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# Verification of an Automated, Digital Dispensing Platform for At-Will Broth Microdilution-Based Antimicrobial Susceptibility Testing

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**With rapid emergence of multidrug-resistant bacteria, there is often a need to perform susceptibility testing for less commonly used or newer antimicrobial agents. Such testing can often be performed only by using labor-intensive, manual dilution methods and lies outside the capacity of most clinical labs, necessitating reference laboratory testing and thereby delaying the availability of susceptibility data. To address the compelling clinical need for microbiology laboratories to perform such testing in-house, we explored a novel, automated, at-will broth microdilution-based susceptibility testing platform. Specifically, we used the modified inkjet printer technology in the HP D300 digital dispensing system to dispense, directly from stock solutions into a 384-well plate, the 2-fold serial dilution series required for broth microdilution testing. This technology was combined with automated absorbance readings and data analysis to determine MICs. Performance was verified by testing members of the *Enterobacteriaceae* for susceptibility to ampicillin, cefazolin, ciprofloxacin, colistin, gentamicin, meropenem, and tetracycline in comparison to the results obtained with a broth microdilution reference standard. In precision studies, essential and categorical agreement levels were 96.8% and 98.3%, respectively. Furthermore, significantly fewer D300-based measurements were outside ±1 dilution from the modal MIC, suggesting enhanced reproducibility. In accuracy studies performed using a panel of 80 curated clinical isolates, rates of essential and categorical agreement and very major, major, and minor errors were 94%, 96.6%, 0%, 0%, and 3.4%, respectively. Based on these promising initial results, it is anticipated that the D300-based methodology will enable hospital-based clinical microbiology laboratories to perform at-will broth microdilution testing of antimicrobials and to address a critical testing gap.**

The rapid emergence of antimicrobial resistance has challenged current susceptibility testing paradigms. Based on complexity and labor-intensiveness, gold standard reference susceptibility methodologies—manual broth macrodilution, manual broth microdilution (BMD), and agar dilution susceptibility testing—are not performed routinely, if ever, by hospital-based clinical laboratories. All require a large number of pipetting steps to create an antimicrobial doubling dilution series for MIC determination.

Therefore, hospital-based clinical laboratories make use of more facile alternative methods, including MIC testing with preformulated antimicrobial dilution panels or MIC surrogate methods. These methods generally work well for common bacterial pathogens and most antimicrobials available in these test formats. Disk diffusion or Etest strip (bioMérieux) testing may be used as a primary or supplementary method for select antimicrobials not available for panel testing methods.

However, during the past decade, there has been a dramatic emergence of multidrug-resistant *Enterobacteriaceae* (1). Limited therapeutic options remain to treat these multidrug-resistant pathogens. Thus, there is often a clinical need to test antimicrobials not available in premade panels or supplementary FDA-cleared methods. Colistin is a prime example of a drug that is effective against >85% of carbapenem-resistant *Enterobacteriaceae* organisms (2) but is not available in FDA-cleared susceptibility panels.

Therefore, there is a significant antimicrobial testing gap where current methodologies have not kept pace with the introduction of new drugs or increasing frequencies of antibiotic resistance. As a result, most hospital-based clinical microbiology laboratories must rely on reference laboratories to perform dilution-based reference testing for these critical, potentially lifesaving antimicrobials, a process that may delay the availability of susceptibility results by an additional 4 to 6 days. In the face of multidrug-resistant pathogens with unpredictable susceptibility profiles, such a delay is clearly unsatisfactory. Just as importantly, the inability to test newer agents at the site of care, and therefore to offer confidence in their efficacy in a timely manner, is likely to have a chilling effect on the use of new antimicrobials and their development.

Therefore, we explored the capability of HP D300 inkjet printing technology to dispense, directly from antimicrobial stock solutions into a 384-well plate, the 2-fold serial dilution antimicrobial quantities required for broth microdilution testing. After addition of bacteria and incubation, this high-capacity format was combined with plate absorbance readings and automated data analysis to determine MICs. As proof of principle, we verified the performance characteristics of this combined digital dispensing method (DDM) by testing representative clinical isolates of the *Enterobacteriaceae* for susceptibility to ampicillin, cefazolin, ciprofloxacin, colistin, gentamicin, meropenem, and tetracycline and comparing the results to those of BMD testing (3). Based on our findings, we believe that DDM will enable hospital-based clinical microbiology laboratories to perform at-will testing of nearly any

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antimicrobial and thereby help to address the antimicrobial testing gap.

