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REVIEW ARTICLE OPEN ACCESS

A Guide Towards the Phenotypic Detection of Extended-spectrum β-lactamases Production in *Enterobacteriaceae*: Alone or in Presence of Other Interfering Enzymes

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Abstract

Antimicrobial Resistance (AMR) has been regarded as a major public health concern as a reason of millions of deaths. Extended-spectrum β-lactamase (ESBL) is considered as a leading factor contributing to this and limiting its treatment. Thus, ESBL producing *Enterobacteriaceae* should be discriminated from those having other mechanism conferring resistance. Several phenotypic methods have been evaluated for this purpose. Some of these are based on conventional method (DDST, CDT, ESBL E-test, Cica-β test) while others depend on automated systems (VITEK 1, VITEK 2, Phoenix, MicroScan). All the conventional methods have been found to be more specific, sensitive and cost effective than any of the automated system though they are easy to perform and interpret. Automated system also fails to detect ESBL in presence of other interfering enzymes such as AmpC, MBL or K1 enzyme. ESBL can be detected by using third-generation cephalosporin (cefotaxime or ceftazidime) or monobactam (aztreonam) in combination with clavulanate. AmpC can be distinguished by using cloxacillin-containing agar, fourth-generation cephalosporin (cefepime) or phenylboronic acid. MBL producers remain unaffected in presence of clavulanate but gets inhibited by carbapenems (imipenem, meropenem) in combination with EDTA. Cefpodoxime-clavulanate and ceftazidime- clavulanate combinations are reliable for K1 enzyme detection but are not suitable for distinguishing bla$_{CTX-M1}$.

Keywords: β-Lactamase, *Enterobacteriaceae*, Extended-spectrum β-lactamases, AmpC, Metallo-β-lactamase, K1 Enzyme, Phenotypic Detection

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INTRODUCTION

Antimicrobial resistance (AMR) has emerged as a serious public health concern causing millions of deaths worldwide in last 2 decades.[1] The factors that mostly contribute to AMR include irrational and overuse of antibiotics.[1] β-lactam antibiotics are those that contain β-lactam ring in their molecular structures are classified as penams, cephems (cephalosporins and cephamycins), monobactams, carbapenems,[2] and carbacephems.[3] Until 2003, in terms of selling quantity, more than half of all commercially available antibiotics in use were β-lactam compounds.[4] Extended-spectrum β-lactamases (ESBLs), the enzyme produced by the microorganism like *Klebsiella* species*, Acinetobacter, Pseudomonas aeruginosa, Citrobacter, Proteus, Enterobacter and E. coli,*are known to be a potent hydrolyzer of β-lactam antibiotics.[5] ESBLs producing bacteria are crucial threat for patients in the hospital, long-term care facilities and the community for their highly disseminating characteristics.[6] ESBL producing *E. coli* strains also show resistance to other classes of antibiotic making the pathogen hard to treat.[7-8]

Extended-Spectrum β-Lactamase (ESBL), comes under Ambler class A and D β–lactamase enzymes,[9] They mediate resistance to extended spectrum (third and fourth generation) cephalosporins (e.g. Ceftazidime, cefotaxime, ceftriaxone, cefepime etc.) and monobactams (e.g. Aztreonam) leaving antimicrobial classes like cephamycins (e.g. Cefoxitin and cefotetan) or carbapenems (e.g. meropenem or imipenem) and are inhibited by β-Lactamase inhibitors such as clavulanate, sulbactam and tazobactam.[10-12] The Clinical and Laboratory Standards Institute (CLSI) in the United States issued national guidelines for laboratory detection of *E. coli*, *Proteus mirabilis*, and *Klebsiella* spp. with ESBL. But, the guideline for the interpretation of ESBL testing results for those species of *Enterobacteriaceae* that are also good AmpC producers (Ambler class C enzyme) is not provided there. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) eases this problem by recommending some tests for the detection of ESBL alone and in presence of AmpC and carbapenemase enzymes.[13] Resistance, at breakpoint, is not fixed to all third- or fourth generation cephalosporins, whether based on disk-diffusion in agar, minimum inhibitory concentration (MIC) determination or automated systems, it varies with the choice of cephalosporin tested,[14] and the recommended guidelines to be followed. Two detection strategies are in common use: one is the detection of ESBL using a third-generation cephalosporin, usually cefotaxime or ceftazidime, and second is the confirmation of the former by detecting the synergy between the extended spectrum cephalosporin and a β-lactamase inhibitor, usually clavulanate. Based on these strategies several phenotypic methods have been developed to detect or confirm ESBL production; some of them depend on manual handling while others on commercially available automated systems.[15]

