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(REVIEW ARTICLE)

Beta-lactam antibiotics and extended spectrum beta-lactamases

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Abstract

Extended spectrum beta-lactamases (ESBLs) are enzymes produced by bacteria, mostly members of the family Enterobacteriaceae commonly *Escherichia coli* and *Klebsiella pneumoniae*. ESBLs hydrolyze the beta-lactam ring of beta-lactam antibiotics making these antibiotics ineffective therefore rendering the bacteria resistance against beta-lactam antibiotics. The global upsurge of ESBLs producing bacteria causing both hospital and community acquired infections mostly urinary tract infections, pneumonia and bloodstream infections, threatens the effectiveness of infectious diseases treatment. ESBL families; TEM, SHV and CTX-M are globally disseminated and frequently detected in clinical isolates as well as colonization and contamination isolates. Various laboratory detection methods of ESBLs producing Gram negative bacteria are available. These methods; phenotypic methods, automated methods and molecular-based methods are varying in sensitivity and specificity, need of technical expertise, and rapidness. Therefore, they should be clearly understood before being employed for routine or research use for detection of ESBLs production among Enterobacteriaceae. In addition, understanding the mode of action and mechanisms of resistance to beta-lactam antibiotics, and the epidemiology of ESBLs producing bacteria is of paramount.

Keywords: Antimicrobial resistance; Molecular detection of ESBL; Multidrug resistance; Phenotypic detection of ESBLs production

1. Introduction

1.1. Beta-lactam antibiotics and mode of action

Beta-lactam antibiotics namely penicillins e.g., ampicillin, amoxycillin, and piperacillin; cephalosporins: first generation (e.g., cefazolin and cephalexin), second generation (e.g., cefotetan, cefoxitin and cefuroxime), third generation (e.g., ceftriaxone, cefotaxime and cefixime), fourth generation (e.g., cefepime) and fifth generation (e.g., ceftaroline); monobactams e.g., aztreonam; and carbapenems e.g., meropenem and imipenem, have a beta-lactam ring in their molecular structure [1-3]. Beta-lactam antibiotics are bactericidal acting by inhibiting the synthesis of bacterial cell wall. Briefly, synthesis of bacterial cell wall involves two major steps; transglycosylation and transpeptidation. The final step of transpeptidation is the formation of peptidoglycan layer which is composed of repeating units of N-acetylglucuronic acid and N-acetylmuramic acid [4]. The cross-linking of gaps between peptides (D-alanyl-D-alanine) attached on N-acetylmuramic acid is facilitated by peptidoglycan transpeptidase enzyme (found in bacterial cell membrane). Beta-lactam ring in beta-lactam antibiotics resemble D-alanyl-D-alanine therefore competitively acts as substrate and covalently binds to peptidoglycan transpeptidase enzyme [5]. This enzymatic reaction is irreversible resulting to bacteriolysis and finally cell death [4, 6, 7].

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1.2. Mechanisms of resistance to beta-lactam antibiotics, and dissemination and epidemiology of ESBLs

Mechanism of resistance towards beta-lactam antibiotics in bacteria is simply by production of beta-lactamase enzymes commonly extended spectrum beta-lactamases (ESBLs) which hydrolyses the beta-lactam ring within beta-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems) rendering these antibiotics ineffective [6, 8]. Therefore, ESBLs are "suicide-inhibitors".

