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Methods for the Phenotypic Detection of Extended Spectrum Beta Lactamase (ESBL)-Producing Bacteria

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Abstract

Extended Spectrum Beta Lactamases (ESBLs) are first reported in *Klebsiella pneumonia* in 1983. These enzymes possess the ability to inactivate susceptible β-lactam antibiotics i.e. penicillins, first, second and third generation cephalosporins and aztreonam, but not cephamycins and carbapenems . Their mode of action is by hydrolyzing the β-lactam ring. Even before the first β-lactam antibiotic (penicillin) was developed, resistance to β-lactam antibiotics was observed . ESBL genes are plasmids- and transposons- mediated, as such, can be spread easily to other species of bacteria. Resistance of ESBL- producing bacteria to the β-lactam antibiotics is a continuing cause of public health problems , it is increasingly being observed in community and nosocomial acquired infections. Detection and identification of these ESBLs in the laboratory is of prime importance for the selection of appropriate antibiotics to be used in the treatment of infections caused by ESBL- producing bacteria. The aim of this review is to explain in detail , several phenotypic methods used in the detection and confirmation of extended spectrum β lactamases.

Keywords: Antibiotic resistance, ESBL, bacteria, phenotypic method
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Introduction

Beta lactam antibiotics are a class of broad spectrum antibiotics that contain a β-lactam ring in their core molecular structures (Maria, 2013). They are among the most commonly prescribed antimicrobial agents worldwide to treat bacterial infections (Bradford, 2001; Shaikh et al*.,* 2015). Prestinaci et. al., (2015), reported that their cost-effectiveness, ease of use and tolerability make them the most widely used antibacterial agents. The effectiveness of these antibiotics has been decreased due to the development of resistant mechanisms in certain bacterial species (WHO, 2014) as a result of indiscriminate consumptions. Production of β-lactamases remains the major mechanism employed by bacteria to resist the effects of antimicrobial agents used against them (Ximin and Jun, 2013) . The continuous exposure of bacterial strains to multitude of β-lactam antibiotics has induced the dynamic and incessant production and mutation of β-lactamases in these bacteria, thereby expanding their activity against the newly developed β-lactam antibiotics (Shaikh et. al., 2015) . This class of enzymes that can break down these newer antibiotics are termed as Extended-Spectrum Beta Lactamases (ESBLs) (Paterson and Bonomo, 2005; Pitout and Laupland, 2008;

Shaikh et. al., 2015). Extended Spectrum Beta Lactamases (ESBLs) are enzymes that mediate resistance to broad spectrum of β-lactam antibiotics such as penicillins, third generation cephalosporins (e.g. ceftazidime, cefotaxime, and ceftriaxone) and aztreonam, but not to cephamycins (cefoxitin and cefotetan) and carbapenems (Bonnet, 2004; Al-Muharrmi et. al., 2008; Shaikhet. al., 2015) but are being inhibited by beta lactamase inhibitors like clavulanic acid (Al-Muharrmi et. al., 2008).

The ability of bacteria to produce enzymes that destroy the B-lactam antibiotics began even before penicillin was developed (Abrahan and Chain, 1940). The first β-lactamase was identified in an isolate of *Escherichia coli* in 1940 (Abrahan and Chain, 1940; Turner, 2005; Gupta, 2007). Although β-lactamases are estimated to have existed for the past 2 billion years, their evolution and spread have been highly correlated to the anthropogenic development and prolificacy of β-lactam antibiotics during the past 60 years (Lachmayr et.al., 2009). Therefore, their detection is a major challenge for the clinical microbiologist because they may appear susceptible to certain B-lactam antimicrobial agents during *in vitro* studies. This could result in treatment failure (Linscott and Brown, 2005) which may lead to high rates of morbidity and mortality in infectious diseases caused by the bacteria producing these enzymes (Kang et. al., 2004). Therefore, the detection and identificatio n of ESBL-producing bacteria and the knowledge of their resistance are of paramount importance in selecting appropriate antimicrobia ls to be used in the treatment of infections caused by MDR bacteria, thereby reducing the spread of antibiotics resistant bacteria (ARB). AmpC β-lactamases belong to the Ambler class C and once expressed at high levels confer resistance to many B-lactam antimicrobials, excluding the fourth generation cephalosporins and carbapenems (Deshpade *et al.,* 2006). Unlike ESBLs, these enzymes are not inhibited by commercial β-lactamase inhibitors (clavunic acid, sulbactam or tazobactam), however, different types of inhibitors, such as boronic acid and cloxacillin have shown good inhibition (Pitout et. al., 2010).

