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Direct-from-Blood-Culture Disk Diffusion To Determine Antimicrobial Susceptibility of Gram-Negative Bacteria: Preliminary Report from the Clinical and Laboratory Standards Institute Methods Development and Standardization Working Group

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ABSTRACT The performance of a disk diffusion test using broth from positive blood cultures as inoculum (direct disk diffusion [dDD]) was evaluated for a collection of 20 challenge isolates of *Enterobacteriaceae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Isolates seeded into human blood were inoculated into Bactec Plus Aerobic/F, VersaTREK Redox 1, and BacT/Alert FA Plus bottles and incubated in the respective automated blood culture systems. Disk diffusion results were compared to reference disk diffusion results. Categorical agreement (CA) values for dDD, after removal of random errors due to natural MIC variation, were 87.8%, 88.4%, and 92.2% for the BacT/Alert, Bactec, and VersaTREK systems, respectively. No very major errors (VME) were observed, and major error (ME) rates were 3.0%, 2.3%, and 1.7%, respectively. Incubation of the dDD test samples for 6 h compared to incubation for 16 to 18 h resulted in 19.9% of tests having too light of growth to allow reading of zones of inhibition. Among the evaluable dDD tests, CA values were 58.9%, 76.6%, and 73.2% for the isolates seeded into the BacT/Alert, Bactec, and VersaTREK systems, respectively. VME rates for isolates seeded into these systems were 2.2%, 1.8%, and 3.0%, respectively, and ME rates were 25.4%, 6.1%, and 2.8%, respectively, at the 6-h reading. The best performance of dDD was found for blood cultures with bacterial concentrations in the range of 7.6×10^7 to 5.0×10^8 CFU/ml; CA values ranged from 94.7 to 96.2% for these concentrations after 18 h of incubation and from 76.9 to 84.1% after 6 h of incubation. These preliminary data demonstrate the potential accuracy of dDD testing by the clinical laboratory.

KEYWORDS blood culture, Gram-negative bacteria, susceptibility testing

Rapid and accurate antimicrobial susceptibility testing (AST) is paramount to the management of patients with serious infections, including sepsis. However, conventional AST methods take 2 days to perform, at minimum, from the time the blood culture becomes positive. There is interest in developing AST methods for bacterial blood isolates that can generate data in a more clinically meaningful time frame.

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Multiplex molecular diagnostic assays are currently available that detect select antimicrobial resistance genes directly from positive blood culture broths (e.g., Verigene [Luminex, Austin, TX] and FilmArray [BioFire, Salt Lake City, UT] assays). While these systems provide actionable data for Gram-positive infections through detection of *mecA*, *vanA*, and *vanB*, the only randomized controlled trial that evaluated these systems, to date (1), documented no impact on the management of Gram-negative infections (2). This is largely due to the limitations associated with detection by molecular means of all resistance genes for the management of Gram-negative infections. Antimicrobial resistance in Gram-negative bacteria is complex and often multifactorial, and few genes or gene groups can be used to predict phenotypic susceptibility in these isolates. For instance, carbapenem resistance among the *Enterobacteriaceae* (3–5) can occur either via the presence of a carbapenemase gene or through a combination of porin defects and extended-spectrum beta-lactamases or AmpC (6). Similarly, isolates with a carbapenemase gene may not have MICs above the susceptibility breakpoint (7). As such, detection of carbapenemase genes alone does not yield sufficient information by which to deescalate therapy, and it may miss significant resistance.

A rapid, phenotypic approach to susceptibility testing for Gram-negative bacteria is therefore desirable. At present, several laboratories in the United States have developed such “direct-from-blood” phenotypic susceptibility tests, which involve manipulation of positive blood culture broth to allow inoculation of AST devices or agar plates with the broth rather than with a suspension made from isolated colonies (8–15). However, data from a poll conducted in 2015 by the Clinical and Laboratory Standards Institute (CLSI) demonstrated little to no standardization of methodology between laboratories. Further, almost none of the laboratories that responded to the poll indicated that results of direct-from-blood AST were reported to the patient’s chart or physician, despite excellent performance data (CLSI Subcommittee on Antimicrobial Susceptibility Testing agenda book, January 2015). Most laboratories indicated that this was due to concern over regulations surrounding use of laboratory-developed tests.

In 2014, the CLSI Subcommittee on Antimicrobial Susceptibility Testing formed an *ad hoc* working group to address the standardization of a direct-from-blood-culture susceptibility test method. The goal of this working group is to develop a method that (i) is based on disk diffusion (DD) and, as such, can be performed in all laboratories, including those without the expertise and resources for expensive and complex molecular testing; (ii) performs at or above U.S. Food and Drug Administration (FDA) standards for AST (i.e., $\geq 90\%$ categorical agreement [CA] and acceptable numbers of very major errors [VME; false susceptible] and major errors [ME; false resistant]); and (iii) is simple to implement into the routine laboratory workflow.

This study documents the initial studies performed by the CLSI *ad hoc* committee to establish the feasibility of a direct-from-blood-culture disk diffusion method for Gram-negative bacteria. An evaluation of 6-h and traditional 18-h incubation periods was also performed to verify if a further reduction in incubation time could be achieved.

