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1 # The performance of a resazurin chromogenic agar plate with a combined disc method for rapid screening of extended-spectrum-β-lactamases, AmpC β-lactamases and co-β-lactamases in *Enterobacteriaceae*.

5 **Running title**: Screening of ESBL and AmpC β-lactamases

6

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ABSTRACT

A resazurin chromogenic agar (RCA) along with combined disc method has been developed as a promising method for rapid screening of extended-spectrum-β-lactamase (ESBL), AmpC β-lactamase, and co-production of ESBL and AmpC. Cefpodoxime (CPD) discs supplemented with and without clavulanic acid (CA), cloxacillin (CX), or CA+ CX were evaluated against 86-molecularly confirmed β-lactamase-producing *Enterobacteriaceae*, including 15 ESBLs, 32 AmpCs, 9 co-producers of ESBL and AmpC, and 30 carbapenemase producers. The CA and CX synergy test successfully detected all ESBL producers (100% sensitivity and 98.6% specificity) and all AmpC producers (100% sensitivity and 96.36% specificity). This assay also exhibited a good performance in the screening for the co-existence of ESBL and AmpC (88.89% sensitivity and 100% specificity). The RCA assay is a simple and inexpensive method that allows observation of results within 7 h. It can be applicable in any microbiological laboratory, especially in the endemic areas of ESBL, AmpC, or co-β-lactamase-producing *Enterobacteriaceae*.

KEYWORDS: Beta-lactamases, cefpodoxime combined disc, *Enterobacteriaceae*, Resazurin chromogenic agar

45 # 1. INTRODUCTION

46 An increasing number of antibiotic-resistant opportunistic pathogens have been
47 documented globally in recent years, resulting in decreasing effective antibiotic
48 availability. Not surprisingly, these problems have created a treatment challenge and
49 pose a serious health risk affecting both hospitalized patients and health care providers
50 (1-3). β-Lactamase-associated resistance is a predominant mechanism of resistance to β-
51 lactam antibiotics in *Enterobacteriaceae*. The dissemination of resistance in these
52 bacteria is frequently facilitated by transferring mobile genetic elements among bacteria
53 (4). Currently, infections caused by multidrug-resistant (MDR) Gram-negative bacteria,
54 in particular extended-spectrum-β-lactamases (ESBL)- producing *Enterobacteriaceae*,
55 are among one of the most serious human health concerns (5). bla_{TEM}, bla_{SHV}, and
56 $bla_{CTX\text{-}M}$ genes are the most common ESBL genotypes among *Enterobacteriaceae*.
57 ESBL-producing isolates characteristically hydrolyze cefotaxime, ceftazidime, cefepime
58 and/or monobactam aztreonam, rendering these antibiotics inactive (6-8). ESBLs are
59 inhibited by β-lactamase inhibitors, namely clavulanate, sulbactam and tazobactam.
60 False-negative ESBL test results using combination disc tests may result from high-
61 level expression of AmpC β-lactamases, which masks the presence of ESBLs. The use
62 of clavulanic acid (CA) and cloxacillin (CX) together allows detection of co-production
63 of ESBL and AmpC (9). In addition, AmpC producers and co-producers of AmpC and
64 ESBL have also been reported to be resistant to third-generation cephalosporins,
65 cephamycins or β-lactam/β-lactamase inhibitor combinations (10). Infections caused by
66 AmpC- producing organisms are typically associated with resistance to multiple
67 antibiotics, including penicillins, oxyimino- 7-α-methoxycephalosporins and
68 monobactams (11, 12). In general, AmpC type enzymes are poorly inhibited by β-

69 lactamase inhibitors, especially CA. Phenotypic AmpC confirmation tests are generally
70 based on inhibition of AmpC by either CX or boronic acid (BA) derivatives. BA also
71 inhibits class A carbapenemases (13), justifying the use of CX in the present study.
72 Moreover, co-expression of ESBL and AmpC β-lactamases results in decreased
73 susceptibility to aztreonam and β-lactam/ β-lactamase inhibitors than those with either
74 ESBL or AmpC β-lactamase alone (14). This makes the selection of an effective
75 antibiotic difficult for the treatment of infections caused by these recalcitrant bacteria.
76

