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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.

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

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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: Resazurin chromogenic agar, cefpodoxime combined disc, phenotypic test, β -lactamases, Enterobacteriaceae.

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1. INTRODUCTION

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

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

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

both CA and CX to screen for and discriminate between ESBL, AmpC, and co-existence of ESBL-AmpC among Enterobacteriaceae.

2. MATERIALS AND METHODS

2.1 Bacterial isolates

The present study employed a total of 86 β -lactamase-producing Enterobacteriaceae isolates to evaluate the performance of the RCA assay in rapid screening and discrimination of ESBL, AmpC, and co-producers of ESBL and AmpC. The organisms used in the present study are summarized in Table 1 (17, 20). The molecular types included 15 Ambler class A ESBL producers (4 CTX-M-types, 3 SHV-types, 3 TEM-types, 1 CTX-M+SHV-type, 3 SHV+TEM-types, and 1 CTX-M+SHV+TEM-type), 32 Ambler class C AmpC producers (6 DHA family, 7 CIT family, 2 MOX family, 11 EBC family, and 6 FOX family) and 9 co-producers of ESBL and AmpC (1 TEM+ACT-type, 4 CTX-M+ACT-types, 1 TEM+SHV+ACT-type, 1 TEM+CTX-M+ACT-type, 1 SHV+ACT type, and 1 SHV+CTX-M-ACT-type). Thirty carbapenemase-producing isolates (8 KPC, 11 MBL, and 11 OXA-48 producers) were also included to validate the performance of the RCA plate assay. A reference strain *E. coli* ATCC 25922 was used as a negative β -lactamase control strain. The following β -lactamase-producing isolates obtained from the American Type Culture Collection (ATCC) and National Collection of Type Cultures (NCTC) were used as controls; *E. cloacae* ATCC BAA-1143 (*bla*_{ACT-32}), *E. coli* NCTC 13352 (*bla*_{TEM-10}) and *E. coli* NCTC 13353 (*bla*_{CTX-M-15}).

2.2 Resazurin chromogenic agar (RCA) plate and disc preparations

RCA plates were prepared according to previous studies (16, 17). For the preparation of the antibiotic- or β -lactamase inhibitor-containing discs, 10 μ g of CPD discs (MAST Group, UK) were supplemented with 10 μ l of 1 mg/mL CA (Sigma-Aldrich, UK), 10 μ l of CX (Sigma-Aldrich, UK) at a concentration of 50 mg/mL, or impregnated with both CA and CX. Meropenem (MER) discs (10 μ g) were prepared by adding 10 μ l of MER (Sigma-Aldrich, UK) at a concentration of 1 mg/mL to blank discs (6.5 mm diameter, MAST Group, UK). Prior to performing disc diffusion susceptibility testing, the discs were air-dried in a biosafety cabinet for 1 h.

2.3 Disc diffusion susceptibility testing

The algorithm for phenotypic screening of ESBL, AmpC, and co-producers of ESBL and AmpC is illustrated in Figure 1. The experimental procedure for disc diffusion susceptibility testing was carried out according to the Clinical Laboratory Standards Institute (CLSI) guidelines (21). Briefly, a sterile swab soaked in a 0.5 McFarland standard of test organism was spread entirely on the surface of the RCA plate. Discs containing CPD alone, CPD plus CA, CPD plus CX, CPD plus CA and CX, and MER alone were placed equidistantly on the RCA's surface. The MER disc was used to screen for carbapenem resistance including carbapenemase production. The inhibition zone diameters were scrupulously measured and interpreted following incubation at 37 °C for 7 h by observing a change in the medium from the original blue (resazurin) colour to pink (resorufin). The interpretation criteria in screening and differentiation of ESBL, AmpC, and co- β -lactamases were based upon a previous report as presented in Table 2 (19). An increase in zone diameter (≥ 5 mm) of CPD supplemented with β -

