C-207

Rapid Identification of Resistance Phenotypes in Gram-Negative Bacilli Using Automated Digital Microscopy

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AMENDED ABSTRACT

Background: *Pseudomonas aeruginosa* (PA) and *Acinetobacter baumannii* (AB) are major causes of nosocomial infection and difficult to manage because of multi-drug resistance. *Enterobacteriaceae* that acquire the KPC carbapenemase are also likely to co-exist with multi-drug resistance in addition to presenting formidable detection challenges. Conventional phenotyping methods require growth of large numbers of bacteria, which increases the total time-to-result. We hypothesized that automated digital microscopy (ADM) can rapidly identify resistance phenotypes using small numbers of immobilized bacteria comparable to numbers directly extractable from patient specimens.

Methods: We used ADM with a 32-channel disposable fluidic cassette to measure growth of immobilized bacteria.[1] We separately tested PA clinical isolates for nonsusceptibility (NS) with amikacin (AN) at 32 μg/mL, and AB isolates with imipenem (IMP) at 8 μg/mL or ceftazidime (CAZ) at 8 μg/mL. We tested *Klebsiella pneumoniae* (KP) clinical isolates for ertapenem (ETP) nonsusceptibility at 16 μg/mL with and without the inhibitors aminophenyl boronic acid (APB) at 300 μg/mL or benzo(b)thiophene-2-boronic acid (BTB) at 50 μg/mL to identify putative KPC-positive strains.

We resuspended colonies from agar plates and incubated them for 2 hours in tryptic soy broth. We pipetted 10 μL aliquots of each 5E+7 CFU/mL inoculum into separate cassette flowcell channels. Each microscope field of view contained 10-100 bacteria immobilized on poly-L-lysine-coated glass. We pipetted the appropriate agent into each flowcell. The system measured growth at 10-minute intervals. We classified any non-fermenter isolate as NS if growth was not arrested after 3 hours in the test antibiotic. Inhibitor tests classified a KP isolate as KPC-positive if growth differed by a criterion amount between uninhibited and inhibited conditions within 3 hours. Non-inhibitor tests classified KP isolates ETP-NS if growth was not arrested after 3 hours. We compared results with CLSI disk diffusion for the non-fermenters; and with the CLSI Hodge test and RT-PCR for KP.

Results: Sensitivity and specificity were, respectively: PA-AN (33/37) 89% and (33/35) 94%; AB-IMP (24/26) 92% and (65/66) 98%; AAB-CAZ (58/59) 98% and (14/17) 82%; KP-ETP (6/6) 100% and (13/13) 100%; KPC/APB (5/6) 83% and (13/13) 100%; KPC/BTB (4/6) 67% and (13/13) 100%.

Conclusions: Direct analysis of small numbers of bacteria using ADM identified resistance phenotypes in non-fermenters and in *K. pneumoniae* within 3 hours. The method shows potential for rapid automated testing with bacteria extracted directly from clinical specimens without prior culturing.

INTRODUCTION

Nosocomial infections due to multi-resistant Gram negative bacteria are increasing in frequency and growing in complexity. For critically ill patients, the likelihood for success is indirectly related to the time required to administer effective antimicrobial therapy. However, standard tests require 2-3 days to characterize antimicrobial resistance patterns using culture-based methods. In contrast, automated digital microscopy (ADM) has the potential to reduce turnaround time by direct detection of antimicrobial resistance phenotypes in bacteria extracted from a clinical specimen. The purpose of our study was to determine the sensitivity, specificity, and speed of automated microscopy to detect major resistance phenotypes associated with multi-drug resistance in significant Gram-negative clinical isolates.

MATERIALS & METHODS

Figure 1: 32-channel flowcell cassette.

We adapted a commercial inverted microscope and camera with custom image analysis software and a purpose-built 32-channel disposable fluidic cassette (Fig. 1). Cassette flowcells had transparent top and bottom surfaces to allow microscope imaging. The bottom surface was coated with poly-L-lysine to immobilize live bacteria.

We tested clinical isolates of *Pseudomonas aeruginosa* (PA), *Acinetobacter baumannii* (AB), and *Klebsiella pneumoniae* (KP). Test agents included amikacin (AN), imipenem (IMP), ceftazidime (CAZ), ertapenem (ETP), aminophenylboronic acid (APB), and benzo(b)thiophene-2-boronic acid (BTB). The boronic acids inhibit the KPC enzyme as well as AmpC. Table 1 summarizes organisms and test conditions. We expressed test results as nonsusceptible (NS) or susceptible (S).