77 A simple, rapid and inexpensive method for screening and discrimination between
78 these enzymes at a phenotypic level could guide clinicians to prescribe an appropriate
79 chemotherapy. The combined disc method has been used extensively because it is
80 relatively easy to prepare and perform. However, this test requires at least 18 h or
81 overnight to obtain the results. A resazurin reduction assay, a colorimetric method, is
82 based upon the ability of active cells to reduce the blue colored resazurin to the pink
83 colored resorufin (15). A colorimetric (resazurin containing) disc susceptibility method
84 exhibited excellent reproducibility (16) and high sensitivity and specificity in detection
85 and differentiation of carbapenemase-producing *Enterobacteriaceae* (17). Cefpodoxime
86 (CPD) is an attractive indicator cephalosporin for detection of ESBL production and
87 may be used for screening according to EUCAST guidelines. There are several
88 diagnostic methods that have been proposed for phenotypic confirmation of ESBL and
89 AmpC β-lactamases, including the Etest, combined disc method e.g. MAST D68C test,
90 double disc synergy test, and automated broth microdilution test. These methods usually
91 take at least 18 h to obtain results (9, 18, 19). Hence, the present study investigated a
92 resazurin chromogenic agar (RCA) method together with CPD discs alone or

93 supplemented with CA, CX, and both CA and CX to screen for and discriminate
94 between ESBL, AmpC, and co-existence of ESBL and AmpC among
95 *Enterobacteriaceae*.

96 # 2. MATERIALS AND METHODS

97 ## 2.1 Bacterial isolates

98 The present study employed a total of 86 β-lactamase-producing *Enterobacteriaceae*
99 isolates to evaluate the performance of the RCA assay in rapid screening and
100 discrimination of ESBL, AmpC, and co-producers of ESBL and AmpC. The organisms
101 used in the present study are summarized in Table 1 (17, 20). The molecular types
102 included 15 Ambler class A ESBL producers (3 CTX-M-3, 1 CTX-M-15, 1 SHV-27, 1
103 SHV-18, 1 SHV, 1 TEM-214, 1 TEM-10, 1 TEM-70, 1 CTX-M-15+SHV-27, 1 SHV-
104 27+TEM-53, 1 SHV-27+TEM-71 , 1 SHV-110+TEM-84, and 1 CTX-M+SHV+TEM),
105 32 Ambler class C AmpC producers (6 DHA family, 7 CIT family, 2 MOX family, 11
106 EBC family, and 6 FOX family) and 9 co-producers of ESBL and AmpC (1
107 TEM+ ACT-type, 4 CTX-M+ ACT-types, 1 TEM+ SHV+ ACT-type, 1 TEM+ CTX-
108 M+ ACT-type, 1 SHV+ ACT type, and 1 SHV+ CTX-M-ACT-type) . Thirty
109 carbapenemase-producing isolates (8 *Klebsiella pneumoniae* carbapenemase (KPC), 11
110 metallo-β-lactamase (MBL), and 11 OXA-48 producers) were also included to validate
111 the performance of the RCA plate assay. A reference strain *E. coli* ATCC 25922 was
112 used as a negative β-lactamase control strain. The following β-lactamase-producing
113 isolates obtained from the American Type Culture Collection (ATCC) and National
114 Collection of Type Cultures (NCTC) were used as controls; *E. cloacae* ATCC BAA-
115 1143 (bla_{ACT-32}), *E. coli* NCTC 13352 (bla_{TEM-10}) and *E. coli* NCTC 13353 ($bla_{CTX-M-15}$).

116

117 **2.2 Resazurin chromogenic agar (RCA) plate and disc preparations**

118 RCA plates were prepared according to previous studies (16, 17). Briefly, 10 mL of
119 sterile 2.5 mg/mL resazurin sodium salt (Sigma, UK) was added to 990 mL of sterile
120 Mueller- Hinton (MH) agar 45-50 °C (Oxoid, UK), then mixed gently and poured into
121 Petri dishes into Petri dishes to achieve an approximately 4 mm depth.

122 For the preparation of the antibiotic- or β-lactamase inhibitor-containing discs, 10 µg of
123 CPD discs (MAST Group, UK) were supplemented with 10 µl of 1 mg/mL CA (Sigma-
124 Aldrich, UK), 10 µl of CX (Sigma-Aldrich, UK) at a concentration of 50 mg/mL, or
125 impregnated with both CA and CX. Meropenem (MER) discs (10 µg) were prepared by
126 adding 10 µl of MER (Sigma-Aldrich, UK) at a concentration of 1 mg/mL to blank
127 discs (6.5 mm diameter, MAST Group, UK). Prior to performing disc diffusion
128 susceptibility testing, the discs were air-dried in a biosafety cabinet for 1 h.