MATERIALS AND METHODS

Bacterial isolates. Gram-negative bacterial isolates evaluated in this study ($n = 20$) are listed in Table 1. These were isolated from the blood of patients with sepsis by the UCLA Clinical Microbiology Laboratory and chosen to include both wild-type and resistant phenotypes. Quality control (QC) strains tested included *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *E. coli* ATCC 35218.

All bacteria were stocked and frozen at -70°C in brucella broth plus 10% glycerol (Hardy Diagnostics, Santa Ana, CA). All isolates were subcultured twice from freezer stocks on sheep blood agar plates (BAP; BD, Sparks, MD). After 18 to 24 h of incubation on BAP, colonies were harvested to prepare a suspension equivalent to a 0.5 McFarland standard in 0.85% saline. One-milliliter saline aliquots of the bacterial suspensions at a 1:10⁶ dilution (i.e., approximately 10² CFU) were seeded into Bactec Plus Aerobic/F (BD, Sparks, MD), VersaTREK Redox 1 (ThermoFisher, Lenexa, KS), and BacT/Alert FA Plus (bioMérieux, Durham, NC) blood culture bottles, along with 10 ml of human blood obtained from a donation center. Bottles were incubated on the respective blood culture systems until they were flagged as positive. Bottles were removed from the system within 8 h of being flagged as positive and used immediately after removal. Blood culture broth was subcultured to a blood agar plate and used to perform colony counts and as an inoculum for the direct disk diffusion method.

TABLE 1 Bacterial isolates used in this study^a

Isolate no.	Species	Resistance phenotype
15-05-01	<i>Klebsiella pneumoniae</i>	CRE (NDM-1)
15-05-02	<i>K. pneumoniae</i>	CRE (KPC)
15-05-03	<i>K. pneumoniae</i>	ESBL (CTX-M-15)
15-05-04	<i>Proteus mirabilis</i>	Wild type
15-05-05	<i>Enterobacter aerogenes</i>	Resistant to cephalosporins III, AmpC overexpression
15-05-06	<i>Enterobacter cloacae</i>	Wild type
15-05-07	<i>E. cloacae</i>	Resistant to cephalosporins III, AmpC overexpression
15-05-08	<i>Citrobacter freundii</i>	None
15-05-09	<i>Escherichia coli</i>	Plasmid for AmpC CMY-2
15-05-10	<i>E. coli</i>	Cefazolin resistant (mechanism not defined)
15-05-11	<i>E. coli</i>	Wild type
15-05-12	<i>E. coli</i>	ESBL
15-05-14	<i>Pseudomonas aeruginosa</i>	Carbapenem resistant
15-05-15	<i>P. aeruginosa</i>	Wild type
15-05-16	<i>P. aeruginosa</i>	Fluoroquinolone resistant
15-05-17	<i>Acinetobacter baumannii</i>	Wild type
15-05-18	<i>K. pneumoniae</i>	Wild type
15-05-19	<i>A. baumannii</i>	Carbapenem resistant (mechanism not defined)
15-05-20	<i>P. aeruginosa</i>	Aminoglycoside resistant
15-05-21	<i>Serratia marcescens</i>	SME
ATCC 25922	<i>E. coli</i>	Wild type
ATCC 35218	<i>E. coli</i>	Beta-lactamase producer
ATCC 27853	<i>P. aeruginosa</i>	Wild type

^aCRE, carbapenem-resistant *Enterobacteriaceae*; ESBL, extended-spectrum beta-lactamase. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *E. coli* ATCC 35218 were used as quality control strains.

DD. Disk diffusion testing (DD) was performed on all isolates from subculture plates according to CLSI standards (16); this test was termed reference DD (rDD) and used as the reference method in the data analyses. In addition, two modifications of the rDD method were evaluated. The first used an alternative inoculum, i.e., broth direct from the positive blood culture. For this direct-from-blood DD (dDD), 4 drops of blood culture broth from a Sub/Venting needle (BD) was applied to a 150-mm Mueller-Hinton agar (MHA) plate (BD) and swabbed in three directions across the plate to yield a lawn of bacteria. Disks were applied, and the plates were incubated at 35°C in ambient air. The second variable evaluated was incubation time. dDD plates were read after 6 h of incubation and again after 18 h of incubation. In these studies, the following antimicrobials were evaluated: amikacin, amoxicillin-clavulanate, ampicillin, aztreonam, cefazolin, cefepime, cefoxitin, ceftazidime, ceftriaxone, ciprofloxacin, ertapenem, gentamicin, imipenem, levofloxacin, meropenem, minocycline, piperacillin-tazobactam, tigecycline, tobramycin, and trimethoprim-sulfamethoxazole. All disks were purchased from BD.

Colony counts in blood culture bottles. At the same time that samples from positive bottles were directly inoculated onto Mueller-Hinton agar, a sample of the blood culture broth fluid was mixed and diluted serially in saline to dilutions of 10⁻², 10⁻³, and 10⁻⁴, the dilutions were vortexed, and 50 μl of each dilution was spread onto a separate blood agar plate. After overnight incubation, the plates were examined for colonies. Colonies from plates with 30 to 300 colonies were counted, and numbers of CFU per milliliter were calculated from the dilution factor (i.e., CFU count/0.05 ml/dilution factor).

