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# Rapid simultaneous identification and quantitation of *Staphylococcus aureus* and *Pseudomonas aeruginosa* directly from bronchoalveolar lavage specimens using automated microscopy [☆],[☆☆]

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## ABSTRACT

Diagnosis of ventilator-assisted pneumonia (VAP) requires pathogen quantitation of respiratory samples. Current quantitative culture methods require overnight growth, and pathogen identification requires an additional step. Automated microscopy can perform rapid simultaneous identification and quantitation of live, surface-immobilized bacteria extracted directly from patient specimens using image data collected over 3 h. Automated microscopy was compared to 1 μL loop culture and standard identification methods for *Staphylococcus aureus* and *Pseudomonas* spp. in 53 remnant bronchoalveolar lavage specimens. Microscopy identified 9/9 *S. aureus* and 7/7 *P. aeruginosa* in all specimens with content above the VAP diagnostic threshold. Concordance for specimens containing targets above the diagnostic threshold was 13/16, with concordance for sub-diagnostic content of 86/90. Results demonstrated that automated microscopy had higher precision than 1 μL loop culture (range ~0.55 log versus ≥1 log), with a dynamic range of ~4 logs (~$10^3$ to $10^6$ CFU/mL).

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## 1. Introduction

Ventilator-associated pneumonia (VAP) is the leading cause of death due to nosocomial infection (Klevens et al., 2007; Kollef, 2005) and can lead to prolonged hospitalization and increased healthcare costs (Kollef et al., 2012; Restrepo et al., 2010). Without microbiological support, clinical diagnosis of suspected VAP has limited accuracy. Distal sampling of pulmonary regions, typically using bronchoalveolar lavage (BAL), combined with quantitative culture and organism identification helps to distinguish true infection from adventitious sampling of upper airway colonizers (Chastre et al., 2010; Jourdain et al., 1997).

Routine clinical quantitative culture methods, however, are most often semi-quantitative and have high variation. They also require at least overnight growth plus additional analysis to identify potential pathogens. Each hour of delay in starting microbiologically appropriate therapy with critically ill patients increases the patient's risk of severe morbidity and mortality (Iregui et al., 2002; Luna et al., 2006). Treatment guidelines (Muscedere et al., 2008), therefore, advise prompt (1–3 h post-diagnosis) initiation of empiric combination broad-spectrum antibiotics pending microbiology results. Physicians must proceed informed only by epidemiological history and patient assessment (Micek et al., 2006). Empiric combinations are recommended because of extensive spread of multiple drug resistance, but these empiric regimens prove inappropriate in as many as 40% of cases (Kaye et al., 2008).

Quantitative identification provides 2 types of actionable information: first, the probability that the patient actually has an infection and, second, whether the likely etiologic organism belongs to a genus or group known to potentially express significant antibiotic resistance. The clinical microbiology laboratory therefore urgently needs much more rapid, precise quantitative pathogen identification to guide selection of appropriate therapy.

Although a number of novel rapid diagnostic commercial products have appeared and more are in late-stage development (Cuzon et al., 2013), none as yet work directly with lower respiratory specimens to

identify organisms and their viable specimen density (CFU/mL). We propose an innovative approach using automated microscopy to simultaneously identify and quantify surface-immobilized live bacterial pathogens extracted directly from patient specimens. By eliminating prior enrichment growth and colony isolation, this strategy has the potential to provide actionable information during the critical 1–3 h time window to improve selection of appropriate antibiotics for initial therapy. This study extends earlier experimental work for assay development that used isolates and remnant BAL specimens that were not controlled for specimen age (Metzger et al., 2010).

The first purpose of this study was to characterize the quantitative precision and dynamic range of automated microscopy, including testing with polymicrobial isolate mixtures. The second purpose was to simultaneously identify and quantify *Staphylococcus aureus* and *Pseudomonas aeruginosa* in BAL remnant samples. The third purpose was to estimate potential turnaround time. To the best of our knowledge, this is the first reported study to perform simultaneous identification and quantitation of live pathogens extracted directly from lower respiratory tract specimens.