This review describes the phenotypic methods for ESBL detection, their advantages, and limitations. In addition, it also describes the detection of ESBL-producing micro-organisms which are co-producers of other enzymes that interfere in ESBL detection. AmpC, Metallo-β-lactamase, and K1 enzyme act as masking agents of ESBL production. ESBL production should, therefore, be detected in the presence of these enzymes. Though phenotypic methods are out of date a bit, they are still performed in various laboratories, especially in low- and middle-income countries where the prevalence of ESBL producing Escherichia coli is high.

Manual Methods of ESBL Detection Test

Double-Disc Synergy Test (DDST)

Double-disc synergy test is a kind of disc diffusion test that detects the effectiveness of an antimicrobial agent in the presence of another antimicrobial agent.[16] This test utilizes two discs on the cultivated agar plate, either infused with a different antimicrobial solution.[16,17] It was initially designed to differentiate between the microorganisms that over produce cephalosporinase, and those that produce ESBLs.[18] In this test two discs are utilized: one containing 30µg of any third-generation cephalosporin (cefotaxime, ceftriaxone, ceftazidime) or monobactam (aztreonam) and another containing amoxicillin–clavulanate (containing 10 µg of clavulanate) usually positioned at a distance of 30 mm (centre to centre) on a cultivated agar plate (Figure 1). DDST for ESBL is considered positive

when reduced susceptibility to third-generation cephalosporin or monobactam shows a prominent increase in inhibition zone of antimicrobial agent towards the clavulanate-containing disk, often resulting in a characteristic shape-zone referred to as 'champagne-cork' or 'keyhole'. The DDST was first used in epidemiological studies to assess the spread of ESBL-producing *Enterobacteriaceae* in French hospitals.[19,20] It has been generally regarded as an effective and reliable method for the detection of ESBLs in a wide range of *Enterobacteriaceae* species, although it is sometimes necessary to adjust the disk spacing. It has been shown that the sensitivity increased even more, to 90%, when this distance was reduced to 20 mm.[21] Modified Double Disc Synergy Test (MDDST) utilizes the 4th generation cephalosporins (cefepime) and an optimum spacing of the discs infused with antimicrobial solutions and inhibitor for the detection of the synergy.[22] But for this spacing modification, disks should be arranged by the help of forceps with narrower distances as several types of marketed disk-dispenser are designed for the routine spacing (i.e. 30 mm).

Combination Disc Test (CDT)

Combination Disc Test is another kind of disc diffusion test that also require the comparison of the effectiveness of two discs infused with antimicrobial solution placed on same cultured agar plate, but in this case both of the discs are having the same antimicrobial agent. For each test, 2 discs are required; One containing cephalosporin/monobactam alone (cefotaxime, ceftazidime, cefpodoxime, aztreonam) while another contains the same antimicrobial in combination with clavulanic acid. The inhibition zone around the cephalosporin/monobactam disc combined with clavulanic acid is compared with the zone around the disc with the cephalosporin/monobactam alone.[23] The test is supposed be positive if the inhibition zone diameter is ≥ 5 mm larger with clavulanic acid than without (Figure 2).[23] The advantage of this test is it is very easy to perform and interpret and has an upper hand over DDST as the latter one lacks sensitivity because of the problem of optimal disc spacing, the need of precision.[5] The sensitivity, specificity, positive predictive value & negative predictive value of CDT were assessed as 100%, 77.9%, 83.1% & 100% respectively in one study by Chowdhury et al.[6] This method is also useful in distinguishing ESBL producers from strains with AmpC enzymes and from K1 enzyme over-expressing the *K. oxytoca*.[15]

ESBL E-Test

Epsilometer test or E-test is defined as the 'exponential gradient' method that determines the antimicrobial resistance of the microorganism.[24] ESBL E-test uses commercially

