Stop waiting for tomorrow: Disk diffusion performed on early growth is an accurate method for antimicrobial susceptibility testing with reduced turnaround time

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Stop Waiting for Tomorrow: Disk Diffusion Performed on Early Growth Is an Accurate Method for Antimicrobial Susceptibility Testing with Reduced Turnaround Time

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ABSTRACT Disk diffusion is a slow but reliable standard method for measuring the antimicrobial susceptibility of microorganisms. Our objective was to improve the turnaround time for this method by reducing the time that cultures are incubated before setting up disk diffusion testing. For initial method development, clinical isolates (n = 13) and quality control strains (n = 8) of bacteria were inoculated on blood agar and were incubated at 35°C for either 6, 10, or 24 h before performing disk diffusion testing, in triplicate, using a panel of clinically appropriate antimicrobial agents. Disk diffusion zone sizes were interpreted using Clinical and Laboratory Standards Institute (CLSI) guidelines. Compared to standard 24 h of incubation, early 6-h growth had 1.3% major errors (MEs) and 1.9% very major errors (VMEs), whereas 10-h growth yielded 0.7% MEs and no VMEs. Categorical agreement with standard incubation was similar for both 6 h (96.7%) and 10 h (96.7%) growth. Inhibitory zone size from 6 h (r² = 0.98) and 10 h (r² = 0.99) growth correlated well with results from standard conditions. Based on these results, we performed disk diffusion under optimized conditions (6 h growth), using 100 additional clinical isolates, demonstrating a high level of categorical agreement (917 of 950 measurements [96.5%]; 95% confidence interval [CI], 95.2 to 97.5%), as well as no VMEs or MEs. Using early growth for disk diffusion testing is a simple and accurate method for susceptibility testing that can reduce time to results by as much as 18 h, compared to standard incubation, with no additional supply costs or equipment/instrumentation.

KEYWORDS disk diffusion, rapid susceptibility, antimicrobial susceptibility testing

Disk diffusion is a method for measuring antimicrobial susceptibility that was first standardized by Bauer et al. in 1966 (1). This method is simple, reproducible, and reliable; and the cost of supplies and materials is low. In addition, Kirby-Bauer disk diffusion provides the laboratory with a great deal of flexibility; different “panels” can be created by the laboratory that are responsive to the hospital formulary, local resistance trends, and antibiogram. Furthermore, when new antimicrobials are approved for clinical use, disk diffusion is frequently the first type of susceptibility method that is cleared and available for clinical laboratories to use (2–6).

Despite its strengths, one potential downside to using disk diffusion testing as a routine antimicrobial susceptibility test (AST) method is that it is relatively slow. The Clinical and Laboratory Standards Institute (CLSI) specifies that the inoculum for the disk diffusion test (0.5 McFarland standard from direct colony suspension) should be prepared from colonies on a nonselective agar plate that have been incubated for 18 to 24 h (7). Depending on the organism-antimicrobial combination, the test is then incubated for an additional 16 to 24 h before reading and interpretation (7). CLSI also describes a broth culture method (growth method) that can be used with nonfastidious organisms other than staphylococci. This method allows isolates to be grown in broth culture to an optical density of 0.5 McFarland (usually 2 to 6 h) before streaking the solution onto Mueller-Hinton agar for AST. The use of

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this method with standard breakpoints hints at the potential for using early bacterial growth for AST.

The recommendation to use 18- to 24-h growth to prepare the initial testing inoculum is largely predicated on the norms of the human workday and the fact that historically most clinical microbiology laboratories were fully operational only during the "day shift." Thus, cultures would be set up or subcultured then incubated overnight before performing AST the following day. As the norms of microbiology testing are changing, it is time to reevaluate the need for 18 h of culture incubation before setting up AST. Reducing the time interval prior to test setup would be a relatively easy and very inexpensive way to provide AST results faster and could be well-suited to AST testing of positive blood cultures, where the time to results can be of critical importance.

In addition, with the deployment of total laboratory automation systems in clinical microbiology, culture plates are transported immediately to incubators, where they remain for the duration of incubation; as a result, colonies are grown more quickly, and cultures are ready for workup sooner (8–10). Thus, with "early" culture reading using total laboratory automation, there is also a desire to set up disk diffusion testing at earlier time points (referred to as early disk diffusion [EDD] testing) (10–12).