129 **2.3 Disc diffusion susceptibility testing**

130 The algorithm for phenotypic screening of ESBL, AmpC, and co-producers of ESBL
131 and AmpC is illustrated in Figure 1. The experimental procedure for disc diffusion
132 susceptibility testing was carried out according to the Clinical Laboratory Standards
133 Institute (CLSI) guidelines (21). Briefly, a sterile swab soaked in a 0.5 McFarland
134 standard of test organism was spread entirely on the surface of the RCA plate. Discs
135 containing CPD alone, CPD plus CA, CPD plus CX, CPD plus CA and CX, and MER
136 alone were placed equidistantly on the RCA's surface. The MER disc was used to
137 screen for carbapenem resistance including carbapenemase production. The inhibition
138 zone diameters were scrupulously measured and interpreted following incubation at 37

139 °C for 7 h by observing a change in the medium from the original blue (resazurin)
140 colour to pink (resorufin). The interpretation criteria in screening and differentiation of
141 ESBL, AmpC, and co-β-lactamases were based upon a previous report as presented in
142 Table 2 (19). An increase in zone diameter (≥ 5 mm) of CPD supplemented with β-
143 lactamase inhibitor compared with CPD alone was considered as synergistic activity. To
144 interpret the results, CA synergy was considered as a positive result for ESBL, while
145 CX synergy and CA plus CX synergy were noted as positive results for AmpC and co-
146 production of ESBL and AmpC, respectively. A zone diameter of MER < 25 mm was
147 used at a cut-off point to screen for the presence of carbapenemases. Sensitivity and
148 specificity of the RCA assay with a combined disc method were calculated by
149 comparing the results with molecular types from PCR and sequencing data. A box-and-
150 whisker plot was analyzed using SPSS statistical analysis program version 18 (SPSS
151 Inc, USA) to elucidate the distribution of zone diameters of discs against different β-
152 lactamase producers.

153 **3. RESULTS**

154 The use of the RCA assay along with a combined disc method for phenotypic
155 confirmation of ESBL, AmpC, and co-expression of ESBL plus AmpC clearly showed
156 the inhibition zone diameters within 7 h (Figure 2). Figure 3 illustrates the distribution
157 of the zone diameters of CPD impregnated with and without CA, CX, or CA plus CX,
158 and MER alone against ESBL, AmpC, co-existence of ESBL and AmpC, and
159 carbapenemase-producing *Enterobacteriaceae*. For screening of ESBL-producing
160 isolates, the median zone diameter of CPD alone was 6.5 mm (range = 6.5-16 mm) and
161 the median diameters of CPD supplemented with CA, CX, or CA plus CX were 22 mm

162 (range = 19-25 mm), 6.5 mm (range = 6.5-17 mm), and 23 mm (range = 21-25 mm),
163 respectively. MER discs exhibited potential activity in inhibition of ESBL producers
164 with a median zone diameter 25 mm (range = 23-27 mm) (Figure 3A). A substantial
165 increase in the zone diameters of CA-containing discs compared with those of discs
166 without CA was only observed in ESBL-producing isolates. The mean zone increase of
167 CPD plus CA compared with CPD alone was 14.6 mm (range = 5-17.5 mm). No
168 marked increase in zone diameter was observed in AmpC producers (mean = 0.48 mm
169 and range = 1.5-3.5 mm), co-producers of ESBL and AmpC (mean = 2.72 mm and
170 range 1.5-8.5 mm), as well as carbapenemase-producing isolates (mean = 0.37 mm and
171 range = 0-5.5 mm). The RCA assay with combined disc method successfully detected
172 all test ESBL producers with 100% sensitivity and 98.6 % specificity (Table 2). A false-
173 positive result was observed in an OXA-48-producing *E. coli.*
174 In AmpC producers, an increase in median zone diameters was seen in CX-containing
175 discs. CPD plus CX and CPD plus CX plus CA had equally a median zone diameter of
176 20 mm and a range of 12-26 mm. The median zone diameter of CPD against these
177 isolates was 7.25 mm (range = 6.5-20 mm) which was similar to CPD plus CA (median
178 = 8 mm and range =6.25-21 mm). MER discs inhibited the growth of AmpC-producing
179 isolates at a median zone diameter of 25 mm and range 23-28mm (Figure 3B). The
180 mean difference of zone diameter of CPD plus CX versus CPD alone was 10.1 mm
181 (range = 5.5 -16.5 mm) against AmpC producers, whilst no dramatic difference in mean
182 zone increase was observed in ESBL producers (mean =0.33 mm and range = 0-2 mm),
183 co-producers of ESBL and AmpC (mean = 4.33 mm and range = 1-7.5 mm), or
184 carbapenemase producers (mean = 0.88 mm and range = 0-9.5 mm). The RCA assay
185 demonstrated an excellence performance in the screening of AmpC-producing strains by