Reference BMD. CLSI reference broth microdilution (BMD) was performed on all isolates. BMD was used as a reference arbiter in resolving apparent outlier errors. Testing was performed on panels prepared in-house at UCLA, using cation-adjusted Mueller-Hinton broth (Difco, BD, Sparks MD), as previously described (17).

Study design and data analysis. dDD results were compared to the rDD results obtained by testing isolated colonies from subcultures of the same bottles, which were used as the reference standard. Categorical agreement was evaluated by using CLSI document M100 breakpoints, excluding any antimicrobials for which the organism has intrinsic resistance (18). As a result, 14 antibiotics were evaluated for *Acinetobacter baumannii*, 16 for *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae*, 19 for *Klebsiella pneumoniae*, 16 for *Serratia marcescens*, and 11 for *P. aeruginosa*. Very major error (VME) (false susceptible) rates were calculated as numbers of results resistant by rDD but susceptible by dDD over the total number of resistant results by the reference method. Major error (ME) (false resistant) rates were calculated as numbers of isolates that tested susceptible by rDD but resistant by dDD over the number of susceptible isolates. Minor errors (mE) were defined as one result yielding an intermediate category and the other either a susceptible or resistant result. All VME and ME were further evaluated by comparing results of all DD and BMD tests performed for a given isolate; if the erroneous result was in agreement with the results of other reference methods (DD and BMD), it was considered to be a random error. Zone sizes for QC strains were evaluated using CLSI QC ranges published in the M100S document. For analysis, colony count data were divided into bins of 0.25-log increments.

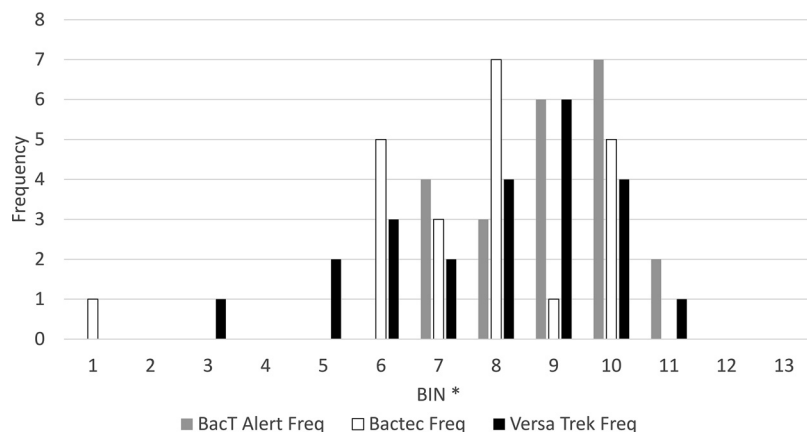


FIG 1 Bacterial concentrations (CFU per milliliter) at the time of positive signal for three blood culture systems. Counts were divided into 0.25-log increments; the number of CFU per milliliter that corresponds to each bin is listed in Table 6.

RESULTS

Colony counts in blood culture bottles at time of inoculation. Twenty challenge isolates (Table 1) were seeded into blood culture bottles and incubated on the respective automated instruments. Bottles were removed from the instruments within 8 h of being flagged as positive, and colony counts were performed. The numbers of bacteria in the bottles spanned a 2.75-log range, from 9.1×10^6 to 3.4×10^9 CFU/ml (Fig. 1). The average number of bacteria present in the positive blood cultures was 8.6×10^8 CFU/ml. Colony counts were divided into 0.25-log increments and plotted by blood culture manufacturer (Fig. 1). The average number of bacteria in the BacT/Alert bottles was approximately 0.25 log higher, at 1.15×10^9 CFU/ml, than the values of 6.9×10^8 and 7.3×10^8 CFU/ml for the Bactec and VersaTREK systems, respectively. No obvious species-specific differences existed in the concentrations of bacteria present in the blood cultures when they were flagged as positive or between members of the *Enterobacteriaceae*, *P. aeruginosa*, and *A. baumannii* (data not shown).

Performance of the direct disk diffusion method. The first variable of the CLSI disk diffusion (DD) method evaluated in this study was use of an alternative inoculum, i.e., broth from a positive blood culture instead of colony growth from a plate. Results were read at the traditional 18-h time point for this set of experiments. Nine hundred seventy-one rDD and dDD data points were recorded for the 20 challenge isolates tested across the three blood culture systems. Two results, both for ciprofloxacin, were not recorded because the disks fell off during incubation: one from a dDD plate inoculated with an *E. cloacae* isolate from a BacT/Alert bottle and the other from an rDD plate inoculated with an *E. coli* isolate from a BacT/Alert bottle. Overall categorical agreement of dDD and rDD results after 18 h of incubation was good, at 87.9%, just under the acceptable 90% limit for susceptibility test systems. There were no obvious performance differences for the dDD method between the three blood culture systems (Table 2). Two VME were observed among 390 resistant results (0.5%), 18 ME among 518 susceptible results (3.5%), and 97 mE (10.0%).

