Automated 4-Hour Detection of Heteroresistant Vancomycin-Intermediate *Staphylococcus aureus* (hVISA)

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AMENDED ABSTRACT

Background: The hVISA phenotype presents a therapeutic dilemma with infected patients, but the extent of the threat remains unknown because these isolates are under-detected at most laboratories. We report and characterize a new method for identification of the hVISA phenotype within 4 hours from clinical isolates.

Methods: 30 isolates were characterized by 48 hr microdilution population analysis profile (PAP) and automated microscopy in the presence of vancomycin (VAN) ranging from 0 to 6 µg/mL in 10 non-doubling steps. AUC for the Mu3 hVISA strain (ATCC 700698) served as the reference. The PAP area under the curve (AUC) was calculated and hVISA isolates were detected using the 0.9x Mu3 AUC threshold criterion. Multiplexed automated digital microscopy (MADM) used bacterial cell suspension of 1e6 CFU/mL for each sample. Cells were introduced into multiple, independent flowcell channels in a fluidic cassette. Bacteria were immobilized and spatially mapped on a transparent surface for observation. A wash displaced the sample fluid with Mueller-Hinton media containing different VAN concentration for each flowcell, from 0.01 to 4 µg/mL, plus one antibiotic-free growth control channel. Image analysis software computed changes in mass of each growing clone. Automated microscopy scanned and analyzed the growth of approximately 1e3 clones. It counted the number of growing clones that increased in mass 4x in 4 hours for each condition, computed the abbreviated AUC and compared to microdilution PAP AUC.

Results: Using PAP, 15 isolates were characterized as hVISA and 15 isolates as VSSA. MADM had one technical failure (organism overload) with a VSSA isolate, leaving 29 total comparisons. The MADM was 93% (14/15) sensitive and 100% (14/14) specific using the derived MADM AUC criterion value when compared to the PAP results. The MADM time-to-result was 4 hours.

Conclusions: Automated MADM can identify the hVISA phenotype within 4 hours using a small number of live immobilized bacteria from clinical isolates. Further studies are required to more fully characterize performance and study clinical utility of the test method.

INTRODUCTION

Infection with heteroresistant organisms can be difficult or impossible to detect by standard antibiotic susceptibility culturing methods using MIC criteria. Of particular concern, heteroresistance by *S. aureus* to vancomycin (VAN) may be emerging as a diagnostic challenge. The magnitude of the problem remains obscure because VAN-heteroresistant *S. aureus* (hVISA) exhibits MICs within the susceptible range but may lead to VAN failure. This leaves the laboratory community unable to perform adequate epidemiological and clinical studies. The purpose of our study was to determine assay criteria for multiplexed automated digital microscopy (MADM) to rapidly identify the hVISA phenotype in individual live organisms using abbreviated population analysis profiles (PAP). Numbers of individual organisms tested fell within the range obtainable directly from lower respiratory specimens.

MATERIALS & METHODS

Customized MADM systems used commercial inverted microscopes with 12-bit monochrome cameras. A PC ran custom image analysis and experiment control software. 32-channel disposable cassettes (Fig. 1) enabled live microbial cell immobilization for microscopy and fluid exchanges for different test media and reagents.[1]

Figure 1: *32-channel flowcell cassette.*

We screened *Staphylococcus aureus* (SA) clinical isolates and isolates from a CDC SA collection using 48-hour broth microdilution abbreviated population analysis profiles (BMD-PAP), which served as the control condition. We also applied BMD-PAP to hVISA reference strain Mu3 (ATCC® 700698), and measured areas under the curve (PAP-AUC) in all tests.

BMD-PAP consisted of serial isolate concentrations from 1 to 10^6 CFU/mL dropped onto sectors of VAN agar plates containing from 0 to 6 µg/mL in 10 steps (non-doubling dilutions) and counting colonies. An isolate met the hVISA+ detection criterion if its BMD-PAP-AUC ≥0.9 Mu3 AUC. This study did not attempt to discriminate between hVISA and VISA, as designated by the plus sign in hVISA+.

For MADM analysis, each independent flowcell channel (Fig. 2) contained 10 µL of 10^6 CFU/mL (pipette introduction). A capture coating immobilized individual organisms for microscope image acquisition. Each flowcell channel received one VAN concentration, using 0, 0.01, 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, or 4.0 µg/mL.

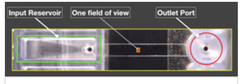

Figure 2: *One flowcell channel.*

The instrument acquired images at 10-minutes intervals for 90 minutes, using three fields of view in each flowcell channel through a 20x objective and darkfield illumination. In each channel, the image analyzer identified approximately 1,000 growing clones. It then counted the number of clones from the same population sample that exhibited at least 4-fold gain in mass by the end of a 4-hour analysis period. Computation normalized the latter count by dividing it by the initial count.