186 detecting all test AmpC producers (100 % sensitivity), but there were two false-
187 positives in KPC-3-producing *K. pnuemoniae* and OXA-48-producing *E. coli* (96.36 %
188 specificity; Table 2).
189 For screening of ESBL and AmpC-co-producing *Enterobacteriaceae*, CPD discs alone
190 exhibited a median zone diameter of 9 mm (range = 6.5-22 mm). CPD plus CA (median
191 = 15 mm and range = 6.5-22 mm) and CPD plus CX (median = 14 mm and range = 10-
192 27 mm) showed a slight increase in median zone diameter compared with CPD alone.
193 CPD plus CA plus CX demonstrated excellent activity in inhibiting the growth of ESBL
194 and AmpC co-producers. The median zone was significantly increased (median = 24
195 mm and range = 21-27 mm) in comparison with those of CPD alone, CPD plus CA, and
196 CPD plus CX. The median zone diameter and zone range of MER against these isolates
197 were 25 mm and 23-26 mm, respectively (Figure 3C). The mean difference in zone
198 diameter of CPD plus CA and CX versus CPD plus CA, or versus CPD plus CX was
199 also calculated. The mean zone increase of CPD plus CA and CX versus CPD plus CA
200 was 11. 61 mm (range=5-19.5 mm). A similar result was observed in CPD plus CA and
201 CX versus CPD plus CX. The sensitivity and specificity of the RCA assay with the
202 combined disc method were 88.89% and 100%, respectively (Table 2). The assay failed
203 to detect ESBL activity in a SHV plus ACT-producing *E. aerogenes*. Furthermore, in
204 carbapenemase-producing isolates, the median zone diameters of CPD with and without
205 CA, CX, or CA and CX were not markedly different, while the ranges did vary. The
206 MER disc alone had a median zone diameter of 17 mm and range 6. 5-25 mm. A
207 reference strain *E. coli* ATCC 25922 was inhibited by a CPD disc alone with zone
208 diameter 25 mm which was in the susceptible range according to the CLSI breakpoint
209 (≥21 mm) (22). The findings of this study demonstrated that the RCA assay with CPD

210 combination discs showed an excellent performance in screening of and differentiation

211 between ESBL, AmpC, and co-production of ESBL and AmpC in *Enterobacteriaceae*.

212

213 4. DISCUSSION

214 The present study proposes a rapid screening method using the RCA assay along with

215 CPD combined disc method to detect the presence of and discriminate between β-

216 lactamases within 7 h. The CA synergy test using the RCA assay with CPD combined

217 discs to confirm the presence of ESBL production in *Enterobacteriaceae* detected all

218 tested ESBL-producing isolates. Only one false-positive was found, which was in an

219 OXA-48-producing isolate. This finding agrees with a previous report published by

220 Derbyshire and colleagues (26). They found that a CA synergy test using CPD was able

221 to detect all 117 ESBL producers indicated by a ≥ 5 mm increase in zone diameter of

222 CPD plus CA in comparison with CPD alone. This synergy test could not detect ESBLs

223 in the co-presence of AmpCs. Similarly, CPD exhibited excellent performance in the

224 screening of ESBL in *K. pneumoniae* and *E. coli*, but poor sensitivity for *K. oxytoca*

225 (24, 25). The presence of ESBLs may also be masked by carbapenemases such as

226 MBLs or KPCs (26). Furthermore, not all OXA-48-variants exhibit significant

227 carbapenemase activity, some OXA-48 variants such as OXA-163 and OXA-405 have

228 been reported to be resistant to either carbapenem antibiotics or to extended-spectrum

229 cephalosporins. These two variants were significantly inhibited by CA (27, 28). We

230 speculate that the OXA-48-producing isolate used in the present study might have low

231 carbapenemase activity as indicated by relatively large zone diameter for MER (22 mm)

232 and might also co-produce ESBL.