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4 137 lactamase inhibitor compared with CPD alone was considered as synergistic activity. To
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6 138 interpret the results, CA synergy was considered as a positive result for ESBL, while
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8 139 CX synergy and CA plus CX synergy were noted as positive results for AmpC and co-
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10 140 production of ESBL and AmpC, respectively. A zone diameter of MER < 25 mm was
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12 141 used at a cut-off point to screen for the presence of carbapenemases. Sensitivity and
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14 142 specificity of the RCA assay with a combined disc method were calculated by
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16 143 comparing the results with molecular types from PCR and sequencing data. A box-and-
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18 144 whisker plot was analyzed using SPSS statistical analysis program version 18 (SPSS
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20 145 Inc, USA) to elucidate the distribution of zone diameters of discs against different β -
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22 146 lactamase producers.
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27 **3. RESULTS**
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30 148 Using the RCA assay along with a combined disc method for phenotypic confirmation
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32 149 of ESBL, AmpC, and co-expression of ESBL plus AmpC clearly showed the inhibition
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34 150 zone diameters within 7 h (Figure 2). Figure 3 illustrates the distribution of the zone
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36 151 diameters of CPD impregnated with and without CA, CX, or CA plus CX, and MER
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38 152 alone against ESBL, AmpC, co-existence of ESBL and AmpC, and carbapenemase-
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40 153 producing Enterobacteriaceae. For screening of ESBL-producing isolates, the median
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42 154 zone diameter of CPD alone was 6.5 mm (range = 6.5-16 mm) and diameters of CPD
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44 155 supplemented with CA, CX, or CA plus CX were 22 mm (range = 19-25 mm), 6.5 mm
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46 156 (range = 6.5-17 mm), and 23 mm (range = 21-25 mm), respectively. MER discs
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48 157 exhibited potential activity in inhibition of ESBL producers with a median zone
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50 158 diameter 25 mm (range = 23-27 mm) (Figure 3A). A substantial increase in zone
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4 160 observed in ESBL-producing isolates. The mean zone increase of CPD plus CA
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6 161 compared with CPD alone was 14.60 mm (range = 5-17.50 mm). No marked increase in
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8 162 zone diameter was observed in AmpC producers (mean = 0.48 mm and range = 1.5-3.5
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10 163 mm), co-producers of ESBL and AmpC (mean = 2.72 mm and range 1.5-8.5 mm), as
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12 164 well as carbapenemase-producing isolates (mean = 0.37 mm and range = 0-5.5 mm).
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14 165 The RCA assay with combined disc method successfully detected all test ESBL
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16 166 producers with 100% sensitivity and 98.6 % specificity (Table 2). A false-positive result
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18 167 was observed in an OXA-48-producing *E. coli*.

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21 168 In AmpC producers, an increase in median zone diameters was seen in CX-containing
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23 169 discs. CPD plus CX and CPD plus CX plus CA had equally a median zone diameter of
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25 170 20 mm and a range 12-26 mm. The median zone diameter of CPD against these isolates
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27 171 was 7.25 mm (range = 6.5-20 mm) which was similar to CPD plus CA (median = 8 mm
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29 172 and range = 6.25-21 mm). MER discs inhibited the growth of AmpC-producing isolates
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31 173 at a median zone diameter of 25 mm and range 23-28mm (Figure 3B). The mean
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33 174 difference of zone diameter of CPD plus CX versus CPD alone was 10.09 mm (range =
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35 175 5.50 -16.50 mm) against AmpC producers, whilst no dramatic difference in mean zone
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37 176 increase was observed in ESBL producers (mean = 0.33 mm and range = 0-2 mm), co-
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39 177 producers of ESBL and AmpC (mean = 4.33 mm and range = 1-7.5 mm), or
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41 178 carbapenemase producers (mean = 0.88 mm and range = 0-9.5 mm). The RCA assay
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43 179 demonstrated an excellence performance in the screening of AmpC-producing strains by
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45 180 detecting all test AmpC producers (100 % sensitivity), but there were two false-
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47 181 positives in KPC-3-producing *K. pneumoniae* and OXA-48-producing *E. coli* (96.36 %
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49 182 specificity; Table 2).
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55 183 For screening of ESBL and AmpC-co-producing Enterobacteriaceae, CPD discs alone
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exhibited a median zone diameter of 9 mm (range = 6.5-22 mm). CPD plus CA (median = 15 mm and range = 6.5-22 mm) and CPD plus CX (median = 14 mm and range = 10-27 mm) showed a slight increase in median zone diameter compared with CPD alone. CPD plus CA plus CX demonstrated excellent activity in inhibiting the growth of ESBL and AmpC co-producers. The median zone was significantly increased (median = 24 mm and range = 21-27 mm) in comparison with those of CPD alone, CPD plus CA, and CPD plus CX. The median zone diameter and zone range of MER against these isolates were 25 mm and 23-26 mm, respectively (Figure 3C). The mean difference in zone diameter of CPD plus CA and CX versus CPD plus CA, or versus CPD plus CX was also calculated. The mean zone increase of CPD plus CA and CX versus CPD plus CA was 11. 61 mm (range=5-19.5 mm). A similar result was observed in CPD plus CA and CX versus CPD plus CX. The sensitivity and specificity of the RCA assay with the combined disc method were 88.89% and 100%, respectively (Table 2). The assay failed to detect ESBL activity in a SHV plus ACT-producing *E. aerogenes*. Furthermore, in carbapenemase-producing isolates, the median zone diameters of CPD with and without CA, CX, or CA and CX were not markedly different, while the ranges did vary. The MER disc alone had a median zone diameter of 17 mm and range 6.5-25 mm. A reference strain *E. coli* ATCC 25922 was inhibited by a CPD disc alone with zone diameter 25 mm which was in the susceptible range according to the CLSI breakpoint (≥ 21 mm) (22). The findings of this study demonstrated that the RCA assay with CPD combination discs showed an excellent performance in screening of and differentiation between ESBL, AmpC, and co-production of ESBL and AmpC in Enterobacteriaceae.