## MATERIALS AND METHODS

**Bacterial strains and antimicrobials.** *Escherichia coli* ATCC 25922, *Enterobacter cloacae* ATCC 13047, *Klebsiella pneumoniae* ATCC 13883, and *Proteus mirabilis* ATCC 702 were obtained from the American Type Culture Collection (Manassas, VA). *K. pneumoniae* BIDMC12A is a previously described, carbapenem-resistant clinical isolate (4) expressing a KPC-3 carbapenemase. The 80 deidentified *Enterobacteriaceae* clinical isolates used for verification studies were collected at our institution under Institutional Review Board (IRB)-approved protocols. All colony-purified strains were minimally passaged and stored at −80°C in tryptic soy broth (BD Diagnostics, Franklin Lakes, NJ) in 50% glycerol (Sigma-Aldrich, St. Louis, MO) prior to use in this study.

Ampicillin and tetracycline were from Thermo Fisher Scientific (Waltham, MA). Cefazolin was from Tokyo Chemical Industry (Portland, OR). Ciprofloxacin was from U.S. Biological (Salem, MA). Colistin sulfate was from Santa Cruz Biotechnology (Dallas, TX). Gentamicin was from Sigma-Aldrich. Meropenem was from ArkPharm (Libertyville, IL). Compounds used in reference BMD testing were dissolved according to CLSI guidelines (5). Antibiotic stock solutions used for the digital dispensing method were dissolved in sterile water containing 0.3% polysorbate 20 (P-20; Sigma-Aldrich), as a surfactant is required for proper aqueous fluid handling by the D300 instrument. The final concentrations of surfactant ranged from $7.5 \times 10^{-7}$% to 0.0015% at the extreme low and high ends of the doubling dilution ranges tested, respectively. All antimicrobials were stored as aliquots at −20°C and discarded after a single use.

**Reference BMD testing.** BMD testing was performed using the colony suspension method according to published guidelines (3, 6). Serial 2-fold dilutions of antimicrobials at double concentration were made in 96-well plates (Evergreen Scientific, Los Angeles, CA) by using single-strength cation-adjusted Mueller-Hinton broth (BD Diagnostics) and a 50-μl volume. Inocula were prepared by suspending several bacterial colonies in cation-adjusted Mueller-Hinton broth and adjusting the optical density at 600 nm ($OD_{600}$) to 0.0006, corresponding to approximately $1 \times 10^6$ CFU $ml^{-1}$ for *E. coli* ATCC 25922. Fifty microliters of adjusted suspension was added to each well, bringing the bacteria to a final concentration of approximately $5 \times 10^5$ CFU $ml^{-1}$, as determined by CLSI-recommended methods for inoculum validation (3), and bringing the antibiotic to the final desired concentration. Panels were incubated at 37°C in ambient air for 18 to 24 h. The MIC was defined as the lowest concentration of antimicrobial resulting in complete inhibition of growth as determined visually (5).

**DDM testing.** Antimicrobials were directly dispensed in the required amounts to create 2-fold dilutions into empty, flat-bottomed, untreated 384-well polystyrene plates (Greiner Bio-One, Monroe, NC) by use of an HP D300 digital dispensing system (Hewlett-Packard, Palo Alto, CA). Bacterial suspensions were prepared as described for BMD and adjusted to an $OD_{600}$ of 0.0003, corresponding to approximately $5 \times 10^5$ CFU $ml^{-1}$ for *E. coli* ATCC 25922, as determined by CLSI-recommended methods for inoculum validation (3). Fifty microliters of suspension was added to each well by use of a multichannel pipette. After incubation, cell growth was quantified by measurement of the $OD_{600}$ without prior agitation, using an Epoch microplate reader (BioTek, Winooski, VT). The cutoff for growth ($OD_{600}$ = 0.08; approximately 2-fold above typical background readings) was chosen to correspond to BMD visual growth determinations. MICs were automatically determined using a custom Python script based on the growth determination for each well. Uninoculated control wells were included in every plate to test for contamination during microplate setup.

