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Identification of *mecA* in *Staphylococcus aureus* Using Small Numbers of Immobilized Cells and the Response to Cefoxitin (FOX) by Automated Growth Analysis

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AMENDED ABSTRACT

Background: Conventional MRSA phenotype-based test methods require large numbers of bacteria, and culturing delays the time to result. We report initial results for a new method that requires less than 500 bacterial cells, enabling rapid MRSA phenotype identification in *S. aureus*, potentially eliminating cultures for many types of patient specimen.

Methods: A microfluidic device used computerized microscopy of immobilized bacteria to measure bacterial growth rates. Isolates having borderline oxacillin MICs were obtained from the Centers for Disease Control (CDC) and tested using cefoxitin (FOX) to predict *mecA* status. 77 strains were *mecA*-positive and 56 were *mecA*-negative. Colonies were resuspended from agar plates and grown for 2 hours. 10 µl of a 1x10^6 CFU/mL inoculum was pipetted into a flowcell. Bacteria were electrokinetically concentrated onto poly-L-lysine coated glass, immobilizing approximately 200-500 cells in the microscope's field of view.

Cation-adjusted Mueller-Hinton broth (MHB) with 1 µg/mL FOX was introduced into the flowcell, cells grew for 1 hour, then 6 µg/mL FOX in MHB followed. An adjacent flowcell contained a growth control. The system acquired images every 10 minutes and computed cell population growth rates. The system classified strains according to differences in growth rates between the FOX-exposed organisms and control organisms. Results were compared to published CDC *mecA* PCR results.

Results: Growth rate differences, used to predict *mecA* status, correctly classified 75 of 77 *mecA*-positive and 56 of 56 *mecA*-negative isolates. Following exposure to 6 µg/mL FOX, growth arrest of *mecA*-negative strains occurred in less than 4 hours. Growth was not arrested for strains interpreted as positive.

Conclusions: Direct measurement of growth rates of small numbers of immobilized bacteria enabled rapid prediction of *mecA* status in *S. aureus*. The method shows promise for rapid MRSA identification using an inoculum size 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. The supernumerary *mecA* gene causes the MRSA phenotype by encoding a variant of PBP2 (PBP2a or PBP2') that has low affinity for ß-lactam antibiotics. Most laboratories cannot afford to use gene analysis to detect *mecA* directly. The CLSI recognizes cefoxitin (FOX) disk diffusion (DD) as a robust phenotyping test for routine clinical prediction of *mecA* expression. However, the culturing delay prevents the lab from reporting results in time to guide initial therapy.

Despite the apparent simplicity of the resistance mechanism, MRSA identification presents challenges because of widespread heteroresistance, which also occurs with other types of antibiotic resistance. New analytical methods must meet such challenges.

The experimental methods tested in this study are intended to become part of a new rapid diagnostic system using bacteria extracted directly from a patient specimen without prior enrichment culturing or colony isolation. The purpose of the study was to determine whether the experimental methods meet objectives for sample cell count, speed, and accuracy for predicting *mecA* status with accuracy similar to that of the FOX DD test but in a much shorter time.

MATERIALS & METHODS

Direct observation of bacterial response to FOX was performed on a disposable fluidic cassette inserted into a custom bench-top instrument that combines automated digital microscopy, motion control, and image analysis software.

Fig. 1: *Dual 16-channel flowcell cassette.*

A collection of oxacillin MIC borderline isolates was provided by the CDC. The collection included 77 *mecA*-positive and 56 *mecA*-negative strains, plus one strain with mutated *mecA* that produced a variant PBP2a protein. We also included the CLSI MRSA QC strain, ATCC® 43300.

The cassette contained multiple independent flowcells (**Fig. 1**). Flowcells were constructed with transparent top and bottom surfaces to allow microscope imaging. Each surface had a transparent electrode coating, forming an electrophoresis chamber. The bottom surface was coated with poly-L-lysine that immobilized bacteria upon surface contact.

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 channel of a multichannel fluidic cassette, and the cassette placed into the instrument.

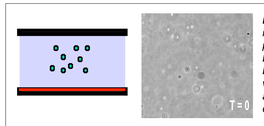


Fig. 2a. *Left: suspension of bacteria inside flowcell, side view. Right: phase contrast image focused on bottom surface after introducing bacteria. Unfocused images of individual cells show that the bacteria are above the surface. Microscope objective is beneath the flowcell.*

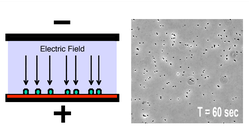
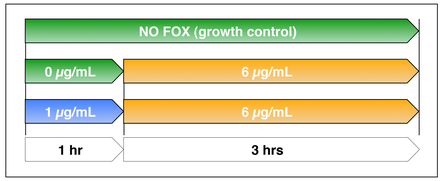


Fig. 2b. *Left: negatively charged bacteria migrate to the positively charged bottom surface. Right: phase contrast image shows sharply focused images of individual bacteria on the surface.*

Application of an electrical field caused bacteria to migrate to the positively-charged lower electrode (**Fig. 2**) during a capture step. The bacteria adhered to the surface coating, permitting subsequent medium exchanges.

Each flowcell contained approximately 200 to 500 founder cells on the surface within the digital microscope's 444 x 592 µm field of view. After capture, the flowcells were washed with cation-adjusted Mueller-Hinton broth (MHB), removing electrokinetic capture buffer.

