Review Article

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Extended Spectrum β-Lactamases: Critical Tools of Bacterial Resistance

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Abstract

Extended-spectrum β-lactamases (ESBLs) is one of the important groups of β-lactamases produced by Gram-negative bacteria including Enterobacteriaceae. This group of enzyme can effectively destroy most of the β-lactam antibiotics including penicillins, the third generation cephalosporins, and aztreonam. The emerging of ESBLs-producing bacteria is widely spread and becomes a major public health concern for limited treatment options. Detection of these enzymes is necessary for identification, protection, and treatment. The detection method of this enzyme can be divided into 2 groups, phenotypic detection and genotypic detection. The first method depends on non-molecular techniques to detect ESBLs-producing bacteria. This method is generally used in clinical laboratory because it is easy to perform, convenient, and inexpensive. However, the phenotypic method cannot be used to identify ESBLs type. The latter method, genotypic detection, uses molecular techniques to detect the enzyme encoding sequence. This method is capable to classify ESBL types and is currently used. This review will summarize the ESBLs in aspect to classification and detection methods.

Key words: Extended spectrum beta lactamases, Resistance, Classification, Detection

INTRODUCTION

The emergence of multi-drug resistant bacteria is becoming increasingly public health problem worldwide. The discovery of penicillin in 1950s and development of extended spectrum penicillin in 1960s gave the hope to eradicate the infectious diseases. However, the bacteria can still develop its resistance mechanisms against all antibiotics in parallel. One of the important resistance mechanisms is production of enzymes destroying the drug. β-lactamase is an enzyme produced by many gram-negative bacteria which can hydrolyze β-lactam antibiotics. This enzyme destroys the drug prior to bind to penicillin binding proteins (PBPs) on bacterial cytoplasmic membrane. To date, several types of β-lactamases have been characterized depending on the characteristic and hydrolytic activity. Extended-spectrum β-lactamases (ESBLs) is one of the important groups of β-lactamases. This group of enzyme can hydrolyze most of the β-lactam antibiotics including penicillins, oxyimino cephalosporins (the third generation cephalosporins), and aztreonam. Detection of these enzymes is highly concerned in order to prevent resistant bacteria spread and to treat the infection. At present, there are 2 groups of methods used for detection ESBLs, phenotypic detection and genotypic detection. Phenotypic detection depends on non-molecular technique for enzyme detection. This method is routinely used in clinical laboratory because it is easy to perform, convenient, and inexpensive. However, this method is not suitable for ESBLs type classification. Another method, genotypic detection, uses molecular techniques to detect the gene encoding enzyme. This new method is capable to classify ESBL types and is currently used instead.

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EXTENDED SPECTRUM β-LACTAMASES (ESBLs)

Bacteria have developed their resistance mechanisms along with the discovery of the first antibiotic, penicillin. Since then, it spreads to other bacterial species including gram-negative bacilli, which are able to transfer genetic material interspecies. The plasmid encoded **β**-lactamase was first reported in 1960s. The plasmid was isolated from a Greek patient name Temoniera and the plasmid was named TEM-1. At present, TEM-1 enzyme has been detected in various bacterial species including *Escherichia coli* and other species in Enterobacteriaceae such as *Klebsiella pneumoniae*. There are also many reports on other types of plasmids encoded **β**-lactamase such as SHV-1. Since the discovery of TEM-1, the new generation of **β**-lactam antibiotics have been developed to increase their spectrum and to be more resistant to **β**-lactamase enzymes. However, the **β**-lactamases have been mutated from a narrow spectrum to an extended-spectrum, which can hydrolyze many types of **β**-lactam antibiotics including the third generation cephalosporins especially oxyimino cephalosporins. According to their ability to hydrolyze many **β**-lactam antibiotics including extended-spectrum cephalosporins, this enzyme was named extended-spectrum **β**-lactamases (ESBLs) (1-2).

ESBLs are a group of enzymes capable to hydrolyze penicillin including the 1st, 2nd, and 3rd generation cephalosporins. The special characteristic of these enzymes is the ability to hydrolyze **β**-lactam structure in all classes of **β**-lactam antibiotics including penicillins, narrow spectrum and extended spectrum cephalosporins such as oxyimino cephalosporins (cefotaxime, ceftazidime), fourth generation cephalosporins (cefipime), and monobactams (aztreonam). These enzymes are sensitive to **β**-lactamase inhibitors (sulbactam, clavulanic acid, and tazobactam). At present, ESBLs are found only in gram-negative bacteria such as *K. pneumoniae, E. coli, Salmonella Typhimurium, Pseudomonas aeruginosa, Neisseria meningitidis*, and Enterobacteriaceae.

