074

C-163. Direct Observation of Inducible Clindamycin Resistance in *Staphylococcus aureus* using Single Live Cell Imaging

S. Metzger, C. Greef, D. Howson, N. Kulprathipanja, M. Lindsey, J. Mascali, T.H. Ngo, J. Schraufnagel, Accelr8 Technology Corporation, Denver, CO

Accelr8 Technology Corporation
Please visit:
www.accelr8.com
Contact:
dhowson@accelr8.com

Abstract

Background: Inducible clindamycin (CLI) resistance can occur in erythromycin (ERY)-resistant staphylococci. Testing of clindamycin by standard susceptibility methods will not detect inducible resistance. The CLSI recommends a disk approximation method for detection of inducible clindamycin resistance referred to as the "D-zone test" that requires overnight incubation.

Methods: An automated microfluidic device using computerized microscopy of immobilized individual bacterial cells and clones was used to rapidly test *S. aureus* strains for the inducible clindamycin resistance (MLS_Bi) phenotype. CLSI recommended reference strains (ATCC™ BAA-976, BAA-977, 25923), two bacterial reference strains (ATCC 12600, 29213), and 7 multi-drug resistant test strains (ATCC BAA-39, BAA-40, BAA-41, BAA-42, BAA-43, BAA-44,27660) were settled on poly-L-lysine coated glass capture surfaces in separate flowcell channels. Immobilized cells were exposed to a sub-inhibitory concentration of ERY (0.07 μg/mL) for 60 minutes (induction phase) followed by addition of 8 μg/ml CLI for a susceptibility testing phase.
The system measured growth rates of each individual cell throughout ERY induction and following CLI exposure. Controls included exposure of the strains to CLI without prior ERY induction. Results of the automated induction tests were compared with CLSI D-zone tests.

Results: All staphylococcal strains grew normally in the presence of 0.07 μg/ml ERY. Following ERY induction, MLS_Bi strains (BAA-977 and 27660) grew normally in CLI. Without ERY induction, growth of the MLS_Bi strains were inhibited by CLI. *S. aureus* strains 12600, 25923, 29213, BAA-42, and BAA-976 were inhibited by CLI with or without induction. *S. aureus* strains BAA-39, BAA-40, BAA-41, BAA-43, and BAA-44 were resistant to CLI with or with out induction. These findings agreed with those of the D-zone test and the instrument results were conclusive in less than 5 hours.

Conclusion: Detection of the MLS_Bi phenotype in *S. aureus* in 5 hours is possible using this approach of direct microscopic analysis of individual immobilized bacterial cells.

Introduction

- Macrolides, lincosamides, and group B streptogramins are important antibiotic classes for the treatment of Gram-positive cocci.
- Resistance mechanisms to these antibiotics have been classified into three categories:
  (1) Interference with the ribosomal drug target
  (2) Efflux pump mechanisms
  (3) Drug inactivation
- Interference with the ribosomal binding target can be mediated by *erm* (erythromycin *r*ibosome *m*ethylase) genes leading to cross-resistance to macrolides, lincosamides, and group B streptogramins; the MLS_B phenotype.
- An induced MLS_B phenotype, MLS_Bi, can mutate to a constitutive form *in vitro* at rates of ~10^{-7} per division.
- The diversity of MLS_B resistance has led to complex phenotypes and reporting difficulties.
- CLSI recommends an overnight double-disk approximation method for detection of inducible clindamycin resistance, referred to as the "D-zone test".

The purpose of this investigation was to determine whether a new automated analytical method based on single-cell and single-clone analysis could achieve results comparable to those of the D-zone test in a much shorter time.

Methods

Direct observation of bacterial induction was performed on an experimental bench top instrument that combines a disposable fluidic cartridge with automated digital microscopy and image analysis software.

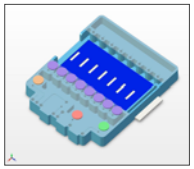
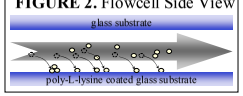


FIGURE 1. 8-flowcell cartridge

- *S. aureus* ATCC™ isolates were resuspended in phosphate buffered saline at a 1 x 10^8 CFU/mL.
- This sample was introduced into each flowcell in a disposable cartridge that contained 8 flowcells.
- Cells adsorbed to the transparent flowcells' capture surface coated with poly-L-lysine.
- Flow cells were then rinsed with growth media. Approximately 500 – 1,000 founder cells remained immobilized on the capture surface (~3000/mm²) within the digital microscope's field of view.
- Time sequence images of multiple fields of view were acquired by the system for each of the 8 flowcells.
- The system performed three assays:
  1. Growth of immobilized cells in tryptic soy broth (TSB) for 2 hours.
  2. Induction assay consisting of:
     a) Growth of immobilized cells in 0.08 μg/mL ERY in TSB below resistant break point concentrations for 1 hour.
     b) Exposure to CLI at 8 μg/mL containing 0.08 μg/mL ERY for an 3 additional hours.
  3. CLI susceptibility test consisting of:
     a) Growth of immobilized cells in TSB for 1hr
     b) Exposure to CLI 8 μg/mL in TSB for an additional 3 hours.
- The assays were used to report each *S. aureus* strain as inducibly resistant to CLI, CLI constitutively resistant, or CLI susceptible.
- Each test required less than 5 hours of run time.
- D-zone tests were performed using CLSI procedures with 18-hour incubation.

