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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.

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

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

A simple, rapid and inexpensive method for screening and discrimination between these enzymes at a phenotypic level could guide clinicians to prescribe an appropriate chemotherapy. The combined disc method has been used extensively because it is relatively easy to prepare and perform. However, this test requires at least 18 h or overnight to obtain the results. A resazurin reduction assay, a colorimetric method, is based upon the ability of active cells to reduce a blue colored resazurin to a pink colored resorufin (15). A colorimetric (resazurin containing) disc susceptibility method exhibited excellent reproducibility (16) and high sensitivity and specificity in detection and differentiation of carbapenemase-producing Enterobacteriaceae (17). Cefpodoxime (CPD) is an attractive indicator cephalosporin for detection of ESBL production and may be used for screening according to EUCAST guidelines. There are several diagnostic methods that have been proposed for phenotypic confirmation of ESBL and AmpC β-lactamases, including the Etest, combined disc method e.g. MAST D68C test, double disc synergy test, automated broth microdilution test. The time to result for these methods usually takes at least 18 h (9, 18, 19). Hence, the present study has investigated a resazurin chromogenic agar (RCA) method together with cefpodoxime (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 µg of CPD discs (MAST Group, UK) were supplemented with 10 µl of 1 mg/mL CA (Sigma-Aldrich, UK), 10 µl of CX (Sigma-Aldrich, UK) at a concentration of 50 mg/mL, or impregnated with both CA and CX. Meropenem (MER) discs (10 µg) were prepared by adding 10 µl 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-

lactamase inhibitor compared with CPD alone was considered as synergistic activity. To interpret the results, CA synergy was considered as a positive result for ESBL, while CX synergy and CA plus CX synergy were noted as positive results for AmpC and co-production of ESBL and AmpC, respectively. A zone diameter of MER < 25 mm was used at a cut-off point to screen for the presence of carbapenemases. Sensitivity and specificity of the RCA assay with a combined disc method were calculated by comparing the results with molecular types from PCR and sequencing data. A box-and-whisker plot was analyzed using SPSS statistical analysis program version 18 (SPSS Inc, USA) to elucidate the distribution of zone diameters of discs against different β-lactamase producers.

3. RESULTS

Using the RCA assay along with a combined disc method for phenotypic confirmation of ESBL, AmpC, and co-expression of ESBL plus AmpC clearly showed the inhibition zone diameters within 7 h (Figure 2). Figure 3 illustrates the distribution of the zone diameters of CPD impregnated with and without CA, CX, or CA plus CX, and MER alone against ESBL, AmpC, co-existence of ESBL and AmpC, and carbapenemase-producing Enterobacteriaceae. For screening of ESBL-producing isolates, the median zone diameter of CPD alone was 6.5 mm (range = 6.5-16 mm) and diameters of CPD supplemented with CA, CX, or CA plus CX were 22 mm (range = 19-25 mm), 6.5 mm (range = 6.5-17 mm), and 23 mm (range = 21-25 mm), respectively. MER discs exhibited potential activity in inhibition of ESBL producers with a median zone diameter 25 mm (range = 23-27 mm) (Figure 3A). A substantial increase in zone diameters of CA-containing discs compared with the discs without CA was only

observed in ESBL-producing isolates. The mean zone increase of CPD plus CA compared with CPD alone was 14.60 mm (range = 5-17.50 mm). No marked increase in zone diameter was observed in AmpC producers (mean = 0.48 mm and range = 1.5-3.5 mm), co-producers of ESBL and AmpC (mean = 2.72 mm and range 1.5-8.5 mm), as well as carbapenemase-producing isolates (mean = 0.37 mm and range = 0-5.5 mm). The RCA assay with combined disc method successfully detected all test ESBL producers with 100% sensitivity and 98.6 % specificity (Table 2). A false-positive result was observed in an OXA-48-producing *E. coli.*