233 For screening of AmpC-producing isolates using CX synergy test, the assay was able to
234 detect all AmpC producers and two-false positive results (100% Sensitivity and 96.36%
235 specificity). This result is consistent with many previous papers reporting a good
236 performance of CPD and CX synergy tests in detection of these enzymes (18, 19, 29). In
237 one such study MAST® D68C successfully detected almost all AmpC producers whilst
238 a few false-positive results were also reported (96.7 % sensitivity and 96.9%
239 specificity). The test could not detect the low production of AmpC β-lactamases in
240 AmpC-producing isolates (19). A similar result was reported by Ingram and colleagues,
241 they found that MAST® D68C exhibited a sensitivity and specificity above 90% in
242 detection of the presence of AmpC β-lactamase in *Enterobacteriaceae* (18). In
243 agreement with a previous study, MAST-4 disc demonstrated good sensitivity (92%)
244 and specificity (86.7%) in the detection of AmpC-producing nosocomial *Klebsiella*
245 isolates (29). Combined activity of ESBL and AmpC in the same strain can result in
246 phenotypic detection failure (30). Co-production with AmpC β-lactamases can mask
247 ESBL production with CLSI confirmatory tests leading to false-negative results (31).
248 Therefore, adding two or more specific β-lactamase inhibitors could exclude different
249 types of β-lactamase in the same strain. In the present study, we used CA plus CX
250 synergy test to discriminate co-producers of ESBL and AmpC. The assay was able to
251 detect 8 co-producers of ESBL and AmpC. Only AmpC was detected in one co-
252 producer of ESBL and AmpC. This false-negative isolate was susceptible to CPD
253 according to the CLSI breakpoint (22). The finding from this study is similar to the
254 result from a previously mentioned study where MAST® D68C was reported to
255 successfully detect all 8 ESBL and AmpC-co-producing isolates (18).

256 To screen carbapenemase-producing isolates, it has been recommended to use a cut-off
257 point lower than 25 mm for MER disc because the zone diameter of MER in some
258 OXA-48 like-producing bacteria is still in the susceptible range (≥ 23 mm) (32, 33). The
259 current study found that MER zone diameters against ESBL, AmpC, and Co-ESBL and
260 AmpC ranged from 23-28 mm, whilst in carbapenemase-producing isolates zone
261 diameters ranged from 6.5 – 25 mm. Only one OXA-48 producing isolate had a zone
262 diameter of 25 mm. Thus, the isolates showing zone diameters < 25mm for 10 µg MER
263 disc should be further investigated to detect the distinct type of carbapenemase (MBLs,
264 KPCs, and OXA-48 like carbapenemases) or AmpC plus porin loss. Carbapenem
265 antibiotics are frequently used for the treatment of the infections caused by AmpC- and
266 ESBL-producing Gram-negative pathogens (34) . However, overuse of carbapenems
267 results in development of carbapenem resistance (13,35) . Colistin, fosfomycin, and
268 tigecycline are among the most frequently used antibiotics for treating these cases (36).
269 Inclusion of the MER disc allows detection of meopenem resistance, so it is therefore
270 important in guiding clinicians to appropriate antibiotic treatments.

271 To summarize, the combined disc test is commonly used in many microbiological
272 laboratories, because it is very simple. The conventional method takes at least 18 h to
273 observe the inhibition zone diameter. In the present study, we support the use of the
274 RCA assay to improve the time to result for the disc diffusion susceptibility test. The
275 result from RCA assay can be observed within 7 h. It also demonstrates excellent
276 sensitivity and specificity for differentiation of ESBL, AmpC, and co-ESBL and AmpC-
277 producing *Enterobacteriaceae*. The RCA assay could be applicable to commercially
278 available discs, including MAST discs (Mast Group, UK) and it can also be applied in
279 CLSI ESBL confirmatory tests and any disc diffusion method. However, a larger

280 sample size of clinical isolates is still required to further validate and establish the
281 robustness of this assay. A rapid phenotypic method that can detect and differentiate the
282 different types of β-lactamase would improve the effectiveness of antibiotic
283 administration and would also help to control the dissemination of the infection caused
284 by these refractory bacteria.

285

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291

292 DISCLOSURE

293 The authors have no conflict of interest to declare.

294

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297 REFERENCES

298 1. Huttner, A., Harbarth, S., Carlet, J., Cosgrove, S., Goossens, H., Holmes, A.,
299 Jarlier, V., Voss, A.,Pittet, D. (2013) Antimicrobial resistance: a global view

300 from the 2013 World Healthcare-Associated Infections Forum. *Antimicrob*
301 *Resist Infect Control* **2**(1): 1-13.

302 2. National Nosocomial Infections Surveillance System (2004) National
303 Nosocomial Infections Surveillance (NNIS) System Report, data summary from
304 January 1992 through June 2004, issued October 2004. *Am J Infect Control*
305 **32**(8): 470-85.