4. DISCUSSION

Resistance to β -lactams, the most potent bactericidal antibiotics commonly used for the treatment of bacterial infections, has been continuously documented throughout the globe. β -Lactamase-mediated resistance is a major mechanism that can potentially render β -lactams inactive by hydrolytically cleaving the amide bond of the β -lactam ring (23). To guide clinicians to prescribe antibiotic therapy appropriately, development of quick phenotypic methods is necessary. The detection of the presence of β -lactamase enzymes in Gram-negative bacteria at the phenotypic level is useful because it is not costly compared with genotypic tests (PCR and sequencing). Also, a phenotypic method does not require skilled and experienced technicians (24). The principle of the phenotypic test is fundamentally based upon a synergistic effect between antibiotics and β -lactamase inhibitors (25). Several phenotypic tests for the detection of β -lactamase enzymes in Gram-negative bacteria have currently been proposed including disc diffusion assays and broth microdilution methods (9, 19, 26, 27). The time to interpret these results usually takes 18 h or overnight incubation (28, 29). In the present study, we propose the rapid screening method using RCA assay along with CPD combined disc method to detect the presence of and discriminate between β -lactamases within 7 h.

CA synergy test using the RCA assay with CPD combined discs to confirm the presence of ESBL production in Enterobacteriaceae was capable of detecting all test ESBL-producing isolates. There was only one false-positive found in an OXA-48-producing isolate. This finding agrees with a previous report published by Derbyshire and colleagues (26). They found that a CA synergy test using CPD was able to detect all 117 ESBL producers indicated by a ≥ 5 mm increase in zone diameter of CPD plus CA in

231 comparison with CPD alone. This synergy test could not detect ESBLs in the co-
232 presence with AmpCs. Similarly, CPD exhibited excellent performance in the screening
233 of ESBL in *K. pneumoniae* and *E. coli*, but poor sensitivity for *K. oxytoca* (30, 31). The
234 presence of ESBLs may also be masked by carbapenemases such as MBLs or KPCs
235 (32). Furthermore, not all OXA-48-variants exhibit significant carbapenemase activity,
236 some OXA-48 variants such as OXA-163 and OXA-405 have been reported to be
237 resistant to either carbapenem antibiotics or to extended-spectrum cephalosporins.
238 These two variants were significantly inhibited by CA (33, 34). We speculate that a
239 OXA-48-like-producing isolate used in the present study might have low
240 carbapenemase activity as indicated by relatively large zone diameter for MER (22 mm)
241 and might also co-produce ESBL.