Table 1: Organisms and Test Conditions

Species	Test	# NS/S	Conditions
PA	AN	37/35	AN 32 μg/mL
AB	IMP	26/66	IMP 8 μg/mL
	CAZ	59/17	CAZ 8 μg/mL
KP	ETP	6/13	ETP 16 μg/mL
	KPC/APB		ETP 16 μg/mL + APB 300 μg/mL
	KPC/BTB		ETP 16 μg/mL + BTB 50 μg/mL

We grew isolates on blood agar, suspended colonies in tryptic soy broth for 2 hours, then centrifuged and resuspended log-phase bacteria in low ionic strength electrokinetic buffer.

The experimental procedure consisted of —

- Pipetting 10 μL aliquots of 5E+7 CFU/mL into separate flowcells for each isolate and test condition.
- Concentrating bacteria with an electrical field to the positively-charged lower surface to immobilize cells and yield 10-100 bacteria per field of view (Fig. 2).

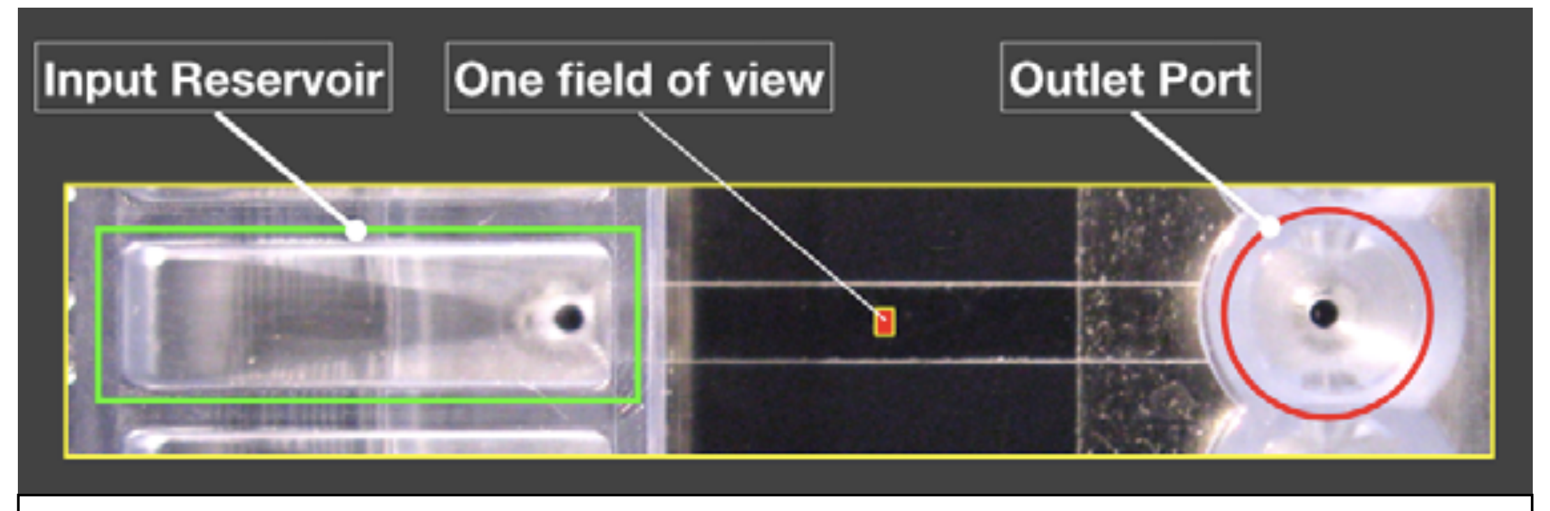

Figure 2: One flowcell in a 32-channel fluidic cassette.

- Testing each isolate in separate flowcell channels with no antibiotic (growth controls).
- Acquiring microscope images at 10-minute intervals.
- Exposing to the test conditions for 3 hours.
- Interpreting results for PA, AB, and KP-ETP as NS if growth had not arrested within 3 hours.
- Interpreting results for both KP-KPC tests as presumptive for KPC if growth differences between the inhibited (with APB or BTB) and uninhibited (ETP alone) exceeded a criterion amount (Fig. 5).
- Comparing results to CLSI disk diffusion for PA and AB; and CLSI Hodge Test and RT-PCR for KP.

RESULTS

Table 2 summarizes assay performance.