In AmpC producers, an increase in median zone diameters was seen in CX-containing discs. CPD plus CX and CPD plus CX plus CA had equally a median zone diameter of 20 mm and a range 12-26 mm. The median zone diameter of CPD against these isolates was 7.25 mm (range = 6.5-20 mm) which was similar to CPD plus CA (median = 8 mm and range =6.25-21 mm). MER discs inhibited the growth of AmpC-producing isolates at a median zone diameter of 25 mm and range 23-28mm (Figure 3B). The mean difference of zone diameter of CPD plus CX versus CPD alone was 10.09 mm (range = 5.50 -16.50 mm) against AmpC producers, whilst no dramatic difference in mean zone increase was observed in ESBL producers (mean =0.33 mm and range = 0-2 mm), co-producers of ESBL and AmpC (mean = 4.33 mm and range = 1-7.5 mm), or carbapenemase producers (mean = 0.88 mm and range = 0-9.5 mm). The RCA assay demonstrated an excellence performance in the screening of AmpC-producing strains by detecting all test AmpC producers (100 % sensitivity), but there were two false-positives in KPC-3-producing *K. pnuemoniae* and OXA-48-producing *E. coli* (96.36 % specificity; Table 2).

For screening of ESBL and AmpC-co-producing Enterobacteriaceae, CPD discs alone

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

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

For screening of AmpC-producing isolates using CX synergy test, the assay was able to detect all AmpC producers and two-false positive results (100% Sensitivity and 96.36% specificity). This result is consistent with many previous works reporting a good performance of CPD and CX synergy test in detection of these enzymes. In one such study MAST® D68C successfully detected almost all AmpC producers whilst a few false-positive results were also reported (96.7 % sensitivity and 96.9% specificity). The test could not detect the low production of AmpC β-lactamases in AmpC-producing isolates (19). A similar result was reported by Ingram and colleagues, they found that MAST® D68C exhibited a sensitivity and specificity above 90% in detection of the presence of AmpC β-lactamase in Enterobacteriaceae. In agreement with a previous study, MAST-4 disc demonstrated good sensitivity (92%) and specificity (86.7%) in the detection of AmpC-producing nosocomial *Klebsiella* isolates (35). Combined activity of 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 < 25mm for 10 μg 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

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

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DISCLOSURE

The authors have no conflict of interest to declare.

REFERENCES

1. Huttner, A., Harbarth, S., Carlet, J., Cosgrove, S., Goossens, H., Holmes, A., Jarlier, V., Voss, A.,Pittet, D. (2013) Antimicrobial resistance: a global view from the 2013 World Healthcare-Associated Infections Forum. *Antimicrob Resist Infect Control* **2**(1): 1-13.

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

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

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

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

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

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

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

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

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

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

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

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

14. Tsui, K., Wong, S.S., Lin, L.C., Tsai, C.R., Chen, L.C.,Huang, C.H. (2012) Laboratory identification, risk factors, and clinical outcomes of patients with bacteremia due to *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum and AmpC type beta-lactamases. *J Microbiol Immunol Infect* **45**(3): 193-9.

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

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

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

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

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

20. Teethaisong, Y., Eumkeb, G., Chumnarnsilpa, S., Autarkool, N., Hobson, J., Nakouti, I., Hobbs, G.,Evans, K. (2016) Phenotypic detection of AmpC beta-lactamases, extended-spectrum- beta-lactamases and metallo-beta-lactamases in Enterobacteriaceae using a resazurin microtitre assay with inhibitor-based methods. *J Med Microbiol* **65**: 1079-87.

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

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

23. Wright, G.D. (2005) Bacterial resistance to antibiotics: enzymatic degradation and modification. *Adv Drug Deliv Rev* **57**(10): 1451-70.

24. Picao, R.C., Andrade, S.S., Nicoletti, A.G., Campana, E.H., Moraes, G.C., Mendes, R.E.,Gales, A.C. (2008) Metallo-beta-lactamase detection: comparative evaluation of double-disk synergy versus combined disk tests for IMP-, GIM-, SIM-, SPM-, or VIM-producing isolates. *J Clin Microbiol* **46**(6): 2028-37.