While there is a clear rationale for setting up disk diffusion testing at earlier time points, there is also promising work showing that the incubation period for disk diffusion testing can be shortened without a significant impact on susceptibility results (10, 13–19). Unfortunately, these modified protocols have yet to be fully standardized by or incorporated into the CLSI AST recommendations for disk diffusion. Here, we have focused on reducing the 18 to 24 h of incubation interval that precedes disk diffusion testing. Thus, our aim was to compare susceptibility results performed on isolates following 6-h (EDD6) and 10-h subculture (EDD10) and compare this with standard disk diffusion testing performed on standard 24 h growth (St24).

**MATERIALS AND METHODS**

**Bacterial isolates and culture medium.** We selected resistant and susceptible strains of bacteria that are representative of bacteria commonly encountered in the clinical microbiology laboratory, including 13 clinical isolates and 8 quality control (QC) strains. Representative organisms included six *Staphylococcus aureus* (two methicillin-susceptible QC strains [ATCC 29213 and ATCC 25923] and four methicillin-resistant clinical strains), three *Enterococcus faecium*, three *Enterococcus faecalis* (ATCC 29212 and ATCC 51299), three *Escherichia coli* (ATCC 25922 and ATCC 35218), three *Pseudomonas aeruginosa* (ATCC 27853), two *Klebsiella pneumoniae* (ATCC BAA-1705), and one *Enterobacter cloacae*.

**Disk diffusion testing.** Subcultures were prepared by quadrant streaking three to five colonies of each clinical isolate or QC strain onto blood agar plates (Hardy Diagnostics, Santa Maria, CA), or in the case of the validation experiments, a 100-μL suspension was prepared at a concentration equal to 0.5 McFarland standard from each of the 100 clinical isolates and was struck onto a blood agar plate. The plates were inverted and incubated at 35 ± 2°C in a Thermo Scientific (Waltham, MA, USA) Forma 3960 incubator for either 6, 10, or 24 h and were then used to make a bacterial suspension equal to 0.5 McFarland standard as measured within the range of 0.5 to 0.63 by a Densichek Plus (bioMérieux) turbidimeter. This suspension was prepared from three to five individual colonies or from a small area of growth in cases in which the colonies were indistinct (e.g., EDD6). Each bacterial suspension was inoculated by sterile cotton swab onto a 150-mm Mueller-Hinton agar plate (Hardy Diagnostics) and was spread into a uniform lawn using a Retro C80 automatic plate inoculator. Excess moisture was allowed to evaporate (3 to 15 min) before applying antimicrobial disks appropriate for the microorganism (Table 1) to the agar surface using a 12-place, BD BBL Sensi-Disc dispenser (Franklin Lakes, NJ); the disks were selected based on availability and were compatible with the BBL dispenser that was used for application. The plates were inverted and incubated at 35 ± 2°C for 16 h in an ambient air incubator or up to 24 h when needed, per CLSI guidelines (2, 7). After incubation, the zone of inhibition around each antibiotic was measured manually by two individuals (the mean of duplicate readings is reported) or by a single individual when reading results from the 100 clinical isolates in the validation study. The process of subculturing, incubating plates, and performing AST was repeated on three separate occasions for each of the 21 clinical and QC isolates. All testing was set up and interpreted per CLSI guidelines (2, 7). The results from each organism/antimicrobial agent were evaluated and reported as an individual result.