The two VME both occurred with *P. aeruginosa* isolates. The first was for imipenem on an isolate from the BacT/Alert system, where the reference rDD zone was 14 mm (resistant) and the dDD zone was 27 mm (susceptible). However, upon review, all other dDD and rDD results for this isolate were susceptible, and the reference broth microdilution (BMD) MIC was 1 μ g/ml (susceptible). As such, the error was considered a random error for the rDD for that isolate (see Table S1 in the supplemental material). The second VME was for levofloxacin on an isolate from the VersaTREK system. In this case, the dDD zone was 19 mm (susceptible) and the rDD zone was 12 mm (resistant). Again, all of the dDD and rDD results for this isolate were either susceptible or

TABLE 2 Performance of direct-from-blood-culture disk diffusion method at 18 h

Agreement type and blood culture system	No. of results from rDD ^a			% CA	No. (%) of:		
	S	I	R		VME	ME	mE
Initial agreement							
BacT/Alert FA aerobic	169	25	129	86.3	1 (0.8)	6 (3.5)	37 (10.7)
Bactec Plus aerobic	174	21	129	87.0	0 (0)	6 (3.5)	36 (10.3)
VersaTREK Redox	175	17	132	90.4	1 (0.8)	6 (3.4)	24 (6.9)
Resolved agreement							
BacT/Alert FA aerobic	168	25	129	87.8	0 (0)	5 (3.0)	37 (10.8)
Bactec Plus aerobic	172	21	129	88.4	0 (0)	4 (2.3)	36 (10.4)
VersaTREK Redox	172	17	132	92.2	0 (0)	3 (1.7)	24 (7.0)

^aS, susceptible; I, intermediate; R, resistant.

intermediate, and the reference BMD MIC was 2 µg/ml (susceptible), suggesting this was a random error for the rDD for this isolate. ME are detailed in Table S1. Similar to what was observed for the VME, 6 of the 18 ME appeared to be attributable to random errors of the rDD, as all other dDD and rDD results were either intermediate or resistant and the BMD result was also intermediate or resistant. Removal of these ME from analysis yielded an overall ME rate of 2.3%, which is within the typically accepted level of 3.0%. Removal of both the VME and ME outlined above and in Table S1 resulted in an overall categorical agreement of 88.7% (not shown) and in categorical agreements of 87.8 to 92.1% for isolates recovered from each respective blood culture system (Table 2).

Of the 97 mE, 87 (89.7%) were due to the dDD result being more resistant than the rDD result, 6 occurred when the dDD result was susceptible and the rDD result was intermediate, and 4 were due to intermediate dDD and resistant rDD results. On average, the dDD zones were 1.9 mm smaller than the rDD zones, with a mode of a 0-mm difference, and ranged from 24 mm smaller to 14 mm larger than the rDD measurements.

Data by antimicrobial, across all three blood culture systems and excluding the errors outlined above, are presented in Table 3. In most cases, higher incidences of ME were observed for antibiotics with smaller numbers of susceptible results, for example,

TABLE 3 Resolved performance of direct-from-blood-culture disk diffusion method at 18 h, by antibiotic

Drug	No. of isolates			% CA	No. (%) of:		
	S	R	VME		ME	mE	
Amikacin	45	13	96.7	0 (0)	0 (0)	2 (3.3)	
Amoxicillin-clavulanate	9	17	88.9	0 (0)	1 (11.1)	2 (7.4)	
Ampicillin	6	9	93.3	0 (0)	0 (0)	1 (6.7)	
Aztreonam	21	28	94.3	0 (0)	0 (0)	3 (5.7)	
Cefazolin	5	18	73.1	0 (0)	2 (40.0)	5 (19.2)	
Cefepime	41	17	91.7	0 (0)	0 (0)	5 (8.3)	
Cefoxitin	10	15	85.2	0 (0)	1 (10.0)	3 (11.1)	
Ceftazidime	25	31	89.8	0 (0)	0 (0)	6 (10.2)	
Ceftriaxone	16	29	87.5	0 (0)	2 (12.5)	4 (8.3)	
Ciprofloxacin	26	27	96.6	0 (0)	0 (0)	1 (1.7)	
Ertapenem	22	12	83.3	0 (0)	0 (0)	7 (16.7)	
Gentamicin	39	18	95.0	0 (0)	1 (2.6)	2 (3.3)	
Imipenem	34	21	68.3	0 (0)	3 (8.8)	15 (25.0)	
Levofloxacin	33	25	91.7	0 (0)	1 (3.0)	3 (5.0)	
Meropenem	37	19	84.7	0 (0)	1 (2.7)	8 (13.6)	
Minocycline	29	11	80.0	0 (0)	0 (0)	9 (20.0)	
Piperacillin-tazobactam	23	30	83.3	0 (0)	0 (0)	10 (16.7)	
Tigecycline	35	3	87.2	0 (0)	0 (0)	5 (12.8)	
Tobramycin	39	17	93.2	0 (0)	0 (0)	4 (6.8)	
Trimethoprim-sulfamethoxazole	17	30	95.8	0 (0)	0 (0)	2 (4.2)	

TABLE 4 Performance of dDD read at 6 h compared to that of rDD read at 18 h^b

Blood culture system	No. of isolates with insufficient growth	No. of evaluable results ^a	No. of evaluable results with rDD result			% CA	No. (%) of:		
			S	I	R		VME	ME	mE
BacT/Alert FA Plus aerobic	80	241	134	17	90	58.9	2 (2.2)	34 (25.4)	63 (26.1)
Bactec Plus aerobic	48	274	147	17	110	76.6	2 (1.8)	9 (6.1)	52 (19.0)
VersaTREK Redox 1 aerobic	64	257	142	16	99	73.2	3 (3.0)	4 (2.8)	60 (23.3)

^aEvaluable results included only plates with sufficient growth to be read by both rDD and dDD.

