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Direct Identification of the ESBL Phenotype in *Enterobacteriaceae* Isolates Using Small Numbers of Immobilized Cells

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AMENDED ABSTRACT

Background: Conventional phenotyping requires large numbers of bacteria, which increases the total time-to-result. We report results for a new method that uses approximately 500 bacterial cells to detect the ESBL phenotype.

Methods: A multi-channel fluidic device used computerized microscopy to measure growth of immobilized bacteria.[1] Colonies from 53 ESBL-positive and 73 ESBL-negative *Klebsiella* spp. and *Escherichia coli* isolates were re-suspended from agar plates and pre-grown for 2 hours in tryptic soy broth. Then 14 µL aliquots of a 7E+5 CFU/mL inoculum were delivered to multiple flowcells. Bacteria were concentrated onto a poly-L-lysine-coated glass surface, capturing approximately 200-500 cells in the microscope's field of view. ESBL detection used separate fluidic channels containing 256 µg/mL of either of two 3rd generation cephalosporins (3GCs) with or without the addition of 4 µg/mL of clavulanic acid (CA). Ceftazidime (CAZ) and cefotaxime (CTX) were the 3GCs tested. A growth control and CA control were run in parallel. The system acquired images every 10 minutes and computed the mass of the cell population throughout the test. The ratio of cell mass in 3GC only to that in the 3GC+CA combination was calculated. The system classified isolates as ESBL-positive if the mass ratio exceeded a threshold criterion. Results were compared to CLSI confirmatory ESBL disk diffusion test results.

Results: The average maximum mass ratio of ESBL-negative isolates was 1.9 ± 1.3 s.d. while the range for ESBL positive isolates was 3.6 to >200. The system correctly classified 51 of 53 ESBL-positive and 70 of 73 ESBL-negative isolates, using a growth inhibition criterion mass ratio of 5, in a total test time of 3 hours. Sensitivity and specificity were, respectively, 93% [CI 82%-98%] and 96% [CI 87%-99%].

Conclusions: Direct measurement of growth of small numbers of immobilized bacteria enabled identification of the ESBL phenotype in *Enterobacteriaceae*. Our method shows promise for rapid testing, with a bacterial sample size that is compatible with direct extraction from clinical specimens.

INTRODUCTION

Infections due to Gram negative bacteria with ESBLs 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, 2-3 days are required to characterize antimicrobial resistance patterns using standard culture-based methods. In contrast, automated microscopy has the potential to reduce turnaround time by direct detection of antimicrobial resistance phenotypes in a clinical specimen. The purpose of our study was to determine the sensitivity, specificity, and speed of automated microscopy to detect ESBL expression in clinically significant isolates of *Enterobacteriaceae*.

MATERIALS & METHODS

Direct observation of bacterial response to antibiotic exposure was performed on a disposable 32-channel fluidic cassette (***Fig. 1***) inserted into a custom bench-top automated instrument that combines digital microscopy, motion control, and image analysis software.[1] Flowcells were constructed with transparent top and bottom surfaces to allow microscope imaging.

All testing used *Klebsiella* spp. and *E. coli* known to be ESBL-positive or -negative. A collection for assay optimization development was obtained from the JMI Laboratories and the American Type Culture Collection (ATCC). The CLSI confirmatory disk diffusion (DD) ESBL test was used to characterize the optimization isolate set. The set included CLSI ESBL QC strains, ATCC® 700603 and 25922. The collection was used to establish an ESBL classification criterion.

Figure 1: *32-channel flowcell cassette.*

A second set of challenge isolates had been previously characterized using CLSI broth microdilution (BMD) methods. Of the ESBL-positive isolates, 6 had MICs ≥256 µg/mL and 10 had MICs of ≤8 µg/mL. 6 ESBL-negative isolates had MICs ≥256 µg/mL and 6 had 1- or 2-dilution differences by BMD.

In addition, the Washington University School of Medicine, Department of Pathology & Immunology Laboratory at Barnes-Jewish Hospital in St. Louis, MO used system prototype to test 44 recent isolates obtained from clinical specimens. The study results include those from this isolate set. ***Table 1*** summarizes the isolate collections.

Table 1

	Optimization	Challenge			Clinical	Total
		MIC ≥256 µg/mL	MIC ≤8 µg/mL	Δ MIC ≤2 dilutions		
ESBL-pos	18	10	6	NA	19	53
ESBL-neg	36	6	0	6	25	73

Colonies from agar plates were resuspended in broth and grown for 2 hours in tryptic soy broth. Log phase cells were resuspended in electrokinetic capture buffer at 7E+5 CFU/mL. A 14 µL sample was pipetted into each flowcell channel, the cassette was placed into the instrument, and the bacteria were electrokinetically concentrated and immobilized on the flowcell surface.

Each flowcell contained approximately 200 to 500 founder cells on the surface in the microscope's 444 x 592 µm field of view. ***Fig. 2*** shows a flowcell layout. After capture, the flowcells were washed with cation-adjusted Mueller-Hinton broth (MHB), removing the capture buffer.