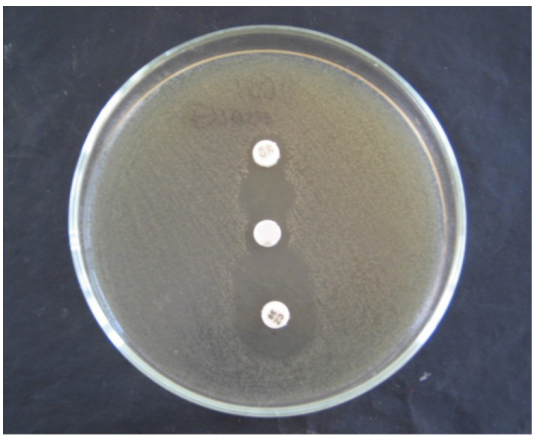

Figure 1. Double-disc synergy test performed on ESBL producing strain. Amoxicillin/clavulanate containing disc placed in between cefotaxime (up) and ceftazidime (down).[24]

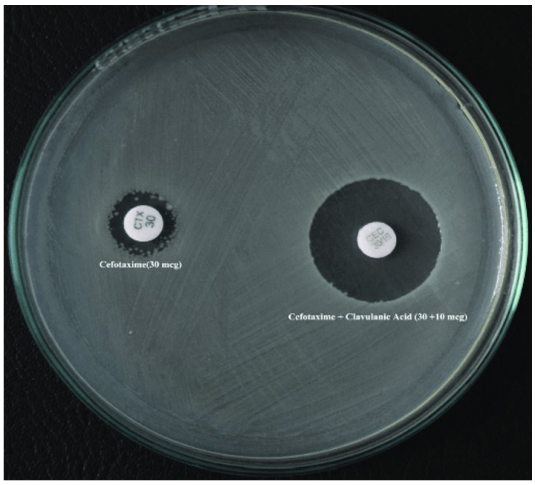

Figure 2. Combination disc test performed on ESBL producing strain. Cefotaxime and cefotaxime/clavulanate containing discs are placed apart from each other.[58]

available rectangular plastic strips containing exponential gradient of cefotaxime, ceftazidime or cefepime alone at one end and in combination with clavulanate (4mg/L) on the other (Figure 3a). The concentration gradient is marked with numerical scale on the strip. These strips are denoted as CT/CTL, TZ/TZL, PM/PML strips respectively for cefotaxime/cefotaxime-clavulanate, ceftazidime/ceftazidime-clavulanate and cefepime/cefepime-clavulanate.[25] A strain is considered ESBL positive when the inhibition ellipse made by the test cephalosporin alone and in combination with clavulanate is compared and the latter shows a reduced MIC ratio by ≥ 8.[25] The test is also considered as positive when there is either a rounded zone (phantom zone) (Figure 3b) just below the lowest concentration of CTL, TZL or PML gradients, or a deformation of the CT, TZ or PM inhibition ellipse at the tapering end independent of the ratios or MICs[24]. A major drawback of this test is interpreting the results of the ESBL E-test strips is delicate and requires expert handling. In addition, it is also difficult to interpret when the MIC values for cephalosporins fall outside the range of MICs available on the test strip and it is also true for the multi-resistant isolates.[26]

Cica-β Test

This test examines for hydrolysis of the chromogenic oxyimino-cephalosporin HMRZ-86 with and without specific inhibitors.[27] This technique is simple and fast; gives result in 15 mins and it is also useful for detecting not only ESBL but also the AmpC and metallo-β-lactamases (MBL). Cica-β Test kit comes with four strips; each containing chromogenic oxyimino-cephalosporin HMRZ-86 with a) no inhibitor to detect hydrolysis of extended-spectrum cephalosporins, b) clavulanic acid to detect ESBL, c) with boronic acid to detect AmpC production and d) sodium mercapto-acetic acid to detect metallo-β-lactamases.[27] A colour change from yellow to red indicates positive result (Figure 4). Garrec et al. in his study, showed that Cica-β test had significantly lower sensitivity in comparison to the other phenotypic detection methods.[28] Not only that, it had some other limitations such as misinterpreting the K1 enzyme and carbapenemase hyper producer as AmpC overproducer.[27]

Three Dimensional Tests (TDTs)