^brDD, reference disk diffusion; dDD, direct-from-blood disk diffusion; VME, very major errors; ME, major errors; mE, minor errors.

amoxicillin-clavulanate (11.1% ME; 9 susceptible results), cefazolin (40.0% ME; 5 susceptible results), and ceftiofur (10.0% ME; 10 susceptible results). Imipenem and levofloxacin were the other two antimicrobials with >3% ME rates, both with more than 30 susceptible results recorded. Imipenem also had a large proportion of mE (25.0%) (Table 3).

Performance of the early-read disk diffusion method. The second variable evaluated was incubation time. Both rDD and dDD plates were read after only 6 h of incubation, in contrast to the traditional 18 h of incubation. Incubation of rDD plates for 6 h resulted in 501 (51.5%) plates being too difficult to read due to light growth. In contrast, only 192 (19.9%) dDD plates were not read due to light growth. In both cases, *P. aeruginosa* results were difficult to read, with 92.4% of dDD and 100% of rDD results yielding insufficient growth to allow evaluation. For the dDD, 68.1% of *C. freundii* and 16.8% of *E. coli* results were not read due to light growth. For rDD, 66.7% of *A. baumannii*, 62.5% of *E. coli*, 25.4% of *K. pneumoniae*, 100% of *Proteus mirabilis*, and 50.0% of *S. marcescens* results were too difficult to read at the 6-h time point.

For the dDD results that were evaluable at 6 h of incubation ($n = 772$), categorical agreement with rDD results read at 18 h was 69.9%, with 2.3% VME, 11.8% ME, and 21.1% mE. Interestingly, CA was significantly ($P < 0.05$) lower for tests performed using broth from BacT/Alert bottles (58.9%) than for those using broth from Bactec (76.6%) and VersaTREK (73.1%) bottles. Strikingly, the number of ME with the BacT/Alert bottles (25.4%) was significantly higher than those observed using the Bactec (6.8%) and VersaTREK (4.2%) bottles (Table 4). Comparing dDD 18- and 6-h read results for isolates with sufficient growth for evaluation at 6 h yielded a 12.5% decrease in CA at the 6-h read for the Bactec system, a 16.4% decrease in CA for the VersaTREK blood culture system, and a 26.5% decrease for the BacT/Alert system. The higher categorical disagreement observed with the BacT/Alert system was due to there being more ME at the 6-h read (25.4%) than at the 18-h read (3.7%) for isolates with evaluable results at 6 h. Twelve of the 34 (35.3%) ME observed for the BacT/Alert system were for the *P. mirabilis* isolate tested, which yielded ME for all antibiotics tested except amikacin and ciprofloxacin (not shown). Other ME for the BacT/Alert system were for *A. baumannii* ($n = 5$), *E. aerogenes* ($n = 6$), *E. cloacae* ($n = 3$), *E. coli* ($n = 1$), *K. pneumoniae* ($n = 6$), and *S. marcescens* ($n = 1$).

As was done for the dDD read at 18 h, all VME and ME were evaluated to assess the results for random errors by the rDD test. Three ME were found to be due to an error of the rDD, wherein all results except the one rDD result were interpreted as resistant. These included a Bactec piperacillin-tazobactam result for *S. marcescens*, a VersaTREK *E. coli* result versus ciprofloxacin, and a VersaTREK *C. freundii* result versus meropenem (Table S2). Removal of these errors from the ME calculations yielded ME rates of 6.2% for the Bactec system and 2.9% for the VersaTREK system (Table 4). Agreement by antimicrobial is shown in Table 5. CA was highest for gentamicin and tobramycin (>90%) (Table 5) and lowest for imipenem and tigecycline (both below 50%). Three of the 13 amikacin-resistant results were read as susceptible at 6 h (23.1% VME), as were 2 of 30 piperacillin-tazobactam results (6.7% VME), 1 of 18 cefazolin-resistant results (5.6% VME), and 1 of 30 trimethoprim-sulfamethoxazole results (3.3% VME). High ME

TABLE 5 Resolved performance of direct-from-blood-culture disk diffusion method at 6 h, by antibiotic