25. Coudron, P.E. (2005) Inhibitor-based methods for detection of plasmid-mediated AmpC beta-lactamases in *Klebsiella* spp., *Escherichia coli*, and *Proteus mirabilis*. *J Clin Microbiol* **43**(8): 4163-7.

26. Derbyshire, H., Kay, G., Evans, K., Vaughan, C., Kavuri, U.,Winstanley, T. (2009) A simple disc diffusion method for detecting AmpC and extended-

spectrum beta-lactamases in clinical isolates of Enterobacteriaceae. *J Antimicrob Chemother* **63**(3): 497-501.

27. Yagi, T., Wachino, J., Kurokawa, H., Suzuki, S., Yamane, K., Doi, Y., Shibata, N., Kato, H., Shibayama, K.,Arakawa, Y. (2005) Practical methods using boronic acid compounds for identification of class C beta-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*. *J Clin Microbiol* **43**(6): 2551-58.

28. Nordmann, P., Dortet, L.,Poirel, L. (2012) Rapid detection of extended-spectrum-beta-lactamase-producing Enterobacteriaceae. *J Clin Microbiol* **50**(9): 3016-22.

29. Osei Sekyere, J., Govinden, U.,Essack, S.Y. (2015) Review of established and innovative detection methods for carbapenemase-producing Gram-negative bacteria. *J Appl Microbiol* **119**(5): 1219-33.

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

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

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

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

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

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

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

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

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

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

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

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). º = mild outlier; * extreme outlier.

Tables

Table 1. Summary of ESBL, AmpC, ESBL+AmpC and carbapenemase-producing isolates used in the present study. 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. CPD=cefpodoxime (10 µg); CA= clavulanic acid (10 µg); CX=cloxacillin (500 µg)

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. pnuemoniae,* 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 ≥ 5 mm and CPD+CA+CX vs CPD+CX ≥ 5 mm	+	-	-	100 (15/15)	98.6 (71/72)
CX synergy test	CPD+CX vs CPD ≥ 5 mm and CPD+CA+CX vs CPD+CA ≥ 5mm	-	+	-	100 (32/32)	96.36 (53/55)
CA+CX synergy test	Both CPD+CA+CX vs CPD+CX ≥ 5 mm and CPD+CA+CX vs CPD+CA ≥ 5 mm	-	-	+	88.89 (8/9)	100 (78/78)

CPD=cefpodoxime (10 µg); CA= clavulanic acid (10 µg); CX=cloxacillin (500 µg)

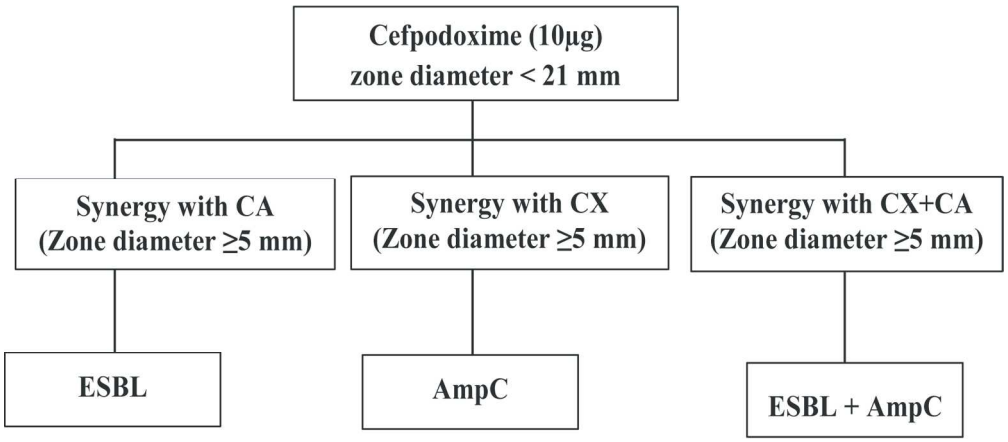

Figure 1. The algorithm for confirmation of and differentiation between ESBL, AmpC, and co-production of ESBL and AmpC in Enterobacteriaceae. CA= clavulanic acid (10 µg); CX = cloxacillin (500 µg).