Production of these enzymes is encoded by genes which may be found on bacterial chromosome or cytoplasmic mobile genetic elements (MGEs) e.g., harboring of conjugative plasmids is common. However, carriage of MGEs is common compared to chromosomal mutations. Conjugative plasmids harboring ESBL encoding genes are always shared either vertically (from mother to daughter cells) or horizontally among same or different bacterial species. Commonly, conjugative plasmids are not only encodes ESBLs genes but also they encodes for other antimicrobial resistance genes (ARGs) i.e., ARGs for quinolones and aminoglycosides resistance [9, 10]. Therefore, conjugative plasmids facilitate effective dissemination of ESBLs and other ARGs genes globally. There are at least four classes of beta-lactamases namely class A beta-lactamases, class B beta-lactamases, class C beta-lactamases and class D beta-lactamases [6]. This chapter will focus on extended spectrum beta-lactamases (ESBLs) categorized in class A beta-lactamases. Class A beta-lactamases known as penicillinase are also known as extended spectrum beta-lactamases (ESBLs). Families CTX-M, TEM, and SHV are widely disseminated globally and commonly found among members of the family *Enterobacteriaceae* mostly *Escherichia coli* and *Klebsiella pneumoniae*. Other rarely ESBLs families exist, including PER, VEB, GES, and IBC, which are mainly found in *Pseudomonas aeruginosa*. ESBLs are effective in hydrolyzing penicillins, cephalosporins (1st, 2nd and 3rd generations) and monobactams but have poor or no effect against methoxy-cephalosporins (i.e., cephamycins) and carbapenems. They are also inhibited by beta-lactamase inhibitors such as sulbactam, tazobactam and clavulanic acid [11]. Being vulnerable to inhibition by beta-lactamase inhibitors it is of advantage for phenotypic detection of ESBLs production.

ESBLs producing bacteria are well disseminated worldwide [12-14]. ESBLs producing bacteria have been reported from causing infections to colonization (among humans and animals) and contaminations of animate and inanimate surfaces mostly in health-care facilities [15-22]. In developed countries the proportion of ESBLs producing bacteria causing infections is below 14% whereby in developing countries the proportion of ESBLs producing bacteria causing diseases is above 50% [23]. For example, studies from Australia, New Zealand, USA, Japan and China reported a proportion of 3.6%, 3.7%, 6.9%, 10.6% and 13.3% of EBSLs producing bacteria respectively among overall bacteria causing infections [23-26]. In developing countries; Burkina Faso, Uganda, India and Tanzania, the proportions of ESBLs producing bacteria from clinical samples is 58%, 62%, 77% and 77% respectively [27-30].

1.3. Drivers for the emergence of ESBLs producing bacteria

Antibiotics selection pressure is the core driver for the emergence of antimicrobial resistant bacteria (ARB). Antibiotics selection pressure is always put on effect whenever antibiotics are used inappropriately such as unnecessary use of antibiotics in health-care facilities mainly intensive care units, extensive antibiotics use in the community, extensive antibiotics use in livestock husbandry and the void of antibiotics discovery which facilitate the overuse of few available antibiotics [31].

Under antibiotic selection pressure, unfit bacteria strains (wild type) are eliminated while the fit strains (non-wild type) survive. The sub-population of non-wild type strains is always the minority compares to sub-population of wild type strains. Therefore, under antibiotic selection pressure from inappropriate and unnecessary use of antibiotics, the non-wild type survivors propagate and establish a population of entirely resistant strains. The resistant bacteria and their ARGs are then transmitted locally (between health-care facilities and communities within the same region or country) and internationally between countries by geographical migrations of carriers e.g., humans and animals colonized with ESBL producing bacteria.

2. Laboratory methods for detection of ESBLs producing Gram negative bacteria

2.1. Phenotypic methods for screening of ESBLs producing Gram negative bacteria

2.1.1. Clinical and Laboratory Standards Institute (CLSI) screening methods

Method I: CLSI disc diffusion method

The CLSI is recommending on the use of beta-lactam antibiotics by disc diffusion method in screening for ESBLs production among gram negative bacteria, particularly *E. coli*, *K. pneumoniae*, *K. oxytoca* and *P. mirabilis* [32]. If bacteria exhibit resistance to cefpodoxime 10 µg (≤17 mm for *E. coli* and *Klebsiella* species and ≤22 mm for *P. mirabilis*), or ceftazidime 30 µg (≤22 mm), or aztreonam 30 µg (≤27 mm), or cefotaxime 30 µg (≤27 mm) or ceftriaxone 30 µg (≤25 mm), these bacteria should be presumed as ESBLs producer (Figure 1). For *P. mirabilis*; cefpodoxime, ceftazidime and cefotaxime discs only should be used. This method should be performed in Mueller Hinton agar (MHA) plate and incubated at 35±2°C in ambient air for 16-18 hours.

