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Rapid Antimicrobial Susceptibility Testing Using Forward Laser Light Scatter Technology

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The delayed reporting of antimicrobial susceptibility testing remains a limiting factor in clinical decision-making in the treatment of bacterial infection. This study evaluates the use of forward laser light scatter (FLLS) to measure bacterial growth for the early determination of antimicrobial susceptibility. Three isolates each (two clinical isolates and one reference strain) of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were tested in triplicate using two commercial antimicrobial testing systems, the Vitek2 and the MicroScan MIC panel, to challenge the BacterioScan FLLS. The BacterioScan FLLS showed a high degree of categorical concordance with the commercial methods. Pairwise comparison with each commercial system serving as a reference standard showed 88.9% agreement with MicroScan (two minor errors) and 72.2% agreement with Vitek (five minor errors). FLLS using the BacterioScan system shows promise as a novel method for the rapid and accurate determination of antimicrobial susceptibility.

Rapid, accurate identification and antimicrobial susceptibility testing (AST) is necessary for optimal clinical decision-making and resource utilization. Timely antibiotic therapy is particularly critical to outcomes in patients with sepsis (1), because each hour of delay within the first 6 h for patients with septic shock results in a 7.6% decrease in survival (2). Long turnaround times (TAT) for either identification or AST result in empirical rather than pathogen-specific therapy. As a result, patients are often given empirical broad-spectrum antibiotics based on clinical suspicion and epidemiology.

Delays in both pathogen identification and AST have led to inappropriate or unnecessary antibiotic use in 20 to 50% of patients in acute-care hospitals in the United States (3–8). Such care provides uncertain clinical benefit and may increase the risk for unexpected side effects, such as *Clostridium difficile* infection, hepatotoxicity, cardiac arrhythmias, or hemolysis (9–12). Standard identification and susceptibility methods based on traditional phenotype testing in the past typically required 24 to 72 h, with an average TAT of 40 h as reported by Kerremans et al. (13). Fortunately, TATs have decreased due to advances in technology. For example, in a recent report by Machen et al., the average TAT for both identification and AST was 11.4 h using the lysis-filtration method for both Vitek MS and VITEK2 compared with 56.3 h for conventional methods (14).

Molecular-based platforms are an increasingly popular option that offers rapid TAT for the detection of genes such as *mecA*, which confers antibiotic resistance to methicillin-resistant *Staphylococcus aureus* (MRSA). These assays may provide results in less than 2 h (15–17). However, molecular detection of the gene targets is far from a perfect solution, targeting only select genetic loci, typically responsible for only a fraction of important resistance patterns. In addition, findings are not always specific. For example, methicillin resistance by genotyping does not always indicate phenotypic resistance. This is evidenced by the finding of "*mecA* dropout" or "empty cassette" strains (17, 18).

There is a widespread effort to improve pathogen-specific antibiotic use. In order to support this goal, the Centers for Disease Control and Prevention (CDC) have recommended that all acute-care hospitals implement antibiotic stewardship programs (ASPs) (8). The goal of ASPs is to optimize appropriate antimicrobial treatment, thereby reducing adverse outcomes (19–21) and preventing further antimicrobial resistance. Technologies have evolved to address these needs, and they include not only molecular methods but also methods based on the use of microfluidics, cell lysis detection, mass spectrometry, rapid cytometry, isothermal microcalorimetry, and magnetic bead rotation, among others (22–25). Forward laser light scatter (FLLS) is another promising emerging technology that depends on electro-optical technology to measure bacterial growth prior to visual assessment. It has the potential of offering the sensitivity of phenotypic methodology with the rapid TAT of a molecular test. This study provides initial proof-of-principle data regarding the use of FLLS technology for AST testing, comparing accuracy and TAT to those of conventional AST systems.