242 For screening of AmpC-producing isolates using CX synergy test, the assay was able to
243 detect all AmpC producers and two-false positive results (100% Sensitivity and 96.36%
244 specificity). This result is consistent with many previous works reporting a good
245 performance of CPD and CX synergy test in detection of these enzymes. In one such
246 study MAST® D68C successfully detected almost all AmpC producers whilst a few
247 false-positive results were also reported (96.7 % sensitivity and 96.9% specificity). The
248 test could not detect the low production of AmpC β -lactamases in AmpC-producing
249 isolates (19). A similar result was reported by Ingram and colleagues, they found that
250 MAST® D68C exhibited a sensitivity and specificity above 90% in detection of the
251 presence of AmpC β -lactamase in Enterobacteriaceae. In agreement with a previous
252 study, MAST-4 disc demonstrated good sensitivity (92%) and specificity (86.7%) in the
253 detection of AmpC-producing nosocomial *Klebsiella* isolates (35). Combined activity of
254 ESBL and AmpC in the same strain can result in phenotypic detection failure (36). Co-

production with AmpC β -lactamases can mask ESBL production with CLSI confirmatory tests leading to false-negative results (37). Therefore, adding two or more specific β -lactamase inhibitors could exclude different types of β -lactamase in the same strain. In the present study, we used CA plus CX synergy test to discriminate co-producers of ESBL and AmpC. The assay was able to detect 8 co-producers of ESBL and AmpC. Only AmpC was detected in one co-producer of ESBL and AmpC. This false-negative isolate was susceptible to CPD according to the CLSI breakpoint (Clinical Laboratory Standards Institute, 2014). The finding from this study is similar to the result from a previously mentioned study where MAST[®] D68C was reported to successfully detect all 8 ESBL and AmpC-co-producing isolates (19).

To screen carbapenemase-producing isolates, it has been recommended to use a cut-off point lower than 25 mm for MER disc because the zone diameter of MER in some OXA-48 like-producing bacteria is still in the susceptible range (≥ 23 mm) (38, 39). The current study found that MER zone diameters against ESBL, AmpC, and Co-ESBL and AmpC ranged from 23-28 mm, whilst in carbapenemase-producing isolates zone diameters ranged from 6.5 – 25 mm. Only one OXA-48 producing isolate had a zone diameter of 25 mm. Thus, the isolates showing zone diameters < 25 mm for 10 μ g MER disc should be further investigated to detect the distinct type of carbapenemase (metallo- β -lactamases, *Klebsiella pneumoniae* carbapenemases, and OXA-48 like carbapenemases) or AmpC plus porin loss.

To summarize, the combined disc test is commonly used in many microbiological laboratories, because it is very simple. The conventional method takes at least 18 h to observe the inhibition zone diameter. In the present study, we support the use of the RCA assay to improve a time to result for the disc diffusion susceptibility test. The

279 result from RCA assay can be observed within 7 h. It also demonstrates excellent
280 sensitivity and specificity for differentiation of ESBL, AmpC, and co-ESBL and AmpC-
281 producing Enterobacteriaceae. The RCA assay could be applicable to commercially
282 available discs, including MAST discs (Mast Group, UK) and it can also be applied in
283 CLSI ESBL confirmatory tests and any disc diffusion method. However, a larger
284 sample size of clinical isolates is still required to further validate and establish the
285 robustness of this assay. A rapid phenotypic method that can detect and differentiate the
286 different types of β -lactamase would improve the effectiveness of antibiotic
287 administration and would also help to control the dissemination of the infection caused
288 by these refractory bacteria.

289

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296 **DISCLOSURE**

297 The authors have no conflict of interest to declare.

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FIGURE LEGENDS

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

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

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

454 **Tables**

455

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

462 **Table 2** Interpretation criteria, sensitivity, and specificity of a combined disc synergy
463 method along with RCA assay for rapid screening of ESBL, AmpC, and co-producers
464 of ESBL and AmpC among Enterobacteriaceae. CPD=cefpodoxime (10 μ g); CA=
465 clavulanic acid (10 μ g); CX=cloxacillin (500 μ g)

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Table 1. Summary of ESBL, AmpC, ESBL+AmpC and carbapenemase-producing isolates used in the present study.