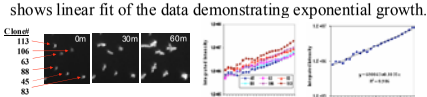
FIGURE 2. Flowcell Side View



Growth Analysis

- Growth of 2- dimensionally growing individual cells and clones was assessed using darkfield integrated intensity methods

FIGURE 2. Darkfield image time series of *Acinetobacter baumannii* showing exponential growth(a). Panel b shows the log of integrated intensity verses time for 6 clones. Panel c shows linear fit of the data demonstrating exponential growth.



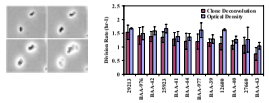
(A) (B) (C)

- Immobilized cells and clones exhibit expected exponential growth in flowcell cartridge.

Growth Analysis with Clone Deconvolution

- 3-Dimensional *S. aureus* clone growth confounds area-based growth analysis techniques, resulting in under-estimation or missed clone growth.

FIGURE 4. Clone deconvolution methods generate doubling times in agreement with conventional optical density based methods.

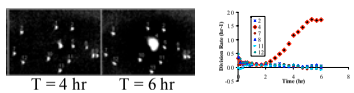


- Deconvolution of serial clone images enables accurate quantitation of growth at the clone level, providing more accurate aggregation of population values.

Individual Clone Growth Measurement

- Automated image analysis enables tracking of individual founder cells and progeny on a clone-by-clone level.
- Clone level analysis detects low levels of heterogenity in clone growth patterns, enabling detection of hetero-resistance.

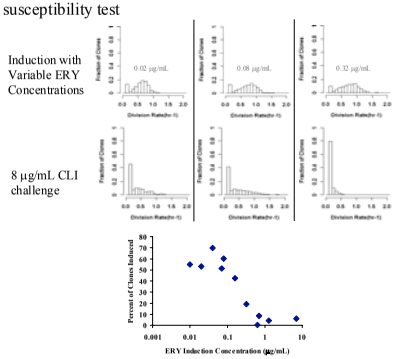
FIGURE 5. Growth rate measurement and differentiation of clone growth phenotype.



Induction Assay Optimization

- CLSI control strain BAA-977 was induced with variable concentrations of ERY (0.01 - 7.0 μg/ml) and the percentage of growing cells and corresponding division rates were used to determine optimum ERY concentration.

FIGURE 6. Distribution of clone division rates for BAA-977 during erythromycin induction phase and clindamycin susceptibility test

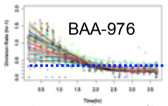


- An ERY concentration of 0.08 μg/mL was used for subsequent in induction assays.

Growth Rate Derived Criteria

- Division rate of a susceptible *S. aureus* strain slows significantly after 3 hours of CLI exposure.
- A 0.33 hr^{-1} division-rate (180 minute doubling time) at 3 hrs of CLI+ERY following 1 hour of induction was established as a classification criterion to differentiate resistant and susceptible clones.

FIGURE 7. Division rate based classification criterion using CLI susceptible *S. aureus* strains during induction assay.

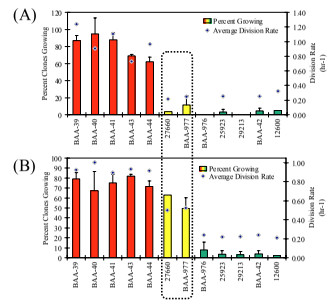


- Majority of susceptible clones do not grow above criterion (blue horizontal line) after 3 hours of CLI exposure.

Results

- Induction and CLI susceptibility assays were run on 12 isolates.
- The percentage of clones growing above breakpoint and the average division rate for all clones was used to classify strains as resistant/susceptible to CLI.

FIGURE 8. Percentage of clones growing above criterion and the average rate for 12 *S. aureus* isolates at the end of the CLI susceptibility assay (A) and at the end of an induction assay (B).



- Inducibly CLI resistant ATCC isolates 27660 and BAA-977 show a significantly higher percentage of growing clones in induction assay relative to the CLI susceptibilty test, characteristic of the MLS_Bi phenotype.
- CLI constitutive resistant ATCC isolates BAA-39, BAA-40, BAA-41, BAA-42, BAA-43, and BAA-44 grow in CLI with or with out induction characteristic of the constitutive CLI resistance phenotype.
- CLI susceptible ATCC isolates BAA-42, BAA-976, 12600, 29213, and 25923 do not grow significantly in the induction or CLI susceptibility assay characteristic of a susceptible phenotype.

Conclusions

- Growth rates for individual cell and clones were assessed in the presence of antibiotics.
- Rapid, direct observation of induced CLI resistance is possible in less than 5 hours.
- Results agree with D-zone tests.