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1 **Direct-from-blood culture disk diffusion to determine antimicrobial susceptibility of Gram-**

2 **negative bacteria: Preliminary report from the Clinical and Laboratory Standards Institute**

3 **Methods Development and Standardization Working Group**

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16 **Abstract**

17 The performance of disk diffusion, using broth from positive blood cultures as inoculum ("direct
18 disk diffusion", dDD), was evaluated for a collection of 20 challenge isolates of
19 Enterobacteriaceae, Acinetobacter baumannii and Pseudomonas aeruginosa. Isolates seeded
20 into human blood were inoculated into BACTEC™ Plus Aerobic/F, VersaTREK REDOX 1 and
21 BacT/ALERT FA Plus bottles and incubated on the respective automated blood culture systems.
22 Disk diffusion results were compared to reference disk diffusion. Categorical agreement (CA) of
23 dDD, after removal of random errors due to natural MIC variation, was 87.8%, 88.4% and
24 92.2%, for BacT/ALERT, BACTEC and VersaTREK systems, respectively. No very major errors
25 (VME) were observed and major error (ME) rates were 3.0%, 2.3% and 1.7%, respectively.
26 Incubation of the dDD test for 6 h, compared to 16-18 h, resulted in 19.9% of tests having too
27 light of growth to read zones of inhibition. Among the evaluable dDD tests, CA was 58.9%,
28 76.6% and 73.2% for the isolates seeded into BacT/ALERT, BACTEC and VersaTREK systems,
29 respectively. VME rates for isolates seeded into these systems were 2.2%, 1.8% and 3.0%, and
30 ME were 25.4%, 6.1% and 2.8%, respectively, at the 6 h reading. The best performance of dDD
31 was found for those blood cultures with bacterial concentrations in the range of 7.6×10^7 - $5.0 \times$
32 10^8 CFU/mL; CA ranged from 94.7 – 96.2% at this concentration after 18 h incubation, and 76.9
33 – 84.1% after 6 h incubation. These preliminary data demonstrate the potential accuracy of
34 dDD testing by the clinical laboratory.

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36 **Introduction**

37 Rapid and accurate antimicrobial susceptibility testing is paramount to the management
38 of patients with serious infections, including sepsis. However, conventional antimicrobial
39 susceptibility testing (AST) methods take at minimum 2 days to perform from the time the
40 blood culture becomes positive. There is interest in developing AST methods for bacterial blood
41 isolates that can generate data in a more clinically meaningful timeframe. Multiplex molecular
42 diagnostic assays are currently available that detect select antimicrobial resistance genes
43 directly from positive blood culture broths (e.g., Luminex Verigene (Austin, TX), BioFire
44 FilmArray (Salt Lake City, UT)). While these systems provide actionable data for Gram-positive
45 infections, through detection of *mecA*, *vanA* and *vanB*, the only randomized controlled trial that
46 has evaluated these systems to date (1) documented no impact on the management of Gram-
47 negative infections (2). This is largely due to the limitations associated with detection, by
48 molecular means, of all resistance genes for the management of Gram-negative infections.
49 Antimicrobial resistance in Gram-negative bacteria is complex and often multifactorial, and few
50 gene or gene groups can be used to predict phenotypic susceptibility in these isolates. For
51 instance, carbapenem resistance among the *Enterobacteriaceae* (3-5) can occur via either the
52 presence of a carbapenemase gene, or through combination of porin defects and extended
53 spectrum beta-lactamases or AmpC (6). Similarly, isolates with a carbapenemase gene may not
54 have MICs above the susceptible breakpoint (7). As such, detection of carbapenemase genes
55 alone does not yield sufficient information by which to deescalate therapy, and may miss
56 significant resistance.

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

68 In 2014, the CLSI Subcommittee on Antimicrobial Susceptibility Testing (AST) formed an
69 ad-hoc working group to address the standardization of a direct-from-blood culture
70 susceptibility test method. The goal of this working group is to develop a method that 1) is
71 based on disk diffusion and as such can be performed in all laboratories, including those
72 without the expertise and resources for expensive and complex molecular testing; 2) performs
73 at or above U.S. Food and Drug Administration (FDA) standards for AST (i.e. ≥90% categorical
74 agreement with acceptable numbers of very major [false susceptible] and major [false
75 resistance] errors); 3) is simple to implement into the routine laboratory workflow.