Group of β -lactamases	EC	KP	EA	ECL	MM	CF	KOX	KOZ
Ambler class A ESBL (n =15)								
CTX-M family (n=4)	4	-	-	-	-	-	-	-
SHV family (n=3)	-	2	-	1	-	-	-	-
TEMfamily (n=3)	3	-	-	-	-	-	-	-
CTX-M + SHV (n=1)	-	1	-	-	-	-	-	-
SHV + TEM (n=3)	-	3	-	-	-	-	-	-
CTX-M + SHV+TEM (n=1)	-	1	-	-	-	-	-	-
Ambler class C AmpC (n =32)								
DHA family (n=6)	2	2	-	-	2	-	-	-
CIT family (n= 7)	4	1	1	-	-	1	-	-
MOX family (n=2)	1	-	-	-	-	1	-	-
EBC family (n=11)	1	1	3	6	-	-	-	-
FOX family (n=6)	2	-	3	1	-	-	-	-
Class A + Class C (n=9)								
TEM+ACT (n=1)	-	-	-	1	-	-	-	-
CTX-M + ACT (n=4)	1	-	1	-	-	2	-	-
TEM+SHV+ACT (n=1)	-	-	1	-	-	-	-	-
TEM+CTX-M+ACT (n=1)	-	-	1	-	-	-	-	-
SHV+ACT (n=1)	-	-	1	-	-	-	-	-
SHV+CTX-M+ACT (n=1)	-	-	1	-	-	-	-	-
Carbapenemase producers (n=30)								
class A KPC (n=8)	2	5	-	-	-	-	1	-
class A MBL (n=11)	1	7	-	2	-	-	-	1
Ambler class D OXA-48 (n=11)	4	5	-	2	-	-	-	-
Total (number of isolates)	25	28	12	13	2	4	1	1

Abbreviation for Organism; EC = *E. coli*, KP = *K. pneumoniae*, EA= *E. aerogenes*, ECL = *E. cloacae*, MM = *M. morganii*, CF = *C. freundii*, KOX = *K. oxytoca*, KOZ = *K. ozaenae*. **Abbreviation for β -lactamase;** ESBL = extended-spectrum- β -lactamase, KPC = *Klebsiella pneumoniae* carbapenemase, MBL = metallo- β -lactamase.

Table 2. Interpretation criteria, sensitivity, and specificity of a combined disc synergy method along with RCA assay for rapid screening of ESBL, AmpC, and co-producers of ESBL and AmpC among Enterobacteriaceae.

Synergy test	Definition of the test	ESBL	AmpC	ESBL+ AmpC	Sensitivity	Specificity
CA synergy test	CPD+CA vs CPD \geq 5 mm and CPD+CA+CX vs CPD+CX \geq 5 mm	+	-	-	100 (15/15)	98.6 (71/72)
CX synergy test	CPD+CX vs CPD \geq 5 mm and CPD+CA+CX vs CPD+CA \geq 5mm	-	+	-	100 (32/32)	96.36 (53/55)
CA+CX synergy test	Both CPD+CA+CX vs CPD+CX \geq 5 mm and CPD+CA+CX vs CPD+CA \geq 5 mm	-	-	+	88.89 (8/9)	100 (78/78)

CPD=cefepodoxime (10 μ g); CA= clavulanic acid (10 μ g); CX=cloxacillin (500 μ g)

