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Rapid antibiotic susceptibility phenotypic characterization of *Staphylococcus aureus* using automated microscopy of small numbers of cells☆,☆☆

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ABSTRACT

Staphylococcus aureus remains a leading, virulent pathogen capable of expressing complex drug resistance that requires up to 2–4 days for laboratory analysis. In this study, we evaluate the ability of automated microscopy of immobilized live bacterial cells to differentiate susceptible from non-susceptible responses of *S. aureus* isolates (MRSA/MSSA, clindamycin resistance/susceptibility and VSSA/hVISA/VISA) to an antibiotic based on the characterization of as few as 10 growing clones after 4 h of growth, compared to overnight growth required for traditional culture based methods. Isolates included 131 characterized CDC isolates, 3 clinical isolates and reference strains. MRSA phenotype testing used 1 h of 1 μg/mL cefoxitin induction followed by 3 h of 6 μg/mL cefoxitin. Clindamycin susceptibility testing used 1 h of induction by 0.1 μg/mL erythromycin followed by 3 h of 0.5 μg/mL clindamycin. An automated microscopy system acquired time-lapse dark-field images, and then computed growth data for individual immobilized progenitor cells and their progeny clones while exposed to different test conditions. Results were compared to concurrent cefoxitin disk diffusion and D-test references. For CDC organisms, microscopy detected 77/77 MRSA phenotypes and 54/54 MSSA phenotypes, plus 53/56 clindamycin-resistant and 75/75 clindamycin susceptible strains. Automated microscopy was used to characterize heterogeneous and inducible resistance, and perform population analysis profiles. Microscopy-based hVISA population analysis profiles (PAPs) were included as an extended proof of concept, and successfully differentiated VSSA from hVISA and VISA phenotypes compared to plate-based PAP.

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1. Introduction

Staphylococcus aureus is a leading cause of serious infection in both the hospital and the community (Landrum et al., 2012; Klevens et al., 2007). Methicillin-resistant *S. aureus* (MRSA) has evolved as a leading cause of nosocomial infections worldwide and has also emerged as a major resistance phenotype in community-acquired infections (Chambers and Deleo, 2009). MRSA strains inherently resist essentially all β-lactam antibiotics, which make up the largest, most effective, and most widely used class of antibiotics. Clinical MRSA strains are frequently multi-drug resistant (MDR) and may express numerous virulence factors (Skrupky et al., 2009), making staphylococcal infections increasingly difficult to treat. *S. aureus* can have additional types of antibiotic resistance including resistance to clindamycin and reduced susceptibility to vancomycin.

Antimicrobial susceptibility testing (AST) is essential to assure appropriate antibiotic choice in treating infection, but typically requires 2–4 days to produce results. Guidelines (Muscedere et al., 2008; Liu et al., 2011) recommend starting empiric broad-spectrum antibiotic therapy within 1–3 h of the time that a critically ill patient exhibits signs of a potentially severe infection. Because of widespread antibiotic resistance, the patient is thus at risk of receiving inadequate therapy during the wait for laboratory results (Ibrahim et al., 2000; Iregui et al., 2002).

Automated microscopy of immobilized individual live bacterial cells is an innovative rapid phenotyping method for AST that analyzes the properties of a small number of cells to infer the phenotypic response of an entire population in hours compared to overnight culturing methods. The first purpose of this study is to evaluate the performance of automated microscopy to detect the MRSA phenotype and clindamycin resistance using induction-based assays on small numbers of cells compared to conventional AST confirmatory methods. Secondly, the ability of the automated microscopy method to characterize heteroresistant and inducible

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☆☆ Findings from preliminary studies related to this study were previously presented in part at the American Society for Microbiology 2008 and 2011 General Meetings (Metzger et al., 2008, 2011).

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strains was evaluated, including the time necessary to reveal a phenotypic response as well as the minimum number of cells required. As an extended proof of principle, a microscopy-based population analysis profile (PAP) to differentiate heterogeneous vancomycin intermediate *S. aureus* (hVISA) and VISA strains from vancomycin susceptible *S. aureus* (VSSA) was evaluated, as this is the best-known use of PAP currently.

2. Materials and methods

2.1. S. aureus isolates

S. aureus isolates included quality control strains specified by the CLSI for the purposes of susceptibility testing (CLSI, 2009): ATCC® 43300 MRSA, ATCC 29213 and 27659 methicillin susceptible *S. aureus* (MSSA), BAA-977 clindamycin (CLI) inducible resistance, BAA-976 CLI susceptible, Mu50/ATCC 700699 VISA, and Mu3/ATCC 700698 hVISA (ATCC, Manassas, VA). The Washington University School of Medicine (St. Louis, MO) Barnes-Jewish Hospital provided three *S. aureus* clinical isolates (1-3C, 1-4C, and 1-6I) that showed growth on 3 μg/mL vancomycin screening agar. The United States Centers for Disease Control and Prevention (CDC, Atlanta, GA) provided 131 characterized *S. aureus* isolates that expressed borderline oxacillin resistance and contained 78 *mecA* positive strains and 53 *mecA* negative strains (Swenson et al., 2007). Thirty-eight of the CDC strains were D-test positive for inducible CLI resistance, 18 strains expressed constitutive CLI resistance, and 75 strains were CLI susceptible (Table 1).

