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Direct Identification of MRSA and MLS_B Phenotypes in *Staphylococcus aureus* Using Small Numbers of Immobilized Cells

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AMENDED ABSTRACT

Background: Conventional phenotyping methods require growth of large numbers of bacteria, which lengthens the total time-to-result. We report results for a new method that uses approximately 100-500 bacterial cells and enables 4-hour MRSA and MLS_B phenotype detection.

Methods: A multi-channel fluidic device used computerized microscopy of immobilized bacteria to measure bacterial growth rates. Borderline oxacillin resistant isolates (CDC) were exposed to cefoxitin (FOX) in one fluidic channel to predict MRSA and, in another channel, erythromycin (ERY) followed by clindamycin (CLI) to identify MLS_B phenotypes. 79 strains were *mecA*-positive and 56 were *mecA*-negative. One strain had a mutated *mecA* and produced a variant PBP2a of unknown clinical significance. 44 of the 79 *mecA*-positive isolates and 14 of the 56 *mecA*-negative isolates exhibited inducible or constitutive CLI resistance as determined by D-test. Colonies were resuspended from agar plates, pre-grown for 2 hours, and then 10 µL of a $7x10^5$ CFU/mL inoculum was delivered to each flowcell. Bacteria were concentrated onto a poly-L-lysine-coated glass surface, capturing approximately 100-500 cells in the microscope's field of view. MRSA detection used 1 µg/mL of FOX followed by 6 µg/mL FOX. MLS_B detection used 0.1 µg/mL ERY followed by 0.5 µg/mL CLI. The system acquired images every 10 minutes and computed growth rates of the cell population throughout the test. The system classified strains according to differences in growth rates between the antibiotic exposed organisms and control organisms. Results were compared to *mecA* PCR results, CLSI FOX disk diffusion (FOX-DD), and D-tests.

Results: The FOX assay correctly classified 78 of 78 FOX-DD positive and 57 of 57 FOX-DD negative isolates in a total test time of 4 hours. The mutated *mecA* strain tested negative by FOX-DD and the experimental method. 43 of 44 of the *mecA*-positive isolates and 14 of 14 of the *mecA*-negative isolates expressing CLI resistance were correctly identified.

Conclusions: Direct measurement of growth rates of small numbers of immobilized bacteria enabled rapid identification of the MRSA and MLS_B phenotypes in *S. aureus*. The method shows promise for rapid testing of a bacterial sample compatible with direct extraction from clinical specimens.

INTRODUCTION

Mechanisms of broad-spectrum resistance to ß-lactam antibiotics present serious clinical challenges, particularly with critically ill patients. Methicillin resistant *S. aureus* (MRSA) has become a major pathogenic phenotype that requires rapid identification in order to assure adequate initial therapeutic coverage. MRSA is associated with multiple drug resistance mechanisms in addition to conferring total β-lactam resistance. Laboratories need new methods to rapidly determine all major antibiotic resistance phenotypes. New methods requiring small numbers of organisms for testing could potentially obviate the need for overnight culturing and enable direct-from-specimen analysis.

Multiplexed direct cellular phenotyping offers a rapid alternative method, requiring relatively small numbers of cells. It has the potential to overcome the inherent limitations of other rapid methods, such as gene detection, for which resistance expression lacks a direct molecular marker correlate. Direct cellular phenotyping shows evidence of meeting analytical challenges such as inducibility and heteroresistance that now complicate antibiotic susceptibility testing.

This study tested multiplexed assay methods intended to enable a new rapid diagnostic system that will use bacteria extracted directly from a patient specimen without prior enrichment or colony isolation. The purpose was to determine whether the novel direct cellular phenotyping methods meet requirements for speed and accuracy in simultaneously identifying two unrelated and clinically important resistance mechanisms in *S. aureus* using small numbers of bacterial cells.

MATERIALS & METHODS

Direct observation of bacterial response to antibiotic exposure was performed on a custom disposable 32-flowcell cassette inserted into an automated digital microscope with customized motion control and image analysis software. Each flowcell was independent. Flowcell top and bottom surfaces had transparent, electrically conductive coatings for electrophoresis and microscopy. The bottom surface was coated with poly-L-lysine that immobilized bacteria upon contact.

A collection of oxacillin borderline-MIC isolates was provided by the CDC. The collection included 78 *mecA*-positive and 56 *mecA*-negative strains, plus one strain with mutated *mecA* that produced a variant PBP2a protein of unknown clinical significance. Tests also included CLSI QC strains (data not shown), ATCC® 43300 (MRSA), BAA-976 (macrolide efflux), BAA-977 (inducible MLS_B phenotype), and 29213 (susceptible control). 44 of the *mecA*-positive and 14 of the *mecA*-negative isolates were either constitutively or inducibly resistant to clindamycin (CLI) according to D-test results. **Table 1** lists the CLI resistance phenotype counts by *mecA* status.