**Precision analysis.** A precision analysis was conducted according to established guidelines, with *Escherichia coli* ATCC 25922 included in all experiments as a control (7, 8). BMD and DDM tests were repeated at least three and five times in triplicate, respectively, for each antimicrobial-organism combination. All testing occurred on separate days with freshly prepared antimicrobial dilutions and independent inocula. Antimicrobial agents were used over the following doubling dilution ranges: ampicillin, 0.06 to 128 μg $ml^{-1}$; cefazolin, 0.008 to 16 μg $ml^{-1}$; ciprofloxacin, 0.004 to 8 μg $ml^{-1}$; colistin, 0.06 to 32 μg $ml^{-1}$; gentamicin, 0.02 to 32 μg $ml^{-1}$; meropenem, 0.004 to 8 μg $ml^{-1}$; and tetracycline, 0.03 to 64 μg $ml^{-1}$. Antibiotic-organism combinations yielding growth at the highest concentration tested or no growth at the lowest concentration tested were considered off-scale. The modal MICs for both methods were determined using on-scale measurements. The distribution of $\log_2$ differences of each measurement from the mode was plotted using Microsoft Excel 2010 (Microsoft, Redmond, WA).

To calculate precision categorical (CA) and essential (EA) agreement levels, the modal MIC from BMD testing was recorded as the reference MIC. Each value determined by DDM testing was compared with the reference MIC, and $\log_2$ differences were recorded. Off-scale measurements were not considered for evaluable EA (8). Results from DDM testing were considered to be in evaluable EA if they were on-scale and yielded an MIC of ±1 dilution from that obtained by BMD testing. Results were considered to be in overall EA if they were (i) in evaluable EA, (ii) both off-scale in the same direction, or (iii) one measurement at the lowest or highest evaluable MIC tested and one measurement off-scale in the same direction. Results were considered to be in CA if both methods yielded the same susceptible/intermediate/resistant (S/I/R) interpretation. CLSI categorical interpretive criteria were used for ampicillin, cefazolin (parenteral), ciprofloxacin, gentamicin, meropenem, and tetracycline (5). EUCAST criteria were used for colistin (9).

**Verification study.** DDM and BMD tests were performed in parallel using the same inoculum preparations. BMD panels were prepared ahead of time and stored at −80°C until use (less than 2 weeks). Prior to use in the verification study, a representative frozen BMD panel was tested against *Escherichia coli* ATCC 25922 to ensure that MIC results were within acceptable quality control ranges. DDM panels were prepared fresh each day of use. Antimicrobial agents were used over the following concentration ranges: ampicillin, 0.13 to 256 μg $ml^{-1}$; cefazolin, 0.03 to 64 μg $ml^{-1}$; ciprofloxacin, 0.02 to 32 μg $ml^{-1}$; colistin, 0.13 to 64 μg $ml^{-1}$; gentamicin, 0.06 to 128 μg $ml^{-1}$; meropenem, 0.02 to 32 μg $ml^{-1}$; and tetracycline, 0.06 to 128 μg $ml^{-1}$.

Accuracy was evaluated using established guidelines (7, 8). EA, overall EA, and CA were evaluated as described for the precision study. Minor errors (MinE) were defined as either (i) a susceptible/resistant result from the test method and an intermediate result from the reference method or (ii) an intermediate result from the test method and a susceptible/resistant result from the reference method. Major errors (ME) were defined as a resistant result from the test method and a susceptible result from the reference method. Very major errors (VME) were defined as a susceptible result from the test method and a resistant result from the reference method.

**Statistical analysis.** Proportions of out-of-range (>1 2-fold dilution difference) to in-range (≤1 2-fold dilution difference) measurements determined during precision analysis for the DDM and BMD tests were compared using Fisher's exact test, with significance defined as a $P$ value of <0.05. Ninety-five percent confidence intervals (95% CI) were calculated for EA and CA based on CLSI recommendations (10). All statistical analyses were performed in JMP 12.0.1 (SAS, Cary, NC).

## RESULTS

The performance of DDM was compared to that of the reference BMD method to establish precision and accuracy, as described in the following.

