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Rapid Identification of Live *Acinetobacter* spp. in Bronchoalveolar Lavage Specimens by Automated Immunofluorescence Microscopy

K. Hance, **D. Howson**, M. Lindsey, T. Ngo, S. Metzger

AccelR8 Technology Corp., Denver, CO

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Ken Hance
khance@accelr8.com

AMENDED ABSTRACT

Background: *Acinetobacter* spp. are responsible for up to 10% of cases of nosocomial pneumonia and present a global challenge. Clinicians need a rapid and reliable assay for identification of this diverse genus. Rapid identification and enumeration of intact live cells would represent a significant advance over current methods.

Methods: Polyclonal antibodies specific for *Acinetobacter* spp. surface antigens were isolated from chicken egg yolk by affinity chromatography. Antibody characteristics were determined with 20 *Acinetobacter* spp., and with 28 non-*Acinetobacter* isolates that included 13 species frequently found in respiratory specimens. *Acinetobacter* spp. strains were spiked into sheep bronchoalveolar lavage fluid (BAL), concentrated, and captured onto a slide surface. Immobilized bacteria were washed in tryptic soy broth containing 1% BSA, stained with antibody, and detected using a fluorescent polyclonal anti-chicken secondary antibody. Fluorescence and phase-contrast images were analyzed to determine percent stained and average intensity. Viability of immobilized cells was determined by direct growth measurement using dark field images.

Results: *Acinetobacter*-specific polyclonal antibody bound to 16 of 20 *Acinetobacter* spp. strains, and did not bind to 25 of 28 non-*Acinetobacter* strains. Staining efficiency and intensity of cells spiked into BAL was similar to staining of log-phase bacteria. BAL interference was not significant. Although both live and dead cells were stained, automated serial image analysis of cell growth after staining allowed determination of viability. Time to result for sample preparation, capture, staining, and viability determination was approximately 120 minutes.

Conclusions: Recovery and identification of *Acinetobacter* spp. by a rapid immunofluorescence method exhibited promising performance, with the added ability to determine cell viability and enumerate intact live cells extracted directly from a specimen.

INTRODUCTION

Hospital acquired infections (HAI), and particularly nosocomial pneumonia, are leading causes of morbidity and mortality in critically ill patients. *Acinetobacter* spp., including *A. baumannii* and several other *Acinetobacter* genomospecies, are important pathogens in the ICU.

Hospital-adapted *Acinetobacter* harbors numerous antibiotic resistance mechanisms and presents serious diagnostic challenges. Because these organisms are often highly drug resistant, their identity and phenotype markedly influence the choice of therapy.

Culture-based systems are able to identify *Acinetobacter* spp. but require initial enrichment culturing and colony isolation. Culturing methods therefore require as long as 48 hours for positive identification and antibiotic susceptibility testing. This is too long for managing critical infectious diseases because initial therapy must assure adequate control of disease progression.

Molecular methods shorten the identification, but cannot differentiate between live and dead, nor intact or fragmented bacteria, nor can they quantify specimen contents. These are important criteria for many types of specimen, particularly in diagnosing pneumonia.

In order to eliminate the delays required for culturing, it would be desirable to analyze live organisms extracted directly from a patient specimen. Such a method would require species identification and enumeration, as well as the ability to determine the viability of individual cells.

The purpose of this investigation was to characterize a method for rapid identification of *Acinetobacter* spp. extracted directly from a mock specimen using fluorescent-labeled antibody paired with automated growth tracking of individual bacteria to determine viability. The experimental methods tested in this study are intended to become part of a new rapid diagnostic system using bacteria extracted directly from a patient specimen without prior enrichment culturing or colony isolation.

MATERIALS & METHODS

Acinetobacter spp. and non-*Acinetobacter* isolates were obtained from ATCC® and JMI Laboratories (N. Liberty, IA). The collection included 19 *A. baumannii* and 1 *Acinetobacter* genomospecies-13, plus 28 non-*Acinetobacter* isolates of species often found in respiratory specimens.

Fig. 1: *Dual 16-channel flowcell cassette.*

Direct observation of bacteria was performed on a disposable fluidic cassette (**Fig. 1**) inserted into a custom bench-top instrument that combines automated digital microscopy, motion control, and image analysis software.