This review aims at explaining in detail , several phenotypic methods used in the detection of extended spectrum β-lactamases. Numerous detection methods of ESBL have been proposed based on clinical microbiology techniques, these techniques include preliminary screening for ESBL production followed by confirmatory tests. The screening is usually done by disk diffusion techniques, while the phenotypic confirmatory methods rely mainly on the action of beta lactamase inhibitors such as clavulanic acid and tazobactam to increase the zone of inhibition. The various methods that will be reviewed in this study include Double Disc Synergy, Three dimensional, Vitek system, Disk combination, ESBL Chrom Agar, Broth Micro dilution, Nordmann/Dortet/Poirel (NDP) test, and E-test.

ESBL Detection Methods

Initial Screening Test for ESBL Production

The Clinical and Laboratories Standard Institute (CLSI) as well as European Committee on Antimicrobial Susceptibility testing (EUCAST) recommended a two-step phenotypic approach in detecting ESBL production followed by confirmatory tests. The initial screening may be carried out by broth microdilution or disk diffusion method while the confirmatory test mainly relies on the action of beta lactamase inhibitors to enhance the zone of inhibition (EUCAST, 2012; Yarima et. al., 2019).

Broth Microdilution

This test has been recommended by both CLSI and EUCAST in screening of ESBL production. In this test, a concentration of 4µg/ mL of Cefpodoxime or 1 µg/mL of Ceftazidime, Aztreonam, Cefotaxime or Ceftriaxone are tested against the test bacteria. After incubation at 37^{o}C for 16 hours. Growth at or above the screening concentrations preliminarily indicates possible ESBL production (i.e. for *Escherichia coli, Klebsiella pneumoniae,* and *Klebsiella. oxytoca*, MIC ≥ 8 µg/mL for cefpodoxime or MIC ≥ 2µg/mL for ceftazidime, aztreonam, ce fotaxime, or ceftriaxone; and for *P. mirabilis*, MIC ≥ 2µg/mL for cefpodoxime, ceftazidime, or cefotaxime). It is recommended that using more than one antimicrobial agent in screening for

ESBL production improves the sensitivity of the detection method.

Disk Diffusion Method

Disk diffusion method detects reduced suscepti bility to two or more indicator antibiotics (Cefotaxime 30µg, Ceftriaxone 30µg,Ceftazidime 30µg, Aztreonam 30µg, and Cefpodoxime10µg). Once an ESBL producer is suspected, it is then confirmed by standardized methods (EUCAST, 2012).

A suspension of the test isolates as well as the standard strains is made using a loopful of its colony in normal saline to achieve a cell turbidity equivalent to the 0.5 McFarland turbidity 1.5×10^8CFU/mL. Then inoculums are aseptically streaked on to the surface of Muller-Hinton agar media using a wire loop. The antibiotic discs are aseptically placed on the surface of Muller Hinton agar using sterile forceps, leaving 15 mm away from the edge of the Petri dish, the disks were separated from each other by a distance not less than 20mm to avoid overlapping zones of inhibition. The inoculated media are then incubated at 37°C for 16 hours. Zones of inhibition around the antibiotic disks are measured to the nearest mm and can be compared with the reference strains of *E. coli* (ATCC25922) and *K. pneumonia* (ATCC-700603) as negative and positive controls respectively (CLSI, 2015)

ESBL Confirmation Methods

E-test Strip (Epsilon test)