**Precision.** Published guidelines suggest testing of precision by use of five separate strains, including characterized control strains and representative multidrug-resistant pathogens (7). Therefore, *Enterobacter cloacae* ATCC 13047, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, and *Proteus mirabilis* ATCC

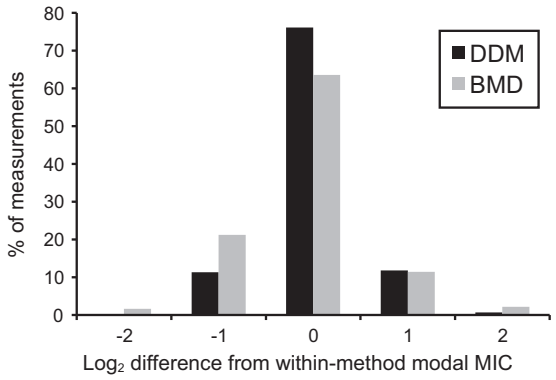


**FIG 1** $Log_2$ variance from the modal MIC. $Log_2$ differences shown represent numbers of 2-fold dilutions away from the modal MIC for all antimicrobials tested during the precision study. A total of 99.3% of the DDM measurements ($n = 432$) and 96.2% of the BMD measurements ($n = 184$) were within one 2-fold dilution of the modal MIC.

702 were selected for precision studies to provide diverse genera and antimicrobial susceptibility patterns. *K. pneumoniae* BIDMC12A was selected as a previously characterized representative carbapenem-resistant member of the *Enterobacteriaceae* (4).

We chose to examine seven drugs, each potentially undermined by distinct antimicrobial resistance mechanisms and, with the exception of colistin, suggested by CLSI for primary or secondary reporting for *Enterobacteriaceae* (5). Specifically, ampicillin, cefazolin, and gentamicin are suggested by CLSI as group A antimicrobials that should be used for primary testing and reporting. Ciprofloxacin and meropenem are CLSI group B antimicrobials recommended for primary testing and selective reporting. Tetracycline is a CLSI group C antimicrobial recommended for supplemental testing and as a predictor of doxycycline and minocycline susceptibility. Colistin is an agent of last resort that may be useful for treatment of infections with carbapenem-resistant *Enterobacteriaceae*.

For precision analysis, the majority of measurements were on-scale for DDM (82.6%) and BMD (82.8%). These measurements were used to create a distribution showing the reproducibility of each method compared to the modal MIC (Fig. 1). Off-scale measurements were not included, however, as $log_2$ differences from the modal MIC could not be calculated. The known colistin heteroresistance of *K. pneumoniae* ATCC 13883 (11) was detected by both DDM and BMD testing. This strain tested alternately as susceptible (MIC of $\leq 0.25\ \mu g\ ml^{-1}$), resistant (MIC $= 8\ \mu g\ ml^{-1}$), or uninterpretable (based on multiple skipped wells). As such, colistin results for this organism were also not included in this or subsequent analyses.

Of 184 on-scale MIC measurements performed with BMD testing, 96.2% fell within 1 doubling dilution of the modal MIC, 2.2% of measurements were 2 dilutions above the modal MIC, and 1.6% of measurements were 2 dilutions below the modal MIC. The average $log_2$ difference from the modal MIC was −0.09, with a 95% confidence interval of −0.19 to 0.012. For 432 on-scale MIC tests performed with DDM testing, 99.3% of results fell within 1 dilution of the modal MIC. A total of 0.7% of measurements were 2 dilutions above the modal MIC, and no measurements were 2 dilutions below the modal MIC. The average $log_2$ difference from the modal MIC was 0.019, with a 95% confidence interval of −0.03 to 0.06. Comparison of the ratios of in-range to out-of-range measurements indicated that DDM was significantly more precise than BMD (Fisher's exact test; $P = 0.01$).

Precision essential agreement (EA) and categorical agreement (CA) were then determined (Table 1). Of all on-scale measurements, 97.8% were considered to be in evaluable EA. The evaluable EA for ampicillin, cefazolin, ciprofloxacin, gentamicin, meropenem, and tetracycline averaged 98.9%. EA for colistin was somewhat lower, at 84.4%. However, all disagreements for colistin occurred in cases where the BMD MIC was ≥2 dilutions below the EUCAST-defined susceptibility breakpoint ($2\ \mu g\ ml^{-1}$) (9). Overall EA was then calculated through inclusion of "off-scale" DDM and BMD measurements. For ampicillin, cefazolin, ciprofloxacin, gentamicin, meropenem, and tetracycline, the overall EA averaged 99.1%. Colistin presented a lower overall EA (88.3%), with all disagreements again occurring at low levels of colistin (≥2 dilutions below the interpretive breakpoint for susceptibility).