76 This study documents the initial studies performed by the CLSI ad hoc committee, to
77 establish the feasibility of a direct-from-blood culture disk diffusion method for Gram-negative

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78 bacteria. An evaluation of a 6 hour and traditional 18 hour incubation period was also

79 performed to verify if a further reduction in incubation time could be achieved.

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80 **Methods**

81 *Bacterial Isolates*

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

87 All bacteria were stocked, and frozen at -70°C in Brucella broth plus 10% glycerol (Hardy
88 Diagnostics, Santa Ana, CA). All isolates were subcultured twice from freezer stocks on sheep
89 blood agar plates (BAP, BD, Sparks, MD). After 18-24 h incubation on BAP, colonies were
90 harvested to prepare a suspension equivalent to a 0.5 McFarland standard in 0.85% saline. A
91 one mL saline aliquot of a 1:10^6 dilution (i.e. approximately 10^2 CFU) of the bacterial
92 suspensions was seeded into BACTEC™ Plus Aerobic/F (BD, Sparks, MD), VersaTREK REDOX 1
93 (ThermoFisher, Lenexa, KS) and BacT/ALERT FA Plus (bioMérieux, Durham, NC) blood culture
94 bottles, along with 10 mL of human blood obtained from a donation center. Bottles were
95 incubated on the respective blood culture systems until they flagged positive. Bottles were
96 removed from the system within 8 hours of flagging positive and used immediately after
97 removal. Blood culture broth was subcultured to a blood agar plate, used to perform colony
98 counts, and as inoculum for the direct disk diffusion method.

99 *Disk Diffusion*

100 Disk diffusion (DD) was performed for all isolates from subculture plates, according to
101 CLSI standards (16), which was termed as reference DD (rDD) and used as the reference

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102 method in the data analyses. In addition, two modifications of the rDD method were evaluated.
103 The first used an alternative inoculum, i.e., broth direct from the positive blood culture. For this
104 direct-from-blood DD (dDD), four drops of blood culture broth from a Sub/Venting needle (BD,
105 Catalogue # 271056) were applied to a 150 mm Mueller Hinton agar (MHA) plate (BD) and
106 swabbed in three directions across the plate to yield a lawn of bacteria. Disks were applied and
107 the plates were incubated at 35°C, in ambient air. The second variable evaluated was
108 incubation time. dDD plates were read after 6 h incubation, and again after 18 h incubation. In
109 these studies, the following antimicrobials were evaluated: amikacin, amoxacillin-clavulanate,
110 ampicillin, aztreonam, cefazolin, cefepime, cefoxitin, ceftazidime, ceftriaxone, ciprofloxacin,
111 ertapenem, gentamicin, imipenem, levofloxacin, meropenem, minocycline, piperacillin-
112 tazobactam, tigecycline, tobramycin and trimethoprim-sulfamethoxazole. All disks were
113 purchased from BD.

114 *Colony Counts in Blood Culture Bottles*

115 At the same time that positive bottles were directly inoculated to Mueller Hinton agar, a
116 sample of the blood culture broth fluid was mixed, diluted serially in saline to dilutions of 10^{-2},
117 10^{-3}, and 10^{-4}, the dilutions vortex mixed, and 50 μL of each dilution spread onto separate blood
118 agar plates. After overnight incubation the plates were examined for colonies. Colonies from
119 plates with 30 – 300 colonies were counted and number of CFU/mL calculated from the dilution
120 factor (i.e., CFU count/0.05mL/dilution factor).

121 *Reference Broth Microdilution*

122 CLSI Reference broth microdilution (BMD) was performed on all isolates. The BMD was used as
123 a reference arbiter in resolving apparent outlier errors. Testing was performed on panels

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124 prepared in-house, at UCLA, using cation-adjusted Mueller Hinton Broth from Difco (BD, Sparks
125 MD), as previously described (17).

