C-145

Direct Detection and Enumeration of Viable Bacteria in Human Bronchoalveolar Lavage Specimens Using Automated Growth Rate Analysis

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AMENDED ABSTRACT

Background: Culturing substantially delays reporting to the physician who treats critically ill patients. To eliminate culturing prior to testing, it is necessary to extract viable bacteria directly from a specimen and present them in a form suitable for analysis.

Methods: 21 random patient specimen remnants of bronchoalveolar lavage fluid (BAL) were analyzed using automated microscopy for growth rate analysis and progeny clone enumeration of bacteria extracted and immobilized in a multi-channel flowcell device. An aliquot of each specimen was also analyzed by quantitative agar culture. Specimens ranged from cloudy, mucus-containing and blood-tainted to a clear water-like appearance. Specimens were flash spun to remove erythrocytes and mucus, then centrifuged on 1.03 g/mL Percoll® to separate bacteria from debris. Elapsed time for specimen preparation was less than 45 minutes. Resuspended bacteria were delivered to the flowcells, electrokinetically concentrated, and immobilized on a poly-L-lysine-coated glass surface. The device incubated the cells at 35°C for 3 hours and acquired images every 10 minutes. Size and growth rate criteria were used to exclude yeasts and residual debris from analysis. Growing clones were enumerated and cell morphology was determined. Cultured isolates were Gram stained, and cell morphology was compared to those in acquired images.

Results: Quantitative cultures confirmed clinically relevant levels of bacteria (10^5 CFU/mL) in 9 specimens. The experimental device detected a significant number of growing clones in 8 of the specimens in less than 1 hour. The device correctly identified 12 of 12 non-diagnostic specimens. Cell morphology of immobilized growing clones was consistent with that of Gram-stained cells. Morphology of immobilized non-growing cells was also consistent with the Gram-stained cells.

Conclusions: The automated system was able to enumerate diagnostic levels of growing bacteria extracted directly from BAL fluid in less than 2 hours after specimen access. The methods are consistent with the requirements for rapid diagnostic analysis of pathogens without prior culturing and isolation. Additional experimentation is required to determine the quantitative relationship between the experimental device and conventional methods.

INTRODUCTION

Delayed initiation of microbiologically adequate antimicrobial therapy is well recognized as a leading risk factor for mortality with infections in critically ill patients. Delay occurs because bacterial cultures require 1-3 days to perform and the physician must begin therapy without guidance. Widespread and complex antibiotic resistance causes initial therapy to fail in a high proportion of cases as a result. Laboratories need new methods to rapidly determine antibiotic resistance phenotypes. New methods requiring small numbers of organisms for testing could potentially obviate the need for overnight culturing and enable direct-from-specimen analysis.

This study tested a rapid method for extracting viable bacteria directly from a patient specimen, assessing cellular morphology, measuring individual cell growth rates, and determining whether a specimen contained bacteria at diagnostic levels.

MATERIALS & METHODS

Specimens. 21 randomly selected, de-identified remnants of bronchoalveolar lavage (BAL) fluids were obtained from a 477 bed tertiary care hospital without regard to the original specimen's purpose or patients' medical conditions. Material was obtained after processing for the usual course of care (UCC). De-identified abstracts from the associated UCC microbiology lab reports provided comparator data. Specimens varied in appearance, including clear water-like, cloudy, heavy mucus, light mucus, and blood-tainted materials.

Quantitative Culturing. An aliquot of each specimen was plated on sheep blood agar plates using standard laboratory procedures for quantitative culturing.

Specimen Preparation. Fig. 1 diagrams the specimen preparation procedure. 1 mL of specimen was flash centrifuged to pellet erythrocytes and other large debris. The supernatant was then aspirated and layered over 0.5 mL of a 1.03 g/mL Percoll® solution. Tubes were centrifuged 30 minutes at 2000xg. Approximately 950 μL of supernatant was aspirated, removing a majority of the specimen debris, leaving 50 μL of supernatant over the pellet that contained cells and residual small debris. The pellet was resuspended by pipette shearing and vortexing in 1 mL of a 1mM L-histidine wash solution. The suspension was then centrifuged for 5 min. at maximum speed on an Eppendorf MiniSpin®, and the pellet resuspended in a low ionic strength electrokinetic capture (EKC) buffer for introduction into the experimental device. Pilot studies had previously determined an average sample preparation procedure loss of approximately 50% (data not shown).