306 3. Payne, D.J. (2008) Microbiology. Desperately seeking new antibiotics. *Science*
307 **321**(5896): 1644-5.

308 4. Bush, K. (2010) Alarming beta-lactamase-mediated resistance in multidrug-
309 resistant *Enterobacteriaceae*. *Curr Opin Microbiol* **13**(5): 558-64.

310 5. Tacconelli, E., Cataldo, M.A., Dancer, S.J., De Angelis, G., Falcone, M., Frank,
311 U., Kahlmeter, G., Pan, A., Petrosillo, N., Rodriguez-Bano, J., Singh, N.,
312 Venditti, M., Yokoe, D.S.,Cookson, B. (2014) ESCMID guidelines for the
313 management of the infection control measures to reduce transmission of
314 multidrug-resistant Gram-negative bacteria in hospitalized patients. *Clin*
315 *Microbiol Infect* **20 Suppl 1**: 1-55.

316 6. Lahlaoui, H., Anis, B.H., Mohamed, K.,Mohamed, B.M. (2012) Emergence of
317 SHV-12 extended spectrum beta-lactamase among clinical isolates of
318 *Enterobacter cloacae* in Tunisia. *Microb Pathog* **53**(2): 64-5.

319 7. Livermore, D.M.,Brown, D.F. (2001) Detection of beta-lactamase-mediated
320 resistance. *J Antimicrob Chemother* **48 Suppl 1**: 59-64.

321 8. Peymani, A., Farivar, T.N., Sanikhani, R., Javadi, A.,Najafipour, R. (2014)
322 Emergence of TEM, SHV, and CTX-M-extended spectrum beta-lactamases and
323 class 1 integron among *Enterobacter cloacae* isolates collected from hospitals of
324 Tehran and Qazvin, Iran. *Microb Drug Resist* **20**(5): 424-30.

325 9. Jeong, S.H., Song, W., Kim, J.S., Kim, H.S.,Lee, K.M. (2009) Broth
326 microdilution method to detect extended-spectrum beta-lactamases and AmpC
327 beta-lactamases in *Enterobacteriaceae* isolates by use of clavulanic acid and
328 boronic acid as inhibitors. *J Clin Microbiol* **47**(11): 3409-12.

329 10. Matsumura, Y., Yamamoto, M., Matsushima, A., Nagao, M., Ito, Y., Takakura,
330 S.,Ichiyama, S. (2012) Cefotaxime for the detection of extended-spectrum beta-
331 lactamase or plasmid-mediated AmpC beta-lactamase and clinical characteristics
332 of cefotaxime-non-susceptible *Escherichia coli* and *Klebsiella pneumoniae*
333 bacteraemia. *Eur J Clin Microbiol Infect Dis* **31**(8): 1931-9.

334 11. Livermore, D.M. (1995) beta-Lactamases in laboratory and clinical resistance.
335 *Clin Microbiol Rev* **8**(4): 557-84.

336 12. Seral, C., Gude, M.J.,Castillo, F.J. (2012) [Emergence of plasmid mediated
337 AmpC beta-lactamasas: Origin, importance, detection and therapeutical options].
338 *Rev Esp Quimioter* **25**(2): 89-99.

339 13. Jacoby, G.A. (2009) AmpC beta-lactamases. *Clin Microbiol Rev* **22**(1): 161-82.

340 14. Tsui, K., Wong, S.S., Lin, L.C., Tsai, C.R., Chen, L.C.,Huang, C.H. (2012)
341 Laboratory identification, risk factors, and clinical outcomes of patients with
342 bacteremia due to *Escherichia coli* and *Klebsiella pneumoniae* producing

343 extended-spectrum and AmpC type beta-lactamases. *J Microbiol Immunol Infect*
344 **45**(3): 193-9.

345 15. O'brien, J., Wilson, I., Orton, T.,Pognan, F. (2000) Investigation of the Alamar
346 Blue (resazurin) fluorescent dye for the assessment of mammalian cell
347 cytotoxicity. *European Journal of Biochemistry* **267**(17): 5421-26.

348 16. Sener, S., Acuner, I.C., Bek, Y.,Durupinar, B. (2011) Colorimetric-plate method
349 for rapid disk diffusion susceptibility testing of *Escherichia coli*. *J Clin*
350 *Microbiol* **49**(3): 1124-7.

351 17. Teethaisong, Y., Eumkeb, G., Nakouti, I., Evans, K.,Hobbs, G. (2016) A
352 combined disc method with resazurin agar plate assay for early phenotypic
353 screening of KPC, MBL and OXA-48 carbapenemases among
354 *Enterobacteriaceae*. *J Appl Microbiol* **121**(2): 408-14.