**Equations and statistics.** GraphPad Prism 8 and R were used for data analysis (21). A regression line was fit to EDD6, EDD10, and St24 inhibitory zones using least-squares regression. Potential bias in EDD versus St24 was evaluated by generating a Bland-Altman plot of the difference versus mean values from the early and standard disk diffusion methods (22). One sample t test comparing mean differences in bacterial species to a hypothetical mean difference of 0 were used to evaluate mean differences in EDD6, EDD10, and St24 with 95% confidence intervals (CIs). Categorical agreement, minor errors (mEs), major errors (MEs), and very
**TABLE 1** Bacteria and antibiotics evaluated via initial (module 1) and optimized (module 2) testing of early isolate growth

<table>
<thead>
<tr>
<th>Study phase</th>
<th>Staphylococcus</th>
<th>Enterococcus</th>
<th>Enterobacteriales</th>
<th>Acinetobacter</th>
<th>Pseudomonas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Module 1: Clinical isolates (count)</td>
<td>S. aureus (4)</td>
<td>E. faecalis (3), E. faecalis (1)</td>
<td>E. coli (1), K. pneumoniae (1), E. cloacae (1)</td>
<td></td>
<td>P. aeruginosa (2)</td>
</tr>
<tr>
<td>Module 1: QC organisms (accession number)*</td>
<td>S. aureus (ATCC 29213), S. aureus (ATCC 25923)</td>
<td>E. faecalis (ATCC 29212), E. faecalis (ATCC 51299)</td>
<td>K. pneumoniae (ATCC BAA-1705), E. coli (ATCC 25922), E. coli (ATCC 35218)</td>
<td></td>
<td>P. aeruginosa (ATCC 27853)</td>
</tr>
<tr>
<td>Module 2: 100 clinical isolates (count)</td>
<td>MRSA (10), MSSA (10)</td>
<td>VRE (10)(^\text{a}), VSE (10)(^\text{a})</td>
<td>E. coli (10), K. pneumoniae (10), E. cloacae (10)</td>
<td></td>
<td>A. baumannii (10)</td>
</tr>
<tr>
<td>Module 1 and 2: antibiotics (disk mass)*</td>
<td>Ciprofloxacin (5 μg), linezolid (10 μg), nitrofurantoin (300 μg), rifampin (5 μg), cefoxitin (30 μg), cefotaxime (30 μg), clindamycin (2 μg), delafloxacin (5 μg), erythromycin (15 μg), TMP/SMX (25 μg)</td>
<td>Ciprofloxacin (5 μg), linezolid (30 μg), nitrofurantoin (300 μg), rifampin (5 μg), ampicillin (10 μg), chloramphenicol (30 μg), doxycycline (30 μg), vancomycin (30 μg)</td>
<td>Cefepime (30 μg), ceftazidime (20 μg), ceftazidime-avibactam (30/20 μg), ciprofloxacin (5 μg), gentamicin (10 μg), meropenem (10 μg), piperacillin tazobactam (100/10 μg), ceftriaxone (30 μg), cefazolin (30 μg), ceftriaxone (30 μg), nitrozofuran (300 μg), TMP/SMX (25 μg)</td>
<td></td>
<td>Cefepime (30 μg), ceftazidime (20 μg), ceftazidime-avibactam (30/20 μg), ciprofloxacin (5 μg), gentamicin (10 μg), meropenem (10 μg), piperacillin tazobactam (100/10 μg), ceftriaxone (30 μg), TMP/SMX (25 μg)</td>
</tr>
</tbody>
</table>

*QC, quality control; MRSA, methicillin-resistant *S. aureus; MSSA, methicillin-susceptible *S. aureus; TMP/SMX, trimethoprim-sulfamethoxazole.

\(^{a}\)VRE, vancomycin-resistant *Enterococcus, including *E. faecium (n = 10).

\(^{b}\)VSE, vancomycin-susceptible *Enterococcus, including *E. faecalis (n = 3) and *E. faecalis (n = 7).

\(^{c}\)Antibiotics supplied by BD Biosciences in optimization study: cefepime, ciprofloxacin, linezolid, meropenem, piperacillin tazobactam, gentamicin. Antibiotics supplied by BD Biosciences in confirmatory study: cefepime, doxycycline, linezolid, meropenem, piperacillin tazobactam, rifampin. Antibiotics supplied by Oxoid in optimization study: cefoxitin. All other antibiotic disks were from Hardy Diagnostics.

**RESULTS**

**Isolate growth.** Examination of blood agar plates incubated for 6 h revealed scant growth, primarily in the first one or two quadrants, while blood agar plates incubated for 10 h demonstrated growth in all four quadrants, as well as the presence of isolated colonies in most cases (representative images are displayed in Fig. 1). Despite limited growth after 6 h, there were adequate bacteria on 6-h plates to make a 0.5 McFarland standard suspension for all 603 disk diffusion tests (Table 1).