The principle behind the ESBL E-test strip is dilution and diffusion. The ESBL E-test strip is a thin plastic, non-porous strip with dimensions 60mm by 5mm, and bearing two shorter gradients aligned in opposite directions on the same strip (Nitin et. al., 2014), The strip is two sided containing a gradient of one of the Oxyiminocephalosporins (e.g. Ceftazidime alone) on one end and a gradient of Cephalosporin + Clavulanic acid (e.g. Ceftazidime plus clavulanate) on the other (Nitin et. al., 2014, Rahman et. al., 2014). The strip is inoculated on a surface of the agar plate and incubated overnight at 37°C. After incubation, any reduction of > 3 log 2 (doubling) dilution is considered as positive (Figure 1) (Vercauteren et al., 1997; Rahman, et. al., 2014). Numerous reports of ESBLs have been confirmed by the use of E-test from different parts of the world including but not limited to Kaur and Aggarwal, 2013; Rahman et. al., 2014; Singh and Lokhendro 2014;Prabha et. al., 2016 .

This method is sensitive, convenient and easy to use, but it is sometimes difficult to read the test when the minimum inhibition concentration (MIC)s of ceftazidime is low because the clavulanate sometimes diffuses over to the side that contains ceftazidime alone (Rahman, 2014).

Figure1: E-test showing enhance zone of inhibition towards cephalosporin plus clavulanic acid (Source: Vercauteren et. al.,1997).

Combination Disk Test (CDT)

In this test, a disk containing cephalosporin alone (cefotaxime 30µg, or ceftazidime 30µg) is placed in opposite direction to a disk containing cephalosporin plus clavulanic acid (20/10µg) with a distance of 15 mm apart on Muller Hinton agar medium (Khosravi et. al.,2013;CLSI, 2014;Anand et. al., 2016; Lohani et. al., 2019). The inoculated media are then incubated at 37°C for 18–24 hours. After incubation, zones of growth inhibition are measured to the nearest mm, a difference of >5 mm for a disk containing cephalosporin plus clavulanic acid compared to a disk containing cephalosporin alone is considered positive as shown in figure 2 (Naseer et. al.,

2007; EUCAST, 2012; Anand et. al., 2016; Shiferaw et. al., 2019;). Shiferaw et. al., (2019) reported that combination disc test is better than double disc synergy test in detecting ESBLs. This method was found to detect ESBL producing *Klebsiella* with 100% sensitivity and specificity and was validated with both BSAC and NCCLS methods Carter et al., (2000) as reported by Livermore and Brown, (2001). Combination disc test is a simple and cost effective method of detecting ESBLs (Jabeen et al., 2003). Several studies (Livermore and Brown, 2001; Giriyapur et. al., 2011;EUCAST, 2012; SinghandLokhendro 2014) recommended that the combination disc test be used to confirm ESBL production.

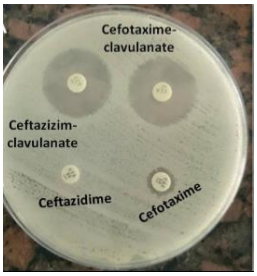

Figure 2: Zone of inhibition around disk with clavulanic acid and disk without clavulanic acid (Source: Mahmoud et. al., 2016).

ESBL Chrom Agar

This test, offers a rapid screening method for the detection of ESBL producing Gram negative bacteria. The test organisms are inoculated on to a CHROMagar using spread plate technique of direct streaking, and then incubated at 37^{o}C for 18-24 hours. Colonies of ESBL producers develop species-specific colors as represented on Table 1 and Figure 3 (Hornsey et. al., 2013; Hassan and Abdalhamid 2014; Prabha et. al., 2016). The advantage of this method is that it is rapid, sensitive and specific ; it also inhibits the growth of other bacteria, including most of those carrying AmpC type resistance (Prabha et. al.,2016). Studies from Uyanga et al., 2019 and Prabha et. al., 2016 reported that ESBL CHROM agar test can be used to confirm ESBL production.

Figure 3: Species-specific colours developed by microorganisms on CHROM agar

Labelled: **1** represent *P. mirabilis;* **2**, *E. faecalis;* **3**, *K. pneumoniae;* **4**, *P. aeruginosa;* **5**, *E. coli;* **6**, *S. aureus* (Samra, et. al., 1998).