MATERIALS AND METHODS

Study design. Three isolates each of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were used, including two delinked clin-

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ical isolates and one reference strain. All isolates were coded and tested blindly in triplicate using FLLS and in singlet using two commercial AST systems. The commercial systems used were the automated Vitek2 (bioMérieux, Marcy l'Etoile, France) and the manual MicroScan (Siemens, Erlangen, German). The commercial AST systems were compared to FLLS using the BacterioScan 216R system (BacterioScan, Inc., St. Louis, MO). Errors were characterized using standard definitions as described in the CLSI M23-A3 document as very major, major, and minor (26).

Cation-adjusted Mueller-Hinton broth (CAMHB) was used to dilute the stock solution of each antimicrobial using a 2-fold increment dilution scheme with concentrations based on CLSI guidelines (M100-S25) (27). Bacterial inocula were prepared as follows. Isolated colonies were picked from an 18- to 24-h agar plate and suspended in saline to achieve a turbidity equivalent to a 0.5 McFarland standard. Within 15 min of preparation, this suspension was further diluted 1:150 in saline to give an approximate concentration of 1×10^6 CFU/ml of organism. A final 2-fold dilution occurred when 1 ml of the adjusted inoculum was added to each tube containing 1 ml of antimicrobial agent in the dilution series. The final volume for each of the bug-drug combination dilutions was 2 ml. The 2-ml aliquot was then used for inoculation of the BacterioScan system. Broth microdilution using the MicroScan panel system and MIC testing using the Vitek2 system were performed at Diagnostic Laboratory Services (DLS). BacterioScan FLLS system testing was performed at the St. Jude Children's Research Hospital.

Forward laser light scatter system. There is a long history of using laser light scatter instrumentation to study microbiological samples, including the effects of antibiotics (28–31). The BacterioScan system uses a laser light source to measure a liquid sample's optical density (OD) as well as the scattered intensity in a direction that passes near the laser beam. This low-angle forward scattered signal allows the instrument to approximate the sample's OD value to levels significantly lower (1 or 2 orders of magnitude) than those obtainable using a simple ratiometric transmittance type measurement. The BacterioScan system uses a low-power laser (typically in the visible wavelength range, i.e., 400 to 700 nm) passing through approximately 25 mm of a liquid sample in a specially designed cuvette to minimize noise signals from various sources. Since typical bacterial samples have sizes in the 100- to 10,000-nm range, the sample size is comparable to the light wavelength; thus, Mie scattering theory is appropriate. Mie scattering theory is named after its developer, German physicist Gustav Mie, and refers to a series solution of Maxwell's equations for scattering by spheres or by infinite cylinders or other geometries where one can write separate equations for the radial and angular dependence of solutions. The formalism is generally used to calculate either how much light is scattered, the total optical cross section, or where it goes in the form factor. The laser beam transmitted through the sample and the low-angle forward-scattered signals are captured on a CMOS 2-dimensional camera sensor. The instrument mathematically processes the sensor image to minimize background noise and clutter, calibrates the measured values against predetermined baselines, and generates values for organism density. Multiple measurements over time are compared to calculate growth rates and generation times.

The BacterioScan model 216 tabletop instrument used for this study uses light scattering to measure the concentration of particles in a liquid sample and can be used to estimate the density of microorganisms in a liquid sample. Initially developed for measurement of actively growing organisms in urine, the instrument performs reliably down to a minimum density of 10,000 CFU/ml, which is commonly considered a diagnostic threshold for bacteriuria. Therefore, the laser microbial growth monitor (LMGM) technology has potential for other applications, such as AST. The system capacity accommodates up to 16 samples, which can be run simultaneously with a volume of 2 to 3 ml each. Samples are maintained at a thermostatically controlled elevated temperature to promote growth (onboard incubation), and repeated measurements are automatically collected at approximately 3-min increments to provide real-time continuous measurement of organism growth.