## 2. Materials and methods

### 2.1. Bacterial isolates

*S. aureus*, *P. aeruginosa*, and *Klebsiella pneumoniae* isolates were obtained from the American Type Culture Collection (ATCC) or JMI Laboratories (North Liberty, IA, USA).

### 2.2. Bacterial immobilization

Automated microscopy tests were performed in disposable multi-channel fluidic cassettes containing 32 independent fluidic channels, each with its own inlet and outlet ports for fluid exchange by pipetting (Fig. 1). Each transparent fluidic channel was approximately 300 μm thick, and a coating of indium tin oxide on the top and bottom inside surfaces of the channel served as electrodes. To prepare an inoculum, bacteria were suspended in electrokinetic buffer containing 10 mmol/L L-DOPA and 1 mmol/L L-histidine at pH 7.0 (reagents used as received from Sigma-Aldrich, St Louis, MO, USA), and 20–30 μL of inoculum was pipetted into each independent fluidic channel. Bacteria were negatively charged in the electrokinetic buffer (data not shown). A 5-min 1.5 V electrical field caused the bacteria to migrate to the lower surface of each fluidic channel where they were immobilized on an additional poly-cationic poly-L-lysine coating (Sigma-Aldrich). After the electric field was stopped, cells remained adherent to the poly-L-lysine coating, allowing the operator to pipette test solutions through each fluidic channel and replace the electrokinetic buffer without detaching the cells.

### 2.3. Automated microscopy

Immobilized bacteria were viewed using a custom microscopy instrument (Accelerate Diagnostics Inc., Tucson, AZ, USA) that consisted of an assembly with an inverted Olympus IX-71 dark-field microscope (Olympus America, Inc., Center Valley, PA, USA) adapted with commercially available accessories. A 12-bit monochrome MicroFire camera (Olympus) captured time-lapse images in each fluidic channel at 10-min intervals over the testing 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 including autofocus and cassette scanning. A heated enclosure maintained the instrument at $35 \pm 2\ °C$.

### 2.4. Image analysis

Time-lapse 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 the 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 and extract time-based morphologic features and measure relative mass based on pixel intensity. To reduce background interference from passive debris, analysis only included image entities ("pixel blobs") that exhibited incremental changes in mass over the imaging period.

### 2.5. Target organism presumptive identification and quantitation

Separate *S. aureus* and *P. aeruginosa* algorithms converted data from each series of clone images into an identification probability
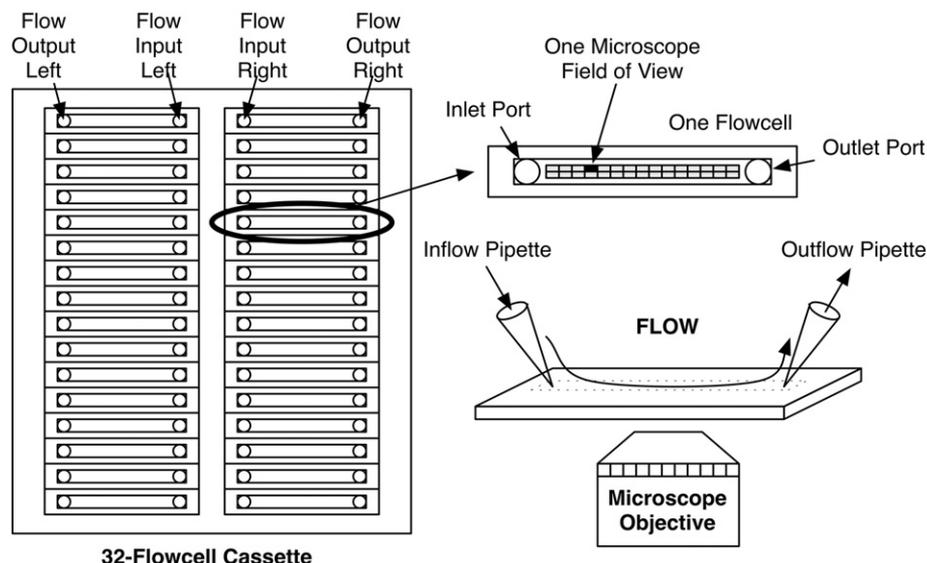