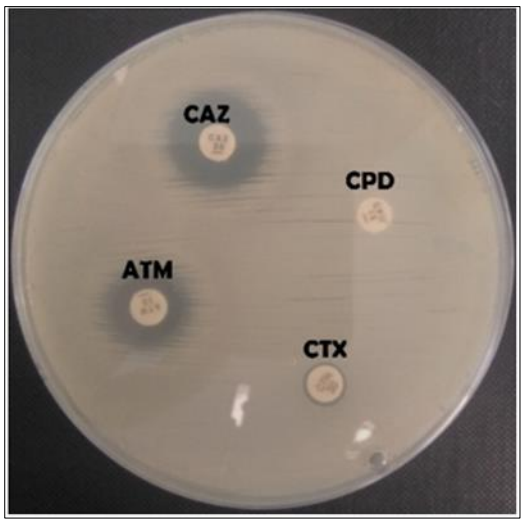

Figure 1 CLSI screening method for ESBLs production. Note the reduced sensitivity towards ceftazidime 30 µg (CAZ) and aztreonam 30 µg, and completely resistance towards cefpodoxime 10 µg (CPD) and cefotaxime 30 µG (CTX)

Method II: CLSI broth microdilution test

CLSI recommends on the use of broth microdilution (minimum inhibitory concentration (MIC)) for screening of ESBLS production among *E. coli*, *K. pneumoniae*, *K. oxytoca* and *P. mirabilis* [32]. A MIC of ≥2 µg/ml for cefpodoxime, ceftazidime and cefotaxime for *P. mirabilis* may indicate ESBLs production. Whereby, a MIC of ≥8 µg/ml for cefpodoxime or a MIC of ≥2 µg/ml for ceftazidime, aztreonam and ceftriaxone for *E. coli*, *K. pneumoniae* and *K. oxytoca* may indicate ESBLs production. Tubes are incubated at 35±2°C in ambient air for 16-20 hours. The limitation of this screening method includes coverage of few members of Enterobacteriaceae (*E. coli*, *K. pneumoniae*, *K. oxytoca* and *P. mirabilis*) and more than one antibiotic disc should be tested as reported by CLSI.

2.1.2. Selective culture medium supplemented with antibiotics

This cost-effective method employs the use of antibiotic-containing agar (for example, MacConkey agar plates supplemented with 1 to 2µg/ml of cefotaxime or ceftazidime) for screening of ESBLs production among members of family Enterobacteriaceae [33, 34]. Plates are then inoculated with 1µl of test bacterial suspension which is equivalent to 0.5 McFarland standard solution [33]. After 18 to 24 hours of aerobic incubation at 37°C plates are interpreted for positive or negative growth. Positive growth is interpreted as positive screening result. Both, the sensitivity and specificity of this method is above 90% [33], however sensitivity is promising when ceftazidime agent is used rather than cefotaxime agent [35, 36]. Therefore, it may be a reliable method which may be used in resources constrained laboratories for screening of large numbers of bacteria as more than one bacterium can be inoculated on one culture plate hence cost effective.

2.1.3. ESBLs chromogenic agar

ESBL chromogenic agar such as Brilliance ESBL agar (Oxoid, UK) and CHROMID ESBL (bioMérieux, France), is the screening medium for the detection of ESBLs producing members of the family Enterobacteriaceae [37, 38]. The medium contains a patent mixture of antibiotics (including cefpodoxime) and chromogenic substrates which enables selective growth of ESBLs producing Enterobacteriaceae and presumptively identifies ESBLs producers to species level mainly *E. coli*, *Klebsiella* species, *Citrobacter* species, *Enterobacter* species, *Proteus* species, *Providencia* species and *Morganella* species [37, 38]. The sensitivity of ESBL chromogenic agars varies from 85% to 99% whereas the specificity varies from 76% to [39, 40].

2.2. Phenotypic methods for confirmation of ESBLs production among Gram negative bacteria

Principle behind phenotypic methods for detection of ESBLs production relies on the inhibition of ESBL enzymes production from bacteria by beta-lactamase inhibitors such as clavulanic acid and tazobactam. Then, this allows beta-lactam antibiotics to kill ESBL producing bacteria, because they become susceptible to these antibiotics as they no longer produce ESBLs enzymes (in the presence of beta-lactamase inhibitors). Recently, antibiotics combination method by CLSI and double disc synergy method are common methods used for confirmation of ESBLs production in Gram negative bacteria.