For CA comparisons, interpretive criteria from CLSI (for ampicillin, parenteral cefazolin, ciprofloxacin, gentamicin, meropenem, and tetracycline) or EUCAST (for colistin) were used to classify each MIC as susceptible, intermediate (where applicable), or resistant. CA was 100% for ampicillin, ciprofloxacin, colistin, gentamicin, meropenem, and tetracycline (Table 1). Frank colistin resistance of *P. mirabilis* ATCC 702 and *E. cloacae* ATCC 13047

**TABLE 1** Precision analysis[a]

| Antimicrobial[b] | No. of measurements with $log_2$ difference from reference MIC[c] | | | | | % agreement (95% CI) | | |
|---|---|---|---|---|---|---|---|---|
| | −1 | 0 | 1 | 2 | 3 | Overall essential | Evaluable essential | Categorical |
| AMP | 11 | 23 | 26 | 0 | 0 | 100 (95.1–100.0) | 100 (93.9–100) | 100 (95.1–100) |
| CFZ | 0 | 21 | 23 | 1 | 0 | 98.7 (92.8–99.8) | 97.8 (88.4–99.6) | 88 (78.7–93.6) |
| CIP | 0 | 22 | 37 | 1 | 0 | 98.7 (92.8–99.8) | 98.3 (91.1–99.7) | 100 (95.1–100) |
| CL | 0 | 22 | 16 | 5 | 2 | 88.3 (77.8–94.2) | 84.4 (71.2–92.2) | 100 (93.9–100) |
| GEN | 15 | 50 | 9 | 1 | 0 | 98.7 (92.8–99.8) | 98.7 (92.8–99.8) | 100 (95.1–100) |
| MEM | 0 | 25 | 34 | 1 | 0 | 98.7 (92.8–99.8) | 98.3 (91.1–99.7) | 100 (95.1–100) |
| TET | 34 | 39 | 2 | 0 | 0 | 100 (95.1–100) | 100 (95.1–100) | 100 (95.1–100) |
| Total *n* (% or %, 95% CI) | 60 (14.3) | 202 (48.1) | 147 (35) | 9 (2.1) | 2 (0.5) | 499 (97.8, 96.2–98.8) | 409 (97.3, 95.3–98.5) | 501 (98.2, 96.7–99.1) |

[a] Precision analysis was performed on *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883, *E. cloacae* ATCC 13047, *P. mirabilis* ATCC 702, and *K. pneumoniae* BIDMC12A.
[b] AMP, ampicillin; CFZ, cefazolin; CIP, ciprofloxacin; CL, colistin; GEN, gentamicin; MEM, meropenem; TET, tetracycline.
[c] Only evaluable comparisons for which both DDM and BMD MIC measurements were within the dilution ranges tested were included.

**TABLE 2** Summary of antimicrobial resistance in 80 strains used for verification study

| Antibiotic | No. (%) of nonsusceptible strains[a] |
|---|---|
| Ampicillin | 65 (81.3) |
| Cefazolin | 51 (65.4) |
| Ciprofloxacin | 35 (44.9) |
| Colistin | 17 (21.5) |
| Gentamicin | 25 (31.3) |
| Meropenem | 14 (17.5) |
| Tetracycline | 34 (42.5) |

[a] Categories were based on CLSI criteria, except for colistin, for which categories were based on EUCAST criteria, as no CLSI interpretive criteria exist for colistin and the *Enterobacteriaceae*.

and susceptibility of *E. coli* ATCC 25922 and *K. pneumoniae* BIDMC 12A were consistently detected.