In the present study, each bacterial strain was tested using serial dilutions of three antimicrobials. Antimicrobials tested by FLLS depended on the organism: for *S. aureus*, clindamycin (0.25 to 4 μg/ml), moxifloxacin (0.5 to 4 μg/ml), and oxacillin (0.25 to 4 μg/ml); for *E. coli*, cefepime (2 to 32 μg/ml), ciprofloxacin (0.5 to 4 μg/ml), and gentamicin (1 to 16 μg/ml); for *P. aeruginosa*, cefepime (2 to 32 μg/ml), ciprofloxacin (0.5 to 4 μg/ml), and gentamicin (1 to 16 μg/ml). After inoculation, growth was measured by FLLS for 24 h at 36.7 to 37.3°C, with light scatter plotted against incubation time. The MIC of each antimicrobial corresponded to the lowest concentration at which all three replicate inocula showed growth inhibition, as defined below (see the Statistical Analysis section). Interpretation of MIC results as susceptible (S), susceptible-dose dependent (SDD), intermediate (I), and resistant (R) were based on CLSI guidelines (27).

Categorization of errors. According to standard terminology listed in CLSI M23-A3, errors were classified as very major, major, and minor. A very major error (VME) occurred when the reference method result was resistant and the test method result was susceptible. A major error (ME) occurred when the reference method result was susceptible and the test method result was resistant. A minor error (mE) occurred when the reference method result was either susceptible or resistant with a test method result of intermediate, or the reference method result was intermediate with a test method result of susceptible or resistant (26).

Statistical analysis. For each sample (representing one replicate of a given bacterial strain at a single concentration of antimicrobial), a dense series of OD measurements was obtained over time. The area under the curve (AUC) of the OD measurements, as a function of time, next was computed for each sample. For each treated sample, the relative area under the curve (rAUC) was computed as the ratio of the complete AUC (cAUC) of the treated sample to the cAUC of the control sample. For each set of three replicates of a particular organism strain treated with a particular agent, bacteria were defined to be inhibited if all three rAUC values were less than or equal to 0.10. In this way, for each organism-agent pair, a series of inhibition calls was obtained as a function of agent dose. The MIC was defined as the lowest dose at which an inhibition call was made. For each treated sample among a set of three replicates declared as inhibited, the partial area under the curve until each time point, pAUC(t), was calculated. For each of these treated samples, the relative partial area under the curve from each time point t, rpAUC(t), was further determined as the ratio of the pAUC(t) for the treated sample to the untreated sample. Time to inhibition was defined as the earliest time point, T, at which all calculated rpAUC(t) values of the set of three replicates were less than 0.10 for all subsequent time points ($t \geq T$). Figures S1 and S2 in the supplemental material illustrate the calculation of the variables used in the statistical analysis. All analyses were performed using SAS, Windows version 9.3.

RESULTS

There were 360 experimental samples and 120 control samples collected for 27 drug-bacterium pairs. Strong bacterial growth was observed for all 120 control samples. One experimental sample was dropped from analysis for *E. coli* 9992 with cefepime at a concentration of 4 μg/ml, due to insufficient length (duration) of data collection. A total of 27 drug-bacterium pairs were investigated, and 19 (70%) revealed inhibition for at least one tested concentration of antimicrobial (Table 1). The remaining 8 drug-bacterium pairs gave a MIC greater than the highest tested concentration of antimicrobial. Data quality was adequate for interpretation for all drug-bacterium pairs tested. The MICs for *S. aureus*, *E. coli*, and *P. aeruginosa* were similar for cefepime, ciprofloxacin, and gentamicin on all three platforms, BacterioScan, Vitek, and MicroScan (Table 1). The expected MICs on Vitek and MicroScan are shown for the ATCC reference strains. Between Vitek and MicroScan, the only disparate results (showing