Three Dimensional susceptibility testing was first proposed by Thomson et al. in 1984.[29] This test came with slight modification in disc diffusion method with a heavy inoculum load that had been thought to provide more information as some workers felt that the light inoculum of some disc techniques fails to portray the *in-vivo* situation.[29] Two types of Three Dimensional susceptibility test are there: a) direct and b) indirect.[30] In direct TDT MHA plates are inoculated with test strains matching 0.5 McFarland turbidity standards. Then this inoculated agar plate is to be stabbed vertically with a sterile scalpel blade touching the bottom of the agar at a predetermined point 3 mm inside

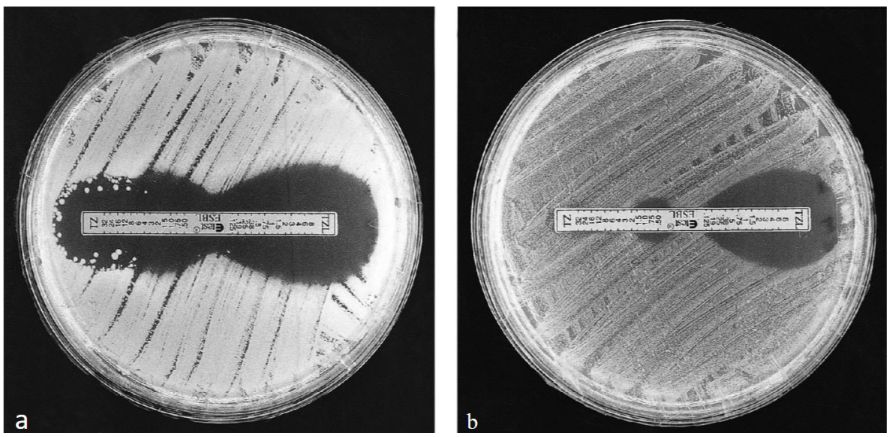

Figure 3. ESBL E-test performed with cefepime and cefepime/clavulanate strip. a) Result shows ESBL negative strain. b) Result shows ESBL positive strain with clear presence of phantom zone.[59]

the position at which the antibiotic discs are to be placed. A circular slit is cut in the agar concentric with the margin of the plate (Figure 5). Slit was inoculated with test strain having $10^9 – 10^{10}$ CFU of cells dispensed in tryptone soy broth. Then plate is to be incubated overnight at 37°C. In indirect TDT MHA plates were seeded with the inoculum of a standard sensitive strain (*E. coli* ATCC 25922) adjusted to McFarland 0.5 standards.[31] With the exception of this, the method is the same as that described for the direct three-dimensional test. Indirect was performed when inhibition zones were small or absent. Distortion of the inhibition zone in the vicinity of its intersection with the circular three-dimensional inoculation indicated positive result. In some cases, crude enzyme extract can also be used instead of using direct bacterial isolate as Courdon et al. showed in his study.[32] Thomson and Sanders compared the combination of the direct and the indirect three-dimensional tests and the DDST with 32 strains of *E. coli* and *K. pneumoniae*, 28 of which produced ESBL. 93% and 82% of the isolates were found to be ESBL positive respectively by TDTs and DDST.[31] Menon et al. performed the same with 70 *Enterobacteriaceae* strains, of which 56 were multidrug resistant and 14 were ESBL producers.[30] They reported detection rates of 86% for the three-dimensional test and 14% for DDST.[30] Sahid et al. in 2004 proposed some modifications in three-dimensional susceptibility testing. He brought the modifications in both the ways stated earlier, i.e. a) using direct bacterial isolates and b) using crude enzyme extracts.[33] The modifications adopted by him were that he put inoculum or enzyme extract into slits, wells or onto the surface as spots at varying distances from the discs. He showed that spot inoculation and well inoculation method gave better results than the conventional method by using whole bacterial isolates and enzyme extract respectively.[33]

Automated Methods of ESBL Detection Test

VITEK 1 ESBL Test

VITEK 1 analysis is an automated system that assess the antimicrobial activity of some cephalosporins with and without clavulanate simultaneously while the test interpretation is based on computerized expert system.[34] VITEK 1 test utilized commercially available cards having four wells containing cefotaxime and ceftazidime alone (at 0.5 mg/ml) and in combination with clavulanic acid (at 4 mg/ml). After inoculation this card is placed inside the VITEK 1 analyzer which then indicates the isolate as either ESBL positive or negative based on the predetermined ratio of reduction in growth of the cefotaxime or ceftazidime well containing clavulanic acid with that in the well containing drug alone.[34]

