*, methicillin resistance gene
Cap	capitis
Epi	epidermidis (replicate)
Haem	haemolyticus
EAC	Extraction-Amplification Control
Mec	*mecA*, methicillin resistance gene
War2	warneri variant
Epi2	epidermidis variant
Aur	aureus
Aur	aureus (duplicate)
COF	Chip Orientation Feature

Capture probes are immobilized to the silicon surface. A diagonal set of control features verifies that chip orientation (COF), DNA **extraction** and **amplification** (EAC), and **detection** (HC, DC) functioned properly, validating test results.

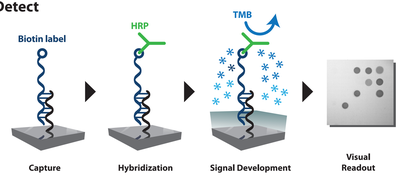
Assay Overview

1 Extract DNA

2 Amplify



3 Detect



Bacterial DNA is **Extracted** by cell lysis, then **Amplified** via Helicase Dependent Amplification (HDA). HDA represents an isothermal alternative to PCR in which strand separation is accomplished by a helicase rather than by heat denaturation.

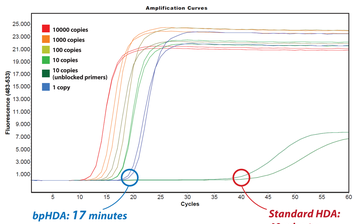
After amplification for 30 min at 65°C, **Detection** occurs: amplified DNA binds to sequence-specific capture probes. After washing, an anti-biotin antibody conjugated to Horse Radish Peroxidase (HRP) produces a visual signal via precipitation of a tetramethylbenzidine cleavage product onto the silicon surface.

GREAT BASIN CORPORATION

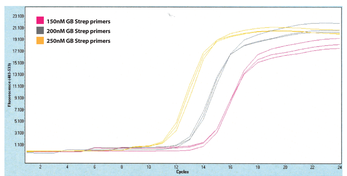
Amplification and Detection Technology

Hot Start Amplification: Blocked primer HDA (bpHDA) uses primers bearing a 3' blocking group to create hot start conditions. Deblocking requires that primers be annealed to the target DNA sequence, resulting in cleavage by RNAse H2, followed by polymerase extension. Because the RNase is inactive at low temperature, primer artifact is suppressed without need for a modified polymerase.

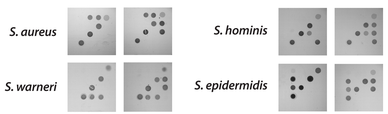
Comparison of blocked primers with unblocked primers. Here, bpHDA amplifies 10 copies of input DNA in 17 minutes. Because lower primer concentrations (100 vs 300 nM) are required to avoid amplification artifacts in standard HDA, the amplification is 23 minutes delayed. In contrast to PCR, which is rate-limited by device heating/cooling rates bpHDA rate is device-independent and limited only by enyzme and primer concentrations. Note 1 cycle=1 minute; there is no thermal cycling.



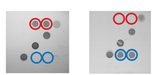
Multiplex Amplification: proof of principle. Primer pairs for 4 different bacterial genes are mixed. Primers for the fourth amplicon, a Group B streptococcus gene, are titrated into the bpHDA reaction at 150, 200, 250 nM each.



Multiplex Detection. After amplifying a single locus within the tuf gene, multiple bacterial species are discriminated using unique capture probes arrayed on the chip surface. Multiple probes, coupled to multiple amplicons, produces panel assays that can detect large numbers of target organisms.



Detection Specificity. Two bacterial species, differing by a single nucleotide in the capture probe region as indicated, are distinguished by hybridization. This allows resolution of closely related species and strains.



Capture Probes
SA = NNNNNNNNNNNNNN**A**NNN
SW = NNNNNNNNNNNNNN**T**NNN

Limit of Detection

Bacterial Species	Gene	Limit of Detection (CFU/mL) Pure	Mixed
S. hominis/novo	tuf	1e3	
S. epidermidis	tuf	3e3	1e5
S. capitis	tuf	1e5	
S. lugdunensis	tuf	1e6	
S. haemolyticus	tuf	1e4	
S. aureus	tuf	3e3	3e4
S. warneri	tuf	1e5	
E. facaelis	tuf	ND	
MRSA/MRSE	mecA	1e3	1e3

Blood culture samples were quantitated to determine CFU/mL, then serially diluted using a blood culture control. Staph ID/R was performed manually in 96-well format. *mecA* detection is more sensitive than species detection due to strength of capture probe for this sequence. LODs are on par with real-time PCR methods. LODs for mixed cultures were determined by placing a fixed amount of one competing bacterial species into a negative blood culture (3 x 10^7 CFU/mL) and then titrating in the species of interest.

Great Basin Cartridge and Analyzer

The Staph ID/R assay is built into a injection-molded card. Reagents are lyophilized or placed in blister packs. In the instrument, optical sensors control motors that propel 10s to 100s of uL through channels and chambers. This mesofluidic-scale design and injection-molded plastic card, in combination with isothermal amplification and human eye-visible signal, enables a low-cost card and instrument.

The operator inserts ~50 uL blood culture into the sample port as shown, inserts the card into the desktop instrument, and initiates the test. Software automatically returns a result within 90 min. The report details the presence of staphylococci, specifically identifies the 12 Staph. species deemed most relevant, and indicates status of the drug resistance gene *mecA*.