Table 2: Test Results

Test	Sensitivity	Specificity
PA-AN	33/37 89% CI 74-96%	33/35 94% CI 79-99%
AB-IMP	24/26 92% CI 73-99%	65/66 98% CI 91-100%
AB-CAZ	58/59 98% CI 90-100%	14/17 82% CI 56-95%
KP-ETP	6/6 100% CI 52-100%	13/13 100% CI 72-100%
KP-KPC/APB	5/6 83% CI 36-99%	13/13 100% CI 72-100%
KP-KPC/BTB	4/6 67% CI 24-94%	13/13 100% CI 72-100%

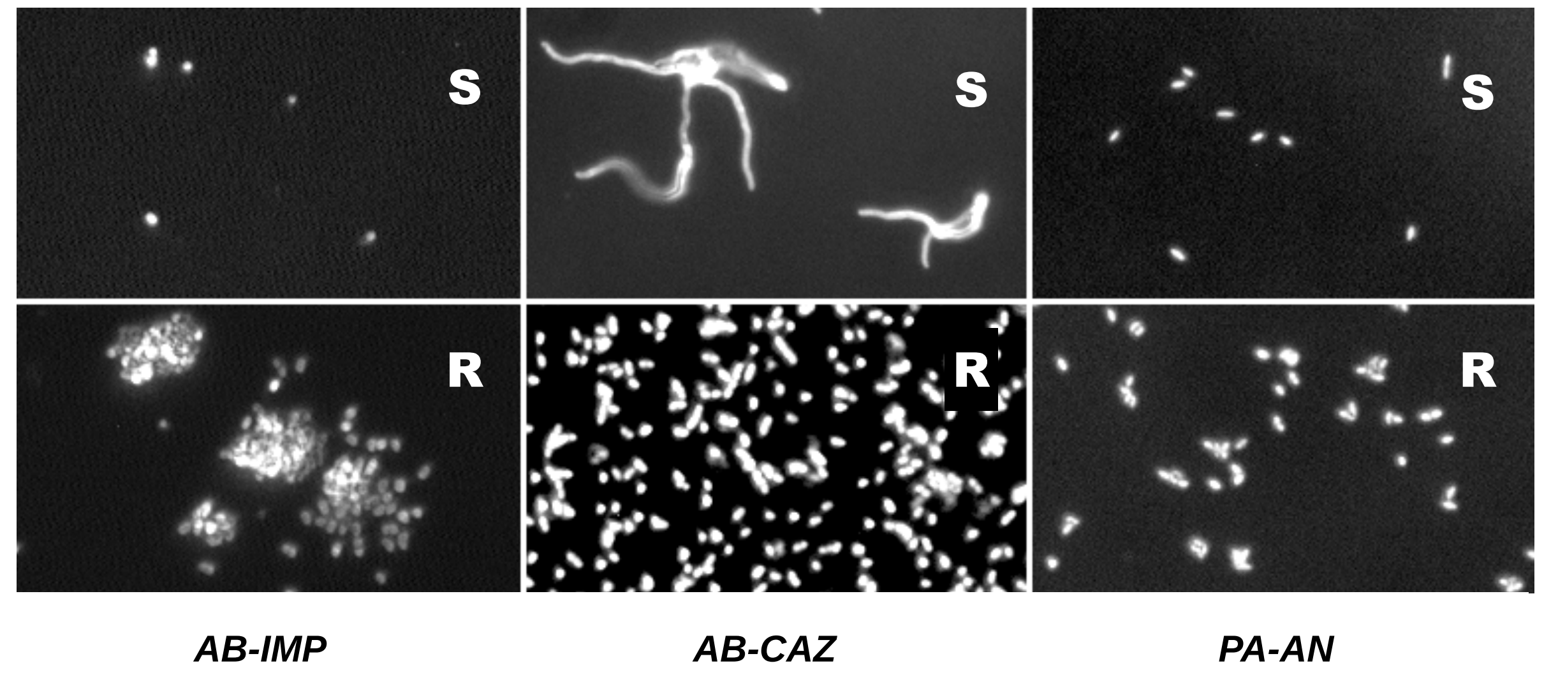

Figure 3: *Non-fermenter clinical isolates at end of 3 hours of drug exposure.* ***Top Row:*** *Susceptible strains.* ***Bottom Row:*** *Resistant strains. Images zoomed in for detail. Antibiotic exposure may cause abnormal growth morphology. Sum of integrated pixel intensities of individual clones closely parallels clone mass and/or cell count from standard methods.*

Fig. 3 shows images of bacteria exposed to antibiotics for 3 hours. Either susceptible or resistant strains may show abnormal morphology during growth in drug-containing media. The detectable difference occurs when a susceptible strain ceases to grow.

Figs. 4 and 5 show images and growth data from KP tests. Clear differences occur between the behaviors in ETP alone and ETP with an enzyme inhibitor added. Images show the same field of view at different times (20 and 90 minutes of drug exposure).