Drug	No. of isolates		% CA	No. (%) of:		
	S	R		VME	ME	mE
Amikacin	45	13	62.2	3 (23.1)	2 (4.4)	12 (26.7)
Amoxicillin-clavulanate	9	17	60.0	0 (0)	1 (11.1)	9 (36.0)
Ampicillin	6	9	69.2	0 (0)	1 (16.7)	3 (23.1)
Aztreonam	21	28	84.2	0 (0)	1 (4.8)	5 (13.2)
Cefazolin	5	18	66.7	1 (5.6)	2 (40.0)	6 (25.0)
Cefepime	41	17	75.6	0 (0)	4 (9.8)	6 (13.3)
Cefoxitin	10	15	68.0	0 (0)	1 (10.0)	7 (28.0)
Ceftazidime	25	31	65.9	0 (0)	4 (16.0)	11 (25.0)
Ceftriaxone	16	29	77.3	0 (0)	3 (18.8)	7 (15.9)
Ciprofloxacin	24	27	57.1	0 (0)	1 (4.2)	16 (39.0)
Ertapenem	22	12	73.7	0 (0)	2 (9.1)	8 (21.1)
Gentamicin	39	18	95.6	0 (0)	0	2 (4.4)
Imipenem	34	21	46.7	0 (0)	6 (17.6)	18 (40.0)
Levofloxacin	33	25	75.6	0 (0)	1 (3.0)	10 (22.2)
Meropenem	36	19	52.3	0 (0)	9 (25.0)	11 (25.6)
Minocycline	29	11	65.9	0 (0)	0	12 (29.3)
Piperacillin-tazobactam	22	30	64.4	2 (6.7)	4 (18.2)	11 (25.0)
Tigecycline	35	3	45.7	0 (0)	3 (8.6)	16 (45.7)
Tobramycin	39	17	95.6	0 (0)	0	2 (4.4)
Trimethoprim-sulfamethoxazole	17	30	86.4	1 (3.3)	2 (11.8)	3 (6.8)

rates were observed for cefazolin (2/5 results [40.0%]), cefoxitin (1/10 results [10.0%]), ceftazidime (4/25 results [16.0%]), ceftriaxone (3/16 results [18.8%]), imipenem (6/34 results [17.6%]), meropenem (9/36 results [25.0%]), piperacillin-tazobactam (4/22 results [18.2%]), and trimethoprim-sulfamethoxazole (2/17 results [11.8%]).

Effect of bacterial concentration in blood culture on performance of direct-from-blood-culture disk diffusion. CA was evaluated for both the 18-h and 6-h dDD results in relation to the concentration of the bacterial inoculum. Data are presented in Table 6. Colony count data were divided into 0.25-log increments, and categorical agreement was calculated. The best performance of dDD was found in the range of 7.6×10^7 to 5.0×10^8 CFU/ml (Table 5), with CA values ranging from 94.7 to 96.2%, although there was no direct correlation between number of CFU per milliliter and CA at this time point. Similarly, CA was highest at this concentration range for the dDD test read at 6 h, with values ranging from 76.9 to 84.1% (Table 5). Not surprisingly, the largest proportion of dDD results with growth deemed too faint to read at 6 h were found at the lower concentrations (7.5×10^6 to 5.0×10^7 CFU/ml). The approximate cell density of a 0.5 McFarland standard is 1.5×10^8 CFU/ml.

TABLE 6 Performances of dDD after 18 and 6 h of incubation, stratified by concentration of bacteria present in blood culture broth inoculum

Bin	CFU range ^a	No. of readings	18-h dDD		6-h dDD	
			% CA	% of plates with growth too light to read	% CA	
1	7.5E+06–1.0E+07	16		100.0		
2	1.1E+07–2.5E+07	0				
3	2.6E+07–5.0E+07	11	100.0	100.0		
4	5.1E+07–7.5E+07	0				
5	7.6E+07–1.0E+08	26	96.2	0		76.9
6	1.1E+08–2.5E+08	124	94.4	0		80.6
7	2.6E+08–5.0E+08	155	93.5	7.1		77.4
8	5.1E+08–7.5E+08	184	87.5	12.0		58.7
9	7.6E+08–1.0E+09	174	93.7	48.3		36.2
10	1.1E+09–2.5E+09	205	79.5	5.4		53.7
11	2.6E+09–5.0E+09	48	79.2	22.9		39.6

^aRanges in bold represent the range of a 0.5 McFarland standard.

DISCUSSION

Over the last few decades, several clinical laboratories have developed methods to perform phenotypic susceptibility testing using blood culture broth as the primary inoculum. Such studies have shown improved times to susceptibility results, by a minimum of 1 day, as subculture of the blood culture broth is not required (8–15). The methods utilized in these studies have varied greatly over the years, leading to the need for a standardized method, which would also facilitate the use of these rapid results for patient care. Despite variability in methods, most studies show a high degree of categorical agreement with reference methods. Our study, which involved the three major blood culture incubation monitoring systems and a challenge set of Gram-negative bacteria, demonstrated 87.4% CA by the 18-h time point but only 69.9% CA at the early-read 6-h time point. This challenge set of 20 isolates provides several thousand data points as preliminary data, which will be the basis for larger studies involving a larger number of isolates at several institutions. Importantly, it demonstrates the feasibility of this approach.