**Fig. 1.** Diagram of 32-channel cassette showing detail of individual fluidic channel and microscope fields of view. Fluid exchange occurs using pipette inflow and outflow ports, and image capture is performed by an inverted microscope positioned beneath the bottom surface of the fluidic channel.

score for each target organism based on variables derived for relative mass, shape, geometric growth pattern, clone surface features, and growth rate over time. Scores that exceeded an empirically established threshold ($\geq 1.0$) resulted in presumptive identification of a target pathogen. For quantitation, the total clone count for each identified target organism was divided by the number of fields of view and the volume for a microscope field of view to derive the original inoculum concentration in growing clones per milliliter (GC/mL). For comparison purposes, growing clones were considered equivalent to colony-forming units in conventional culturing. Calculations also adjusted for sample volume dilution or concentration that occurred during inoculum preparation.

### 2.6. Dynamic range study

*S. aureus* (ATCC-29213) and *P. aeruginosa* (ATCC-27853) isolates were subcultured on sheep's blood agar (Becton Dickinson, Sparks, MD, USA) overnight. Ten to twenty well-isolated colonies selected from a fresh overnight subculture plate were suspended by vortexing in tryptic soy broth (Becton Dickinson) and incubated for 2 h at $35 \pm 2$ °C to achieve log phase growth. The bacterial suspension was centrifuged ($12,000 \times g$ for 4 min) and resuspended in 1 mmol/L L-histidine buffer at pH 7.2 to create an inoculum of $1 \times 10^8$ CFU/mL. This solution was combined with electrokinetic buffer to create 8 serial dilutions ranging from approximately $10^2$ to $10^6$ CFU/mL. Each dilution was plated from low to high concentration using quantitative culturing. Each suspension (30 μL) was loaded into a separate automated microscopy system fluidic channel, and bacteria were immobilized using electrokinetic concentration. Additional suspension (30 μL) was then added to each fluidic channel followed by electrokinetic concentration 4 additional times for each fluidic channel, such that immobilization cycles were performed in each channel a total of 5 times. Automated microscopy was performed on each fluidic channel using 3–10 fields of view depending on the inoculum concentration. Three fields of view were used for the 3 highest concentrations, 6 fields of view were used for the middle concentration, and 10 fields of view were used for the 4 lowest concentrations. Images were collected every 10 min over a 90 min test period.

### 2.7. Polymicrobial and precision study

*S. aureus* (JMI-309) and *K. pneumoniae* (ATCC-49472) isolates were removed from overnight blood agar plates (Becton Dickinson) using a sterile swab and resuspended in a solution of 1 mmol/L L-histidine at pH 7.2. Bacteria were centrifuged and washed in additional solution, ending with resuspension in electrokinetic buffer to create an inoculum of approximately $5.0 \times 10^4$ CFU/mL containing *S. aureus* alone or a mixture of *S. aureus* and *K. pneumoniae*. Each inoculum (pure or mix) was tested a total of 25 times using automated microscopy and 1 μL loop culture. Automated microscopy used image data from 37 fields of view collected over a 3-h test period for analysis.

### 2.8. BAL specimens

After institutional review board approval, a single-blinded study used 53 fiber-optic de-identified remnant BAL specimens obtained and run in Barnes-Jewish Hospital, a 1250-bed tertiary care hospital affiliated with the Washington University School of Medicine. Specimens were collected from intensive care unit patients from July 2010 to March 2011 and tested on-site within 9 days of collection. Twenty-six specimens were tested within 24 h of collection. Specimens were selected for *S. aureus*, *P. aeruginosa*, or non-target bacterial content and blinded to personnel who supervised automated image analysis.