126 *Study Design and Data Analysis*

127 dDD results were compared to the rDD result obtained by testing isolated colonies from
128 the subculture of the same bottle, as the reference standard. Categorical agreement was
129 evaluated, using M100S 27th Ed. breakpoints, and excluding any antimicrobials for which the
130 organism has intrinsic resistance (18). As a result, 14 antibiotics were evaluated for
131 *Acinetobacter baumannii,* 16 for *Citrobacter freundii*, *Enterobacter aerogenes, Enterobacter*
132 *cloacae*, 19 for *Klebsiella pneumoniae*, 16 for *Serratia marcescens* and 11 for *P. aeruginosa.*
133 Very major error (VME) (false susceptible) rates were calculated as results resistant by the rDD
134 but susceptible by dDD method, over the total number of resistant results by the reference
135 method. Major error (ME) (false resistance) rates were calculated as the number of isolates
136 that tested susceptible by rDD but resistant by dDD, over the number of susceptible isolates.
137 Minor errors were defined as one result yielding an intermediate category and the other either
138 a susceptible or resistant result, over the total number of isolates tested. All VME and ME were
139 further evaluated by comparing results of all DD and BMD tests performed for a given isolate; if
140 the erroneous result was in agreement with other reference methods (DD and BMD), it was
141 considered to be a random error. Zone sizes for quality control strains were evaluated using
142 CLSI QC ranges published in M100S 27th Ed. For analysis, colony count data were divided into
143 bins of 0.25 log increments.

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144 **Results**

145 *Colony Counts in Blood Culture Bottles at Time of Inoculation*

146 Twenty challenge isolates (Table 1) were seeded into blood culture bottles and
147 incubated on the respective automated instruments. Bottles were removed from the
148 instruments within 8h of flagging positive, and colony counts performed. The number of
149 bacteria in each bottle spanned a 2.75 log range, from 9.1 x 10^6 to 3.4 x 10^9 CFU/mL (Figure 1).
150 The average number of bacteria present in the positive blood cultures was 8.6 x 10^8 CFU/mL.
151 Colony counts were divided into 0.25 log increments and plotted by blood culture manufacturer
152 (Figure 1). The average number of bacteria in the BacT/ALERT bottles was approximately 0.25
153 log higher, at 1.15 x 10^9 CFU/mL, as compared to 6.9 x 10^8 and 7.3 x 10^8 CFU/mL for the BACTEC
154 and VersaTREK systems, respectively. No obvious species-specific differences existed in the
155 concentration of bacteria present in the blood cultures when they flagged positive, nor
156 between members of the *Enterobacteriaceae*, *P. aeruginosa* and *A. baumannii* (data not
157 shown).

158 *Direct Disk Diffusion Method Performance*

159 The first variable of the CLSI disk diffusion (DD) method evaluated in this study was use
160 of an alternative inoculum, i.e., broth from a positive blood culture instead of colony growth
161 from a plate. Results were read at the traditional 18 h time point for this set of experiments.
162 Nine-hundred seventy-one rDD and dDD data points were recorded for the 20 challenge
163 isolates tested across the three blood culture systems. Two results, both for ciprofloxacin, were
164 not recorded because the disks fell off during incubation: one from a dDD plate inoculated with
165 an *E. cloacae* from a BacT/ALERT bottle, and the other on a rDD for an *E. coli* isolated from a

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166 BacT/ALERT bottle. Overall categorical agreement of dDD with rDD after 18 h incubation was
167 good, at 87.9%, just under the acceptable 90% limit for susceptibility test systems. There were
168 no obvious performance differences for the dDD method between the three blood culture
169 systems (Table 2). Two VMEs were observed among 390 resistant results (0.5%), 18 MEs among
170 518 susceptible results (3.5%) and 97 minor errors (mE, 10.0%).