**Quality control.** The disk diffusion results for *S. aureus* QC isolates were acceptable per CLSI standards in 28 of 30 (93.3%) of EDD6, 28 of 30 (93.3%) of EDD10, and 27 of 30 (90%) of St24. QC results were abnormal for two replicates of EDD6 growth tested with nitrofurantoin, two replicates of EDD10 growth tested with delafloxacin, two replicates of St24 growth tested with nitrofurantoin, and one replicate of St24 evaluated with delafloxacin. Susceptibility results for *E. coli* (ATCC 25922, ATCC 35218), *P. aeruginosa* (ATCC 27853), and *K. pneumoniae* (ATCC BAA-1705) were acceptable for all 69 EDD6 tests, 69 EDD10 tests, and 69 St24 QC tests. Overall, the test results for QC isolates were acceptable, per CLSI standards (2), for 97 of 99 (98%) of EDD6, 97 of 99 (98%) of EDD10, and 96 of 99 (97%) of St24.

**Categorical agreement between methods.** Disk diffusion zones were interpretable for all 603 EDD6 tests, 603 EDD10 tests, and 603 St24 tests. One-third (122 of 366) of clinical tests were resistant to the evaluated antimicrobials under standard conditions. A comparison of AST results for early and standard growth disclosed that 583 of 603 tests (96.7%; 95% CI, 94.9 to 97.8) were concordant between EDD6 and St24, and an equal number of tests (583 of 603) were concordant between EDD10 and St24 (Table 2 and 3). EDD6 had 1.9% VME (95% CI, 0.5 to 5.6%) and 1.3% ME (95% CI, 0.6 to 2.9%) compared to St24 (Table 2), whereas EDD10 had no VME and 0.7% ME (95% CI, 0.2 to 1.9%) (Table 3). The three VME results for EDD6 included a 1-mm difference between EDD6 and St24 for ceftodin inhibition of *S. aureus* and 4- and 6-mm differences between EDD6 and St24 replicates for nitrofurantoin inhibition of *E. cloacae*.

**Quantitative agreement between methods.** A comparison (linear regression) of early versus standard disk diffusion revealed that both EDD6 (\(r^2 = 0.98\)) and EDD10 (\(r^2 = 0.99\)) were highly correlated with disk diffusion testing of early growth (Fig. 2A to C). Bland-Altman
analysis (Fig. 2D) showed a relatively small bias toward lower EDD6 (mean difference, −0.46 mm; 95% CI, −0.56 to −0.35) and EDD10 (mean difference, −0.42 mm; 95% CI, −0.51 to −0.33) compared to the standard disk diffusion (EDD St24). As shown in Fig. 3, the difference between early and standard disk diffusion methods was due to smaller antimicrobial zone sizes in EDD6 among *E. faecium* (mean difference, −0.51 mm; 95% CI, −0.81 to −0.22) and *S. aureus* isolates (mean difference, −1.14 mm; 95% CI, −1.33 to −0.96), as well as smaller antimicrobial zone sizes in EDD10 among *E. coli* (mean difference, −0.70 mm; 95% CI, −0.90 to −0.50), *E. faecalis* (mean difference, −0.22 mm; 95% CI, −0.40 to −0.04), and *S. aureus* isolates (mean difference, −0.81 mm; 95% CI, −0.96 to −0.65).

When the results were evaluated by bacterial species, there was no more than 1-mm difference in median antimicrobial inhibition between EDD6, EDD10, and St24. Of note, five of seven bacterial species had the same median zone size when comparing EDD6 or EDD10 with standard disk diffusion (Fig. 3). Zone sizes for isolate-drug combinations were concordant within ±1 mm (mode) for 67 of 69 (97%) of AST results from EDD6 growth (Table S1a) and 66 of 69 (96%) of results from EDD10 growth (Table S1b) compared to St24.

