Diagnostic Microbiology and Infectious Disease 79 (2014) 160–165

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

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

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ARTICLE INFO

Article history:
Received 21 October 2013
Received in revised form 27 November 2013
Accepted 29 November 2013
Available online 7 December 2013

Keywords:
Rapid identification
Quantitative identification
Live-cell
Automated microscopy
Polymicrobial infection
BAL
HAP
VAP
ICU
Nosocomial pneumonia

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).

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

☆ Conflicts of interest: Author SM is an employee of Accelerate Diagnostics. Participation by the Washington University School of Medicine was funded by grants from Accelerate Diagnostics to the study site institution and not to investigators RAF or WMD.

☆☆ Data in this study were partially reported at the 2011 Interscience Conference on Antimicrobial Agents and Chemotherapy (Metzger and Dunne, 2011).

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http://dx.doi.org/10.1016/j.diagmicrobio.2013.11.029

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 multichannel 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 ± 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

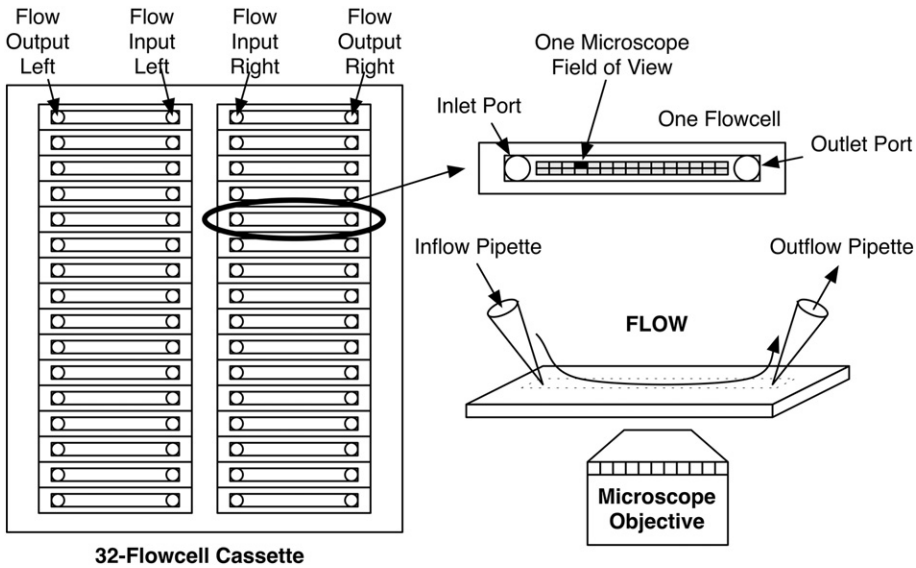

Fig. 1. Diagram of 32-channel cassette showing detail of individual fluidic channel and microscope fields of view. Fluid exchange occurs using pipette inflow and outflow ports, and image capture is performed by an inverted microscope positioned beneath the bottom surface of the fluidic channel.