171 The two VMEs both occurred in *P. aeruginosa* isolates. The first was for imipenem on an
172 isolate from the BacT/ALERT system, where the reference rDD was 14 mm (resistant) and the
173 dDD was 27 mm (susceptible). However, upon review, all other dDD and rDD results for this
174 isolate were susceptible, and the reference broth microdilution (BMD) minimum inhibitory
175 concentration (MIC) was 1 µg/mL (susceptible). As such, the error was considered a random
176 error for the rDD for that isolate (Table S1). The second VME was for levofloxacin on an isolate
177 from the VersaTREK system. In this case, the dDD was 19 mm (susceptible) and the rDD was 12
178 mm (resistant). Again, all of the dDD and rDD results for this isolate were either susceptible or
179 intermediate, and the reference BMD was 2 µg/mL (susceptible), suggesting this was a random
180 error for the rDD for this isolate. MEs are detailed in Table S1. Similar to what was observed for
181 the VMEs, 6 of the 18 MEs appear to be attributable to random errors of the reference rDD,
182 where all other dDD and rDD results were either intermediate or resistant and BMD was also
183 intermediate or resistant. Removal of these ME from analysis yielded an overall ME rate of
184 2.3%, within the typically accepted 3.0%. Removal of both the VME and MEs outlined above
185 and in Table S1, resulted in an overall categorical agreement of 88.7% (not shown), and of 87.8-
186 92.1% for isolates recovered from each respective blood culture system (Table 2).

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

192 Data by antimicrobial, across all three blood culture systems, and excluding the errors
193 outlined above, are presented in Table 3. In most cases, a higher incidence of ME was observed
194 for antibiotics with a lower number of susceptible results, for example amoxicillin-clavulanate
195 (11.1% ME, 9 susceptible results), cefazolin (40.0% ME, 5 susceptible results), and cefoxitin
196 (10.0% ME, 10 susceptible results). Imipenem and levofloxacin were the other two
197 antimicrobials with a greater than 3% ME rate, both with more than 30 susceptible results
198 recorded. Imipenem also had a high proportion of mE at 25.0% (Table 3).

199 *Early Read Disk Diffusion Method Performance*

200 The second variable evaluated was incubation time. Both rDD and dDD plates were read
201 after only 6 h incubation, compared to the traditional 18 h incubation. Incubation of rDD plates
202 for 6 h resulted in 501 (51.5%) being too difficult to read due to light growth. In contrast, only
203 192 (19.9%) dDD were not read due to light growth. In both cases, *P. aeruginosa* was difficult to
204 read, with 92.4% of dDD and 100% of rDD results yielding insufficient growth to evaluate. For
205 the dDD, 68.1% of *C. freundii* and 16.8% of *E. coli* results were not read due to light growth. For
206 rDD, 66.7% of *A. baumannii*, 62.5% of *E. coli*, 25.4% of *K. pneumoniae*, 100% of *P. mirabilis* and
207 50.0% of *S. marcescens* results were too difficult to read at the 6 h time point.

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208 Of the dDD results that were evaluable at 6 h incubation (n=772), categorical agreement
209 with rDD read at 18 h was 69.9%, with 2.3% VME, 11.8% ME and 21.1% mE. Interestingly, CA
210 was significantly ($p<0.05$) worse for tests performed using broth from the BacT/ALERT bottles
211 (58.9%) compared to BACTEC (76.6%) and VersaTREK (73.1%) bottles. Strikingly, the number of
212 ME using the BacT/ALERT bottles (25.4%) was significantly higher than observed using the
213 BACTEC (6.8%) and VersaTREK (4.2%) bottles (Table 4). Comparing dDD 18 to 6 h read results
214 for isolates with sufficient growth to evaluate at 6 h yielded a 12.5% decrease in CA at the 6 h
215 read for the BACTEC, a 16.4% decrease in CA for the VersaTREK blood culture system, and
216 26.5% for the BacT/ALERT system. The higher categorical disagreement observed with the
217 BacT/ALERT system was due to there being more ME at the 6 h read (25.4%) as compared to
218 the 18 h read (3.7%) for those isolates with evaluable results at 6 h. Twelve of the 34 (35.3%)
219 ME observed for the Bact/ALERT system were for the *P. mirabilis* isolate tested, which yielded a
220 ME for all antibiotics tested except amikacin and ciprofloxacin (not shown). Other ME for the
221 BacT/ALERT system were for *A. baumannii* (n=5), *E. aerogenes* (n=6), *E. cloacae* (n=3), *E. coli*
222 (n=1), *K. pneumoniae* (n=6) and *S. marcescens* (n=1).