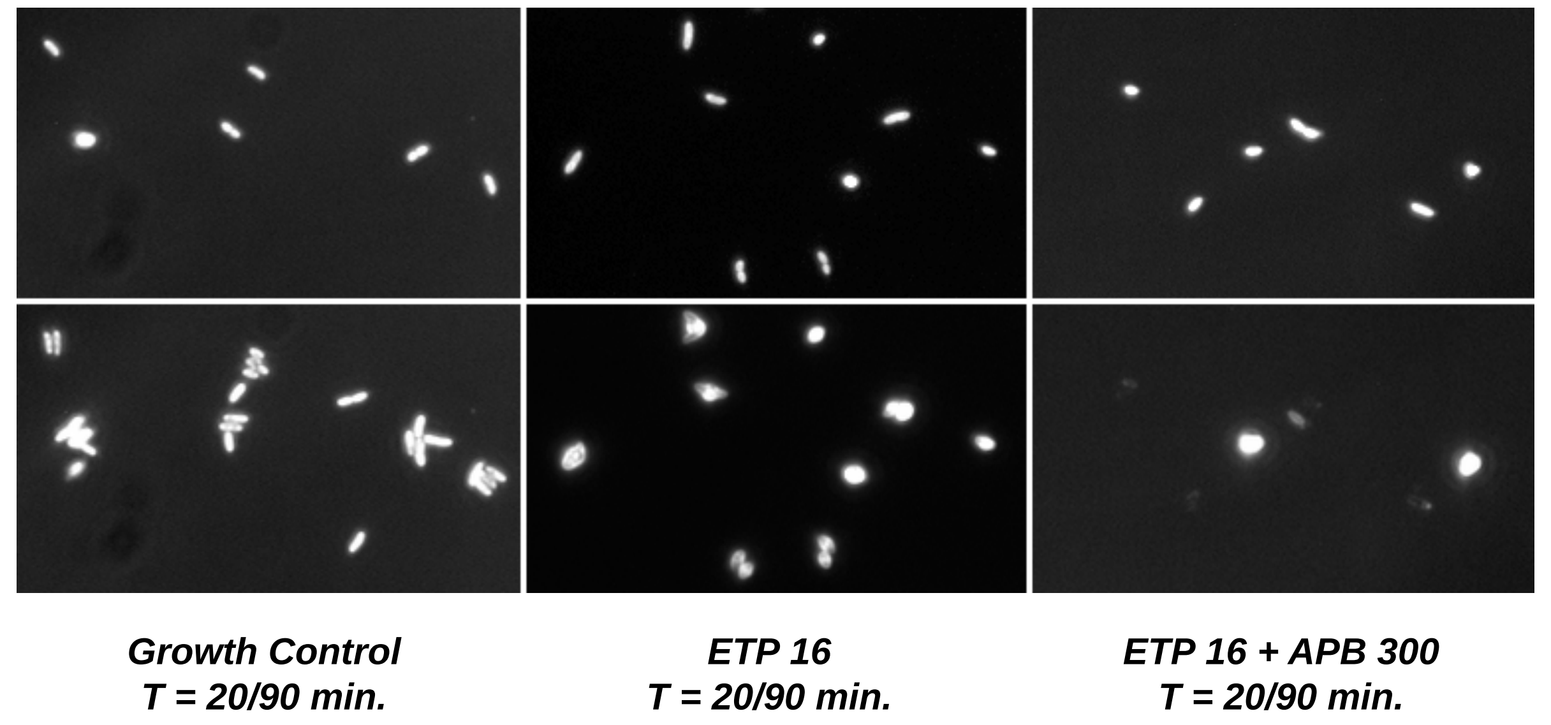

Figure 4: *KPC clinical isolate.* ***Top Row:*** *20 min. after start of drug exposure.* ***Bottom Row:*** *90 min after start. Images zoomed for detail. ETP exposure causes abnormal growth morphology. Sum of integrated pixel intensities of individual clones closely parallels clone mass and/or cell count. Difference between ETP alone and ETP+APB shows enzyme inhibition and decreased resistance, hence KPC-positive interpretation.*