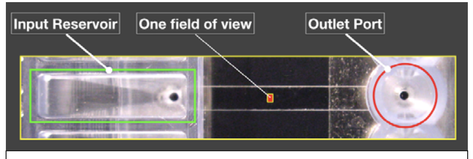


Figure 2: *One flowcell in a 32-channel fluidic cassette.*

The system performed 6 concurrent assays in separate flowcells for each isolate, using two 3rd-generation cephalosporins (3GCs), plus a control for the enzyme inhibitor alone. The 6 test conditions were —

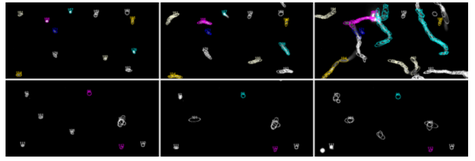
- Growth control (nutrient medium only).
- β-lactamase inhibitor control, 4 µg/mL clavulanic acid (CA) only.
- 256 µg/mL cefotaxime (CTX).
- 256 µg/mL CTX with 4 µg/mL CA (CTX/CA).
- 256 µg/mL ceftazidime (CAZ).
- 256 µg/mL CAZ with 4 µg/mL CA (CAZ/CA).

The instrument acquired darkfield images in each flowcell at 10-minute intervals for 3 hours. It performed a clone-by-clone analysis for one field of view in each flowcell and constructed a population model for each isolate under each test condition.

The system classified ESBL status by comparing growth in each 3GC with or without CA. Inhibition was defined as the population mass in 3GC alone exceeding 5 times that for the same isolate in 3GC/CA in any observation interval. The system classified an isolate as ESBL-positive if the mass ratio exceeded 5 for either drug at any time.

RESULTS

Optimization studies determined that a high 3GC concentration was necessary to detect β-lactamase activity in positive isolates having high MICs (≥256 µg/mL). Studies also determined that β-lactamase activity was detected with low-MIC (≤8 µg/mL) isolates using the same concentration. In the low-MIC ESBL-positive example of ***Fig. 3***, cells began to lyse in CTX later than 160 minutes, but the MIC was 4 µg/mL.



T = 0 min. ***MR = 1.6*** | ***T = 80 min.*** ***MR = 3.2*** | ***T = 160 min.*** ***MR = 14.1***

Figure 3: *ATCC 700603.* ***Top:*** *CTX 256 µg/mL.* ***Bottom:*** *CTX 256 µg/mL + CA 4 µg/mL. Each frame is 74x148 µm (4% of one field of view), zoomed to show detail of individual clones and the image analyzer clone assignments of cells (color) during growth. MICs were CTX 4 µg/mL and CAZ 64 µg/mL (BMD).* ***MR*** *is the mass ratio measured at the designated times.*

Fig. 4 shows growth curves for a low-MIC ESBL-positive and an ESBL-negative strain. The curve flattening of the ESBL-positive strain indicates the start of delayed response to the high drug concentration (6 dilutions >MIC) The average maximum mass ratio of ESBL-negative isolates was 1.9 ± 1.3 s.d. while the range for ESBL-positives was 3.6 to >200. Isolates were correctly characterized in 51 of the 53 ESBL-positive (sensitivity 96% [CI 86%-99%]) and of the 70 of the 73 ESBL-negative (specificity 96% [CI 88%-99%]) isolates.

Tests ended 3 hours after bacterial capture. However, ESBL-positive isolates frequently exceeded the criterion significantly earlier than the endpoint (as in ***Fig. 4***). The median time-to-result was under 2 hours.

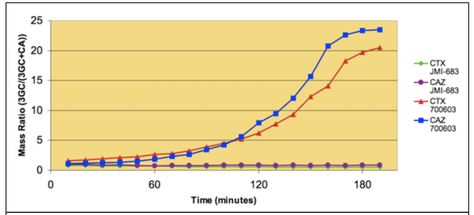


Figure 4: *ESBL-positive (700603) and -negative (JMI-683) strains. JMI-683 MICs were 0.5 µg/mL for both CAZ and CTX (BMD).*

CONCLUSIONS

The experimental method met the objectives of using a small number of cells, achieving rapid results, and having accuracy similar to that of standard tests in identifying the ESBL phenotype. 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 the ESBL phenotype using a single challenge concentration of each antibiotic. The analytical speed of the automated system was consistent with that required for guiding initial empiric therapy in critically ill patients, if combined with direct bacterial extraction and immobilization.[2,3]

References

1. Metzger S., *et al.* 2008. Direct Identification of MRSA and MLS$_B$ Phenotypes in *Staphylococcus aureus* Using Small Numbers of Immobilized Cells. 108th ASM General Meeting, Poster C-005.
2. Hance K., *et al.* 2007. Rapid Identification of Live *Acinetobacter* spp. in Bronchoalveolar Lavage Specimens by Automated Immunofluorescence Microscopy. 47th ICAAC, Poster K-392.
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.