**Evaluation of clinical strains with optimized methodology.** Based on the results from this initial method optimization, we sought to evaluate the accuracy of 6-h early disk diffusion testing among a larger set of 100 clinically relevant isolates, tested against appropriate antimicrobial panels (isolate-drug combinations shown in Table 1; antibiogram shown in Fig. S1). Overall, 38.1% of the 360 Gram-positive isolate-drug combinations were antibiotic-resistant when tested using standard methods (St24), and 41.9% of the 590 Gram-negative isolate-drug combinations were resistant (St24 method). Comparison of AST results from early (EDD6) and standard growth (St24) revealed a high level of categorical agreement (917 of 950 [96.5%]; 95% CI, 95.2 to 97.5%), as well as a low level of very major errors (0 of 306 [0%]; 95% CI, 0 to 1.2%), major errors (0 of 644 [0%]; 95% CI, 0 to 0.6%), and minor errors (33 of 950...
A comparison of zone sizes for each of the 88 isolate-drug combinations demonstrated that 85 of 88 (96.6%) of isolate-drug combinations had a less than 2-mm median difference between EDD6 and St24, and only three isolate-drug combinations had differences of 2 mm or more, including chloramphenicol-VRE, nitrofurantoin-E. cloacae, and erythromycin-methicillin-susceptible S. aureus (MSSA), which had median differences of 2, 2, and 1.25 mm (EDD6 - St24), respectively.

### TABLE 3 Comparison of disk diffusion testing from 10-h early growth (EDD10) versus standard 24-h growth method with clinical and QC organisms

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Organism</th>
<th>R^a</th>
<th>S^b</th>
<th>Total</th>
<th>Agreement, n/n (%)^c</th>
<th>Very major^d</th>
<th>Major^e</th>
<th>Minor^f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical isolates 10-h incubation</td>
<td>S. aureus</td>
<td>14</td>
<td>106</td>
<td>120</td>
<td>119/120 (99.2)</td>
<td>0/14 (0)</td>
<td>0/106 (0)</td>
<td>0/120 (0)</td>
</tr>
<tr>
<td></td>
<td>E. faecalis</td>
<td>11</td>
<td>13</td>
<td>24</td>
<td>24/24 (100)</td>
<td>0/11 (0)</td>
<td>0/13 (0)</td>
<td>0/24 (0)</td>
</tr>
<tr>
<td></td>
<td>E. faecium</td>
<td>36</td>
<td>36</td>
<td>72</td>
<td>69/72 (95.8)</td>
<td>0/36 (0)</td>
<td>0/36 (0)</td>
<td>3/72 (4.2)</td>
</tr>
<tr>
<td></td>
<td>P. aeruginosa</td>
<td>18</td>
<td>24</td>
<td>42</td>
<td>40/42 (95.2)</td>
<td>0/18 (0)</td>
<td>1/24 (4.2)</td>
<td>1/42 (2.4)</td>
</tr>
<tr>
<td></td>
<td>K. pneumoniae</td>
<td>24</td>
<td>12</td>
<td>36</td>
<td>36/36 (100)</td>
<td>0/24 (0)</td>
<td>0/12 (0)</td>
<td>0/36 (0)</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>9</td>
<td>27</td>
<td>36</td>
<td>35/36 (97.2)</td>
<td>0/9 (0)</td>
<td>0/27 (0)</td>
<td>1/36 (2.8)</td>
</tr>
<tr>
<td></td>
<td>E. cloacae</td>
<td>10</td>
<td>26</td>
<td>36</td>
<td>31/36 (86.1)</td>
<td>2/10 (0)</td>
<td>0/26 (0)</td>
<td>3/36 (8.3)</td>
</tr>
<tr>
<td>Total</td>
<td>122</td>
<td>244</td>
<td>366</td>
<td></td>
<td>354/366 (96.7)</td>
<td>3/122 (2.5)</td>
<td>4/244 (1.6)</td>
<td>9/366 (2.5)</td>
</tr>
<tr>
<td>QC 10-h incubation</td>
<td>S. aureus</td>
<td>0</td>
<td>60</td>
<td>60</td>
<td>58/60 (96.7)</td>
<td>0/0</td>
<td>2/60 (3.3)</td>
<td>0/60 (0)</td>
</tr>
<tr>
<td></td>
<td>E. faecalis</td>
<td>5</td>
<td>43</td>
<td>48</td>
<td>46/48 (95.8)</td>
<td>0/5 (0)</td>
<td>0/43 (0)</td>
<td>2/48 (4.2)</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>0</td>
<td>72</td>
<td>72</td>
<td>72/72 (100)</td>
<td>0/0</td>
<td>0/72 (0)</td>
<td>0/70 (0)</td>
</tr>
<tr>
<td></td>
<td>P. aeruginosa</td>
<td>0</td>
<td>21</td>
<td>21</td>
<td>21/21 (100)</td>
<td>0/0</td>
<td>0/21 (0)</td>
<td>0/21 (0)</td>
</tr>
<tr>
<td></td>
<td>K. pneumoniae</td>
<td>27</td>
<td>9</td>
<td>36</td>
<td>36/36 (100)</td>
<td>0/27 (0)</td>
<td>0/9 (0)</td>
<td>0/36 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>205</td>
<td>237</td>
<td></td>
<td>213/237 (91.7)</td>
<td>0/32 (0)</td>
<td>2/205 (1.0)</td>
<td>2/237 (0.8)</td>
</tr>
<tr>
<td>Total: Clinical and QC isolates</td>
<td>All organisms</td>
<td>154</td>
<td>449</td>
<td>603</td>
<td>583/603 (96.7)</td>
<td>3/154 (1.9)</td>
<td>6/449 (1.3)</td>
<td>11/603 (1.8)</td>
</tr>
</tbody>
</table>