Note that cefazolin demonstrated a lower CA (88%) when it was assessed using current parenteral breakpoints (5). This contrasted with the 97.8% evaluable EA. All CA errors were minor and occurred for the two strains with reference MICs lying on a cefazolin breakpoint: the susceptibility breakpoint of 2 $\mu$g ml$^{-1}$ for *E. coli* ATCC 25922 and the intermediate breakpoint of 4 $\mu$g ml$^{-1}$ for *P. mirabilis* ATCC 702. Notably, the cefazolin MIC distribution for *Enterobacteriaceae* strains in general (http://www.eucast.org/mic_distributions_and_ecoffs/), as well as the quality control range for *E. coli* ATCC 25922 (1 to 4 $\mu$g ml$^{-1}$) (5), straddles the susceptible-intermediate demarcation. Therefore, the relatively lower CA for cefazolin can be explained by selective examination in the precision study of strains straddling breakpoint cutoffs. If alternative oral cefazolin breakpoints for uncomplicated urinary tract infection were used (susceptible MIC, $\leq$16 $\mu$g ml$^{-1}$) (5), categorical agreement was 100% across all strains tested.

**Accuracy.** The verification study evaluated the accuracy of DDM testing by utilizing a curated collection of 80 minimally passaged, deidentified clinical strains from our institution. Based on BMD testing, 93.8% of our strains showed nonsusceptibility to $\geq$1 antimicrobial tested, and 43.8% were multidrug resistant, based on the definition of acquired resistance to $\geq$3 antimicrobial classes (13). A summary of the resistance spectrum for the antimicrobials tested is shown in Table 2.

Antibiotic concentrations chosen for evaluation of ampicillin, cefazolin, ciprofloxacin, gentamicin, meropenem, and tetracycline ranged from 3 dilutions above the CLSI-defined resistance breakpoint to 6 dilutions below the susceptibility breakpoint. These ranges exceeded those suggested by the FDA (8) to accommodate the goal of understanding how well DDM and BMD testing correlated at extreme ends of the dilution range. Colistin concentrations ranged from 4 dilutions above the EUCAST resistance breakpoint to 4 dilutions below the susceptibility breakpoint. Further dilutions of colistin were not made due to known binding to plastic at low concentrations, resulting in unreliable MIC determinations (14, 15).

Using these extended ranges, the majority of measurements (68.8%) for all antimicrobials tested by BMD testing ($n$ = 555) were on-scale despite the high incidence of resistance in our strain set. The majority (80.6%) of the high off-scale results ($n$ = 100) were for ampicillin, cefazolin, or ciprofloxacin. All low off-scale results ($n$ = 49) were attributable to ciprofloxacin and meropenem, an expected result given the large splay between the susceptibility breakpoint for these drugs (1 $\mu$g ml$^{-1}$) and the typically very low MICs for susceptible strains (modal MIC of 0.03 $\mu$g ml$^{-1}$ for meropenem and $\leq$0.02 $\mu$g ml$^{-1}$ for ciprofloxacin).

In aggregate, 94.0% of evaluable DDM MICs were in EA ($\pm$1 dilution from the reference method results). When off-scale measurements were included in the calculation, overall EA was 91.0%. Taking the data together, the average evaluable EA for ampicillin, cefazolin, ciprofloxacin, gentamicin, meropenem, and tetracycline was 95.3%, and overall EA was 93.8%. Colistin showed less agreement, with an evaluable EA of 90% and an overall EA of 73.7%. The average CA was 96.6%, and CA ranged from 92.3% to 100% for the antimicrobials tested (Table 3). There were no major (ME) or very major (VME) errors identified. The minor error (MinE) rate was 3.4% ($n$ = 19). Notably, despite the lower EA for colistin, the CA for this antimicrobial was 100%.

## DISCUSSION

Here we present verification data for a digital dispensing technology that enables the generation of custom microdilution antimicrobial susceptibility testing panels. Importantly, we found that this 384-well-format method performed almost identically to BMD testing for seven different types of antimicrobials tested against several *Enterobacteriaceae* species. Specifically, precision EA (97.3%) and CA (98.2%) were well within the recommended >95% threshold suggested by Cumitech 31A (7) and FDA guidance documents (8). In addition, DDM testing demonstrated significantly less variation from the modal MIC during repeat measurements, suggesting enhanced reproducibility. For accuracy studies, the rates of EA, CA, VME, ME, and MinE were 94%,