A. Detection chip
B. Amplification chamber
C. Sample input
D. Blister packs with reagents

Results

Limit of detection was determined for various *Staphylococcal* strains using tuf (species) and *mecA* (methicillin-resistance) duplex amplification reactions, and ranged from 3 x 10^3 to 1 x 10^6 CFU/mL Analytical reactivity was demonstrated for 25 unique *Staphylococcus* strains representing 19 different species (data not shown). All amplified species reacted with the *Staphylococcus* genus control set except *S. lentus* and *S. succinus*, as expected. Each species-specific probe reacted with cognate *Staphylococcal* species amplicon with high specificity. The probe set described here showed no cross-reactivity with a panel of non-cognate bacteria except *E. facaelis*, which reacted with the *Staphylococcus* genus probe set. This was expected and an *E. facaelis*-specific probe was created to distinguish it from *Staphylococcus* in the panel.

In a blinded study, 103 frozen blood culture samples positive for Gram positive cocci in clusters (GPCC) were tested. The distribution of species including methicillin-resistance determination is shown in Table 2. There was agreement on 93/98 samples from mono-microbial GPCC positive blood cultures between Staph ID/R and MicroScan. Resolution of discordant results with *RpoB* gene sequencing correlated in all cases with Staph ID/R.

Table 1.
Identification of Staphylococcus species: Micro Scan versus Portrait Staph ID/R

Resolved Result	Call by Test	
Species (n)	**Portrait**	**Micro Scan**
S. aureus (19)	19	19
MRSA (6)	6	6
S. epidermidis (8)	8	8
MRSE (40)	40	40
S. hominis (2)	2	2
MR S. hominis (4)	4	4
MR S. haemolyticus (1)	1	0
S. warneri/ pasteuri (1)	2	1
MR S. warn/past (7)	6	6
S. lugdunensis	0	0
S. capitis/caprae (2)	2	1
S. genus spp unknown (1)	1	0
Non Staph *E. facaelis* (2)	2	2
Non *Staph* (5)	5	3
# Correct (%)	**98 (100)**	**93 (94.9)**

For poly-microbial GPCC positive blood cultures, Staph ID/R found multiple *Staphylococcus* or *E. facaelis* organisms in each sample and was concordant with *RpoB* gene sequencing in all cases but one. MicroScan found only one *Staphylococcus* or *E. facaelis* organism in each sample. In two poly-microbial cases, MicroScan reported MRSA only whereas Staph ID/R detected a mixture of *S. aureus, S. haemolyticus*, and *mecA* in one and *S. aureus, S. epidermidis*, and mecA in another (Table 3). In one sample, mauve colonies were observed on ChromAgar MRSA, indicating the presence of MRSA. In the other case, mauve colonies were observed on ChromAgar SA, indicating *S. aureus* was present, but on ChromAgar only white colonies were observed. Sequencing of this colony indicated that the methcillin-resistant organism was *S. epidermidis*.

Table 2. Analysis of Poly-microbial Specimens

Test Results			Sub-culture Results				Sequencing Results
ID	MicroScan	Staph ID/R	TSA (unique colonies)	ChromAgar SA	ChromAgar MRSA	MRSA Screen	RpoB gene
10	MR *S. epidermidis*	*S. epidermidis* *S. haemolyticus* *mecA*	2	blue/green	blue/green	Mod	*S. epidermidis* *S. haemolyticus*
16	*E. facaelis*	*E. facaelis* *S. epidermidis* *mecA*	2	blue	blue	Mod	*E. facaelis* *S. epidermidis*
44	MRSA	*S.aureus* *S. haemolyticus* *mecA*	1	mauve	mauve and white	Light	*S. haemolyticus* *S.aureus*
58	MRSA	*S. epidermidis* *S. aureus* *mecA*	1	mauve	white	No	*S. epidermidis* *S. aureus*
102	*E. facaelis* *K. pneumoniae*	*E. facaelis* *S. epidermidis* *S. hominis* *mecA*	2	blue	blue and white	No	*S. epidermidis* *S. cohnii* *E.facaelis*

Conclusions

The Staph ID/R test was shown to rapidly identify the major *Staphylococcus* species and the mecA gene, which confers resistance to methicillin, directly from positive blood culture aliquots with high concordance compared to the standard of RpoB and 16S gene sequencing. Additionally, the test demonstrated the ability to detect more than one organism in a poly-microbial positive blood culture bottle.

Information about the species present and whether or not it harbors the mecA gene can aid in patient management. In the case of MRSA/MSSA tests the value has been clearly shown that a rapid test result lead to shorter hospital length of stay and lower treatment costs due to the initiation of appropriate therapy sooner.

By additionally providing knowledge about the potential methicillin-resistance status of coagulase-negative Staphylococcus, treatment can be adjusted to beta-lactam drugs for methicillin-sensitive infections which lead to improved outcomes compared with treatment with vancomycin, the generally accepted drug of choice.

Typically, ~30% of positive blood cultures are contaminants, not true infections. With the knowledge of species information in positive blood cultures, Staph ID/R could be used as an approach to distinguish true infections from contaminant blood cultures with high predictive value from just two positive culture bottles.