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Direct-From-Remnant-Specimen Quantitative Identification Using Automated Microscopy

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AMENDED ABSTRACT

Background: Conventional specimen quantitation and identification are slow and labor-intensive, typically requiring 2-3 days. We report the results of a novel automated microscopy method to analyze live bacteria extracted directly from a specimen in 150 minutes.

Methods: Isolates used for identification assay development ("training set") included 49 *Staphylococcus aureus* (STAU) and 101 *Pseudomonas aeruginosa* (PSAE) as targets. It included 313 different isolates from 45 relevant species as non-targets. Isolates were spun and resuspended in a buffer to yield 1E+06 CFU/mL. 20 μL inocula were pipetted into each flowcell channel of a multichannel fluidic cassette. An electrical field concentrated bacteria to a surface of the flowcell channels where a capture coating immobilized the bacteria. An automated microscope acquired images of the immobilized bacteria every 10 minutes. Fluorescent antibody labeling was followed by growth measurement for 90 minutes. Identification inputs included bacteria growth rate, morphology, and fluorescence intensity. Counts were compared to quantitative plates. 31 remnant mini-BAL specimens were then used to challenge the assay method. The analyzer counted organisms and calculated the sample concentration of each target and non-target.

Results: Isolate sensitivity/specificity for STAU and PSAE were 95%/98% and 85%/85% respectively. The mean difference between the automated microscopy method and quantitative plates was -0.02 log CFU/mL (R^2 = 0.82). 10 mini-BAL specimens were positive for bacteria above 1E+04 CFU/mL. 3 specimens contained STAU and 3 contained PSAE. Microscopy correctly analyzed all specimens except missing 1 of 2 *H. influenzae* (non-target). The range of specimen concentration for the 6 targets was 1.9E+04 to 1.25E+06 CFU/mL. All testing was complete in 150 minutes, including specimen preparation.

Conclusions: Automated analysis of live immobilized bacteria using automated microscopy has the potential to reliably quantify and identify organisms directly from lower respiratory tract specimen in as little as 150 minutes.

INTRODUCTION

Nosocomial infections due to multiple drug resistant (MDR) bacteria are increasing in frequency and growing in complexity. For critically ill patients, resistance can render initial therapy ineffective, delaying the start of effective antimicrobial therapy. Standard diagnostic cultures require 2-3 days to identify MDR-prone organisms and characterize antimicrobial resistance. In contrast, automated microscopy (AM) has the potential to reduce turnaround time by rapidly analyzing bacteria extracted directly from a clinical specimen. The purpose of our study was to determine AM sensitivity, specificity, and speed for identifying individual live organisms extracted directly from specimens, and to assess quantitation accuracy in measuring specimen concentrations of each targeted organism using culture methods as the comparators.

Custom AM systems used commercial microscopes and accessories, plus custom image analysis and control software. 32-channel disposable cassettes (Fig. 1) enabled live microbial cell immobilization for microscopy and fluid exchanges for different test agents in Mueller-Hinton broth by pipetting.[1]

MATERIALS & METHODS

Figure 1a: *32-channel flowcell cassette.*

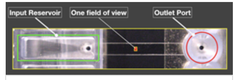

Figure 1b: *One flowcell channel.*

Antibody development used 49 STAU isolates and 101 PSAE isolates to produce chicken IgY antibodies against target species. 313 non-target isolates (including 45 clinically relevant species) provided specificity selection. In addition to antibody tagging, complete identification algorithms included scoring variables derived for cell morphology, clone growth morphology, clonal growth rate, and growth at normal (35º C) and elevated temperature (42º C).

Extraction of live bacteria consisted of sample cleanup (Fig. 2) followed by introduction of the sample into the cassette's fluidic channels. A 5-minute low-voltage electrical field concentrated bacteria to the lower surface of each flowcell where a capture coating immobilized the bacterial cells. Following a brief wash, antibodies were introduced into the flowcells and the system acquired images every 10 minutes for 90 minutes using 10 fields of view in each flowcell channel. Images included initial epifluorescence for antibody detection and dark field for all other analyses.

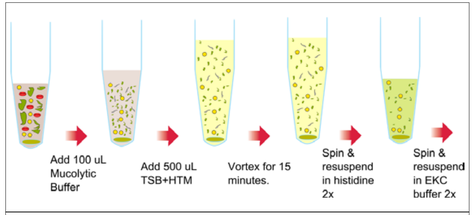

Figure 2: *Specimen cleanup.*

The system applied the identification algorithms to each individual immobilized cell that exhibited growth and measured growth rates of each progeny clone for the 90-minute duration. Identification consisted of computing probability scores for clone morphology, growth rates, and antibody labeling, then classifying as STAU, PSAE, or non-target. Quantitation consisted of counting identified clones and computing original specimen CFU/mL.

31 remnant mini-BAL specimens from ICU patients were tested using the same methods and procedures developed with isolates. An Institutional Review Board approved the study.