**TABLE 3** Results of verification study

| Antimicrobial[a] | No. of strains with $\log_2$ difference from reference MIC[b] | | | | | % agreement (95% CI) | | |
|---|---|---|---|---|---|---|---|---|
| | −2 | −1 | 0 | 1 | 2 | Overall essential | Evaluable essential | Categorical |
| AMP | 0 | 2 | 10 | 19 | 3 | 95.0 (87.8–98.0) | 91.2 (77.0–97.0) | 95 (87.8–98.0) |
| CFZ | 0 | 9 | 34 | 5 | 0 | 100 (95.3–100.0) | 100 (92.6–100) | 92.3 (84.2–96.4) |
| CIP | 0 | 2 | 12 | 10 | 0 | 94.9 (87.5–97.9) | 100 (86.2–100) | 98.7 (93.1–99.8) |
| CL | 1 | 3 | 17 | 7 | 2 | 73.7 (63.2–82.1) | 90 (73.6–96.4) | 100 (95.4–100) |
| GEN | 1 | 12 | 36 | 22 | 6 | 90.0 (81.5–94.8) | 91 (82.4–95.5) | 97.5 (91.3–99.3) |
| MEM | 0 | 4 | 32 | 17 | 1 | 96.3 (89.5–98.7) | 98.1 (90.2–99.7) | 97.5 (91.3–99.3) |
| TET | 1 | 5 | 41 | 17 | 5 | 86.3 (77.0–92.1) | 91.3 (82.3–96.0) | 95 (87.8–98.0) |
| Total $n$ (% or %, 95% CI) | 3 (0.9) | 37 (11.0) | 182 (54.2) | 97 (28.9) | 17 (5.0) | 505 (91.0, 88.1–93.0) | 315 (94.0, 91.0–96.1) | 536 (96.6, 94.7–97.8) |

[a] AMP, ampicillin; CFZ, cefazolin; CIP, ciprofloxacin; CL, colistin; GEN, gentamicin; MEM, meropenem; TET, tetracycline.
[b] Only evaluable comparisons for which both DDM and BMD measurements were within the dilution ranges tested were included.

96.6%, 0%, 0%, and 3.4%, respectively, i.e., within the recommended target value of >89.9% for CA and EA (7, 10) and below the combined threshold of ≤3% for ME and VME and the combined threshold of ≤7% for minor and major errors (7). Therefore, the precision and accuracy of DDM testing were verified by generally accepted criteria.

We also examined the performance of testing for each antimicrobial individually to identify issues that might not be apparent in aggregate analysis. Not unexpectedly, issues with EA were identified for colistin in both precision and accuracy studies. Colistin is a lipopeptide antibiotic with a strong affinity for plastics used in antimicrobial susceptibility testing (15). The majority of colistin EA discrepancies (77.2%) occurred well below the susceptibility breakpoint, as observed in prior studies (14, 16), and did not affect CA, which was 100%. Our findings may relate, at least in part, to differential adsorbance of low levels of colistin in 384-well (test method) versus 96-well (reference method) plates (3, 5, 16).

Note that aqueous dispensing from the D300 instrument requires inclusion of polysorbate 20 (P-20) in stock solutions. Previous studies indicated that 0.002% polysorbate 80 (P-80), a structurally different surfactant (17), lowers colistin MIC values in broth microdilution assays (14, 15). However, the concentrations of P-20 used for DDM testing were 40-fold lower than this P-80 percentage at the 2-μg $ml^{-1}$ colistin susceptibility breakpoint. P-20 concentrations used for DDM testing did not appear to have major impacts on the mode and median DDM MICs compared to those for BMD testing performed in the absence of surfactant (Table 3). Furthermore, the D300 instrument can alternatively dispense dimethyl sulfoxide (DMSO)-based stock solutions without the use of surfactant should P-20 inclusion affect the results for any antimicrobial. Our observation is that DDM testing using DMSO stock solutions performs equivalently except for highly polar antimicrobials (i.e., aminoglycosides), in which case DMSO solubility becomes limiting (18). Final DMSO concentrations in assay wells are always <1%, consistent with CLSI BMD recommendations (5).

In terms of practical implementation of DDM in the clinical laboratory, it is useful to review (i) technology, (ii) work flow, (iii) capacity, (iv) assay cost, (v) reagent availability, (vi) quality control, and (vii) regulatory issues in turn.