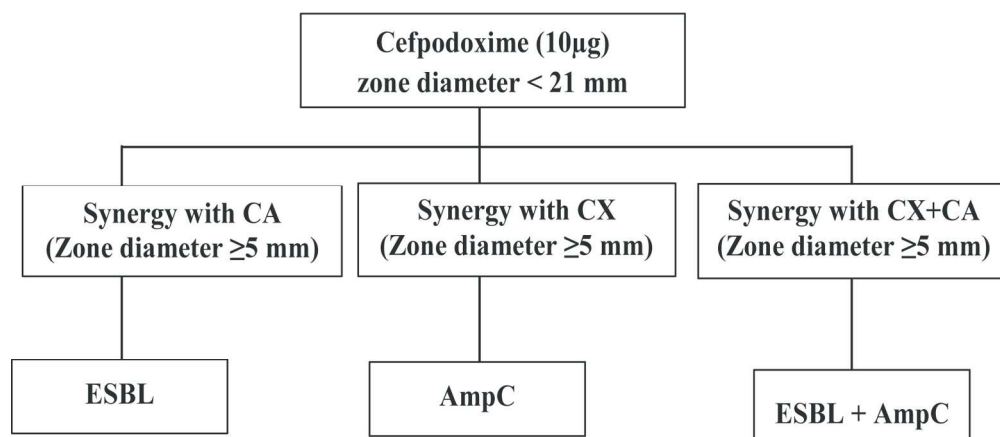


Figure 1. The algorithm for confirmation of and differentiation between ESBL, AmpC, and co-production of ESBL and AmpC in Enterobacteriaceae. CA= clavulanic acid (10 µg); CX = cloxacillin (500 µg).

156x67mm (300 x 300 DPI)

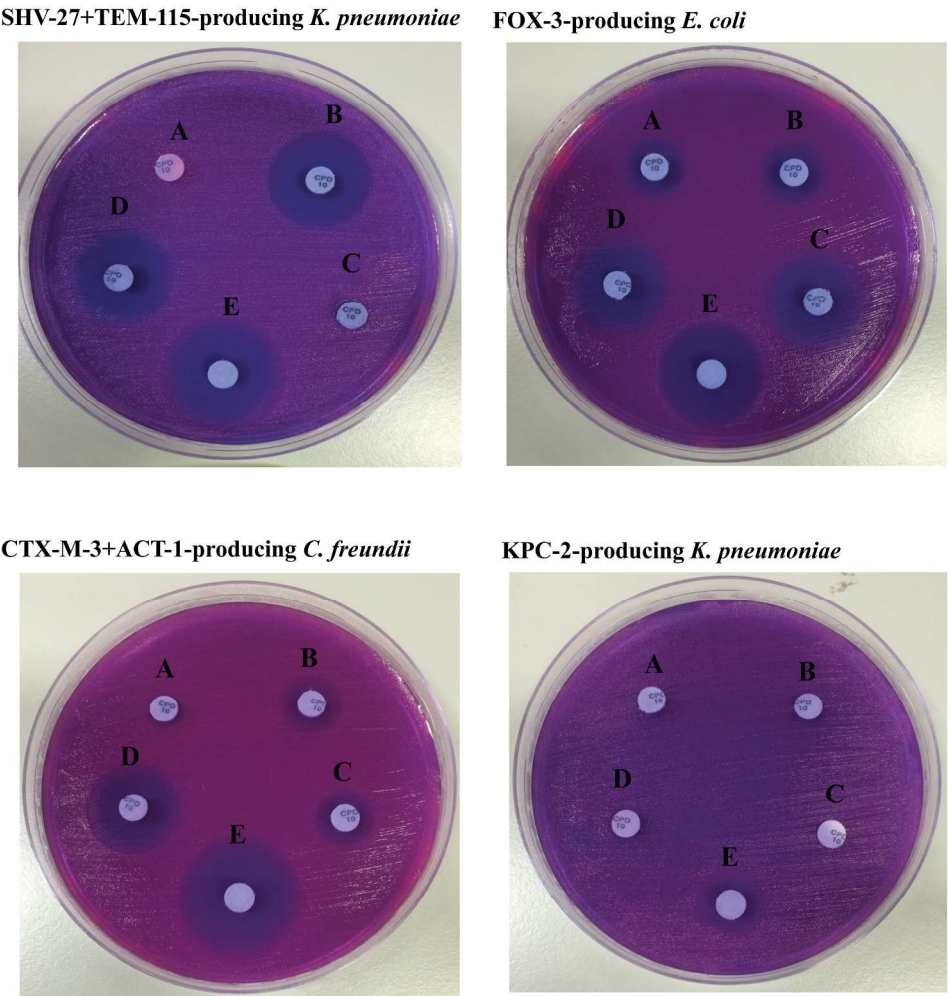


Figure 2. Phenotypic results from RCA plate assay with a combined disc method at 7 h. A = cefpodoxime (10 µg); B = cefpodoxime (10 µg) + clavulanic acid (10 µg); C = cefpodoxime (10 µg) + cloxacillin (500 µg); D= cefpodoxime (10 µg) + clavulanic acid (10 µg) + cloxacillin (500 µg); E = meropenem (10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