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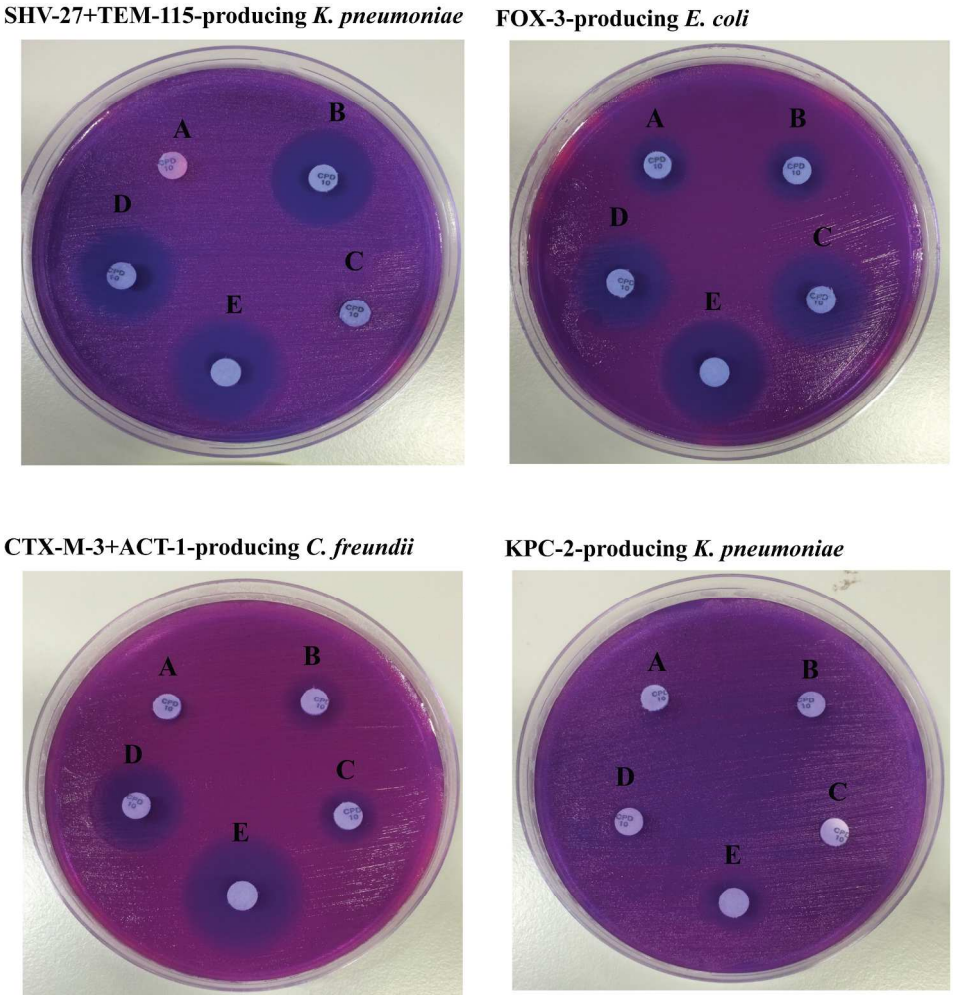

Figure 2. Phenotypic results from RCA plate assay with a combined disc method at 7 h. A = cefpodoxime (10 µg); B = cefpodoxime (10 µg) + clavulanic acid (10 µg); C =cefpodoxime (10 µg) + cloxacillin (500 µg); D= cefpodoxime (10 µg) + clavulanic acid (10 µg) + cloxacillin (500 µg);E = meropenem (10 µg).