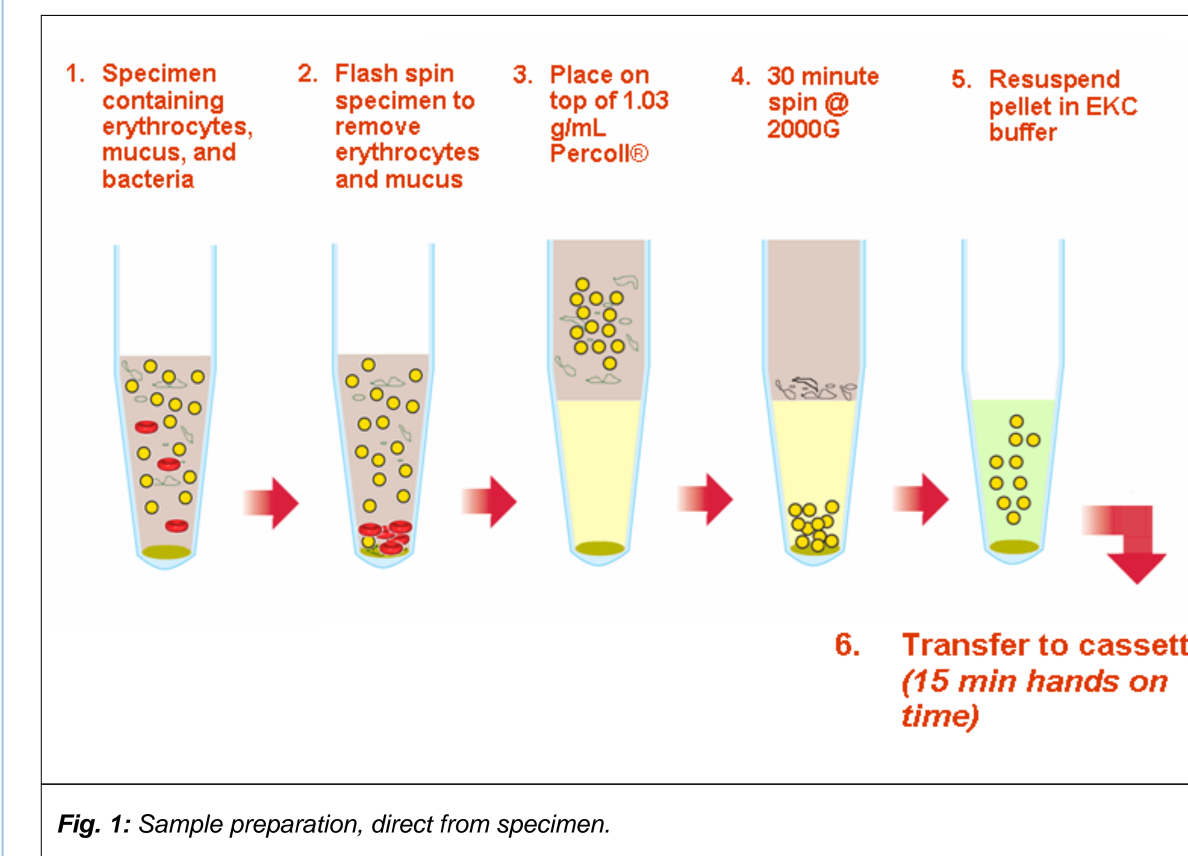

Fig. 1: *Sample preparation, direct from specimen.*

Automated Instrumentation. Direct observation of bacterial cells was performed on a custom disposable 32-flowcell cassette inserted into an automated digital microscope with customized motion control and image analysis software. Each flowcell was independent. Flowcell top and bottom surfaces had transparent, electrically conductive coatings for electrophoresis and microscopy. The bottom surface was coated with poly-L-lysine that immobilized bacteria upon contact. The system used darkfield illumination, and was enclosed in a 35°C incubator.

Application of an electrical field for 3 minutes concentrated all bacteria within a flowcell to the capture surface, enabling fluid exchanges. The EKC buffer was then displaced with cation-adjusted Mueller-Hinton broth. The automated system acquired images every 10 minutes, using a single registered field of view of 444 x 592 μm for each flowcell.

