C-1140

Rapid Quantitation and Identification of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Acinetobacter baumannii* In Bronchoalveolar Lavage Fluid

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AMENDED ABSTRACT

Background: *P. aeruginosa*, *S. aureus*, and *A. baumannii* together account for approximately half of pathogenic bacterial strains isolated from patients with nosocomial pneumonia. We report a new method to count and identify these pathogens within 2½ hours from BAL fluid using automated microscopy.

Methods: We used 20 isolates for each of the target species plus 20 additional non-target isolates. We spiked remnant BAL specimens with bacteria in concentrations from 10^4 to 10^7 CFU/mL. We used mucolytic treatment and centrifugation to extract live bacteria from the spiked specimens. We tested 5 additional un-spiked remnant BAL specimens the same way. After introducing sample aliquots into each flowcell, low-voltage electrophoresis concentrated bacteria onto a poly-L-lysine coating on lower flowcell surfaces. Affinity-purified chicken IgY antibodies, followed by a fluorescent secondary antibody, stained immobilized bacteria. The system acquired flowcell images every 10 minutes. Image analysis software computed an identification score using morphologic data and immunostaining results. The software counted each growing clone, and computed the original specimen concentration for each target organism. We compared results with those from standard culturing techniques.

Results: Tests on spiked samples had a sensitivity/specificity for *P. aeruginosa* 95%/96%, *S. aureus* 95%/100%, and *A. baumannii* 90%/93%. The system correctly identified target organisms in 2 un-spiked remnant BAL that contained them. The remaining un-spiked remnant specimens were microbiologically negative. Quantitation was within ±½ log of cultures. Sample preparation required 45 min. Remaining procedures required ~100 min.

Conclusions: Automated microscopy successfully identified and quantified three of the most common pneumonic nosocomial pathogens directly from BAL specimens in less than 2½ hours.

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. But standard diagnostic cultures require 2-3 days to identify MDR-prone organisms and characterize antimicrobial resistance. In contrast, multiplexed automated digital microscopy (MADM) 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 the sensitivity, specificity, and speed of a MADM system for identifying individual live organisms extracted directly from specimens, and to quantify specimen concentrations of each targeted organism. The analytical methods adapted well-accepted standard test principles, including immunoassay, selective media, growth measurement, and morphology assessment.

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 tested organism recovery, quantitation, and identification from BAL specimens by pooling de-identified remnant bronchoscopic bronchoalveolar lavage specimens (BAL) from ICU patients and spiking aliquots with culture broths that contained cultured isolates. Tested aliquots contained between 10^4 and 10^7 CFU/mL determined by dilution of 0.5 McFarland broths. Isolates included 20 each of *Pseudomonas aeruginosa* (PA), *Acinetobacter baumannii* (AB), and *Staphylococcus aureus* (SA), plus 20 isolates of non-target pathogens commonly found in lower respiratory tract specimens, including fastidious organisms. We also analyzed 5 individual original specimens (not spiked, not pooled). Controls used standard culturing methods.

Brief specimen preparation to release bacteria and reduce imaging background consisted of mucolytic treatment in 30 mg/mL Na-citrate and 0.5 mg/mL saponin, centrifugation, wash, and resuspension of the bacterial pellet in low ionic strength buffer. 20 µL samples were pipetted into independent flowcell channels (Fig. 2) and a low-voltage electrical field was applied for 5 minutes. The electrical field concentrated bacteria onto a poly-L-lysine film that immobilized the bacteria on the lower flowcell surface.

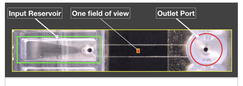

Figure 2: *One flowcell channel.*

A wash and room temperature incubation with custom chicken IgY antibodies, followed by a tagged goat monoclonal anti-chicken antibody labeled individual cognate target cells for epifluorescence microscopy. Each flowcell channel received only one target-specific antibody.

After displacing the solution in each channel with appropriate test reagents in nutrient media the cassette was placed into the instrument. Each flowcell channel received only one type of test reagent solution that contained a specific antibody, and a selective agent if required (AB channels only, sulbactam 32 µg/mL). Each tested sample was split into four 20 µL aliquots for introduction into two separate flowcells in each of two separate cassettes. One cassette was run at 35ºC (AB tests) and the other at 42ºC (SA and PA tests).

The instrument acquired images at 10-minutes intervals for 90 minutes, using three fields of view in each flowcell channel through a 20x objective. Imaging used darkfield illumination except for an initial epifluorescence antibody labeling image. Identification variables included antibody labeling, response to selective agents (AB with sulbactam), cell morphology, growth rate, and growth at elevated temperature (42º).