The D300 platform is based on inkjet printer technology that allows precise delivery of antimicrobials in quantities ranging from 11 pl to 10 μl per the manufacturer's technical specifications (19). In this way, antimicrobial stock solutions can be used to set up doubling dilution series directly over a wide range of concentrations, without requiring serial dilution. Furthermore, the currently available T8+ compound dispense-head cassettes are functionally sterile and can be loaded with up to 8 antimicrobials, each in a separate channel. Each channel is capable of creating multiple 2-fold dilution series, limited only by the total liquid-holding capacity (10 μl). Individual channels can be used independently and at different times.

In terms of work flow, setting up DDM doubling dilution series for a single antimicrobial requires only a single micropipetting step. The process of pipetting stock solution into a T8+ cassette channel, loading the cassette into the D300 instrument, recalling a protocol, and dispensing antimicrobials takes approximately 2 min. In contrast, CLSI document M100-S26 (specifically, Table 8A of this document) (5) suggests performing BMD by creating 4 dilutions from a stock solution followed by combination with three different volumes of medium to create a 13-step dilution series. In total, this requires 24 micropipetting and 13 serological pipetting steps and the use of 17 micropipette tips, 13 conical tubes, and a serological pipette. The BMD steps are estimated to take approximately 14.5 min.

To increase the capacity for susceptibility testing, we verified functionality in a 384-well plate format. However, the D300 instrument is equally capable, per specifications and based on our experience, of dispensing into either 96-well or 1,536-well microplates. We also verified that the system can dispense into dry plates, which can be used immediately (as in this study) or frozen and used at a later time (data not shown). Therefore, it is possible to use digital dispensing technology to create custom MIC panels containing multiple antibiotics for either immediate or later use. Given that DDM is effectively an operator-independent method (i.e., automated dispensing and plate reading) (7), it is not surprising that DDM was more precise than BMD (Fig. 1).

In terms of cost, the D300 system itself has an approximate price of <$40,000. A standard microplate reader, if not already present in the clinical laboratory, costs <$8,000. Alternatively, the D300 instrument can dispense into 96-well plates, which can then be read visually. A calibrated analytical balance is also required to accurately weigh out antimicrobial powder. The foregoing expenses may make the system most appropriate for larger facilities treating patients with antibiotic-resistant infections.

After the initial capital purchase of these instruments, the cost of D300 consumables is relatively low. The cost of a single T8+ channel is <$9. Note, however, that multiple dilution series can be set up from the same channel, allowing significant economies of scale. For example, taking the example of meropenem and plating into a 384-well plate, using a range of 3 dilutions below and 2 dilutions above the susceptible and resistance breakpoints, respectively, and an aqueous stock solution of 6.25 mg $ml^{-1}$ (used in this study), approximately 39 dilution series can be created. Alternative use of a high-capacity D4+ cassette (250 μl) allows creation of 144 dilution series from an aqueous stock solution.

The use of DDM testing assumes the availability of reagents. Commercially available, cation-adjusted Mueller-Hinton broth and most antimicrobial agents can be purchased from a number of suppliers. However, recently approved antimicrobials may be available only directly from antimicrobial manufacturers and may be obtained only with some effort. Furthermore, DDM testing is considered a laboratory-developed test and therefore requires appropriate verification prior to clinical implementation. Quality control testing for *Enterobacteriaceae* or other organisms should be performed during each testing run, unless an internal quality control plan verifies the suitability of less frequent testing (20). Quality control recommendations for even recently approved antimicrobials are published by CLSI and/or available in antimicrobial package inserts and should be followed strictly. Media should be quality controlled according to standards, such as CLSI documents M07 and M100 (3, 5).

In essence, DDM provides a highly automated way to set up a reference broth microdilution equivalent and therefore, we predict, should perform adequately in most, if not all, situations where BMD is used. We further predict that its use should extend to MIC testing of diverse types of organisms, such as fungi and mycobacteria, and should include both traditional and direct susceptibility testing of primary specimens and blood cultures. Lastly, we believe that the D300 instrument may simplify testing of com-

bination agents coming to market through the use of two separate D8+ channels concurrently, as each individual agent can be dispensed, as appropriate, at either fixed ratios or with the concentration of the second agent held constant.

This study provides proof of concept for DDM testing. We expect that this methodology will allow clinical laboratories to rapidly create custom panels of antimicrobials at will, including those not available in commercially available panels or formats. It will thereby enable hospital-based clinical laboratories to address the current, clinically unacceptable antimicrobial testing gap.

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