223 As was done for the dDD read at 18 h, all VME and ME were evaluated to assess for
224 random errors by the rDD test. Three ME were found to be due to an error of the rDD, wherein
225 all results except the one rDD were interpreted as resistant. These included a BACTEC
226 piperacillin-tazobactam result for *S. marcescens*, a VersaTREK *E. coli* versus ciprofloxacin, and a
227 VersaTREK *C. freundii* versus meropenem (Table S2). Removal of these errors from the ME
228 calculations yielded a ME rate of 6.2% for the BACTEC and 2.9% for the VersaTREK (Table 4).
229 Agreement by antimicrobial is shown in Table 5. CA was best for gentamicin and tobramycin

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230 (>90%, Table 5), and worst for imipenem and tigecycline, both of which were below 50% CA.
231 Three of the 13 amikacin-resistant results were read as susceptible at 6 h (23.1% VME), as were
232 2 of 30 piperacillin-tazobactam results (6.7% VME), 1 of 18 cefazolin resistant results (5.6%),
233 and 1 of 30 trimethoprim-sulfamethoxazole results (3.3% VME). High ME rates were observed
234 for cefazolin (2/5, 40.0%), cefoxitin (1/10, 10.0%), ceftazidime (4/25, 16.0%), ceftriaxone (3/16,
235 18.8%), imipenem (6/34, 17.6%), meropenem (9/36, 25.0%), piperacillin-tazobactam (4/22,
236 18.2%) and trimethoprim-sulfamethoxazole (2/17, 11.8%).

237 *Effect of Bacterial Concentration in Blood Cultures on Performance of Direct-from-Blood Culture*
238 *Disk Diffusion*

239 CA was evaluated for both the 18 hour dDD and 6 hour dDD in relation to the
240 concentration of the bacteria inoculum. Data are presented in Table 6. Colony count data was
241 divided into 0.25 log increments and categorical agreement was calculated. The best
242 performance of dDD was found in the range of 7.6×10^7 and 5.0×10^8 CFU/mL (Table 5), where
243 CA ranged from 94.7 – 96.2%, although there was no direct correlation between CFU/mL and
244 CA at this time point. Similarly, CA was best at this concentration range for the dDD when read
245 at 6 h, ranging from 76.9 – 84.1% (Table 5). Not surprisingly, the largest proportion of dDD
246 results with growth deemed too faint to read at 6 h were found at the lower concentrations,
247 7.5×10^6 – 5.0×10^7 CFU/mL. The approximate cell density of a 0.5 McFarland is 1.5×10^8
248 CFU/mL.

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249 **Discussion**

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

263 One of the major contributors to discrepancies between direct from blood culture positive
264 broth and reference disk diffusion appears to be the concentration of bacteria within blood
265 bottles after incubation. The difference in bacterial concentrations between the three
266 commercial systems evaluated spanned nearly three logs. The BacT/ALERT blood cultures
267 flagged positive at approximately 0.25 log higher concentration of bacteria (1.15 x 10^9 CFU/mL)
268 as compared to the BACTEC and VersaTREK systems (6.9 x 10^8 and 7.3 x 10^8 CFU/mL
269 respectively). The BacT/ALERT concentration is significantly higher than the 0.5 McFarland
270 standard used in the reference disk diffusion method, (i.e., 1.5 x10^8 CFUs/ml). In order to

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271 improve CA, differences in bacterial concentration between systems could be compensated for
272 by adjusting the inoculum concentrations, as has been attempted in various studies. However,
273 in busy laboratories, especially in evening and night shifts, manual steps required to standardize
274 a blood culture inoculum are unlikely to receive widespread adoption, as is the case for the
275 manual methods to perform rapid identification from positive blood culture broth, such as with
276 matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry in the
277 U.S.