^aR, number of resistant disk diffusion test results from standard 24-h growth.
^bS, number of susceptible disk diffusion test results from standard 24-h growth.
^cCategorical agreement between the early (EDD6) and standard growth methods.
^dNumber of very major discrepancies divided by total resistant organisms as determined by the standard growth method.
^eNumber of major discrepancies divided by total susceptible organisms as determined by the standard growth method.
^fNumber of minor discrepancies divided by total organisms tested.
DISCUSSION

Faster antimicrobial susceptibility testing can lead to improved clinical outcomes and better antimicrobial stewardship (24). This need for speed has prompted recent developments, including rapid phenotypic and genetic susceptibility testing, staffing of the microbiology laboratory around the clock, as well as total laboratory automation. Despite these advances, there

FIG 2 Comparison of inhibitory zone from early and standard disk diffusion testing. Clinical isolates and quality control (QC) strains were inoculated on blood agar and were incubated at 35°C for either 6 h (EDD6), 10 h (EDD10), or 24 h (St24) before performing disk diffusion testing in triplicate with 24 clinically appropriate antimicrobial agents. (A, B) Comparison of EDD6 (A) and EDD10 (B) with St24 culture incubation; solid shapes denote QC organisms. (C) comparison of disk diffusion zone size between EDD6 and St24 across a range of antibiotic classes. Least-squares linear regression was used to fit the values in panels A to C; goodness of fit is denoted by $r^2$. (D) Bland-Altman graph of early (EDD6 and EDD10) versus standard 24-h disk diffusion methods. Mean bias and $\pm 1.96$ standard deviation (SD) are illustrated by a solid line and broken lines, respectively.

FIG 3 Tukey box-whisker plot of differences in inhibitory zone size between early and standard disk diffusion methods. Central lines denote median differences, boxes surround the interquartile range (IQR), whiskers extend to the farthest nonoutliers, and data points represent outliers that are 1.5 times the IQR.
FIG 4 Inhibitory zone size relative to susceptibility breakpoints for 100 clinical isolates tested with optimized conditions. Comparison of zone sizes relative to antimicrobial breakpoints for disk diffusion testing performed on 6-h (EDD6) versus standard 24-h (St24) (Continued on next page)
remains a pressing need for a dependable, low-cost method of AST that can deliver results faster than traditional methods and can be deployed in laboratories with a wide variety of resources. Herein, we describe a simple and cost-effective modification of disk diffusion testing with the potential to expedite the reporting of AST results.