236x234mm (300 x 300 DPI)

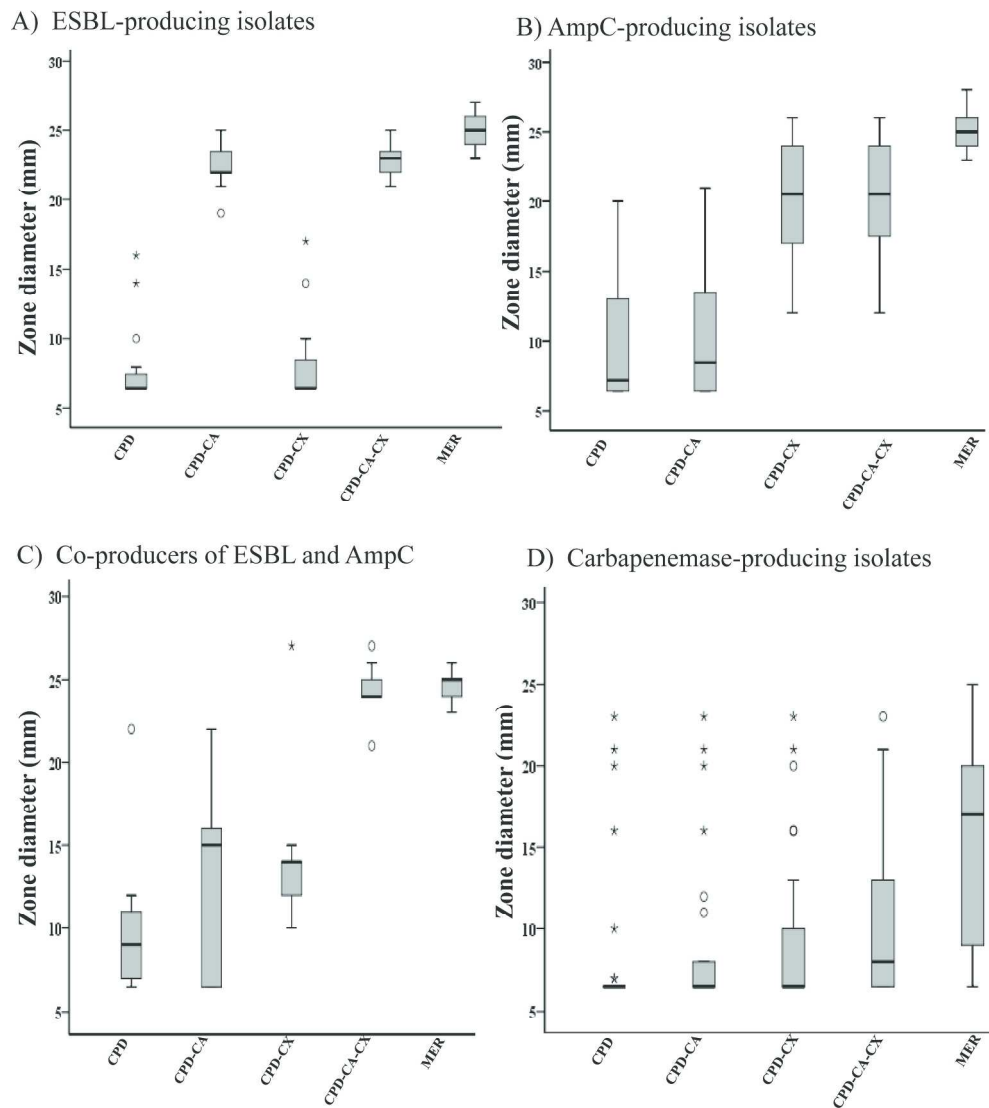


Figure 3. Distribution of zone diameters of cefpodoxime (CPD) alone, CPD with clavulanic acid, CPD with cloxacillin, CPD with both clavulanic acid and cloxacillin and meropenem alone. A = ESBL producers (n=15); B = AmpC producers (n=32); C = co-producers AmpC and ESBL (n=9); D = carbapenemase producers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

243x276mm (300 x 300 DPI)