278 In our study, we also attempted to shorten the incubation time for DD. This alteration
279 resulted in an increase rate of ME, a finding observed by others (14, 19). This observation is
280 consistent with the dynamics of drug diffusion from the disk, the height of the gradient being
281 very steep in the first 6 hours (i.e., drug present at high concentration but close to the disk) and
282 very shallow at later time points (lower concentrations at greater distance from the disk). The
283 inflection point, for penicillin G, is at around 6-8 hours (20). Thus, small zone sizes measured at
284 the earlier time point are consistent with incomplete diffusion of antimicrobial through the
285 agar. As DD breakpoints are correlated to an MIC, one option would be to develop an 'early
286 read' DD breakpoint, which would likely be smaller in diameter than the breakpoints for the
287 rDD. However, these breakpoints would be limited to those species proven to have sufficient
288 growth by the early time point. At 6 hours, 92.3% of the *P. aeruginosa* and 68.1 % of *C. freundii*
289 were unreadable due to light growth, indicating that breakpoints for these species may not be
290 appropriate for an early read. Additionally, some isolates of more common species had poor
291 growth at 6 hours, although for *E. coli*, the most prevalent clinical isolate, 83.2% of our isolates
292 actually yielded adequate growth at this early time point. These data parallel those observed in

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293 1984 by Coyle and colleagues, who noted that 78% of Gram-negative isolates had readable disk
294 diffusion results after a 6 h incubation, ranging from 64% of *P. aeruginosa* to 92% of
295 *Enterobacter* species (13). An important factor to consider is the variability between the readers
296 evaluating dDD plates. Use of digital cameras and automation may aid to resolve this variability.
297 This study also evaluated isolates directly from blood culture broth, although the authors used
298 a sterile swab dipped into the blood culture as opposed to the 4 drop inoculum, used in the
299 present study (14). A recent study by van den Bijllaardt and colleagues evaluated readability of
300 the standard DD method for the *Enterobacteriaceae*, using hourly, automated imaging of rDD
301 plates post-inoculation. In their study, 95.8% of isolates were readable at 6 hours and the
302 increase in incubation to 7 h yielded readable growth for all isolates. Further incubation to a
303 total of 10 hours minimized errors by the current rDD breakpoints (21). Such data suggest that
304 the use of smart incubator systems may further increase the readability of short-incubation disk
305 diffusion methods (19). To this end, CLSI will pursue an 8-10 hour 'early read' time point to
306 further evaluate the dDD method.

307 A second theme that arose in this study are the errors associated with beta-lactams. High
308 mE and ME rates remained at 18 hours for amoxicillin-clavulanate, cefazolin, cefoxitin,
309 ceftazidime, ceftriaxone, imipenem, meropenem, and piperacillin-tazobactam. Beta-lactams
310 translocate into the bacterial cell via porins to act on the cell synthesis pathway. One
311 explanation could involve the inhibition of this translocation due to the blood components
312 present in the inoculum. Older studies on direct-from-blood disk diffusion observed overall high
313 CA, but many of these were completed prior to availability of advanced generation

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314 cephalosporins or carbapenems, antimicrobial classes that we found to be particularly
315 problematic (3, 9, 11).

316 Developing and validating a standardized direct susceptibility method from positive blood
317 cultures could benefit laboratories and clinicians and ultimately advance patient care. Providing
318 the correct identification and susceptibility profile even a single day earlier may impact length
319 of stay, appropriate use of antibiotics, and development of antibacterial resistance. This
320 phenotypic method overcomes the inability to identify all specific resistance genes for Gram-
321 negative bacteria and is more affordable compared to platforms currently available on the
322 market.

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389 **Figure Legends**

390 Figure 1. Bacterial concentration (CFU/mL) at the time of positive signal in three blood culture
391 systems. Counts were divided into 0.25 log increments, the CFU/mL that corresponds to each
392 bin is listed in Table 6

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395 Table 1. Bacterial isolates used in study