### 2.9. BAL remnant study

0.5 mL aliquots of BAL specimens were centrifuged at $12,000 \times g$ for 4 min at room temperature to produce a bacterial pellet. Resuspension in Mueller-Hinton broth (Becton Dickinson) supplemented with *Haemophilus* Test Medium (Becton Dickinson) produced a suspension that was homogenized by pipette shearing and horizontal vortexing for 20 min at 35 °C. The bacteria were washed twice using centrifugation and resuspension with 1 mmol/L L-histidine buffer followed by an additional 2 centrifugation and resuspension cycles using electrokinetic buffer. The final resuspension provided the test inocula. Inocula were loaded into 3 fluidic channels, and automated microscopy was performed on each using 40 fields of view per fluidic channel, for a total of 120 fields of view per sample. Images were taken every 10 min over a 3-h test period. Original unprocessed BAL specimen aliquots were used for a 1 μL semi-quantitative loop culture method previously verified in the Barnes-Jewish clinical microbiology laboratory (data not shown) and paired with standard organism identification methods as the comparator. Performance assessment of the microscopy method used the generally accepted diagnostic threshold of $\geq 1 \times 10^4$ CFU/mL per target species for BAL specimens (ATS/IDSA, 2005).

### 2.10. Statistical methods

The Pearson correlation coefficient was calculated to assess correlation between automated microscopy and quantitative culture. Measurements for quantitative precision were presented in quartile box plots. Significance for differences between quantitation methods was tested with the Mann–Whitney U test at the $P \leq 0.05$ level. Descriptive statistics included SD and coefficients of variation. Positive and negative agreement for quantitation and identification above the VAP diagnostic threshold of $1 \times 10^4$ CFU/mL for BAL specimens was calculated to assess the performance of automated microscopy compared to quantitation by 1 μL loop culture and identification by standard methods for the BAL remnant study.

## 3. Results

### 3.1. Dynamic range of automated microscopy and correlation with quantitative culture

Quantitation by automated microscopy compared to quantitative culture had a Pearson correlation coefficient of $\rho > 0.99$ for both *S. aureus* and *P. aeruginosa* (Fig. 2). Total target clone counts ranged from 1 to 2805, resulting in a dynamic range of approximately $10^3$ to $10^6$ GC/mL (4 logs) for the automated microscopy method. Results were obtained from image data collected in 90 min.