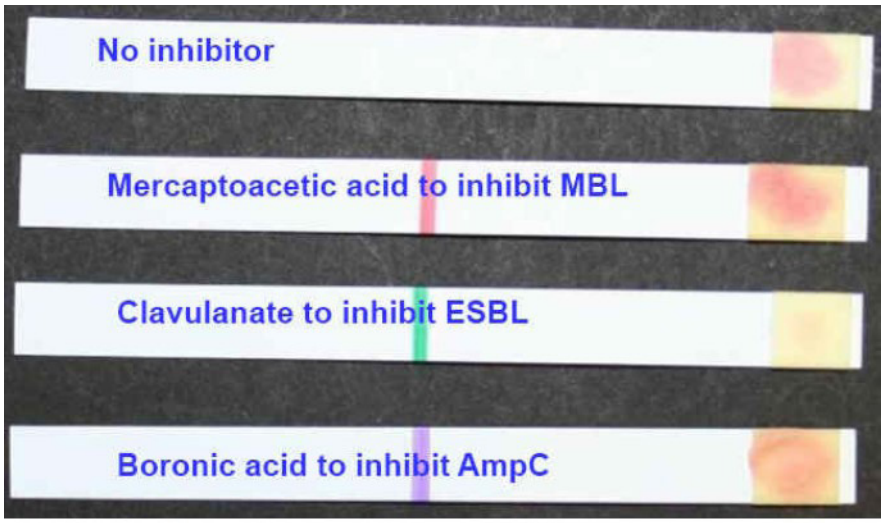

Figure 4. Cica-β test perfomed on ESBL producing strain. Presented by David Livemore at ECCMID 2010 in Vienna

VITEK 2 ESBL Test

VITEK 2 ESBL test is a modified version of VITEK 1 ESBL test; this test uses three antimicrobial drugs instead of two including and excluding clavulanic acid. Different sets of VITEK 2 cards are commercially available. Some cards contain cefepime (1.0 mg/L), cefotaxime and ceftazidime (0.5 mg/L) either alone or associated with 10 or 4 mg/L of clavulanate, respectively,[35] while other cards contain ceftriaxone, cefepime, and aztreonam (0.5 mg/L) with and without clavulanate (4 mg/L). The latter is currently only validated for detection of ESBLs in *E. coli*, *K. pneumoniae*, and *K. oxytoca.*[28]

Phoenix ESBL Test

The automated Phoenix ESBL test relies on the growth response to selected extended-spectrum cephalosporins, with or without clavulanic acid.[36] Phoenix ESBL panel is composed of five wells containing cefpodoxime, ceftazidime, ceftazidime with clavulanic acid, cefotaxime with clavulanic acid and ceftriaxone with clavulanic acid respectively.[36] After inoculation, the panel is placed in the instrument for continuous growth monitoring and result is interpreted through BDXpert system. This BDXpert system depends on three rules: a) Rule no. 1505: "*Enterobacteriaceae* with ESBLs are resistant to all β-lactam drugs, except carbapenems.";b) Rule no. 1502: "*Enterobacteriaceae* that are susceptible to a carbapenem and resistant to ureidopenicillins and 3rd generation cephems or cefpodoxime or aztreonam are also resistant to all β-lactam, except carbapenems."; c) Rule no. 106 may be provided: "Screening test suggests a possible ESBL producer; confirmatory testing is recommended." No rule is supplied if test result is negative.[26]

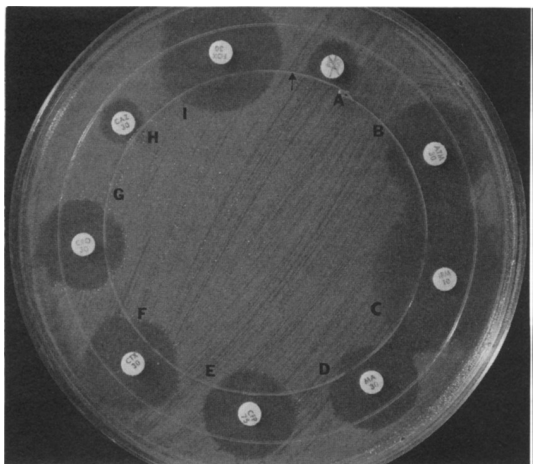

Figure 5. Direct three-dimensional test of CAZ-2-producing E. coli MISC 234. The three-dimensional inoculation slit is represented by the arrow. Result shows minor distortions indicating antibiotic inactivation of cefamandole (D), cefoperazone (E), cefotaxime (F), and ceftriaxone (G). No distortions occur in tests with aztreonam (B), imipenem (C), or cefoxitin (I). The inhibition zones were too small to interpret result with piperacillin (A) and ceftazidime (H).[31]