TABLE 1 MIC from BacterioScan, MicroScan, and Vitek

Bacterium, ID no., and antibiotic	MIC in μg/ml (result) by:			AST interpretive criteria (μg/ml)				Quality control range[b] (MIC, in μg/ml)
	BacterioScan	MicroScan	Vitek[a]	S	SDD	I	R	
E. coli (ESBL)								
3267								
Cefepime	32 (R)	8 (SDD)	No MIC (R)	≤2	4–8		≥16	
Ciprofloxacin	>8 (R)	>2 (R)	≥4 (R)	≤1		2	≥4	
Gentamicin	≤4 (S)	2 (S)	≤1 (S)	≤4		8	≥16	
9992								
Cefepime	>64 (R)	>16 (R)	No MIC (R)	≤2	4–8		≥16	
Ciprofloxacin	>8 (R)	>2 (R)	≥4 (R)	≤1		2	≥4	
Gentamicin	≤4 (S)	≤1 (S)	≤1 (S)	≤4		8	≥16	
P. aeruginosa								
2700								
Cefepime	32 (R)	>16 (R)	≥64 (R)	≤8		16	≥32	
Ciprofloxacin	≤1 (S)	≤0.5 (S)	No MIC (I)	≤1		2	≥4	
Gentamicin	≤4 (S)	4 (S)	≤1 (S)	≤4		8	≥16	
9018								
Cefepime	64 (R)	16 (I)	16 (I)	≤8		16	≥32	
Ciprofloxacin	2 (I)	2 (I)	≥4 (R)	≤1		2	≥4	
Gentamicin	32 (R)	>8 (R)	8 (I)	≤4		8	≥16	
S. aureus (MRSA)								
3032								
Clindamycin	>8 (R)	≥4 (R)	≥8 (R)	≤0.5		1–2	≥4	
Moxifloxacin	>8 (R)	4 (R)	≥8 (R)	≤0.5		1	≥2	
Oxacillin	>8 (R)	≥2 (R)	≥4 (R)	≤2			≥4	
6172								
Clindamycin	>8 (R)	≥4 (R)	≥8 (R)	≤0.5		1–2	≥4	
Moxifloxacin	4 (R)	2 (R)	1 (I)	≤0.5		1	≥2	
Oxacillin	>8 (R)	≥2 (R)	≥4 (R)	≤2			≥4	
E. coli (ATCC)								
25922								
Cefepime	≤4	≤2	≤1					0.015–0.12
Ciprofloxacin	≤1	≤0.5	≤0.25					0.004–0.015
Gentamicin	≤4	≤1	≤1					0.25–1.0
P. aeruginosa (ATCC)								
27853								
Cefepime	≤4	4	≤1					0.5–4.0
Ciprofloxacin	≤1	≤0.5	≤0.25					0.25–1.0
Gentamicin	≤4	2	≤1					0.5–2.0
S. aureus (ATCC)								
29213								
Clindamycin	≤1	0.5	≤0.25					0.06–0.25
Moxifloxacin	≤1	≤2	≤0.25					0.015–0.12
Oxacillin	≤1	≤0.25	0.5					0.12–0.5

[a] No MIC refers to an expert interpretation using the Vitek2 instrument.
[b] Ranges for cation-adjusted Mueller-Hinton broth (see Table 5A in reference 27).

>2 dilution difference) were seen for the *P. aeruginosa* isolate 27853 (cefepime) and for the *S. aureus* isolate 29213 (moxifloxacin).

A comparison of the interpretative results across the platforms is included in Tables 1 and 2. The overall agreement between BacterioScan and Vitek was 72.2% (13/18), and the overall agreement between BacterioScan and MicroScan was 88.9% (16/18). There was one minor error (mE) for the *E. coli* 3267 extended-spectrum beta-lactam (ESBL), where BacterioScan indicated that the organism was resistant to cefepime but MicroScan

TABLE 2 Summary of minor errors[a]

Test comparison	ID no.	Bacterium	Antibiotic
BacterioScan vs. MicroScan	3267	*E. coli*	Cefepime
BacterioScan vs. MicroScan	9018	*P. aeruginosa*	Cefepime
BacterioScan vs. Vitek	9018	*P. aeruginosa*	Cefepime
BacterioScan vs. Vitek	9018	*P. aeruginosa*	Gentamicin
BacterioScan vs. Vitek	2700	*P. aeruginosa*	Ciprofloxacin
BacterioScan vs. Vitek	9018	*P. aeruginosa*	Ciprofloxacin
BacterioScan vs. Vitek	6172	*S. aureus*	Moxifloxacin

[a] No very major or major errors were seen.