Table 1

mecA Status	MLS_B Phenotype						Total
	D	D+	HD	R	NEG	S	
Positive	24	3	8	9	12	23	79
Negative	10	2	1	1	3	39	56

*Phenotypes as described by Steward et al. (2005): **D**=D-test pos. **D+**=D-test pos. plus ingrowth; **HD**=D-test pos. with haze overgrowth; **R**=constitutively resistant to CLI ; **NEG**=susceptible to CLI; **S**=susceptible to CLI and ERY. All phenotypes except the **S**-type were ERY resistant.*

Colonies from agar plates were resuspended in broth and grown for 2 hours. Log phase *S. aureus* were resuspended in electrokinetic capture buffer at $1x10^6$ CFU/mL. A 10 µL sample was pipetted into each flowcell of the cassette, and the cassette placed into the instrument.

Electrophoresis for 5 minutes concentrated bacteria to the flowcell surface. Bacteria adhered to the capture coating, permitting subsequent medium exchanges. Each 444 x 592 µm field of view contained approximately 100-500 bacterial cells. All assays used Mueller-Hinton broth (MHB) as a wash medium and reagent vehicle.

For each isolate, the system performed concurrent assays in separate flowcells: a growth control, a non-induction FOX test, a FOX-induced FOX test, a non-induction CLI test, and an ERY-induced CLI test. Prior studies had established 1 hr. of 1 µg/mL FOX followed by 3 hrs. of 6 µg/mL FOX as standard conditions. Other studies had established 1 hr. of 0.1 µg/mL ERY followed by 3 hrs. of 0.5 µg/mL CLI as standard.

The instrument acquired images for each of the flowcells at 10-minute intervals. It performed growth rate measurements on the entire bacterial population within each field of view.

Prior studies established growth-rate interpretation criteria after the challenge period. For MRSA identification, *mecA*-positive isolates had growth rates greater than 0.1 divisions per hour (div/hr), and *mecA*-negative isolates had rates less than 0.1 div/hr. For MLS_B identification, CLI-resistant isolates had growth rates greater than 0.4 div/hr and CLI-susceptible isolates had rates less than 0.4 div/hr.

RESULTS

Growth began after bacterial immobilization and MHB wash without an appreciable lag time (<10 min.).

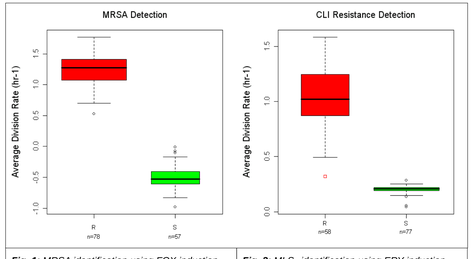

***Fig. 1:** MRSA identification using FOX induction. Negative growth rate signifies cell lysis (loss of cell mass).*

***Fig. 2:** MLS_B identification using ERY induction and CLI challenge. Note difference in Y-axis scale compared to Fig. 1.*

78 of the 79 *mecA*-positive strains were classified as MRSA, and 56 of the 56 *mecA*-negative strains as MSSA (Fig. 1). As with all test methods reported by the CDC (Swenson *et al.*, 2007), the experimental method classified the mutated *mecA* strain (BS-089) as susceptible. This strain was classified MSSA in the tabulated results based on CLSI FOX-DD results.

CLI resistance was correctly characterized in 43 of the 44 *mecA*-positive and 14 of the 14 *mecA*-negative isolates (Fig 2). The division rate for the incorrectly classified strain is plotted separately as a red square.

DISCUSSION

The experimental method met the objectives of minimal starting cell count, rapid time to result, and demonstrated accuracy comparable to that of FOX-DD and D-zone tests in identifying the MRSA phenotype and CLI resistance in this oxacillin MIC borderline collection.

Further optimization of the induction concentration and challenge concentration may further decrease the total assay time.

CONCLUSIONS

The required number of cells (100-500) for MRSA and MLS_B identification was small compared to the number (approx. 10^4-10^5) required by conventional microbiological methods. If combined with compatible concentration and *in situ* identification methods, the rapid direct phenotyping method has the potential to eliminate the need for overnight culturing and colony isolation with patient specimens such as bronchoalveolar lavage fluid, wound swabs, and other high-titer specimens. The analytical speed of the automated system was consistent with that required to guide initial empiric therapy in critically ill patients.

Independent validation studies are underway in outside laboratories.

References

Steward CD, *et al.* "Testing for induction of clindamycin resistance in erythromycin-resistant isolates of *Staphylococcus aureus*." J Clin Microbiol. 2005, 43: 1716-21.

Swenson JM, *et al.* "Detection of *mecA*-mediated resistance using reference and commercial testing methods in a collection of *Staphylococcus aureus* expressing borderline oxacillin MICs." Diagn Microbiol Infect Dis. 2007, 58: 33-9.

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