These results demonstrate that disk diffusion performed on 6- and 10-h growth has good categorical and quantitative agreement with standard disk testing when applied to 21 clinical and QC isolates as well as 100 clinical isolates (6-h growth) from clinically relevant Staphylococcus, Enterococcus, Enterobacterales, and Pseudomonas isolates. The categorical agreement (CA) of EDD6 and EDD10 with St24 when tested among the initial set of 21 isolates was well above the threshold (CA of 90% or more) provided by the FDA Class II Special Controls Guidance for AST systems. Likewise, AST results from EDD6 and EDD10 met the FDA recommended threshold of ME of 3% or less and VME upper and lower 95% CIs less than or equal to 7.5 and 1.5%, respectively.

Although the assay results were highly concordant between early and standard disk diffusion methods, we observed a small (less than 0.5 mm) bias toward reduced zone sizes with EDD compared to St24. Overall, the results of EDD6 and EDD10 were highly concordant with standard disk diffusion testing. Early disk diffusion testing performed well across a range of microorganisms, antibiotic classes, and resistance patterns. The diversity of test parameters was exemplified by the selection of organisms, which included both routine QC strains and more challenging clinical isolates with inhibitory zones near the established breakpoints.

This method also demonstrated a low rate of technical failure. All of the replicates from EDD6, EDD10, and St24 were evaluable and had no technical failures, such as nonconfluent lawn growth or growth of discrete colonies within the zone of inhibition.

Based on these results (testing 21 isolates and appropriate antibiotics in triplicate for EDD6 and EDD10), we performed disk diffusion testing with optimized methodology resulted in no VME or ME and had a 96.5% categorical agreement with results from standard 24-h incubation, suggesting that this is an accurate method for antimicrobial susceptibility testing with reduced turnaround time.

Inexperience with antimicrobial disk diffusion testing is a potential barrier to implementation of EDD in countries such as the United States where disk diffusion testing has more limited use compared to other regions globally where the method has been more widely

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**TABLE 4** Comparison of disk diffusion testing with optimized, 6-h early growth method (EDD6) versus standard 24-h growth method with 100 clinical isolates

<table>
<thead>
<tr>
<th>Organism</th>
<th>R</th>
<th>S</th>
<th>Total</th>
<th>Agreement, n/n (%)</th>
<th>Categorical agreement between the early (EDD6) and standard growth methods.</th>
<th>Number of very major discrepancies divided by total resistant organisms as determined by the standard growth method.</th>
<th>Number of major discrepancies divided by total susceptible organisms as determined by the standard growth method.</th>
<th>Number of minor discrepancies divided by total organisms tested.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. baumannii</td>
<td>46</td>
<td>44</td>
<td>90</td>
<td>88/90 (97.8)</td>
<td>0/46 (0)</td>
<td>0/44 (0)</td>
<td>2/90 (2.2)</td>
<td></td>
</tr>
<tr>
<td>E. cloacae</td>
<td>33</td>
<td>87</td>
<td>120</td>
<td>114/120 (95)</td>
<td>0/33 (0)</td>
<td>0/87 (0)</td>
<td>6/120 (5)</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>50</td>
<td>70</td>
<td>120</td>
<td>112/120 (93.3)</td>
<td>0/50 (0)</td>
<td>0/70 (0)</td>
<td>8/120 (6.7)</td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>49</td>
<td>71</td>
<td>120</td>
<td>119/120 (99.2)</td>
<td>0/49 (0)</td>
<td>0/71 (0)</td>
<td>1/120 (0.8)</td>
<td></td>
</tr>
<tr>
<td>MRSA</td>
<td>24</td>
<td>76</td>
<td>100</td>
<td>99/100 (99)</td>
<td>0/24 (0)</td>
<td>0/76 (0)</td>
<td>1/100 (1)</td>
<td></td>
</tr>
<tr>
<td>MSSA</td>
<td>10</td>
<td>90</td>
<td>100</td>
<td>100/100 (100)</td>
<td>0/10 (0)</td>
<td>0/90 (0)</td>
<td>0/100 (0)</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>37</td>
<td>103</td>
<td>140</td>
<td>134/140 (95.7)</td>
<td>0/37 (0)</td>
<td>0/103 (0)</td>
<td>6/140 (4.3)</td>
<td></td>
</tr>
<tr>
<td>VRE</td>
<td>42</td>
<td>38</td>
<td>80</td>
<td>76/80 (95)</td>
<td>0/42 (0)</td>
<td>0/38 (0)</td>
<td>4/80 (5)</td>
<td></td>
</tr>
<tr>
<td>VSE</td>
<td>15</td>
<td>65</td>
<td>80</td>
<td>75/80 (93.8)</td>
<td>0/15 (0)</td>
<td>0/65 (0)</td>
<td>5/80 (6.2)</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>306</td>
<td>644</td>
<td>950</td>
<td>917/950 (96.5)</td>
<td>0/306 (0)</td>
<td>0/644 (0)</td>
<td>33/950 (3.5)</td>
<td></td>
</tr>
</tbody>
</table>