Table 1: Differential colours of the various ESBL- producing bacterial genera on Chrom agar.

S/ No	**Microorganism**	**Appearance of the colony**
1	*Proteus mirabilis*	Brown halo
2	*Escherichia coli*	Dark pink to reddish
3	*Klebsiella pneumonia*	*Mucoid,*^{x}Metallic blue

4	*Enterobacter aerogenes*	Metallic blue
5	*Citrobacter freundii*	*Metallic blue*[y]
6	*Acinetobacter sp*	Cream, Nontransparent,
7	*Pseudomonas aeruginosa*	Translucent, yellow serrated edges
8	*Stenotrophomonas*	Colourless
9	*Morganellamorganii*	Clear diffusible beige on beige background
10	*Torulopsis glabrata*	Creamy, very small, indistinct
11	*Enterococcus faecalis*.	Dry, turquoise
12	*S. saprophyticus*	Pink, Opaque
13	*Staphylococcus aureus*	Opaque, white to yellowish
14	*Candida*albicans	Creamy, wet convex
15	*Pseudomonas aeruginosa*.	Transparent, yellow serrated edges, diffuse[z]
16	*Streptococcus sp*	Small, translucent; diffuse light blue within agar
17	*Corynebacterium sp*	Colorless, small, undifferentiated
18	*Lactobacillus sp*.	Scanty, light blue within agar

Legend; [x]Slight pink halo around the periphery after 24 to 36 hours.
[y]Strong purple-pink halo (diffuse) after 24 to 36 hours.
[z]Green after 24 to 36 hours.
(Source: Samra**,** et al., 1998;Prabha et. al.,2016**)**

Double Disk Synergy Test (DDST)

In this assay, an overnight broth culture of the test bacteria corresponding to 0.5 McFarland turbidity standard is aseptically streaked onto the surface of Mueller-Hinton medium (Rahman et. al., 2014). A susceptibility disk containing amoxicillin plus clavulanate (20/10µg) is aseptically placed in the center of the plate, and the cephalosporins disks (ceftriaxone, cefpodoxime, cefotaxime, or ceftazidime) are then placed around it at a distance of 15 mm from the center of the amoxicillin plus clavulanate disk (Farzana et. al., 2013; Yarima et. al., 2019). The inoculated media are then incubated overnight at 37°C (Kaviyarasan, et. al.,2018). An increase in the zone of inhibition towards the centrally placed disk will be considered positive for ESBL production (Peter-Getzlaff., 2011; Caurasia et al., 2015) as shown in Figure 4.This test is reliable for the detection of ESBLs provided that the sensitivity disks are placed at the recommended distance (Rahman et. al.,2014). Jabeen et. al., (2003) recommended that ESBL could be detected by the use of double- disk synergy test, as the method is cost effective and simple. Several other studies (Rahman et. al., 2014; Singh and Lokhendro 2014; Chauhan et. al., 2015 Shu'aibu et.al., 2016; Falodun et. al., 2018: Yarima et. al.,2019) also buttressed the finding that DDST can be used to confirm ESBL production.

Figure 4: Zone of inhibition toward the centrally placed amoxicillin plus clavulanic acid disc between ceftazidime and cefpodoxime discs as exhibited by an ESBL producing bacterium (Source: Mangaiyarkarasi et. al., 2013)