2.2.1. Disc combination test (DCT)

This method is approved by the CLSI and it can be performed by disc diffusion or broth microdilution method [32]. For disc diffusion method, antibiotic discs of ceftazidime (30 µg) and cefotaxime (30 µg) alone and in combination with clavulanic acid (10 µg) are seeded on susceptibility testing plate after inoculation of test bacteria suspension equivalent to 0.5 McFarland standard turbidity solution. After 16-18 hours of incubation in ambient air at 37°C, a ≥5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid against the zone diameter of the antimicrobial agent tested alone confirms ESBLs production (Figure 2) [32, 41]. The sensitivity and specificity of this test is 96% and 100% respectively [42].

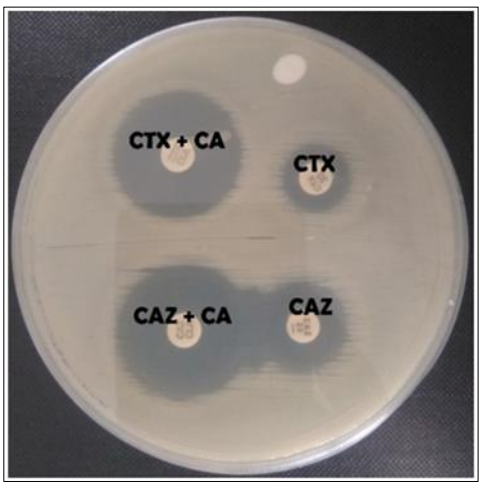

Figure 2 Antibiotics combination method: note the proportional decrease in zone of inhibition towards cephalosporins alone (CTX and CAZ) as compared to cephalosporins with clavulanic acid (CTX + CA and CAZ + CA)

2.2.2. CLSI broth microdilution

Test bacterium is inoculated in broth containing tubes with ceftazidime 0.25-128 µg/ml alone and ceftazidime with clavulanic acid 0.25/4-128/4 µg/ml or cefotaxime 0.25-64 µg/ml alone and cefotaxime with clavulanic acid 0.25/4-64/4 µg/ml. A ≥3 twofold concentration decrease in MIC for either antimicrobial agent tested in combination with clavulanic acid and antimicrobial agent tested alone confirms ESBLs production [32]. The sensitivity and specificity of this method is reported at 100%, respectively [43].

2.2.3. Double disc synergy test (DDST)

The DDST method uses two beta-lactam cephalosporin discs (ceftazidime 30 µg and ceftriaxone 30 µg) and one beta-lactam penicillin combined with clavulanic acid (amoxicillin-clavulanic acid 30/10 µg) [44]. When amoxicillin-clavulanic acid disc is diffused on the surface of susceptibility testing plate after being inoculated with test bacteria, the

bacteria cells around the disc will be inhibited from producing ESBLs enzymes. Therefore, these bacteria cells, around amoxicillin-clavulanic acid disc, will be susceptible to ceftriaxone and ceftazidime discs placed side-by-side with amoxicillin-clavulanic acid disc. The distance between amoxicillin-clavulanic acid and ceftriaxone or ceftazidime discs should be limited between 15 to 20 mm for effective performance of the test [44]. Plates are incubated in ambient air at 35±°C for 16-18 hours. Enhanced zones of inhibition of ceftriaxone and/or ceftazidime discs towards amoxicillin-clavulanic acid disc confirms ESBLs production (Figure 3) [44]. The sensitivity of DDS method is reported ranging between 83.1% and 92.9% [45, 46] whereas its specificity is reported at 100% [45, 46]. The limitation of DDST is failure to detect ESBL phenotypes among co-producers of ESBLs and Amp-*C* β-lactamases [44].