*R, resistant; S, susceptible.

Very major: Categorical agreement between the early (EDD6) and standard growth methods.

Minor: Number of very major discrepancies divided by total resistant organisms as determined by the standard growth method.

Major: Number of major discrepancies divided by total susceptible organisms as determined by the standard growth method.

Minor: Number of minor discrepancies divided by total organisms tested.
adopted. Fortunately, the method is simple and is easy to adopt, and its low cost and potential for automation (specimen processing, imaging, and zone size measurements) support broader use of disk diffusion testing, including EDD and other related methods for rapid AST.

Direct-from-blood-culture disk diffusion testing, also known as the rapid antimicrobial susceptibility test (RAST), is a related method for antimicrobial susceptibility testing in which disk diffusion testing is performed directly from positive blood culture bottles. A 2018 report from the CLSI showed that this method had a categorical agreement with standard disk diffusion that ranged from 86.3 to 90.4% (resolved categorical agreement of 87.8 to 92.2%) when performed on 20 Gram-negative isolates, spiked into three commonly used blood culture systems (Bact/Alert, Bactec, and VersaTREK) (19). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) went on to develop method-specific breakpoints (RAST) that improved the performance of direct disk diffusion testing. Using RAST breakpoints, the authors demonstrated that the zone diameter could be measured in 88, 96, and 99% of samples read 4, 6, and 8 h after blood culture positivity, yielding less than 1.1% VMEs, less than 2.8% MEs, and less than 1.5% mEs (averaged by region) when performed in 55 laboratories in northern and southern Europe (25, 26).

Direct disk diffusion using RAST breakpoints promises to be a rapid and accurate method of AST, which will likely gain broader acceptance in the United States and throughout the world. However, this method changes the laboratory workflow and relies on alternative breakpoints that have only recently been adopted in Europe. In contrast, EDD changes only the length of time for subculture growth (18- to 24-h incubation time prior to disk diffusion setup to 6- to 10-h incubation time prior to disk diffusion set up) and uses established antimicrobial breakpoints, which are regularly updated and are broadly available through package inserts, CLSI guidelines, and EUCAST publications. As a result, it is easy to incorporate EDD into existing laboratory workflows. This method provides a high level of accuracy, as was demonstrated by testing of early (6 h) growth on 100 clinical isolates, which resulted in no VME, no ME, and 3.5% mE. With further study and proper validation, this approach might be extended to AST testing of early growth directly from primary isolation plates (i.e., use with positive blood cultures). However, mixed cultures are often not apparent at 6 h; therefore, a mechanism would need to be in place to cancel and dispose of any AST plates that were prepared from mixed cultures.

Disk diffusion testing is a simple and highly reliable method of susceptibility testing that is widely considered to be a standard method for AST. EDD provides a means of improving the turnaround for disk diffusion while retaining the beneficial attributes of this well-known method. We recommend that future work be aimed at evaluating the performance of EDD when incorporated into a clinical workflow.

In conclusion, using early growth for disk diffusion testing is a simple and accurate method of antimicrobial susceptibility testing that can reduce time to results by as much as 18 h while adding no additional cost to the testing method. This approach should be considered by laboratories that are incorporating total laboratory automation or are routinely reading plates on the second and third shifts.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.03 MB.
SUPPLEMENTAL FILE 2, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 3, PDF file, 0.2 MB.
SUPPLEMENTAL FILE 4, PDF file, 0.3 MB.

REFERENCES