One of the major contributors to discrepancies between direct-from-blood-culture-positive broth and reference disk diffusion results appears to be the concentration of bacteria within blood bottles after incubation. The differences in bacterial concentrations between the three commercial systems evaluated spanned nearly 3 log. The BacT/Alert blood cultures were flagged as positive at an approximately 0.25-log higher concentration of bacteria (1.15×10^9 CFU/ml) than those for the Bactec and VersaTREK systems (6.9×10^8 and 7.3×10^8 CFU/ml, respectively). The BacT/Alert concentration is significantly higher than the 0.5 McFarland standard used in the reference disk diffusion method (i.e., 1.5×10^8 CFU/ml). In order to improve CA, differences in bacterial concentration between systems could be calibrated by adjusting the inoculum concentrations, as attempted in various studies. However, in busy laboratories, especially during evening and night shifts, manual steps required to standardize a blood culture inoculum are unlikely to receive widespread adoption, as is the case for manual methods to perform rapid identification from positive blood culture broth, such as with matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry in the United States.

In our study, we also attempted to shorten the incubation time for DD. This alteration resulted in an increased rate of ME, a finding observed by others (14, 19). This observation is consistent with the dynamics of drug diffusion from the disk, with the height of the gradient being very steep in the first 6 h (i.e., drug present at a high concentration but close to the disk) and very shallow at later time points (lower concentrations at greater distances from the disk). The inflection point for penicillin G is around 6 to 8 h (20). Thus, small zone sizes measured at the earlier time point are consistent with incomplete diffusion of the antimicrobial through the agar. As DD breakpoints are correlated to an MIC, one option would be to develop an early-read DD breakpoint, which would likely be a smaller diameter than the breakpoints for the rDD. However, these breakpoints would be limited to species proven to have sufficient growth by the early time point. At 6 h, 92.3% of *P. aeruginosa* plates and 68.1% of *C. freundii* plates were unreadable due to light growth, indicating that breakpoints for these species may not be appropriate for an early read. Additionally, some isolates of more common species had poor growth at 6 h, although for *E. coli*, the most prevalent clinical isolate, 83.2% of our isolates actually yielded adequate growth at this early time point. These data parallel those observed in 1984 by Coyle and colleagues, who noted that 78% of Gram-negative isolates had readable disk diffusion results after 6 h of incubation, ranging from 64% of *P. aeruginosa* isolates to 92% of *Enterobacter* sp. isolates (14). An important factor to consider is the variability between readers evaluating dDD plates. Use of digital cameras and automation may aid in resolving this variability. The above-mentioned study also evaluated isolates directly from blood culture broth, although the authors used a sterile swab dipped into the blood culture as opposed to the 4-drop inoculum used in the present study (14). A recent study by

van den Bijllaardt and colleagues evaluated the readability of the standard DD method for the *Enterobacteriaceae*, using hourly, automated imaging of rDD plates postinoculation. In their study, 95.8% of isolates were readable at 6 h, and an increase in incubation time to 7 h yielded readable growth for all isolates. Further incubation to a total of 10 h minimized errors by the current rDD breakpoints (21). Such data suggest that the use of smart incubator systems may further increase the readability of short-incubation disk diffusion methods (19). To this end, CLSI will pursue an 8- to 10-h early-read time point to further evaluate the dDD method.

A second theme that arose in this study was the errors associated with beta-lactams. High mE and ME rates remained at 18 h for amoxicillin-clavulanate, cefazolin, cefoxitin, ceftazidime, ceftriaxone, imipenem, meropenem, and piperacillin-tazobactam. Beta-lactams translocate into the bacterial cell via porins to act on the cell synthesis pathway. One explanation for the errors may involve inhibition of this translocation due to the blood components present in the inoculum. Older studies on direct-from-blood disk diffusion observed overall high CA values, but many of these were completed prior to the availability of advanced-generation cephalosporins or carbapenems, antimicrobial classes that we found to be particularly problematic (3, 9, 11).

Developing and validating a standardized direct susceptibility method for positive blood cultures could benefit laboratories and clinicians and ultimately advance patient care. Providing the correct identification and susceptibility profile even a single day earlier may have impacts on the length of stay, appropriate use of antibiotics, and development of antibacterial resistance. The phenotypic method described here overcomes the inability to identify all specific resistance genes for Gram-negative bacteria and is more affordable than platforms currently available on the market.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.01678-17>.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