RESULTS

Assay development with isolates achieved sensitivity/specificity for STAU and PSAE of 95%/98% and 85%/85% respectively. The mean concentration difference between microscopy and culturing was -0.02 ±0.79 (s.d.) log CFU/mL (R^2 0.82). The lower limit of quantitation was less than 1E+04 CFU/mL.

Average age of remnant specimens was 14 days. Cleanup removed substantial debris, enabling high recovery and clone tracking.

Using the standard diagnostic threshold of 1E+04 CFU/mL for mini-BAL, 10 of 31 remnant clinical specimens were diagnostically positive. The 10 culture-positives included 3 STAU, 3 PSAE, and 4 non-targets. The microscopy system correctly identified all target-positive specimens, and 3 of 4 non-targets as quantitative positives. The system missed 1 of 2 *H. influenzae*, using Mueller-Hinton broth (MHB). For the 6 target-positive specimens, the concentrations ranged from 1.9E+04 to 1.25E+06 CFU/mL.

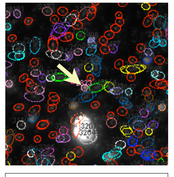

Figure 3: *Computer-imposed colored ellipses indicate potential organisms (pixel blobs) tracked during analysis. Entities that grow are classified as microbial cells (arrow).*

The system reliably tracked growing progeny clones against complex background (Fig. 3) and imposed a filter to ignore non-growing microbe-like pixel blobs. A single microscopy field of view typically contained 1 to 100 growing clones (10-1,000/channel). Growth sufficient for analysis occurred in 30-60 minutes. STAU appeared as relatively bright cocci in dark field (Fig 4a.), some of which grew more slowly than the majority. Mean growth rate for STAU in MHB was 1.8 div/hr. PSAE appeared as dim, under-sized rods (Fig. 4b) relative to *Enterobacteriaceae* in dark field. PSAE exhibited a mean growth rate of about 1 div/hr in MHB (Fig. 5). Progenitor cells extracted directly from mini-BAL specimens exhibited essentially no lag time after immobilization. Fluorescent antibody labeling augmented identification of individual progenitor cells (Fig. 6).

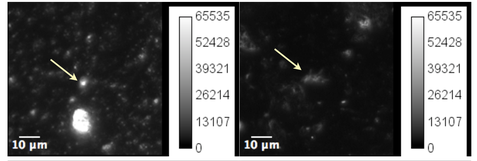

Figure 4a: *STAU, fixed grayscale, single progenitor cell. Dark field.*

Figure 4b: *PSAE, same fixed grayscale, twinned progenitor cells. Dark field.*

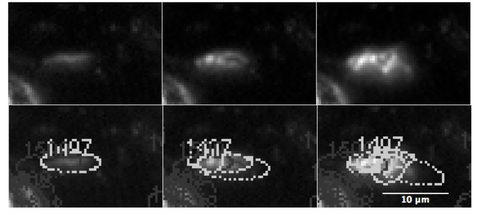

Figure 5: Upper *– PSAE growth (contrast enhanced) at 0, 40, and 90 minutes.* ***Lower*** *– same, with computer-identified cells (ellipses) within one progeny clone. Image numbering for the individual putative organism or clone being tracked. Dark field images.*

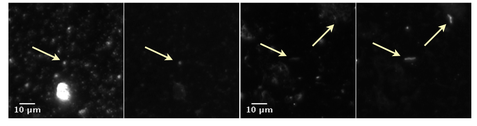

Figure 6a: *STAU, fixed grayscale, single progenitor cell.* ***Left*** *– dark field.* ***Right*** *– fluorescent antibody label.*

Figure 6b: *PSAE, same fixed grayscale, twinned progenitor cells.* ***Left*** *– dark field.* ***Right*** *– fluorescent antibody label.*

The majority of the 60-minute sample preparation time was consumed by vortexing and centrifugation.

DISCUSSION

Automated microscopy enabled successful multiplexed testing to identify and count live bacterial cells concurrently extracted directly from mini-BAL clinical specimens in less than 2½ hours. The small number of cells required is compatible with the bacterial concentration at a BAL and mini-BAL diagnostic threshold of 1E+04 CFU/mL. Diagnostic and quantitative accuracy using cell-by-cell identification enables rapid analysis of polymicrobial specimens without first requiring physical isolation of different species. Sophisticated image analysis allowed prolonged tracking and growth measurement of individual clones as well as detailed morphology analysis. The combination of detailed analyses of cell morphology, growth morphology and growth rate measurements yielded relatively accurate identification of major pathogens. Supplementation with antibody labeling further improves identification without having to rely on antibody performance alone. Immobilization permitted initial identification while avoiding the need to repeat identification as clones grow. This strategy further enables continued analysis of individual clones for drug resistance phenotyping[2,3] with each subpopulation of identified organisms.

Fluid exchanges, image acquisition, and image analysis can be fully integrated and automated as a single instrument.

References

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2. Cooper S., *et al.* 2008. Potential Impact of Rapid Phenotype Identification on Antimicrobial Prescribing. 48th ICAAC. Poster D-4013.
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