Isolate Number	Species	Resistance phenotype
15-05-01	*Klebsiella pneumoniae*	CRE (NDM-1)
15-05-02	*K. pneumoniae*	CRE (KPC)
15-05-03	*K. pneumoniae*	ESBL (CTX-M-15)
15-05-04	*Proteus mirabilis*	Wild Type
15-05-05	*Enterobacter aerogenes*	Resistant to cephalosporins III, AmpC overexpression
15-05-06	*Enterobacter cloacae*	Wild Type
15-05-07	*E. cloacae*	Resistant to cephalosporins III, AmpC overexpression
15-05-08	*Citrobacter freundii*	None
15-05-09	*Escherichia coli*	Plasmid AmpC CMY-2
15-05-10	*E. coli*	Cefazolin resistant (mechanism not defined)
15-05-11	*E. coli*	Wild-type
15-05-12	*E. coli*	ESBL
15-05-14	*Pseudomonas aeruginosa*	Carbapenem resistant
15-05-15	*P. aeruginosa*	Wild Type
15-05-16	*P. aeruginosa*	Fluoroquinolone resistant
15-05-17	*Acinetobacter baumannii*	Wild type
15-05-18	*K. pneumoniae*	Wild type
15-05-19	*A. baumannii*	Carbapenem resistant (mechanism not defined)
15-05-20	*P. aeruginosa*	Aminoglycoside resistant
15-05-21	*Serratia marcescens*	SME
ATCC 25922	*E. coli*	Wild type
ATCC 35218	*E. coli*	Beta-lactamase producer
ATCC 27853	*P. aeruginosa*	Wild type

396 CRE= Carbapenem Resistant *Enterobacteriaceae*; ESBL, Extended Spectrum Beta-Lactamase
397 *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 35218.

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399 Table 2. Performance of direct-from-blood culture broth disk diffusion method at 18 hours

	No. results from rDD			%CA	VME		ME		mE	
Blood Culture System	S	I	R		No.	%	No.	%	No.	%
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

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401 Table 3. Resolved performance of direct-from-blood culture broth disk diffusion method by

402 antibiotic at 18 hours

Drug Name	# S	# R	%CA	VME		ME		mE	
				No.	%	No.	%	No.	%
Amikacin	45	13	96.7	0	0	0	0	2	3.3
Amoxacillin-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

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404 Table 4. Performance of dDD read at 6 hours, as compared to rDD read at 18 hours

Blood Culture System	No. with insufficient growth[1]	No. evaluable	rDD result for evaluable			% CA	VME		ME		mE	
			S	I	R		No.	%	No.	%	No.	%
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

405 1 Evaluable results included only those with sufficient growth to be read by both rDD and dDD

406 rDD, reference disk diffusion; dDD, direct-from-blood disk diffusion; VME, very major error; ME, major error; mE, minor error

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428 Table 5. Resolved performance of direct-from-blood culture broth disk diffusion method, by antibiotic at 6 hours

Drug Name	# S	# R	%CA	VME		ME		mE	
				No.	%	No.	%	No.	%
Amikacin	45	13	62.2	3	23.1	2	4.4	12	26.7
Amoxacillin-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

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432 Table 6. Performance of dDD after 18 and 6 hour incubation, stratified by concentration of
433 bacteria present in blood culture broth inoculum

				18 h dDD	6 h dDD	
Bin	CFU range		# readings	%CA	% too light	%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

434 Boxed values represent range of the 0.5 McFarland inoculum

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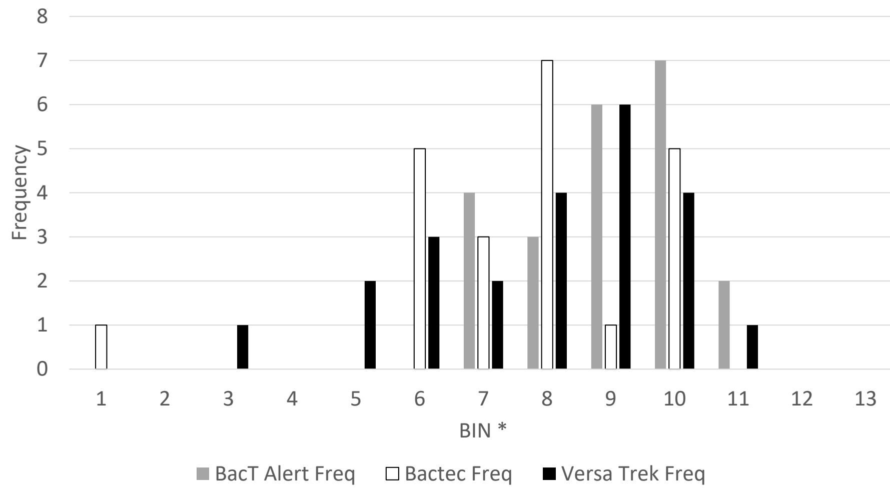

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