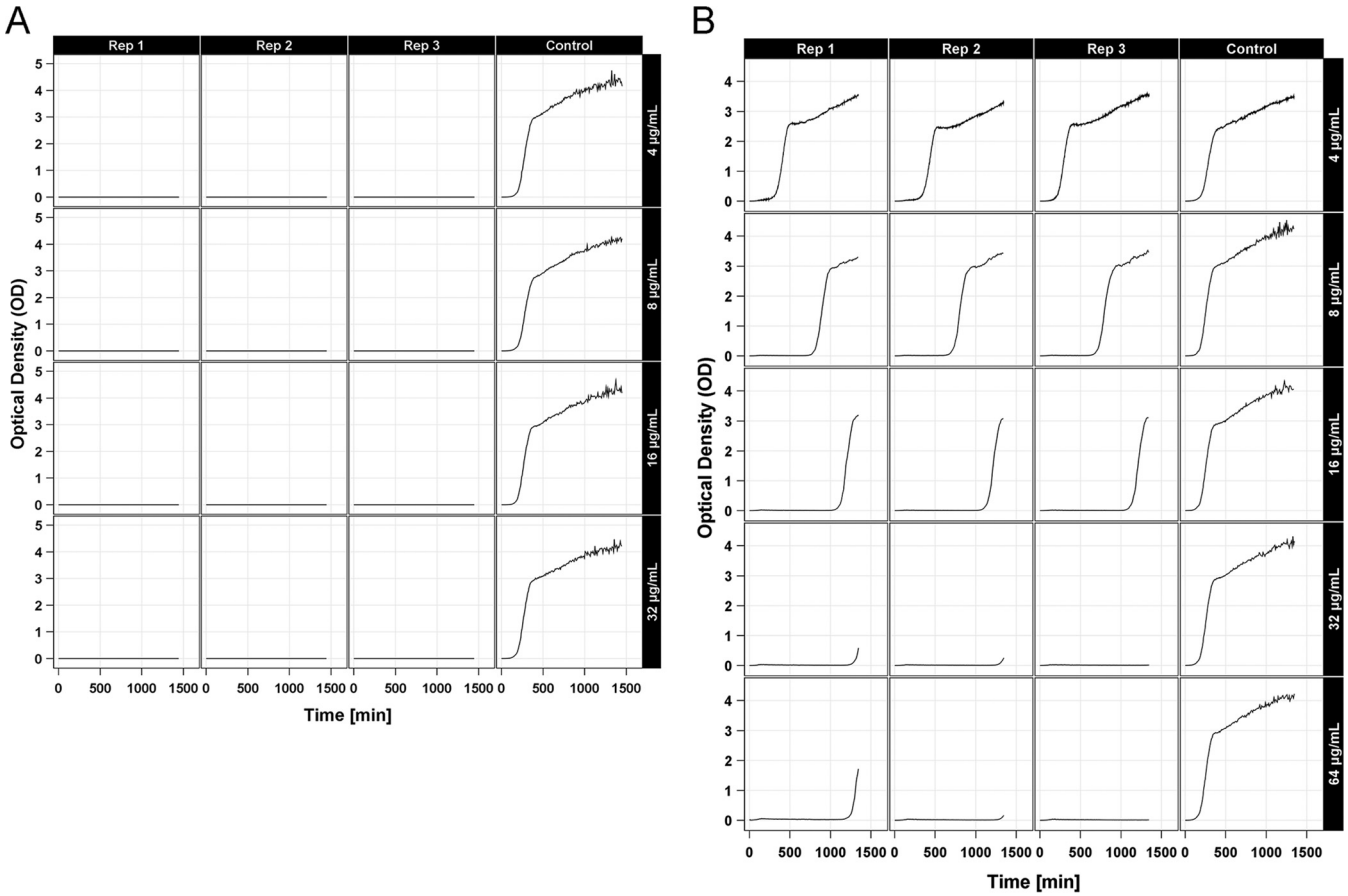

FIG 1 Optical density (OD) plots for treated replicates and controls for *E. coli* 3267 with gentamicin (A) and cefepime (B).

indicated it was susceptible, dose dependent. There was also one mE with cefepime for *P. aeruginosa* 9018 where the MicroScan result was intermediate and the BacterioScan result was resistant. There was a high degree of concordance for bacterial resistance with Vitek with only five mEs (Tables 1 and 2). These mEs occurred with ciprofloxacin for *P. aeruginosa* 2700 and 9018, with cefepime and gentamicin for *P. aeruginosa* 9018, and with moxifloxacin for *S. aureus* 6172.

Optical density plots for *E. coli* 3267 are shown in Fig. 1 for both gentamicin (susceptible) and cefepime (resistant). The triplicate growth curves for cefepime at 4 μg/ml were identical to the control with no antimicrobial agent. It should be noted that as the dose of cefepime increased, the growth curves shifted to the right until growth was inhibited with a cefepime dose of 32 μg/ml. The triplicate growth curves for all concentrations of gentamicin show no growth, while the control with no antimicrobial agent showed rapid growth. The remainder of the optical density plots can be seen in the supplemental material (see Fig. S3).