### 3.2. Precision with polymicrobial samples

Box plots showing the quartile distributions for replicate *S. aureus* counts using the *S. aureus* isolate alone or mixed with *K. pneumoniae* are shown in Fig. 3. Differences between pure and mixed conditions were non-significant ($P > 0.05$), and differences between medians for loop and microscopy methods were non-significant ($P > 0.05$). Microscopy variation had an average range of 0.53 logs, and loop culture variation had an average range of 1.05 logs. The SDs for 1 μL loop culture were $\pm 0.32$ and $\pm 0.48$ logs for pure and mixed conditions, respectively, compared to $\pm 0.19$ logs for both conditions for microscopy. 1 μL loop culture had a coefficient of variation of 7% for pure and 10% for mixed conditions, compared to 4% for both conditions for microscopy. Results were obtained from image data collected in 3 h.
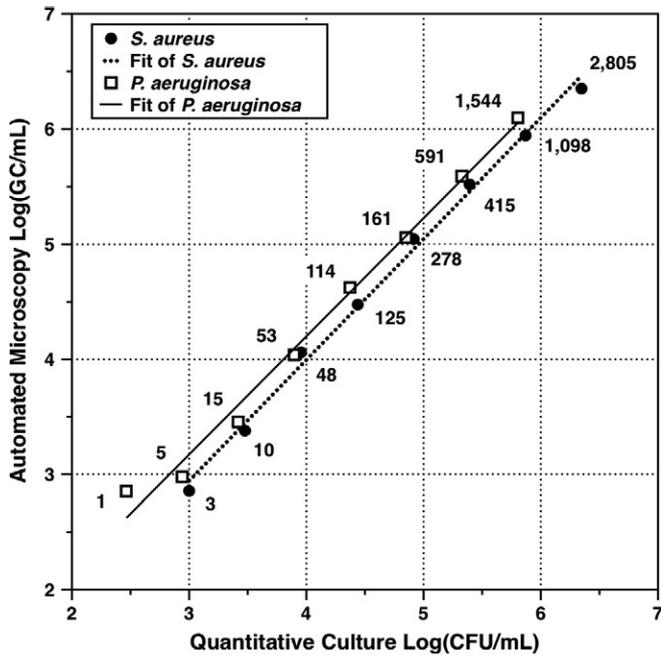
**Fig. 2.** Linearity and dynamic range of automated microscopy method compared to quantitative culture for *S. aureus* (ATCC-29213) and *P. aeruginosa* (ATCC-27853) isolates. Clone counts used to calculate microscopy values are displayed by each point. Microscopy showed high correlation ($\rho > 0.99$) for both species compared to quantitative culture, and a dynamic range of up to 4 logs contrasted with 1 log for quantitative culture (4 different inoculum dilutions were plated to achieve quantitative culture results). Growing clones for microscopy results are considered equivalent for comparative purposes to colony-forming units for culture results.

### 3.3. BAL remnant study results

Fig. 4 shows organism growth for target and non-target species over 3 h to exemplify microscopy images used for time-lapse analysis.

Diagnostic-positive specimens had target organism content equal to or above the BAL diagnostic threshold of $1 \times 10^4$ CFU/mL. Presumptive identification scores ranged from 0 to 1.8 in this study. Automated microscopy presumptively identified target organisms in 9/9 *S. aureus* and 7/7 *P. aeruginosa* diagnostic-positive specimens, resulting in 100% agreement (Table 1A). Microscopy incorrectly reported 3 samples below diagnostic threshold and 4

samples above diagnostic threshold. Automated microscopy agreement with 1 μL loop culture in 106 test runs was 13/16 for diagnostic-positive specimens and 86/90 for diagnostic-negative specimens (Table 1B). Table 2 summarizes discordant results. Automated microscopy detected between 13 and 6990 target clones per specimen, resulting in computed specimen quantitation values ranging from $2 \times 10^3$ to $1 \times 10^6$ GC/mL. Results were obtained from image data acquired in 3 h.

## 4. Discussion

This study demonstrated the ability of the automated microscopy method to simultaneously identify and quantify immobilized live cells of *S. aureus* and *P. aeruginosa* extracted directly from remnant BAL specimens. Time from the start of BAL specimen manual preparation to acquisition of the final time-lapse images was less than 4 h. The dynamic range isolate study, however, demonstrated that 90 min of organism growth was sufficient for analysis. The latter finding suggests potential for the method to achieve results within the critical 1–3 h time frame for initiating antimicrobial therapy. In contrast, current quantitation methods require overnight culture for quantitation and separate identification of selected colonies using a biochemical assay or an automated instrument.

Culture plating techniques rely upon manual counting of approximately 25–300 colonies per plate for reliable quantitation and a microbiologist to manually select different species for identification based on colony appearance or other features. Automated microscopy, in contrast, uses an algorithm to presumptively identify each growing clone as a target species or non-target and performs automated counts over a dynamic range of up to 4 logs. While in some cases only 1 growing clone was used for detection, up to 6990 clones were automatically counted for a single specimen sample in the BAL remnant study. $\rho > 0.99$ in Fig. 2 indicates equivalence between growing clones and colony-forming units used in conventional culturing.