REFERENCES

- Banerjee R, Teng CB, Cunningham SA, Ihde SM, Steckelberg JM, Moriarty JP, Shah ND, Mandrekar JN, Patel R. 2015. Randomized trial of rapid multiplex polymerase chain reaction-based blood culture identification and susceptibility testing. *Clin Infect Dis* 61:1071–1080. <https://doi.org/10.1093/cid/civ447>.
- Banerjee R, Teng CB, Cunningham SA, Ihde S, Steckelberg J, Moriarty JP, Shah ND, Mandrekar J, Patel R. 2016. Reply to Idelevich and Beck. *Clin Infect Dis* 62:269–270. <https://doi.org/10.1093/cid/civ826>.
- Mirrett S, Reller LB. 1979. Comparison of direct and standard antimicrobial disk susceptibility testing for bacteria isolated from blood. *J Clin Microbiol* 10:482–487.
- Idelevich EA, Schule I, Grunastel B, Wullenweber J, Peters G, Becker K. 2014. Acceleration of antimicrobial susceptibility testing of positive blood cultures by inoculation of Vitek 2 cards with briefly incubated solid medium cultures. *J Clin Microbiol* 52:4058–4062. <https://doi.org/10.1128/JCM.02400-14>.
- Funke G, Funke-Kissling P. 2004. Use of the BD Phoenix automated microbiology system for direct identification and susceptibility testing of gram-negative rods from positive blood cultures in a three-phase trial. *J Clin Microbiol* 42:1466–1470. <https://doi.org/10.1128/JCM.42.4.1466-1470.2004>.
- Miller S, Humphries RM. 2016. Clinical laboratory detection of carbapenem-resistant and carbapenemase-producing *Enterobacteriaceae*. *Expert Rev Anti Infect Ther* 14:705–717. <https://doi.org/10.1080/14787210.2016.1206815>.
- Humphries RM, Hindler JA, Epton E, Horwich-Scholefield S, Miller LG, Mendez J, Martinez JB, Sinkowitz J, Sinkowitz D, Hershey C, Marquez P, Bhauria S, Moran M, Pandes L, Terashita D, McKinnell JA. 1 November 2017. Carbapenem-resistant *Enterobacteriaceae* (CRE) detection practices in California: what are we missing? *Clin Infect Dis*. <https://doi.org/10.1093/cid/cix942>.
- Wimmer JL, Long SW, Cernoch P, Land GA, Davis JR, Musser JM, Olsen RJ. 2012. Strategy for rapid identification and antibiotic susceptibility testing of gram-negative bacteria directly recovered from positive blood cultures using the Bruker MALDI Biotyper and the BD Phoenix system. *J Clin Microbiol* 50:2452–2454. <https://doi.org/10.1128/JCM.00409-12>.
- Wegner DL, Mathis CR, Neblett TR. 1976. Direct method to determine the antibiotic susceptibility of rapidly growing blood pathogens. *Antimicrob Agents Chemother* 9:861–862. <https://doi.org/10.1128/AAC.9.5.861>.
- Waites KB, Brookings ES, Moser SA, Zimmer BL. 1998. Direct susceptibility testing with positive BacT/Alert blood cultures by using MicroScan overnight and rapid panels. *J Clin Microbiol* 36:2052–2056.
- Fay D, Oldfather JE. 1979. Standardization of direct susceptibility test for blood cultures. *J Clin Microbiol* 9:347–350.
- Doern GV, Scott DR, Rashad AL, Kim KS. 1981. Evaluation of a direct blood culture disk diffusion antimicrobial susceptibility test. *Antimicrob Agents Chemother* 20:696–698. <https://doi.org/10.1128/AAC.20.5.696>.
- de Cueto M, Ceballos E, Martinez-Martinez L, Perea EJ, Pascual A. 2004. Use of positive blood cultures for direct identification and susceptibility testing with the Vitek 2 system. *J Clin Microbiol* 42:3734–3738. <https://doi.org/10.1128/JCM.42.8.3734-3738.2004>.
- Coyle MB, McGonagle LA, Plorde JJ, Clausen CR, Schoenkecht FD. 1984. Rapid antimicrobial susceptibility testing of isolates from blood cultures by direct inoculation and early reading of disk diffusion tests. *J Clin Microbiol* 20:473–477.
- Chapin KC, Musgnug MC. 2003. Direct susceptibility testing of positive blood cultures by using Sensititre broth microdilution plates. *J Clin Microbiol* 41:4751–4754. <https://doi.org/10.1128/JCM.41.10.4751-4754.2003>.
- CLSI. 2015. Performance standards for antimicrobial disk susceptibility tests; approved standard, 12th ed, vol M02-A12. Clinical and Laboratory Standards Institute, Wayne, PA.
- Bobenchik AM, Deak E, Hindler JA, Charlton CL, Humphries RM. 2015.

- Performance of Vitek 2 for antimicrobial susceptibility testing of Enterobacteriaceae with Vitek 2 (2009 FDA) and 2014 CLSI breakpoints. *J Clin Microbiol* 53:816–823. <https://doi.org/10.1128/JCM.02697-14>.
18. CLSI. 2017. Performance standards for antimicrobial susceptibility testing, 27th ed. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA.
 19. Froding I, Vondracek M, Giske CG. 2017. Rapid EUCAST disc diffusion testing of MDR *Escherichia coli* and *Klebsiella pneumoniae*: inhibition zones for extended-spectrum cephalosporins can be reliably read after 6 h of incubation. *J Antimicrob Chemother* 72:1094–1102. <https://doi.org/10.1093/jac/dkw515>.
 20. Lorian V. 1996. Antibiotics in laboratory medicine, 4th ed. Williams and Wilkins, Baltimore, MD.
 21. van den Bijllaardt W, Buiting AG, Mouton JW, Muller AE. 2017. Shortening the incubation time for antimicrobial susceptibility testing by disk diffusion for Enterobacteriaceae: how short can it be and are the results accurate? *Int J Antimicrob Agents* 49:631–637. <https://doi.org/10.1016/j.ijantimicag.2016.12.019>.