Each strain was recovered from −80 °C frozen storage and subcultured on sheep's blood agar (Becton Dickinson, Sparks, MD). Five to ten colonies selected from a fresh overnight subculture plate were suspended by vortexing in tryptic soy broth (Becton Dickinson) and incubated for 2 h at 35 ± 2 °C to achieve log phase growth. The bacterial suspension was centrifuged (12,000 × *g* for 4 min), washed in 1 mM L-histidine buffer at a pH of 7.2, and resuspended in a low ionic strength electrokinetic buffer containing 10 mM L-DOPA and 1 mM L-histidine at a pH of 7.0 (Accelerate Diagnostics, Tucson, AZ) to create an inoculum of approximately 1×10^6 CFU/mL.

2.2. Bacterial immobilization

Automated microscopy tests were performed in a custom disposable multichannel fluidic cassette containing 32 independent fluidic channels, with each channel having its own independent inlet and outlet ports for fluid exchange by pipetting (Fig. 1). Each fluidic channel consisted of a rectangular conduit formed by a die-cut double-adhesive polyester mask approximately 300 μm thick enclosed by transparent top and bottom covers. The top cover was also polyester, while the bottom cover consisted of a standard 1 × 3 inch glass microscope slide. The top and bottom surfaces of each fluidic channel had a transparent, electrically conductive coating of indium tin oxide. The bottom surface had an additional coating of poly-L-lysine. Bacteria were negatively-charged in the electrokinetic buffer.

After pipetting 20–30 μL of bacterial inoculum into each independent fluidic channel, a 5-min low voltage electrical field applied across the two indium tin oxide layers concentrated individual cells in a random dispersion on the bottom surface. After the current was stopped, the negatively-charged cells remained adherent to the poly-cationic poly-L-lysine layer through electrostatic binding. This allowed the operator to pipette test solutions into each fluidic channel to replace the electrokinetic buffer without detaching the cells. All antibiotic test solutions were prepared using cation-adjusted Mueller–Hinton broth (Becton Dickinson).

2.3. Automated microscopy

Immobilized bacteria were viewed using a custom microscopy instrument (Accelerate Diagnostics Inc., Tucson, AZ) that consisted of an assembly with an inverted IX-71 microscope (Olympus America, Inc., Center Valley, PA) adapted with commercially available accessories and a 20 × 0.45 NA objective lens that viewed each fluidic channel from below. Transmitted dark-field illumination was generated using a halogen light source (Olympus). A 12-bit monochrome MicroFire camera (Olympus) with a 1600 × 1200 pixel frame size captured dark-field time-lapse images in each fluidic channel at 10-min intervals over the antibiotic exposure period. Each field of view covered an area of $592 \times 444\ \mu m^2$ and the observation zone accommodated up to 42 fields of view per fluidic channel (Fig. 1). A PC running custom experiment control software (Accelerate Diagnostics) executed all automated operations. An XY stage (Prior Scientific, Cambridge, UK) driven by stepper motors enabled cassette scanning for synchronous image acquisition. A heated enclosure maintained the entire instrument setup at 35 ± 2 °C.

2.4. Image analysis

Once collected, the dark-field image sequences were analyzed offline using custom image analysis software (Accelerate Diagnostics). The software assigned unique individual spatial XY coordinates to each immobilized progenitor cell within each fluidic channel. As each progenitor cell grew into a clone of daughter cells, the assigned coordinates enabled the software to locate each individual growing clone throughout a series of time-lapse images. The software measured each clone's intensity as a metric of clone mass in each image. Analysis algorithms calculated a growth probability score for each growing clone derived from coefficients of a cubic polynomial fitted to a plot of the natural log of the clone mass *vs.* time. The growth probability score transformed the shape of each curve into a numerical score ranging between 0 and 1 that represented the probability of the clone continuing to grow, to arrest, or to lose mass (lyse). The slope of the cubic polynomial was used to calculate instantaneous division rates for individual clones at specified time points. For AST classification, a growth analysis algorithm multiplied the growth probability for each clone by its 4 h endpoint integrated intensity (which is proportional to total cell count at 4 h) and summed for all clones. This weighted growth probability was then divided by the summed intensity of all clones to derive an AST probability score for each isolate.

2.5. MRSA phenotype detection

Triplicate automated microscopy runs were performed on 131 CDC borderline isolates for MRSA phenotype detection. For each test, a separate growth control channel contained the same growth medium without any drug. A second channel used 1 μg/mL cefoxitin for 1 h of induction followed by 3 h of challenge with 6 μg/mL cefoxitin. A third channel used drug-free medium for 1 h of growth followed by 3 h of challenge with 6 μg/mL cefoxitin (no induction) as a reference to assess the magnitude of induction effects. A fourth channel used 1 μg/mL cefoxitin for 1 h followed by 3 h in drug-free medium as a control. Images were obtained every 10 min for the 3 h challenge period. AST probability scores above or below the criterion level of 0.4 classified an isolate as MRSA or MSSA, respectively. Concurrent standard CLSI

Table 1
Characteristics of CDC *S. aureus* isolates.

	mecA status	Cefoxitin disk diffusion result		D-test result		
		MRSA	MSSA	cCLI-R[a]	iCLI-R[b]	CLI-S[c]
n = 131	78 positive	77	1	17	27	34
	53 negative	0	53	1	11	41

[a] cCLI-R = constitutive clindamycin resistance.
[b] iCLI-R = inducible clindamycin resistance.
[c] CLI-S = clindamycin susceptible.

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