The cassette contained multiple independent flowcells. Flowcells were constructed with transparent top and bottom surfaces to allow microscope imaging. Each surface had a transparent electrode coating, forming an electrophoresis chamber. The bottom surface was coated with poly-L-lysine to immobilize bacteria upon surface contact.

Colonies from agar plates were resuspended in tryptic soy broth (TSB) and grown for 2 hours. Mock specimens were made by spiking log phase bacteria (approx. $5x10^6$ CFU/mL) into bronchoalveolar lavage (BAL) fluid from non-infected sheep. A specimen was then centrifuged on Percoll® to reduce debris, washed and resuspended in electrokinetic capture buffer (**Fig. 2**), and pipetted into a cassette's sample wells.

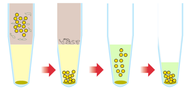

Fig 2: *BAL sample preparation. Centrifuge the specimen on Percoll®. Bacteria pellet while debris do not, concentrating and cleaning the sample. Resuspend the pellet in electrokinetic capture buffer (EKB), and centrifuge again. Resuspend in EKB and pipette into cassette sample well.*

Tests were also performed on isolates without BAL. For experiments on live/dead mixtures, live organisms were mixed with formalin-killed bacteria in a 1:1 ratio (McFarland standard).

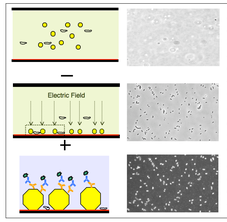

Fig 3. *Illustrations not to scale.* ***Top left:*** *suspension of bacteria and residual debris flows into flowcell from the left. The microscope objective is beneath the flowcell.* ***Top right:*** *phase contrast, unfocused images show that bacteria are not in contact with the surface.*

Center left: *an electrical field concentrates bacteria and residual debris on the surface.* ***Center right:*** *phase contrast, bacteria and residual debris in focus on the surface.*

Bottom left: *zoomed-in view of surface-bound bacteria following specific antibody binding.* ***Bottom right:*** *fluorescence image showing antibody-labeled bacteria.*

Application of an electrical field caused bacteria to migrate to the positively-charged lower electrode (**Fig. 3**) during a capture step. The bacteria adhered to the surface coating, permitting subsequent medium exchanges. Each flowcell contained approximately 100 to 500 founder cells on the surface within the digital microscope's 444 x 592 μm field of view. After capture, the flowcells were rinsed with TSB, removing electrokinetic concentration buffer.

Polyclonal antibodies were developed in chickens and isolated using acid precipitation of yolk proteins followed by tangential flow filtration using a 100 kDa filter. Antibodies specific for *Acinetobacter* surface antigens were isolated from the yolk preparation by affinity purification.

Antibody staining of immobilized bacteria was performed by incubation in the affinity-purified IgY for 5 minutes in a 1% BSA/TSB staining solution. Primary antibody binding was followed by washing and detection of bound IgY using 5-minute incubation in goat anti-chicken antibody conjugated to Alexa-555. Quantitative image analysis computed the mean intensity of cell staining and the percentage of cells that stained above a threshold level criterion.

The instrument acquired time sequenced images for each of the flowcells at 10-minute intervals. For growth measurement, the image analyzer computed mass changes using dark field imaging mode. Clones were considered to be growing if they exhibited at least 50% increase in integrated intensity over the 40 minute growth period.

To test feasibility for polymicrobial multiplexing, 1:1 mixed species of live *Acinetobacter* and *Pseudomonas aeruginosa* were spiked into BAL. Staining for *P. aeruginosa* used rabbit O-typing antisera and goat anti-rabbit antibody conjugated to Alexa-488.

RESULTS

Anti-*Acinetobacter* antibody labeled 16 of 20 strains of *Acinetobacter* spp. and did not label 25 of 28 strains of non-*Acinetobacter* species commonly found in respiratory specimens (**Table 1**).