The system performed 3 concurrent assays in separate flowcells: a growth control, a non-induction test, and an induction test —



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

Classification of *mecA*-positive or -negative status was possible using induction conditions. A growth rate criterion after FOX induction and challenge was defined from pilot studies. Positive strains had growth rates greater than zero, and negative strains had rates less than zero after three hours of challenge. Results were compared to those for *mecA* status provided by the CDC with the isolate collection.

Growth analysis of individual clones (**Fig. 3**) helped to assess the heteroresistant population fraction, assisting test optimization.

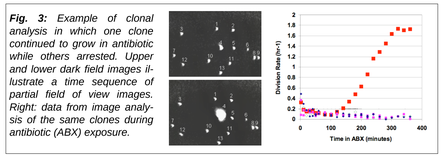


Fig. 3: *Example of clonal analysis in which one clone continued to grow in antibiotic while others arrested. Upper and lower dark field images illustrate a time sequence of partial field of view images. Right: data from image analysis of the same clones during antibiotic (ABX) exposure.*

Additional analysis compared the effects of induction on individual strains. A strain was classified as inducible if its mean population division rate increased at least 2x the non-induced rate during challenge.

Tests were run at least in duplicate and the results averaged. Isolates that exhibited discrepant results were tested with the Oxoid PBP2' Latex Agglutination Test and FOX disk diffusion (FOX DD).

RESULTS

Time to capture was fixed at 300 seconds. Electrokinetic transport moved all bacteria above the capture area to the surface, determined by focusing at different levels above the surface. Growth began after MHB wash without an appreciable lag time (<10 min.).

Optimization studies yielded the expected inverse correlation between FOX challenge concentration and mean population growth rate (**Fig. 4**). Consistent with published findings in other studies (*e.g.* Swenson *et al.*, 2007), 6 µg/mL FOX was selected as the tested challenge concentration.

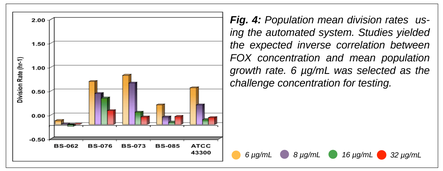


Fig. 4: *Population mean division rates using the automated system. Studies yielded the expected inverse correlation between FOX concentration and mean population growth rate. 6 µg/mL was selected as the challenge concentration for testing.*

Similarly, optimization studies (**Fig. 5**) demonstrated elevated population growth rates after induction in some strains. For a few strains, induction changed the growth rate from zero or negative to strongly positive at the criterion challenge interval. Limited testing indicated 1 µg/mL as suitable for induction. 15 of the 77 *mecA*-positive strains (19%) exhibited inducibility. None of the *mecA*-negative strains exhibited inducibility.

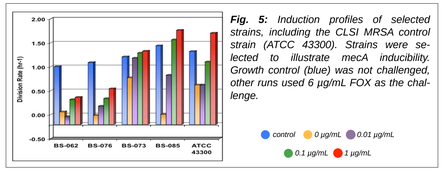


Fig. 5: *Induction profiles of selected strains, including the CLSI MRSA control strain (ATCC 43300). Strains were selected to illustrate mecA inducibility. Growth control (blue) was not challenged, other runs used 6 µg/mL FOX as the challenge.*

75 of the 77 *mecA*-positive strains were classified as resistant, and 56 of the 56 *mecA*-negative strains as susceptible in this collection of MIC borderline isolates (**Fig. 6**). Tests ended 4 hours after bacterial capture.

As determined by PBP2a latex agglutination and FOX DD, none of the discrepancies between the experimental method and reported *mecA* status were inconsistent with their CDC-reported characteristics, demonstrating strain genotypic and phenotypic stability. 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 has unknown clinical significance and was excluded from tabulated results.

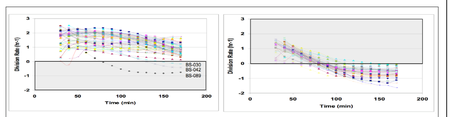


Fig. 6.*The automated system measured division rates for all strains during challenge, and compared them with the classification criterion.* ***Left:*** *Mean population division rates of 78 mecA-positive strains. The shaded area represents division rates for negative mecA calls. Strain numbers indicate experimentally discrepant strains (false negatives). Strain BS-089 carries a mutated mecA gene of unknown clinical significance.*

Right: *Individual strain results for 56 mecA-negative strains. The shaded area represents division rates for positive mecA calls (there were no false positives).*

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 in identifying the MRSA phenotype in this oxacillin MIC borderline collection.

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

Note added in further support of conclusions: Further experimentation with moxalactam (MOX) as an inducer prior to the FOX challenge, resulted in the correct call for a previously reported false negative strain. Further studies are planned to compare a larger panel of inducers and to optimize operating parameters.

CONCLUSIONS

The required number of cells (200-500) for *mecA* prediction was small compared to the number (approx. 10^4-10^5) required by most conventional microbiological methods. If combined with a compatible concentration and identification method, the rapid phenotyping method would eliminate the need for overnight culturing and/or colony isolation with high-titer patient specimens such as bronchoalveolar lavage fluid, endotracheal aspirates, and potentially other critical specimens. The analytical speed of the automated system was consistent with that required for guiding initial empiric therapy in critically ill patients.

References

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(1):33-39.

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