Custom image analysis software measured growth of each cell and its progeny clone. Prior studies had established a detection threshold of 5 growing clones, which corresponded to approximately 1.2×10^5 CFU/mL in the original specimen. For BAL, the diagnostic threshold from culturing is typically 10^5 CFU/mL.

RESULTS

Growth began after bacterial immobilization and MHB wash without an appreciable lag time (<10 min.). To illustrate growth tracking, **Fig. 2** shows an image sequence magnified to 115x70 μm, at 30-min. intervals. Visual inspection suggests coccobacilli. **Fig. 3** shows examples of additional cellular and clonal morphologies after longer growth.

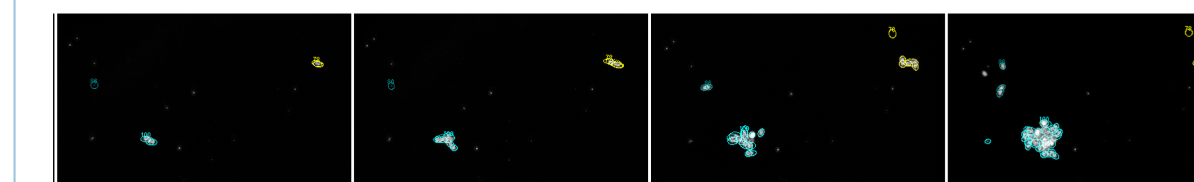

Fig. 2: *30-min. time interval images of individual growing clones as identified by image analysis algorithms. Analysis software tracked each clone throughout the observation period. Partial field of view.*

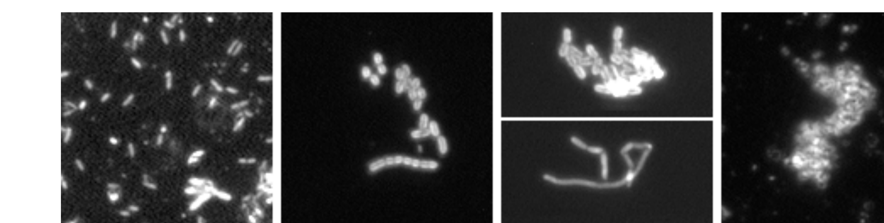

Fig. 3: *Examples of additional cellular and clonal morphologies.*

The experimental method identified 8 of the 9 diagnostically positive specimens. Median division rates ranged from 0.67 to 2.5 div/hr. The system correctly classified the 12 non-diagnostic specimens. Visually assessed morphology was consistent with the UCC lab abstracts.

Table 1 lists the growing clone counts in a single field of view for each of the 9 culture-positive specimens (>10^5 CFU/mL).

Table 1

Specimen ID	Clones/ Field	Specimen ID	Clones/ Field	Specimen ID	Clones/ Field
1	512	4	80	7	<5
2	62	5	31	8	31
3	30	6	144	9	44

DISCUSSION

This study evaluated the ability of a novel direct bacterial extraction method using automated instrumentation to provide actionable information in less than 2 hours after specimen access, without prior enrichment culturing and colony isolation. The new method demonstrated potential for detecting diagnostic levels of bacteria, measuring individual cell and clone growth rates, and providing image exemplars for assessing planktonic and clonal growth morphologies.

Although this study used single fields of view to estimate bacterial titer, the system can include multiple fields of view to increase quantitative sensitivity. With refinement in sample preparation, such as mucolysis and eukaryotic cytolysis, the system has the potential to provide quantitative bacterial titers for the original specimen.

Further development of image analysis algorithms also has the potential to automate morphological analysis.

If combined with compatible *in situ* identification and antibiotic susceptibility testing methods, the rapid bacterial immobilization method thus has the potential to eliminate the need for overnight culturing and colony isolation with high-titer patient specimens. The analytical speed of the automated system was consistent with that required to guide initial empiric therapy in critically ill patients.

CONCLUSIONS

By analyzing live, immobilized bacteria extracted directly from patient specimens, the automated system classified 8 of 9 BAL specimens as containing diagnostic levels of growing bacteria, and 12 of 12 as lacking diagnostic levels. It measured individual cellular and clonal growth rates in approximately 1 hour of analysis time following 45 minutes of sample preparation time. It provided exemplar images for visual assessment of planktonic and growth morphologies. The methods therefore show potential for reporting actionable information to the physician in less than two hours after specimen access.

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