In the polymicrobial study, the presence of non-target organisms (*K. pneumoniae*) did not significantly affect quantitation or variability, and the BAL remnant study demonstrated high concordance in the presence of background flora. Automated microscopy identifies and tracks each individual growing clone, separately counts clones for each identified species, and either ignores or combines non-target counts. In the BAL remnant study, some specimen samples with 13 target clones were detected, resulting in a lower limit of quantitation
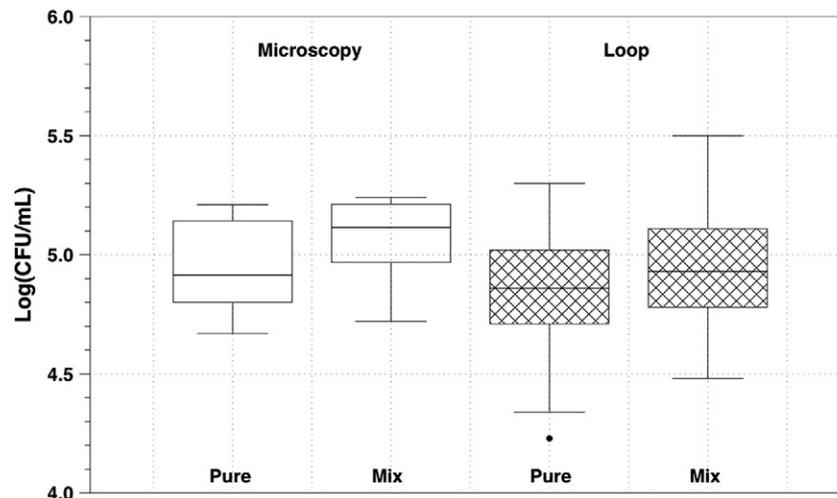


**Fig. 3.** Box plots showing the quartile distributions of the log CFU/mL of 1 μL loop culture (Loop) or log GC/mL for automated microscopy (Microscopy) for replicate counts of *S. aureus* target using only the *S. aureus* isolate (JMI-309) (Pure) or *S. aureus* mixed with *K. pneumoniae* (ATCC-49472) (Mix). Growing clones for microscopy results are considered equivalent for comparative purposes to colony-forming units for culture results.
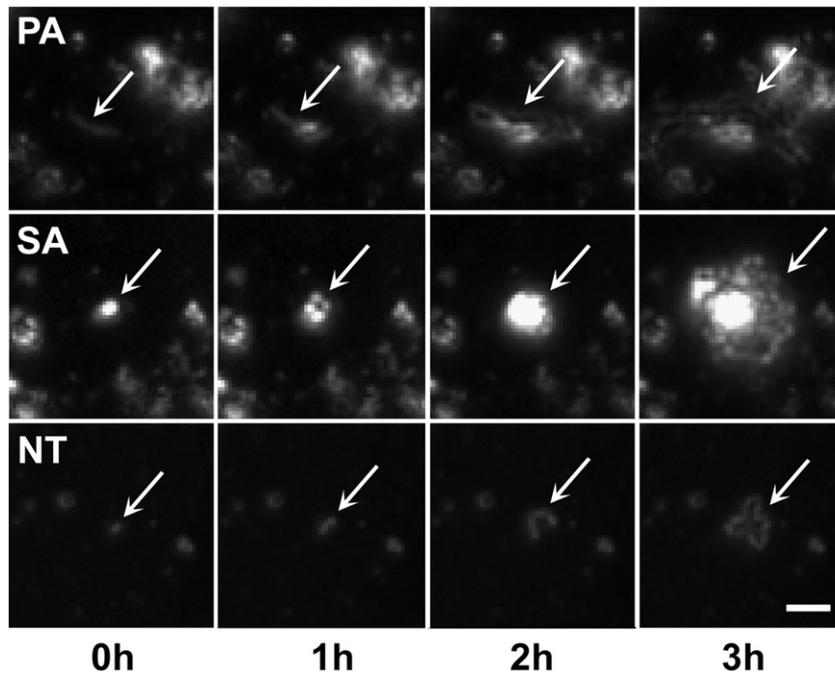
**Fig. 4.** Darkfield images showing morphologies of growing clones (indicated by arrows) over 0–3 h. *P. aeruginosa* from BAL sample 21. *S. aureus* from BAL sample 27. Non-target organism from BAL sample 2. Static pixel blobs are debris and not included in analysis. Scale bar at lower right is 5 μm.