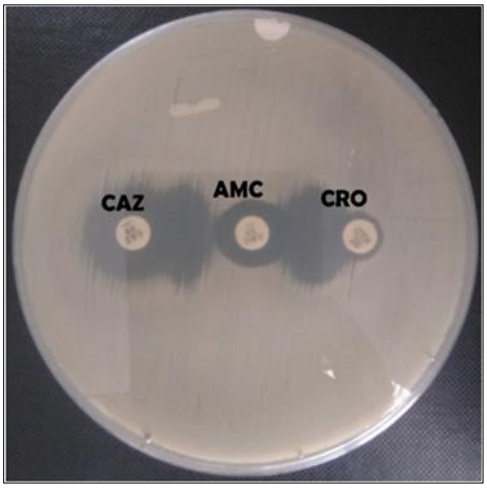

Figure 3 Double disc synergy (DDS) test: note enhanced zones of inhibition of cephalosporins (CAZ and CRO) towards amoxicillin-clavulanic acid (AMC)

2.2.4. Modified double disc synergy test (MDDST)

The DDST is modified by the addition of cefepime 30 µg and tazobactam (beta-lactamase inhibitor) containing disc such as piperacillin-tazobactam on MHA plate [44]. Cefepime and piperacillin-tazobactam discs are placed side-by-side at a distance between 22- and 25-mm. MHA plate is incubated aerobically at 35±2°C for 18-24 hours. Zone of inhibition of cefepime disc enhanced towards piperacillin-tazobactam confirms ESBLs production from Amp-C beta lactamase co-producers [44]. The sensitivity and specificity of MDDS test is reported at 100% and 100% respectively [45].

2.3. Molecular-based methods for detection of ESBLs producing Gram negative bacteria

2.3.1. Polymerase chain reaction (PCR)

PCR, a molecular based technique can be performed qualitatively i.e., conventional PCR or quantitatively i.e., real-time PCR to amplify a target(s) gene(s) e.g., $bla_{\text{CTX-M}}$, bla_{TEM} or bla_{SHV} into thousands to millions of copies. In both cases, a single target (e.g., singleplex PCR assay or real-time PCR assay) or multiple targets (e.g., multiplex PCR assay or multiplex real-time PCR assay) can be amplified and detected [47, 48]. The amplification of target gene(s) involves three major PCR steps namely denaturation: unzipping of the double stranded DNA into two single strands; annealing: specific attachment of primer at each 5' end of the target gene; and extension: addition of dNTPs by polymerase enzyme to produce a complimentary strand. For real-time PCR, amplification and quantitative detection of PCR products is internally and simultaneously. For conventional PCR, qualitative detection of PCR product is externally by gel electrophoresis under UV light using agarose gel 1-2% stained by ethidium bromide or redsafe [41, 48]. Despite its high sensitivity (86-98%) [49-51] and specificity (98-100%) [49-51], PCR is not routinely used in diagnostic laboratories especially in low- and middle-income countries (LMICs) because of its high expense and the needy for technical expertise [52].

3. Automated methods for detection of ESBLs producing bacteria

3.1. Vitek 2 ESBL testing method

The Vitek 2 ESBL testing system use cards with wells containing nutrient broth for bacterial growth, and growth inhibitors i.e., cephalosporins [53]. Bacterial growth in card's wells is quantitatively monitored by optical scanner [53]. Some wells contain cephalosporins alone (e.g., cefepime at 1.0 µg/ml, cefotaxime at 0.5 µg/ml, and ceftazidime at 0.5 µg/ml) and other wells contain cephalosporins with clavulanic acid (CA), (e.g., cefepime/CA at 1.0/10 µg/ml, cefotaxime/CA at 0.5/4 µg/ml, and ceftazidime/CA at 0.5/4 µg/ml) [53]. The proportional reduction of bacterial growth in wells containing cephalosporins with CA compared to wells containing cephalosporins alone suggests ESBLs production [53]. This method is documented high sensitivity (91.8-98.1%) and specificity (99.7-100%) [53, 54].

3.2. MicroScan WalkAway method

This method uses a computer software known as LabPro Expert system which contain rules for screening of ESBLs production in GNB [55]. This system screens potential ESBL producing bacteria by growing in cephalosporins (cefpodoxime 4 µg/ml and ceftazidime 1 µg/ml) containing broth [32, 55]. Although, a positive result from this method requires a confirmatory test. The sensitivity of this method is ranging between 80.5 and 84% [56, 57]. This method is also reported as extremely time consuming and labour intensive [52].