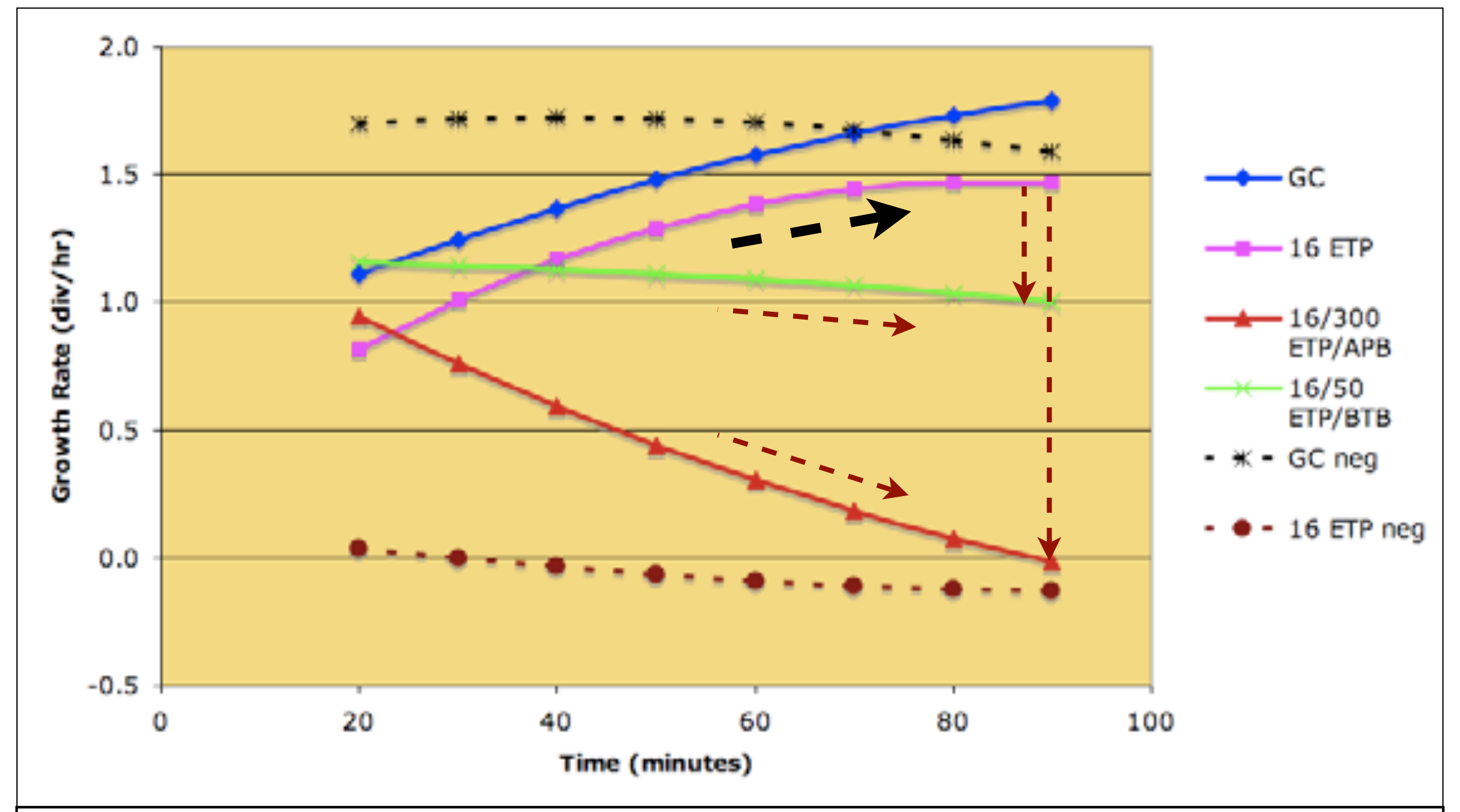

Figure 5: *KPC assays, growth rate vs. exposure time. "GC"=growth control, one for KPC-positive strain (same as in* ***Fig. 4****) and one for KPC-negative strain (dotted line). For KPC-positive, the difference between growth curves for ETP alone and ETP with a boronic acid enzyme inhibitor (APB or BTB) determines the interpretation. Susceptible strains show no differences, and also fail to grow in ETP at 16 μg/mL (lower dotted line). The dashed red arrows indicate growth curves that exceeded difference criteria (from the heavy dashed black arrow) for a positive interpretation (enzyme positive ETP alone vs. ETP+inhibitor).*

CONCLUSIONS

The experimental method met the objectives of using a small number of cells, achieving rapid results, and having accuracy approaching those of standard tests in identifying major resistance phenotypes, including difficult-to-detect KPC-positive organisms. Cell number was consistent with that previously shown adequate to rapidly identify pathogens[2,3] from organisms extracted directly from a polymicrobial patient specimen. Further optimization may further decrease the total assay time and improve test performance.

Assay kinetics enabled sensitive, specific, and rapid detection of each phenotype using a single challenge concentration of each antibiotic.

References

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3. 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.