Three Dimensional Test

In this method, two types of inocula are prepared, namely; three dimensional inoculum (10^9 to 10^{10} CFU/mL) and a standard disk diffusion test inoculum (0.5 McFarland turbidity standard) (Nitin et al., 2014). The surface of the Muller-Hinton Agar is inoculated by standard disk diffusion testing method, and a slit is made into the agar using a sterile scalpel blade, then a three dimensional inoculum is pipetted in to the slit. Subsequently, antibiotic disks are placed on the

surface of the plate at a distance of 3 mm outside of the inoculated circular slit (Rawat and Nair, 2010; Nitin et. al., 2014). While standard disk diffusion susceptibility test results are measured according to the recommendations of CLSI, the distortion or discontinuity in the expected circular inhibition zone is considered positive for ESBL production (Nitin et. al., 2014). Three dimensional test may be direct or indirect. Indirect modified three dimensional test, as reported by Shaikh et al., (2016) is better than double disc synergy test for ESBL detection. This test is very sensitive in detecting ESBL, but it is technically challenging and more labour intensive than other methods (Thomson and Sanders, 1992). Reports from Rupp and Fey, (2003) and Rahman et.al., (2014) shows that this method is used in detecting ESBL production.

Broth Micro dilution test

In this assay, a standard broth dilution procedure is employed using Ceftazidime 0.25–128 µg/mL, Ceftazidime plus clavulanate 0.25/4–128/4 µg/mL and Cefotaxime 0.25–64 µg/mL, Cefotaxime plus clavulanate 0.25/4–64/4 µg/mL as recommended by CLSI. Following incubation at 37^{o}C for 16 hours, phenotypic confirmation is considered as a ≥ 3 twofold serial dilution decrease in Minimum Inhibitory Concentration (MIC) of either cephalosporin in the presence of clavulanic acid compared to its MIC when tested alone. For confirmatory tests, both cefotaxime and ceftazidime alone and in combination with clavulanate can be used (CLSI, 2015). Swenso et. al., (2004), Hoon et. al., (2009) , EUCAST, (2012) , CLSI, (2015) and Abdelmoktader and Talal, (2019) asserted that Broth Microdilution has been used to detect ESBL production.

Vitek System

This is an automated microbial identification and antibiotic susceptibility testing that can be used to confirm ESBLs in members of the family *Enterobacteriaceae* (Dashti et. al., 2006; Shah et. al., 2016). Several other studies (Rupp and Fey, 2003; Spnau et. al., 2006; Putra et. al., 2020) show that Vitek 2 system can be used to detect ESBL Production. This assay relies on card wells containing 1.0 mg/L of cefepime, or 0.5 mg/L of cefotaxime or ceftazidime, either alone or in combination with 10 or 4 mg /L of clavulanate, respectively (Drieux et. al., 2008), following incubation, cards are introduced into the VITEK 2 machine, and for each antibiotic tested, turbidity is measured at regular intervals. An isolate is considered ESBL positive, if a predetermined reduction in growth in wells containing clavulanic acid compared to those without clavulanic acid is observed (Drieux et. al.,2008; Rahman et. al., 2014). Computer algorithms in the vitek system have been used to categorize the beta lactamases present in Gram negative clinical isolates based on the phenotype of susceptibility patterns with various β-lactam antibiotics. This method is fast, sensitive and specific (Rahman et. al., 2014) ; the method helps to identify *Enterobacteriaceae* up to genus and species levels (Dashti et. al., 2006) and the susceptibility tests using this method are expressed as MIC values and interpreted as susceptible, intermediate or resistant with reference to a CLSI (Livermore and Brown, 2001; Dashti et. al., 2006; Shah et. al., 2016).

Nordmann/Dortet/Poirel (NDP) test

This test identifies ESBL producers based on biochemical detection of the hydrolysis of the β-lactamring of cefotaxime. The procedure of Poirelet. al., (2016) as described by Afolabi et. al., (2017) is as follows. Briefly, 50µl of a 10% Triton solution is added into Eppendorf tubes labeled A, B, and C containing 0.5ml of blood culture and the mixture is vortexed and incubated at room temperature for 5min followed by centrifugation at 13000g for 2min. The supernatant is discarded and the pellet is re-suspended in 500µl distilled water, the bacterial suspension is centrifuged again at 13,000 g for 2 min, the supernatant is discarded again and the bacterial pellet re suspended in 100µl of 20 mmol l^{-1}Tris-HCl lysis buffer. Ten microliters (10µl) of 40 $mgmL^{-1}$ tazobactam solution is added into tube C; and then 100µl phenol red (0.5%, w/v) into tube A and 100 µl phenol red supplemented with cefotaxime 6 $mgmL^{-1}$ into tubes B and C. The tubes are incubated at 37^{o} C for 15 min, change in colour from red to yellow/orange is positive for ESBL production as shown in Figure 5 below (tube B) which contains cefotaxime alone while the tube containing cefotaxime supplemented with tazobactam remained red (unchanged).