By plotting abbreviated PAPs for these normalized counts, we then selected an AUC value for the abbreviated PAP region that yielded the best discrimination between hVISA+ and VSSA strains determined by BMD-PAP AUC and the Mu3 reference AUC.

RESULTS

BMD-PAP detected 15 hVISA+ isolates (3 CDC strains, 12 screened clinical isolates) and 15 VSSA (12 CDC strains). One MADM test with a VSSA strain accidentally contained too many organisms to count and was censored as a technical error. MADM correctly classified 14/15 hVISA+ strains, and 14/14 VSSA strains. As shown in the plot of MADM-PAP-AUC *vs.* BMD-PAP-AUC (Fig. 3), one discrepant hVISA+ strain exhibited a MADM AUC value below the classification criterion. Test sensitivity was 93% (CI95 66%-100%) and specificity was 100% (CI95 73%-100%).

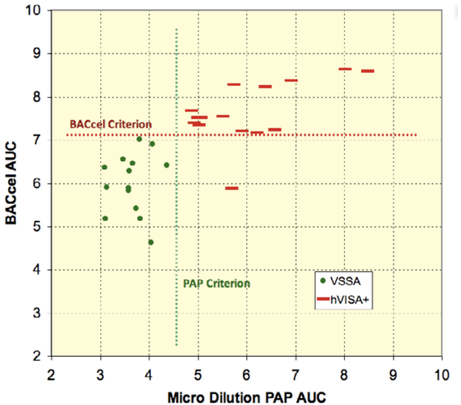

Figure 3. *Scatter plot of MADM (BACcel) AUC for abbreviated PAP vs. BMD-PAP-AUC, arbitrary units for areas. Normalized data. Horizontal dotted line shows the BACcel criterion AUC level, and vertical dotted line shows BMD-PAP AUC detection criterion.*

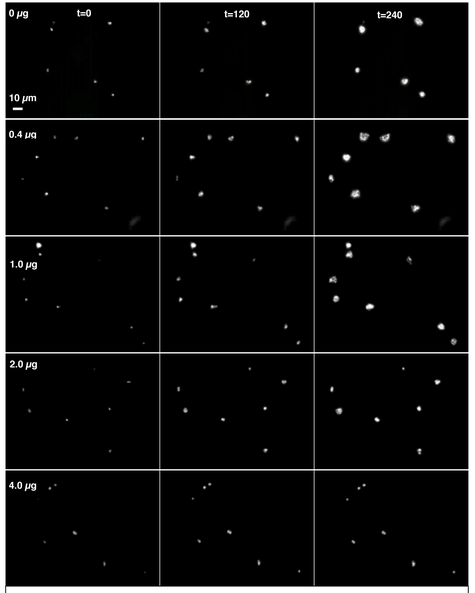

Figure 4. *hVISA strain 2-9B, images at 0, 120, and 240 minutes (horizontal labels and columns) in different VAN concentrations (µg/mL, rows). Zoomed to visualize clone images. Scale bar in upper left image.*

Zoomed images(Fig. 4) show an example for one hVISA+ strain at several VAN concentrations used to derive the abbreviated MADM PAP. Increase in individual clone mass appears as brightening and increase in the clone's 2-dimensional footprint area. Integrated pixel intensity enables computation of growth rates over time using a series of time lapse images acquired at 10-minute intervals. A count of 4-hours clines that exhibit at least 4-fold growth, divided by the initial count of growing clones in the same fields of view, yields normalized PAPs.

DISCUSSION

Growth analysis by MADM revealed an identification criterion for using abbreviated PAPs of individual clones growing in the presence of different VAN concentrations to identify non-susceptible *S. aureus* subpopulations in isolates obtained from various sources. The comparator method used an analogous PAP with broth cultures and the generally accepted classification criterion against a stable reference strain (Mu3).

MADM PAPs for positive strains had down-sloping characteristics of heteroresistance as did the BMD-PAPs. This study identified a narrow range of VAN concentrations to use for expanded studies, enabling efficient and rapid automation. At its present state, MADM appears applicable for use with clinical isolates to identify hVISA+ within 4 hours. This enables replication with larger screening studies to help estimate phenotype prevalence as well as characterizing statistical performance.

The small number of cells required is also compatible with the number available from lower respiratory tract specimens at the diagnostic threshold. Additional research with polymicrobial specimens will determine potential for inclusion in a practical rapid diagnostic system.

CONCLUSIONS

Multiplexed automated digital microscopy (MADM) identified hVISA+ isolates in 4 hours with 93% sensitivity and 100% specificity in a collection of 29 isolates characterized by a broth microdilution methods for population analysis profiling.

References

1. Metzger S., *et al.* 2007. Direct Detection and Enumeration of Viable Bacteria in Human Bronchoalveolar Lavage Specimens Using Automated Growth Rate Analysis. 108th ASM General Meeting, Poster C-145.