355 18. Ingram, P.R., Inglis, T.J., Vanzetti, T.R., Henderson, B.A., Harnett,
356 G.B.,Murray, R.J. (2011) Comparison of methods for AmpC beta-lactamase
357 detection in *Enterobacteriaceae*. *J Med Microbiol* **60**(Pt 6): 715-21.

358 19. Nourrisson, C., Tan, R.N., Hennequin, C., Gibold, L., Bonnet, R.,Robin, F.
359 (2015) The MAST(R) D68C test: an interesting tool for detecting extended-
360 spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*. *Eur J Clin*
361 *Microbiol Infect Dis* **34**(5): 975-83.

362 20. Teethaisong, Y., Eumkeb, G., Chumnarnsilpa, S., Autarkool, N., Hobson, J.,
363 Nakouti, I., Hobbs, G.,Evans, K. (2016) Phenotypic detection of AmpC beta-
364 lactamases, extended-spectrum- beta-lactamases and metallo-beta-lactamases in

365 *Enterobacteriaceae* using a resazurin microtitre assay with inhibitor-based
366 methods. *J Med Microbiol* **65**: 1079-87.

367 21. Clinical Laboratory Standards Institute (2010) Performance standards for
368 antimicrobial disk susceptibility tests; approved standard CLSI document M02-
369 A10, 10 edn. Wayne,PA,USA.

370 22. Clinical Laboratory Standards Institute (2014) Performance standards for
371 antimicrobial susceptibility testing;Twenty-fourth informational supplement
372 CLSI document M100-S24*,* 10 edn. CLSI, Wayne,PA, USA

373 23. Derbyshire, H., Kay, G., Evans, K., Vaughan, C., Kavuri, U.,Winstanley, T.
374 (2009) A simple disc diffusion method for detecting AmpC and extended-
375 spectrum beta-lactamases in clinical isolates of *Enterobacteriaceae*. *J*
376 *Antimicrob Chemother* **63**(3): 497-501.

377 24. Thomson, K.S. (2001) Controversies about extended-spectrum and AmpC beta-
378 lactamases. *Emerg Infect Dis* **7**(2): 333-36.

379 25. Thomson, K.S.,Sanders, C.C. (1997) A simple and reliable method to screen
380 isolates of *Escherichia coli* and *Klebsiella pneumoniae* for the production of
381 TEM- and SHV-derived extended-spectrum beta-lactamases. *Clin Microbiol*
382 *Infect* **3**(5): 549-54.

383 26. Antunes, N.T., Lamoureaux, T.L., Toth, M., Stewart, N.K., Frase,
384 H.,Vakulenko, S.B. (2014) Class D beta-lactamases: are they all
385 carbapenemases? *Antimicrob Agents Chemother* **58**(4): 2119-25.

386 27. Dortet, L., Oueslati, S., Jeannot, K., Tande, D., Naas, T.,Nordmann, P. (2015)
387 Genetic and biochemical characterization of OXA-405, an OXA-48-type
388 extended-spectrum beta-lactamase without significant carbapenemase activity.
389 *Antimicrob Agents Chemother* **59**(7): 3823-28.

390 28. Poirel, L., Castanheira, M., Carrër, A., Rodriguez, C.P., Jones, R.N.,
391 Smayevsky, J.,Nordmann, P. (2011) OXA-163, an OXA-48-related class D β-
392 lactamase with extended activity toward expanded-spectrum cephalosporins.
393 *Antimicrob Agents Chemother* **55**(6): 2546-51.

394 29. Mansour, S.a.E.-A., El-Sharkawy, A.a.-R., El-Kady, L.M.,Esmaeel, N.E.-S.
395 (2013) Detection of extended-spectrum & plasmid-mediated AmpC beta-
396 lactasmases in nosocomial *Klebsiella* isolates. *J Microbiol Infect Dis* **3**(1): 24-
397 30.

398 30. Goossens, H.,Grabein, B. (2005) Prevalence and antimicrobial susceptibility
399 data for extended-spectrum beta-lactamase- and AmpC-producing
400 *Enterobacteriaceae* from the MYSTIC Program in Europe and the United States
401 (1997-2004). *Diagn Microbiol Infect Dis* **53**(4): 257-64.

402 31. Munier, G.K., Johnson, C.L., Snyder, J.W., Moland, E.S., Hanson,
403 N.D.,Thomson, K.S. (2010) Positive extended-spectrum-beta-lactamase (ESBL)
404 screening results may be due to AmpC beta-lactamases more often than to
405 ESBLs. *J Clin Microbiol* **48**(2): 673-74.