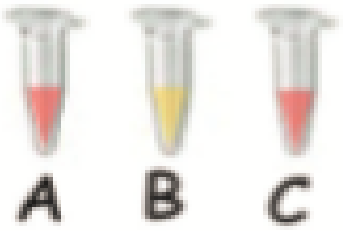

Figure 5: ESBL production exhibited by inoculum in tube B containing cefotaxime alone (Source: Affolabiet. al.,(2017)).

Conclusion

Infections caused by ESBL- producing bacteria often limits therapeutic options, leading to high disease burden. Therefore, diagnostic laboratories are in need of reliable, cost efficient and less labour intensive methods to use in the detection of ESBL- producing bacteria. Bacteria harbouring ESBL genes are capable of spreading these plasmids to other bacteria via horizontal gene transfer. The public health implications of this are disturbing thus the need to rapidly detect these pathogens in the laboratory. Several techniques of ESBL detection and confirmation have been comprehensively explored in this review and the choice of the testing method depends on the preferences and requirements of the laboratory professionals as well as the availability of the testing material. There is the need for reliable but simple phenotypic tests in the laboratory for the detection of these ARB, which do not require highly skilled personnel, this is in order to ensure a swift response in the management and control of these pathogens. Policy makers in the relevant sectors need to implement strategies to ensure the rapid detection of ESBL- producing bacteria in clinical and environmental samples.

S/N	Methods		Merits/demerits	References
1	E-test	Merits	sensitive and easy to use	Rahman et. al., (2014); Nitinet. al., (2014); Prabha et al.,(2016)
		Demerits	Difficult to interpret, less sensitive compare to double-disk test	Rahman et. al.,(2014)
			E-test is expensive	Prabha et. al., (2016);
2	Combination disk test	Merits	This method is better than DDST in the detection of ESBLs	Dejenie Shiferaw Teklu et. al., (2019)
3	ESBL Chrom agar	Merits	Rapid, sensitive and specific	Prabha et. al., (2016)
		Demerits	CHROM agar test is expensive	
4	Double Disk synergy test (DDST)	Merits	Reliable, Easy to use and interpret	Rahman et. al., (2014)
		Demerits	Distance of disk placement not standardized DDST was less effective than E-test and CHROM agar.	(Jabeen et. al.,(2003)
5	Three dimensional test	Merits	simultaneous determination of antibiotic susceptibility and β-lactamase detection (but not specific for ESBLs)	Singh and Kumar, (2013); Rupp and Fey, (2003); Nitin et al., (2014)
			Sensitive in ESBL detection	Thomson and sanders, (1992); Bradford, (2001)
		Demerits	Non-specific for ESBLS, labor intensive	Rahman et. al.,(2014)
6	Broth micro dilution	Merits	Results can be expressed quantitatively	Alizade et al., (2016)
		Demerits	labor intensive	
7	Vitek system	Merits	automated microbial identification and antibiotic susceptibility testing	Dashti et. al.,(2006); Shah et. al.,(2016)
			Fast, sensitive, specific	Rahman et. al.,(2014)
			Identifies *Enterobacteriaceae* up to genus and species level	Dashti et. al.,(2006)
		Demerits	Reduced sensitivity	Rahman et. al.,(2014)
8		Merits	Simple, Rapid, cost-effective, sensitive, specific and reliable	Kumar et. al., (2018), Sadek and Nordmann, (2019)

	Nordmann/Dortet/Poirel (NDP) test	Demerits	The ability of the enzyme to hydrolyze the substrate, the level of expression of the corresponding gene and the affinity of the enzyme for the substrate	Sadek et. al., (2019)

Table 2: Brief summary of the merits and demerits of the methods used in detection of ESBL

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