of $1 \times 10^3$ CFU/mL. The ability to analyze polymicrobial samples combined with its low limit of quantitation below the BAL diagnostic threshold allows automated microscopy to be used for direct specimen analysis. The ability to detect target organisms below the diagnostic threshold could also be clinically useful for the detection of early VAP when a patient shows clinical signs of the disease (Flanagan, 1999).

Automated microscopy demonstrated qualitative identification concordance of 100% for BAL remnant samples (n = 16) relative to identification comparators with specimens having target pathogen density above the diagnostic threshold as determined by the quantitative culture comparator. Current commercially available automated identification methods are expected to have accuracies above 90% (Petti et al., 2011). Rapid molecular identification methods cannot differentially quantify viable organisms in most cases and may report marker detection from non-viable bacteria or free marker debris in addition to live pathogenic species (Schrenzel, 2011). Automated microscopy demonstrated presumptive identification using growth rates, cell morphology, and clone growth morphology to distinguish bright cocci growing in clusters (staphylococci) and thin, dim, slowly-growing rod bacilli (*Pseudomonas*) from non-target organisms (Fig. 4).

1 μL loop culture is a common method in clinical microbiology laboratories that exhibits variable results (Augustin and Carlier, 2006; Jacobs et al., 2000; Pfaller et al., 1988; Sutton, 2011). This variability, especially with low colony counts, means that errors near

the diagnostic threshold of $1 \times 10^4$ CFU/mL should be frequent (Altman, 1999). 1 μL of $1 \times 10^4$ CFU/mL inoculum produces an average count of 10 colonies, which is below the reliable quantitation range of 25–300 colonies for quantitative cultures. Colony counts from 1 μL loop cultures may be spread out on either side of the diagnostic threshold, such that borderline quantitative culture results are not recommended to be used alone to make a diagnosis (Baselski and Wunderink, 1994; Jacobs et al., 2000). Sampling techniques can cause variability in the volume sampled by a loop (Jacobs et al., 2000). Specimen heterogeneity may further contribute to variability, such as bacterial cell distribution being biased between solid and liquid fractions. Additional confounding factors include physical properties of BAL fluid such as viscosity and surface tension. In comparison, automated microscopy shows superior precision with a range of ~0.55 log, but with better efficiency and faster time to result. The microscopy method used in this study reduced confounding factors to yield a relatively homogeneous inoculum suspension. Discordant quantitation is expected when comparing independent methods near the diagnostic threshold (CLSI EP05-A2) and is related to counting small numbers of clones or colonies. The low precision of the 1 μL loop culture comparator method could additionally account for some of the discordant results in the BAL comparison. A substantial proportion of the counts for both automated microscopy and 1 μL loop culture were clustered near or above the diagnostic threshold. 3/7 discordant results (BAL specimens 14, 29, and 32) fell within

**Table 1A**
Performance characteristics of automated microscopy compared to 1 μL loop culture for qualitative presence or absence above the BAL VAP diagnostic threshold of $1 \times 10^4$ CFU/mL.

| Target organism | Qualitative identification (presence or absence) | | | |
|---|---|---|---|---|
| | Positive agreement | | Negative agreement | |
| *S. aureus* | 9/9 | 100% | 42/44 | 95% |
| *P. aeruginosa* | 7/7 | 100% | 44/46 | 96% |
| Total: | 16/16 | 100% | 86/90 | 96% |

**Table 1B**
Performance characteristics of automated microscopy compared to 1 μL loop culture for quantitative identification above the BAL VAP diagnostic threshold of $1 \times 10^4$ CFU/mL.