The identification algorithms applied to each individual immobilized bacterial cell. The system measured the amount of change in mass over time to compute growth rates. Identification consisted of computing and combining probability scores for clone morphology, growth rates, and antibody labeling to produce receiver operating characteristic (ROC) curves.

RESULTS

Quantitation by microscopy achieved ±½ $\log_{10}$ accuracy, which is comparable to that of quantitative culture plates. Frequencies for clonal counts had a flatter distribution than culture plates because of the variation in smaller numbers of clones used with microscopy (Fig. 3).

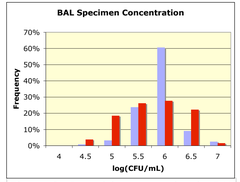

Figure 3: *Bacterial concentration in BAL specimen.* ***Blue:*** *by culture plate colony counts.* ***Red:*** *by microscopy clone counts.*

A field of view typically contained 10 to 30 growing clones.

Dark field illumination revealed specimen matrix residues with a broad range of size and morphology. The system distinguished live organisms by requiring measurable growth as well as morphologic criteria.

Static cell morphology scoring included shape, size, and brightness profiles. Growth analysis included clonal growth morphology and growth rate time profile. Antibody labeling scores used criteria for brightness relative to background.

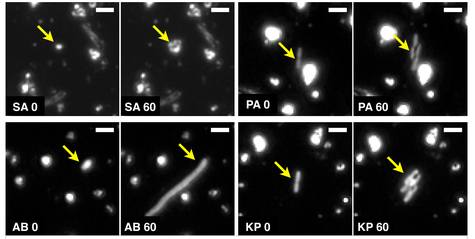

Figure 4. *Darkfield images, scale bars 5 µm. Time 0 and 60 minutes. AB in 32 µg/mL sulbactam. KP is Klebsiella pneumoniae (non-target). Non-growing pixel blobs are debris.*

Images of AB in sulbactam (Fig. 4) show an example of characteristic filamenting growth morphology that improves test specificity. Sulbactam susceptible and resistant AB strains grew with similar morphology. Susceptible strains arrested and dimmed markedly after about 60 minutes of sulbactam exposure.

PA had substantially lower darkfield intensity than almost all other organisms observed. SA grew significantly faster than most coagulase negative *Staphylococci*. AB had a distinctive clonal growth shape profile. Variables such as these augmented identification accuracy.

Using the complete identification scoring algorithms, sensitivity and specificity were, respectively, 95%/100% for SA, 95%/96% for PA, and 90%/93% for AB. The ROC curves (Fig. 5) show how the morphology and antibody scores combined to improve total identification accuracy.

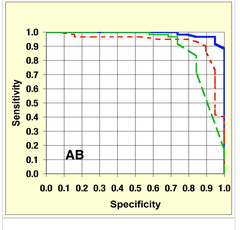

Figure 5. *ROC curves for AB identification.* ***Dashed green:*** *Antibody labeling score.* ***Dotted red:*** *morphology score.* ***Solid blue:*** *combined scores, overall identification score.*

Of the five original BAL specimens examined separately, two were positive for PA (above diagnostic threshold of 10^4 CFU/mL). Other specimens were microbiologically negative. The microscopy results for positive and negative specimens matched those from standard culture methods.

DISCUSSION

Growth sufficient for analysis occurred in 30-60 minutes. Labeling required less than 45 minutes using primary and secondary antibodies for a total analysis time of less than 2 hours. A tagged primary antibody and significantly less label incubation time would be practical. As performed in this study, sample preparation time was about 45 minutes. The preparation methods have, however, been steadily reduced during ongoing development, and are now approximately 30 minutes.

The small number of cells required is compatible with the bacterial concentration at BAL diagnostic threshold of 10^4 CFU/mL. Diagnostic accuracy using cell-by-cell identification is consistent with requirements for analysis of polymicrobial specimens. Additional research with polymicrobial specimens will determine potential for inclusion in a practical rapid diagnostic system.

CONCLUSIONS

Multiplexed automated digital microscopy (MADM) identified and quantified live immobilized bacteria extracted using simple preparation methods for bronchoalveolar lavage fluid. Quantitation range and identification accuracy appear compatible with diagnostic requirements with total analysis time of 2½ hours or less after starting with a specimen. All procedures after sample preparation are amenable to automation.

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.