406 32. Giske, C.G., Gezelius, L., Samuelsen, O., Warner, M., Sundsfjord,
407 A.,Woodford, N. (2011) A sensitive and specific phenotypic assay for detection
408 of metallo-beta-lactamases and KPC in *Klebsiella pneumoniae* with the use of

409 meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid
410 and cloxacillin. *Clin Microbiol Infect* **17**(4): 552-56.

411 33. Van Dijk, K., Voets, G.M., Scharringa, J., Voskuil, S., Fluit, A.C., Rottier,
412 W.C., Leverstein-Van Hall, M.A.,Cohen Stuart, J.W. (2014) A disc diffusion
413 assay for detection of class A, B and OXA-48 carbapenemases in
414 *Enterobacteriaceae* using phenyl boronic acid, dipicolinic acid and temocillin.
415 *Clin Microbiol Infect* **20**(4): 345-49.

416 34. Hurst, M.,Lamb, H.M. (2000) Meropenem: a review of its use in patients in
417 intensive care. Drugs 59(3): 653-80.

418 35. Perez, F., Endimiani, A., Hujer, K.M.,Bonomo, R.A. (2007) The continuing
419 challage of ESBLs. *Curr Opin Pharmacol.* **7**(5): 459-69

420 36. Lee, C.-S.,Doi, Y. (2014) Therapy of Infections due to Carbapenem-Resistant
421 Gram-Negative Pathogens. *Infect Chemother* **46**(3): 149-64.

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430 **FIGURE LEGENDS**

431 **Figure 1.** The algorithm for confirmation of and differentiation between ESBL,
432 AmpC, and co-production of ESBL and AmpC in *Enterobacteriaceae.* CA= clavulanic
433 acid (10 µg); CX = cloxacillin (500 µg).

434 **Figure 2.** Phenotypic results from the RCA plate assay with a combined disc method
435 at 7 h. A = cefpodoxime (10 µg); B = cefpodoxime (10 µg) + clavulanic acid (10 µg);
436 C =cefpodoxime (10 µg) + cloxacillin (500 µg); D= cefpodoxime (10 µg) + clavulanic
437 acid (10 µg) + cloxacillin (500 µg);E = meropenem (10 µg).

438 **Figure 3.** Distribution of zone diameters of cefpodoxime (CPD) alone, CPD with
439 clavulanic acid, CPD with cloxacillin, CPD with both clavulanic acid and cloxacillin
440 and meropenem alone. A = ESBL producers (n=15); B = AmpC producers (n=32); C=
441 co-producers AmpC and ESBL (n=9); D = carbapenemase producers (n=30).
442 CPD=cefpodoxime (10 µg); CA= clavulanic acid (10 µg); CX=cloxacillin (500 µg);
443 MER = meropenem (10 µg). º = mild outlier; * extreme outlier.
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445 **TABLES**

446

447 **Table 1**. Summary of ESBL, AmpC, ESBL+AmpC and carbapenemase-producing
448 isolates used in the present study. Abbreviation for Organism; EC = *E. coli*, KP = *K.*
449 *pneumoniae*, EA= *E. aerogenes*, ECL = *E. cloacae*, MM= *M. morganii*, CF=*C. freundii*,
450 KOX= *K. oxytoca*, KOZ= *K. ozaenae*. Abbreviation for β-lactamase; ESBL=extended-
451 spectrum-β-lactamase, KPC= *Klebsiella pneumoniae* carbapenemase, MBL = metallo-
452 β-lactamase

453 **Table 2** Interpretation criteria, sensitivity, and specificity of the RCA plate assay with a
454 combined disc method for rapid screening of ESBL, AmpC, and co-producers of ESBL
455 and AmpC among *Enterobacteriaceae*. CPD=cefpodoxime (10 µg); CA= clavulanic
456 acid (10 µg); CX=cloxacillin (500 µg).

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458 **List of Abbreviations**

ATCC	American Type Culture Collection
BA	Boronic acid
CPD	Cefpodoxime
CA	Clavulanic acid
CLSI	Clinical Laboratory Standards Institute
CX	Cloxacillin
ESBL	Extended-spectrum-β-lactamase
KPC	*Klebsiella pneumoniae* carbapenemase
MBL	Metallo-β-lactamase
MER	Meropenem
NCTC	National Collection of Type Cultures
RCA	Resazurin chromogenic agar

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