3.3. Phoenix system

Phoenix system uses Expert system with rule for detection of ESBLs production just like MicroScan WalkAway system but instead of screening for bacterial growth in the presence of cephalosporins only, it quantitatively determines bacteria growth in the presence of cephalosporins (cefpodoxime, ceftazidime, ceftriaxone, and cefotaxime) with and without clavulanic acid (CA) [56]. The proportional reduction in bacterial growth in the presence of cephalosporins with CA as compared to cephalosporin without CA is interpreted as positive production of ESBLs [56]. This method is highly sensitive (99%) and its results are comparable to broth microdilution [52, 56].

4. Other methods for detection of ESBL production

4.1 Matrix-Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrophotometry (MALDI-ToF-MS)

The principle of MALDI-ToF-MS: this analytical technique ionizes sample (or analyte) into charged molecules by using matrix-assisted laser desorption/ionization (MALDI) whereby the ratio of ions mass-to-charge (m:c) can be measured [58, 59]. The first part, MALDI, is the source of ion and the second part, ToF, is the mass analyzer which separate molecules based on their m:c ratio depending on time it takes for each molecule to reach the detector through the time-of-flight tube [58, 59]. Therefore, ions of different kinetic energy will be detected at the detector producing peaks of different intensity (Figure 4).

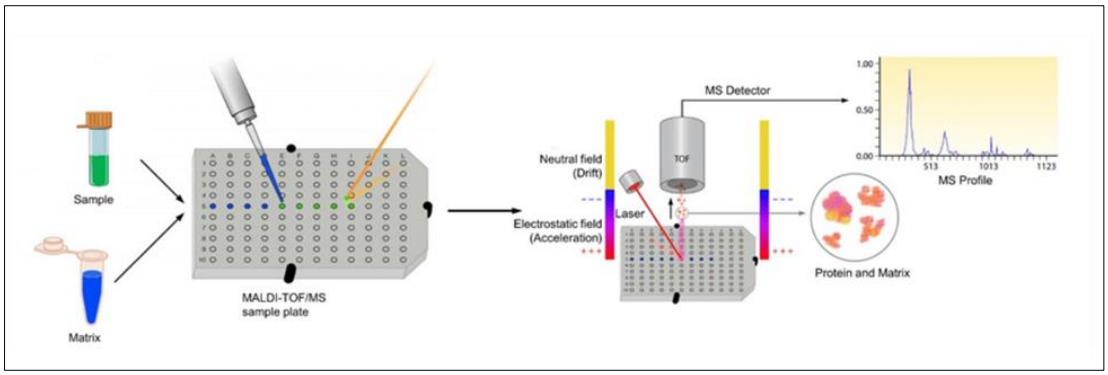

Figure 4 MALDI-ToF-MS procedure and principle (photo by Creative-proteomics *[59]*)

Detection of ESBLs production: protein is extracted from fresh overnight growth of test bacteria colonies. One microliter of supernatant of each extracted protein (sample) is applied to each spot on MALDI-ToF-MS steel sample plate which contains 96-spots [60, 61]. Then, 1 µl of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) is added on the same spots containing samples [60, 61]. Then, MALDI-ToF-MS analysis is performed as per above principle whereby peaks of ESBLs proteins above cut-off value is interpreted

as positive results. MALDI-ToF-MS is rapid (~30 minutes) and has high sensitivity (95-100%) and specificity (100%) [52, 62, 63].

4.2 Immunochromatographic assay

The recently invention of immunochromatographic assays (ICAs) which uses similar principle of antibody-antigen interaction, has reduced the turn-around-time for detection of ESBL enzymes production in Enterobacteriaceae. For example, NG-Test CTX-M MULTI assay, can detect CTX-M producing Enterobacteriaceae within 15 minutes with sensitivity and specificity of 100% [64].

5. Conclusion

Availability of a wide range of reliable laboratory methods for screening and confirmation of ESBLs production in GNB provides equal opportunity of laboratories (including those with limited resources) for detection of ESBLs production. In clinical settings, detection of ESBLs production should be considered as routine diagnostic service and it should be part of multidrug resistance surveillance programmes.

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