Table 1. Antibody Staining Results

Species	# Tested	# Pos. (%)	# Neg. (%)
A. baumannii	19	15 (79)	4 (21)
Acinetobacter gsp. 13	1	1 (100)	0 (0)
Total	**20**	**16 (80)**	**4 (20)**
Pseudomonas aeruginosa	7	0 (0)	7 (100)
Stenotrophomonas maltophilia	4	0 (0)	4 (100)
Haemophilus influenzae	1	0 (0)	1 (100)
Klebsiella pneumoniae	4	1 (25)	3 (75)
Escherichia coli	3	0 (0)	3 (100)
Enterobacter aerogenes	1	0 (0)	1 (100)
Enterobacter cloacae	2	1 (50)	1 (50)
Staphylococcus aureus	1	0 (0)	1 (100)
Staphylococcus epidermidis	1	0 (0)	1 (100)
Staphylococcus haemolyticus	1	0 (0)	1 (100)
Streptococcus pneumoniae	1	0 (0)	1 (100)
Streptococcus pyogenes	1	0 (0)	1 (100)
Streptococcus salivarius	1	1 (100)	0 (0)
Total	**28**	**3 (11)**	**25 (89)**

Capture time was fixed at 300 seconds. Electrokinetic transport moved all bacteria above the capture area to the surface, determined by focusing at different levels above the surface. Growth of immobilized bacteria began after TSB wash without an appreciable lag time (<10 min.).

Antibody did not detectably bind to BAL debris. Over 90% of live cells extracted from the live control mock BAL specimen met the growth criterion, indicating that sample preparation capture, and labeling did not adversely affect viability. None of the spiked dead cells exhibited growth. A mixture of live and formalin-killed cells resulted in staining of both live and dead cells (**Fig. 4**).

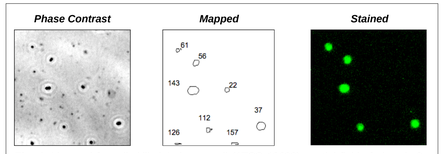

Fig. 4. *Live and formalin-killed A. baumannii (ATCC 19606), partial field of view.* ***Left:*** *phase contrast, with debris.* ***Center:*** *outlines of image analyzer mapped contours of all presumptive cells.* ***Right:*** *fluorescence, antibody stained.*

Growth measurement (**Fig. 5**) clearly differentiated between growing and non-growing individual clones after approximately 30 minutes of growth measurement. In the mixed live/dead mock specimen, 33% of clones met the viability criterion.

A mixture of live *Acinetobacter* and *Pseudomonas* exhibited the expected staining with respective antibodies. Of 344 total cells observed, 221 stained with *Acinetobacter* antibody and 123 stained with *P. aeruginosa* antibody (**Fig. 6**). None of the cells remained unstained, and none stained with both antibodies. Bacteria in control flowcells containing each strain alone were stained using their primary antibodies, and no cross-reactivity was observed for either one. Bacteria in separate control flowcells containing each strain alone did not stain with either secondary antibody in the absence of primary antibody.

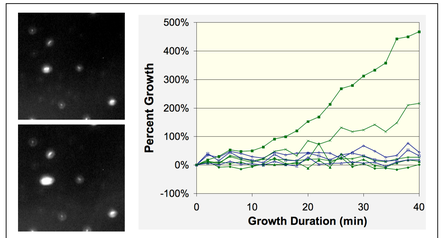

Fig 5*. Example of individual clones of ATCC® 19606 Acinetobacter, live plus dead cells. Time sequence, dark field, partial field of view.* ***Top left:*** *start of interval.* ***Bottom left:*** *after 40 min. of growth.* ***Right:*** *quantitation of individual clone growth from the images.*

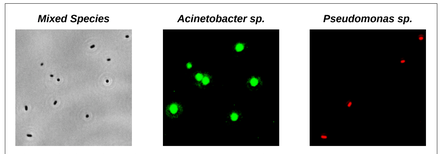

Fig. 6. *Mixed species, A. baumannii ATCC 19606 and P. aeruginosa ATCC 35554, partial field of view.* ***Left:*** *phase contrast.* ***Center:*** *staining with anti-Acinetobacter.* ***Right:*** *staining with anti-Pseudomonas.*

This set of conditions demonstrated the feasibility of concurrent color-multiplexing with multiple antibodies.

CONCLUSIONS

Polyclonal antibody developed against surface antigens of *Acinetobacter* spp. showed the potential for multiplexed identification in the presence of interfering species commonly seen in respiratory specimens. Electrokinetic immobilization and species immuno-identification did not significantly affect cell viability. The experimental methods were able to quantify the ratio of live cells in a mock specimen. Immuno-identification combined with automated growth tracking of immobilized bacteria represents a rapid and potentially powerful approach to identifying and differentiating intact live *Acinetobacter* spp. cells from dead or dormant cells directly from high-titer specimens.