MicroScan Analysis

The Siemens MicroScan technology was introduced as the first advanced technology that was based on true minimum inhibitory concentration.[37] The MicroScan WalkAway-96 SI System comes with automated features including automated maintenance and result interpretation. Dehydrated inoculation panels are provided by the company to be inoculated overnight, placed inside the WalkAway-96 SI instrument for result and interpretation. The following antimicrobial agents (concentration ranges in mg/L) are used in the MicroScan ESBL plus panel: aztreonam (0.5–16); cefepime (1–32); cefotetan (1–32); cefpodoxime (0.5–64); cefotaxime(0.5–128); cefotaxime/clavulanate (0.125/4–16/4); cefoxitin (2–32); ceftazidime (0.5–128); ceftazidime/clavulanate (0.125/4–16/4); ceftriaxone (1–64); imipenem (0.5–16); meropenem (0.5–16); piperacillin (16–64)[38] and results are interpreted according to the CLSI guidelines.[37]

There are many studies that have evaluated the ability of automated systems to detect ESBL-producing *Enterobacteriaceae* and some of these studies are summarized below: Leverstein-van hall et al., in his study, worked with the 74 multi-resistant *Enterobacteriaceae* isolates (34 *E. coli*, 26 *K. pneumoniae*, and 14 *K. oxytoca* isolates) and 17 controls to evaluate the efficacy of the VITEK 1, VITEK 2, and Phoenix automated system. The three automated systems detected ESBL production with a sensitivity ranging from 78% (VITEK 2) to 83% (VITEK 1) to 89% (Phoenix).[26] Concerning multi-resistant isolates the VITEK 2 performed worse than the Phoenix as it gave highest percentage of

indeterminate test results.[26] Wiegand et al. took three automated system into his consideration i.e. VITEK 2, Phoenix and MicroScan automated system. He performed his experiment using 147 *Enterobacteriaceae* isolates (*E. coli*, 62 isolates; *K. pneumoniae*, 29 isolates; *E.cloacae*, 17 isolates; *K. oxytoca*, 16 isolates; *Enterobacter aerogenes*,6 isolates; *Proteus mirabilis*, 6 isolates; *Morganella morganii*, 5 isolates; *C. freundii*, 4 isolates; *Proteus vulgaris*, 1 isolate; *Serratia marcescens*, 1 isolate) of which 85 were ESBL positive. The Phoenix automated system showed highest sensitivity for the detection of ESBLs with 99%, followed by the VITEK 2 (86%) and the MicroScan (84%); while in the case of specificity assessment, result was more variable. VITEK 2 showed highest specificity (78%) followed by a specificity of 72% for MicroScan and 52% for Phoenix automated system.[37] Thomson et al., in their study, they used 76 ESBL producing and 26 ESBL non-producing *Enterobacteriaceae* strains to compare Phoenix automated system with VITEK 2. They found that the Phoenix ESBL confirmatory test and expert system exhibited 96% sensitivity and 81% specificity for ESBL detection initially. Activation of the two additional rules i.e. rules 325 and 1437, increased sensitivity to 99% but unfortunately reduced the specificity to 58%. The VITEK 2 ESBL confirmatory test exhibited 91% sensitivity, which was reduced to 89% sensitivity by its expert system, while its specificity was ranging from 85% to 88%.[39]

Detection of ESBL in the Presence of Other Interfering Enzymes

Combination of Several Tests for Detection of ESBL in Strains Overproducing AmpC

The production of AmpC enzyme in gram-negative bacteria is controlled by either chromosomal gene (inducible) or by a plasmid (stable production).[40] Several bacterial species (*Enterobacter spp., K. aerogenes, C. freundii, M. morganii, P. stuartii, and S. marcescens*) have inducible chromosomally encoded AmpC cephalosporinase and are considered in serious infections.[41] Expression of inducible AmpC is regulated by AmpR. *E coli* lacks this AmpR. Thus, expresses this enzyme at a low level consecutively.[42] Other bacterial species, such as *K. pneumoniae*, which lack chromosomal genes, can acquire plasmid genes. *E coli* may harbor plasmid carrying AmpC.[42] AmpC enzymes confer resistance to oxyimino-cephalosporins, α-methoxy-β-lactams or cephamycins (cefoxitin and cefotetan), and monobactams and are poorly inhibited by clavulanic acid.[40] Thus, AmpC productions can mask the detection of ESBL.