| Target organism | Quantitative identification (above or below $10^4$ CFU/mL threshold) | | | |
|---|---|---|---|---|
| | Positive agreement | | Negative agreement | |
| *S. aureus* | 8/9 | 89% | 42/44 | 95% |
| *P. aeruginosa* | 5/7 | 71% | 44/46 | 96% |
| Total | 13/16 | 81% | 86/90 | 96% |

**Table 2**
Summary of discordant quantitation results between automated microscopy and 1 μL loop culture comparator for BAL remnant study.

| Target | Call | Specimen | Microscopy (GC/mL)[a] | Loop (CFU/mL)[a] |
|---|---|---|---|---|
| S. aureus | False Negative | 32 | $4.5 \times 10^3$ | $1.3 \times 10^4$ |
| | False Positive | 8 | $2.1 \times 10^4$ | $2.0 \times 10^3$ |
| | | 41 | $1.6 \times 10^4$ | $2.0 \times 10^2$ |
| P. aeruginosa | False Negative | 50 | $2.2 \times 10^3$ | $>1 \times 10^4$ |
| | | 14 | $3.9 \times 10^3$ | $2.6 \times 10^4$ |
| | False Positive | 29 | $1.7 \times 10^4$ | $4.0 \times 10^3$ |
| | | 26 | $3.9 \times 10^4$ | 0 |

[a] Growing clones correspond in concept to colony-forming units in conventional culturing.

0.5 log of the diagnostic threshold, which is within the margin of error for both quantitation methods.

One false-positive *P. aeruginosa* (BAL specimen 26) occurred due to a focusing error. This type of technical error would be eliminated with improved autofocus. One false-negative *P. aeruginosa* (BAL specimen 50) occurred in the presence of high levels of debris that obscured 90% of the growing clones and led to an undercount during image analysis. Debris assessment prior to device inoculation or after bacterial immobilization could reject or statistically compensate for this type of interference, as could sample cleanup improvement. Specimen appearance in this study varied widely, from lightly turbid water-like consistency to semi-solid matrix inclusions, blood, and pus. Specimen preparation did, however, adequately reduce background, enabling quantitation of target organisms.

One partial limitation of this study was specimen age. Approximately half of the specimens (26/53) were over 24 h old. Clinical laboratory practice standards (Garcia, 2010) and direct investigation with respiratory specimens indicate organisms' loss of viability with time and temperature. A recent study on respiratory specimen stability (Kneidinger et al., 2013) established a safe limit for BAL of 24 h at 4 °C. Frozen specimens and those stored at room temperature exhibited substantially less viable content at 24 h. The aged specimens in this study did, nevertheless, retain diagnostic levels of viable pathogens and also retained viable background flora. Future studies that only use fresh specimens would assure more accurate performance assessment by eliminating potential target organism viability bias. A second limitation was the relatively small number of specimens analyzed. Background flora and non-target organisms did provide identification specificity, although future studies should include samples containing a larger range of target specimens. A third limitation was the number of target organisms. The feasibility of identifying additional species has been determined, but not as a part of this study (data not shown). Future studies will use fluorescence *in situ* hybridization (FISH) to expand the number of target organisms covered and improve identification specificity.

In conclusion, this study demonstrated that automated microscopy of immobilized live bacteria can perform simultaneous identification and quantitation on clinically relevant densities of *S. aureus* and *P. aeruginosa* directly from BAL remnant specimens in 4 h or less, compared to overnight growth required for 1 μL loop culture. Automated microscopy can analyze polymicrobial samples without prior isolation of different species, thus crossing a critical hurdle for potential direct-from-specimen application. In contrast with molecular methods, the microscopy method counts live organisms, ignoring non-viable bacteria and debris. The ability to perform analysis directly on specimens following a short preparation step suggests that results might be achieved in the critical 1–3 h window for initial therapy.

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