The distribution of time to inhibition for *E. coli*, *P. aeruginosa*, and *S. aureus* is shown in Fig. 2. The minimum time to inhibition was 32 min for *S. aureus*, and the maximum time to inhibition was 1,014 min for *P. aeruginosa*. Note that 80% of organisms were inhibited within 346 min (less than 6 h), and 95% of organisms were inhibited within 598 min (less than 10 h). There was more variability in the time to inhibition for *P. aeruginosa* with more than 20% of *P. aeruginosa* organisms inhibited beyond 350 min (Fig. 2 and Table 3).

DISCUSSION

This study demonstrates proof of principle for the use of forward laser light scatter to rapidly determine MICs for several bacterial isolates of high clinical importance. Representative Gram-positive and Gram-negative pathogens, tested against a variety of antimicrobial agents, demonstrated close agreement with

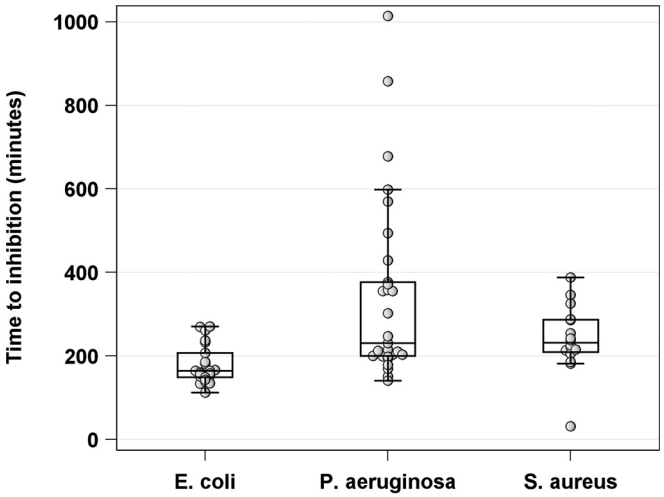

FIG 2 Distribution of time to inhibition.