Several modifications in DDST and CDT have been introduced to detect AmpC production in strains in which ESBL is masked by the overproduction of AmpC cephalosporinases. Those modifications can be summarized as follows:

- Cefepime, a fourth-generation cephalosporin, is less rapidly hydrolysed by AmpC enzyme than ESBL. Tzelepi et al. reported a sensitivity of 16% only when placing cefotaxime, ceftriaxone, ceftazidime and aztreonam discs adjacent (30mm) to amoxicillin–clavulanate containing disc but an increased sensitivity by 61% and 90% when used cefepime disc and placed this disc at a distance of 20mm respectively.[21]
- Clavulanic acid, sulbactam, and tazobactam have an effect in inhibiting ESBLs but AmpC β–lactamases are poorly inhibited by these inhibitors.[43] Cloxacillin, oxacillins however are proved to be good inhibitors of AmpC β–lactamases.[44] Performing the DDST on cloxacillin containing agar (200 mg/L) has been shown to enhance the ability of the test to detect ESBL in *Acinetobacter baumannii*[45] and *Pseudomonas aeruginosa*.[46]
- A small set of boronic acids act as low nanomolar inhibitors of AmpC β-lactamase but do not affect ESBL.[47] Nagarathnamma et al. reported identification of AmpC β-lactamase and ESBL co-producers correctly by CDT method using cefotaxime (CTX), cefotaxime plus clavulanic acid (CTX/CA), cefotaxime plus boronic acid (CTX/BA) and cefotaxime plus clavulanic acid with boronic acid (CTX/CA/BA) discs.[48] But this method fails to detect inducible AmpC (iAmpC). Nagarathnamma overcame this limitation by placing imipenem disc adjacent to the earlier mentioned discs as imipenem had been known to be a potent inducer of iAmpC.[48]
- Drieux et al. recommended the use of the ESBL E-test on cloxacillin-containing agar when the MIC values are higher than those measurable on the strips.[35] He had shown a

significant reduction in MIC value when using cloxacillin-containing agar rather than using Mueller–Hinton agar (MHA)and a further reduction in MIC in presence of clavulanate.[35]

Modification of Some Phenotypic Tests to Distinguish Metallo-β-Lactamases Producers from Carbapenemase Producing *Enterobacteriaceae* and Detection of ESBL in It

Carbapenem-resistant *Enterobacteriaceae* (CRE) can be defined by their resistance to any carbapenems such as imipenem, meropenem, or ertapenem. Carbapenemase class A such as KPC enzyme[49] hydrolyzes a broad range of β-lactam antibiotics including pencillins, cephalosporins and carbapenems. This enzyme also efficiently hydrolyzes cefotaxime but only poorly hydrolyzes ceftazidime[50] and can be inhibited by enzyme inhibitors such as clavulanic acid and tazobactam. Carbapenemase class B enzymes (IMP, VIM, GIM and SPM-1) or metallo-β-lactamases (MBL) generally hydrolyze third generation cephalosporins as well as carbapenems, but not aztreonam[35] and can be inhibited by ethylene diamine tetra-acetic acid (EDTA) and mercaptopropionic acid.[51] Several modifications were adapted for detecting MBL from other carbapenemase producers in these manners:

- Liao et al. used imipenem discs with and without EDTA for the detection of MBL.[51] A difference of 5mm in the zone of inhibition created by those two discs clearly indicated the production of MBL. But in his study, he inoculated the test strain onto the imipenem disc instead of inoculating the disc in test strain suspension indicating that the strains were inoculated onto the MHA plate following the routine disc diffusion procedure (simple EDTA synergistic carbapenem inactivation method or esCIM). He reported that esCIM performs better than eCIM in detecting MBL with 91% sensitivity and 100% specificity.[50]
- Wei et al. developed rapid EDTA-modified carbapenem inactivation method (reCIM) combined with modified rapid carbapenem inactivation method (mrCIM) that might give result within 4 hours.[52] For this purpose, he used meropenem discs incubated with test strain for 45mins. He placed these discs inside the Eppendorf tube containing indicator *E. coli* ATCC 25922 already incubated for 2.5 hours. The growth of indicator *E. coli* with OD > 2.0 indicated carbapenemase production, whereas OD < 1.0 could be considered as carbapenemase negative. Interpretation reCIM was same as mrCIM but in this case OD>2.0 indicated presence of MBL.[52]
- If an isolate shows resistance to imipenem and ceftazidime simultaneously then there are four possibilities of being either class A or class A carbapenemase and ESBL positive or metallo-β-lactamase plus ESBL positive or only class B carbapenemase positive. If this same isolate shows resistance to aztreonam too, it can be considered as class A or KPC enzyme producer or co producer of class A carbpenemase and ESBL or producer of MBL with ESBL as only MBL is susceptible to aztreonam.[35] Now if this same strain confers resistance to EDTA, it can be considered as co-producer KPC enzyme and ESBL or the producer of KPC enzyme alone. The presence of ESBL in MBL producing strain can be demonstrated by detecting synergy between clavulanate & EDTA containing disc and third generation cephalosporin or cefepime.[35] KPC enzyme production can be detected by a combined disc test using aminophenylboronic acid (400µg/disc) with and without imipenem.[53]

False –Positive ESBL Detection with K1 Enzyme Hyperproducers

K1 enzyme is also called KOXY enzyme, named as it is frequently encountered in some isolates of *K. oxytoca.*[54] Hyperproduction of this chromosomal β-lactamase results in false positive detection of ESBL as it also shows resistance to some extended spectrum cephalosporins and monobactams. KOXY enzyme is characterized by resistance to aztreonam and cefuroxime, moderate resistance to ceftriaxone, cefotaxime and cefepime and susceptibility to ceftazidime.[36,55] This resistance pattern is little bit different from ESBL. For this reason, ceftazidime must be included in the panel of antibiotics for routine susceptibility testing[56,57] for ESBL. However, CTX-M ESBL also shows susceptibility to ceftazidime making it difficult to differentiate from K1 enzyme.[55] Potz

et al., reported only one false positive ESBL result among the 25 *K. oxytoca* isolates hyperproducing K1 β-lactamase but lacking ESBLs when performed agar dilution method with ceftazidime-clavulanate.[55] In addition, cefpodoxime discs with and without clavulanate were consistently give negative results for the K1 hyperproducers proving that Cefpodoxime–clavulanate combination disc tests were reliable in distinguishing ESBL producers from hyperproducers of K1 enzyme.

CONCLUSION

ESBLs are becoming more complex, diverse and widespread day by day that a single susceptibility testing approach for detection must be diminished. A wide genetic diversity including the presence of other enzymes that confer broad spectrum drug resistance makes it more difficult to be diagnosed. Conventional phenotypic methods sometimes remain difficult in practice. Therefore, some modifications and modulations have been adapted. Nowadays, some kits are commercially available in the market for the detection of ESBL alone and in combination with other enzymes by CDT. All the above-mentioned studies unanimously evidenced that though these automated methods were easy to perform as all the panel card were commercially available and analyzed by a previously set computerized system but were less effective in detecting simultaneous expression of the different β-lactamases, possibly in combination with outer membrane porin changes or overproduction of AmpC or K1 enzyme that might mask the ESBL production. Performance of the DDST, CDT and ESBL E-Test showed better result than those of the automated systems. Use of single cephalosporin as an indicator has been known for the outcome with low sensitivity while the use of two or more antimicrobials of this category enhances both sensitivity and specificity. Although, it is difficult to attain 100% sensitivity with any of these conventional methods. Molecular assay is always regarded as the gold standard method. PCR can detect accurately the presence of resistance-conferring genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M1}$, $bla_{CTX\text{-}M2}$, $bla_{CTX\text{-}M9}$ etc.) though it is time consuming whereas Microarray can detect simultaneously several enzyme-encoding genes from a single strain. The approach of gene sequencing remains helpful in detecting mutation that makes it more resistant to newer antibiotics. However, phenotypic methods are regarded as more convenient, easy to perform and cost-effective methods for screening in most of the clinical laboratories.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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