236x234mm (300 x 300 DPI)

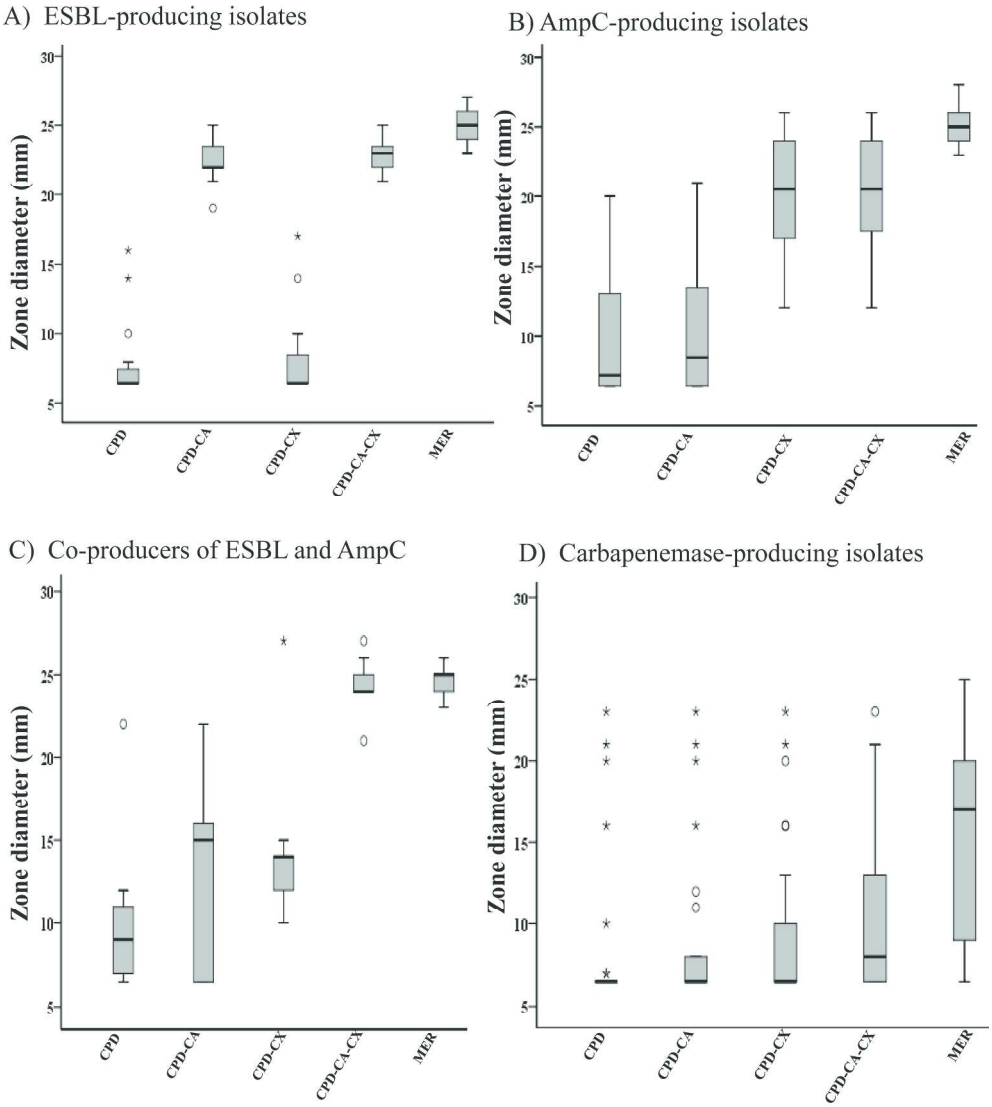

Figure 3. Distribution of zone diameters of cefpodoxime (CPD) alone, CPD with clavulanic acid, CPD with cloxacillin, CPD with both clavulanic acid and cloxacillin and meropenem alone. A = ESBL producers (n=15); B = AmpC producers (n=32); C= co-producers AmpC and ESBL (n=9); D = carbapenemase producers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

243x276mm (300 x 300 DPI)