TABLE 3 Summary of starting times for inhibition (minutes)

Bacterium	Starting time (min) by % inhibition					
	0% (minimum)	50% (median)	80%	90%	95%	100% (maximum)
Overall	32	211	346	429	598	1,014
E. coli	112	164	233	262	270	271
P. aeruginosa	141	231	494	678	858	1,014
S. aureus	32	232	288	346	388	388

results achieved by more conventional testing modalities, with 7 categorical errors (all minor errors). Results were obtained for most organism-drug pairs in less than 6 h. These results suggest promise for this technology to markedly reduce the time to susceptibility testing results in the clinical laboratory.

While a fairly stringent metric was used to determine MIC, in most cases the drop-off at MIC was marked and could be determined by casual visual inspection of the light scatter curves, or by using a much more liberally defined cutoff, without affecting results. However, there were some cases where this was not observed. Moreover, the rapidity of time to result was also not completely uniform. Although most isolates produced rapid results, 5% required greater than 10 h and rarely up to 12 h. *Pseudomonas aeruginosa* appeared to take the longest to produce a result, with one bug-drug combination requiring nearly 17 h to generate a final MIC. The latter finding may represent a weakness of the system, in that a potentially significant proportion of isolates may require read time approaching that of conventional methods. However, most results easily challenged the longer conventional MIC turnaround time (TAT). Given that accuracy and reproducibility were maintained and no read times exceeded those of conventional methods, one could envision using such a system for a majority of testing and reserving conventional MIC testing for use as a reflex method.

Several methods for rapid AST have been described in the literature (22–25). Those in clinical use are primarily genotypic and sensitive and offer a rapid TAT. However, correlation between genotype and phenotype is imperfect, and as genotypic mechanisms of resistance continue to rapidly evolve, it is difficult for manufacturers to keep pace with new assays. Moreover, such modalities target relatively few genes in a well-defined subset of bacteria for which clear genotypic mechanisms are responsible for a large number of cases of clinically significant antibiotic resistance. Therefore, it remains unlikely that even a majority of bacterium-drug-mechanism combinations can be detected through clinical genotypic tests. On the other hand, rapid phenotypic tests have been increasingly seen as having the potential to decrease TAT while not compromising sensitivity of resistance detection or clinical correlation. FLLS is one of the newest entries in this field, and the data here suggest that this technology will prove to be a flexible and accurate alternative to broth microdilution or other traditional methods.

This pilot study was limited by the narrow selection of bacteria and antibiotics used and the fact that only a few clinical strains of each bacterial species were included in the testing. Further studies with a broader range of bacterial strains, antimicrobials, and previously defined genotypes will be necessary to assess the applicability of this method, relative error rates, and the potential advantage in TAT. Unlike some methods, this technique is not inherently limited by the number of optical channels, the production and validation of specific probes, or the definition of proteomic profiles or other surrogate markers of metabolism or growth. It may well be amenable to improving the speed of MIC for determination in a wide variety of clinical settings. This would include routine Gram-positive and -negative isolates as well as even more challenging bacteria, such as nontuberculous mycobacteria (NTM).

We used AUC metrics to quantitatively characterize the observed growth patterns in this study. Future research that explores the utility of other quantitative metrics of the growth patterns may yield algorithms for calling resistance or sensitivity that improve clinical performance. A practical limitation of the AUC metric is that it cannot be computed until after the entire series has been acquired. A more practical metric for real-time utilization may be the time until the OD measurements exceed a specific threshold. Future research should seek to determine the best threshold value.

The speed and accuracy of this innovative system holds great potential for clinical use in expediting AST results, improving the choice of appropriate antimicrobial treatment, and supporting antibiotic stewardship. Findings reported in this study suggest a broader evaluation of clinical isolates and antimicrobials to further explore the correlation between current methods of MIC determination and rapid phenotypic testing by FFLS. In addition, clinical outcome studies will be critical to demonstrate the potential clinical impact of such methods on clinical decision making, resource utilization, and clinical outcome.

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