There are 2 systems used to categorize ESBLs: Amber molecular classification scheme (3) and Bush-Jacoby-Medieros functional classification system (4). In Amber scheme, ESBLs are classified into 4 classes, A to D, by its homology of amino acid at the active site. At the beginning, there were 2 classes, serine-**β**-lactamase (class A) contained serine at the active site and metallo **β**-lactamase (class B) which needed bivalent cation, usually Zn^{2+}, for their activity. Due to the discovery of new types of serine **β**-lactamases, this class was separated to class C (5) and class D (6) (Table 1, 7-8).

Table 1. Classification of **β**-lactamases according to Amber molecular scheme (7,8).

	Class	β - lactamases	Examples
Serine β - lactamases	A	Broad spectrum β - lactamases	TEM-1, TEM-2, SHV-1
		ESBL TEM – type	TEM-3
		ESBL SHV – type	SHV-5
		ESBL CTX-M – type	CTX-M1, CTX-M9
		Carbapenemases	KPC
	C	AmpC cephamycinases (chromosomal encode)	AmpC
		AmpC cephamycinases (plasmid encode)	CMY, DHA
	D	Broad spectrum β - lactamases	OXA-1, OXA-9
		ESBL OXA – type	OXA-2, OXA-10
		Carbapenemases	OXA-48, OXA-23
Metallo β - lactamases	B	Metallo β - lactamases	VIM, IMP

In 1995, Bush – Jacoby – Medeiros proposed the new system to categorize ESBLs using enzymatic activity. They classified ESBLs into 4 groups (1-4) and 5 subgroups (a-f) (Table 2).

Group 1 involves cephalosporinases that resist to clavulanic acid, comparable with class C in Amber scheme.

Group 2 consists of penicillinases and cephalosporinases that can be inhibited by clavulanic acid, comparable with class A and D in Amber classification. The TEM and SHV were originally classified in this group. Due to the increase in number of TEM- and SHV- derived **β**-lactamase, this group was separated into 2 subclasses, 2a and 2b. The member of subclass 2a is only penicillinases, while 2b is composed of a broad spectrum **β**-lactamases which can inactivate penicillins and cephalosporins at the same rate. Moreover, there are sub-subgroups, 2be and 2br, separated from subgroup 2b.

Sub-subgroup 2be (e = extended spectrum of activity) is composed of ESBL that can hydrolyze the 3rd generation cephalosporins (cefotaxime, ceftazidime, and cefodoxime) and monobactams (aztreonam). Sub-subgroup 2br (r = reduced binding to clavulanic acid and sulbactam) contains enzymes that are resistant to clavulanic acid and sulbactam. This group is called inhibitor-resistant TEM derivative enzymes. However, this group is still sensitive to tazobactam.

Subgroup 2c was separated from subgroup 2b later because the members of this group could inactivate carbenicillin more efficiently than benzylpenicillin and had slight effect on cloxacillin. Subgroup 2d is able to hydrolyze cloxacillin more than benzylpenicillin and has similar activity on carbenicillin. This group is slightly inhibited by clavulanic acid. In addition, subgroup 2f, which consist of serine-based carbapenamases, was designated later. This group is different from zinc-based carbapenamases in group 3.

Group 3 consists of zinc-based or metallo-**β**-lactamases comparable with Amber class B. This group is the only group that requires metal ion, usually Zn^{2+}, for their activity. These enzymes can hydrolyze penicillins, cephalosporins, and carbapenams. Therefore, carbapenams can be inhibited by either group 2f (serine-based mechanism) or group 3 (zinc-based mechanism).

Group 4 is composed of penicillinases that resist to clavulanic acid and non-comparable with any groups in Amber classification.

ESBLs are classified into class A and D in Amber molecular scheme and in group 2be in Bush-Jacoby-Medieros functional classification system. These enzymes can hydrolyze oxyimino **β**-lactams at least 10% more than benzylpenicillin and are inhibited by clavulanic acid (4, 10). Originally, the narrow spectrum TEM and SHV are the most prevalence ESBLs (11). However, the new types of CTX-M were discovered later especially in *E. coli* and *K. pneumoniae* and appeared to be the most prevalence ESBLs during the last 5 years (12-14).

CLASSIFICATION OF ESBLs

Most ESBLs can be classified into 3 major groups; TEM, SHV, and CTX-M. However, the other groups of ESBLs such as VEB, PER, GES, TLA, IBC, SFO-1, BES-1, and BEL-1 have been reported.

TEM β-lactamases

TEM-1 is the most prevalence in this group. It has been reported that this enzyme is responsible for more than 90% of ampicillin-resistant *E. coli*. TEM-1 is also involved in the resistance of ampicillin and penicillin in *Hemophilus influenzae* and *Neisseria gonorrhoeae.* TEM-2 is a derivative of TEM-1, which possesses an amino acid substitution. TEM-3 is the first TEM-type **β**-lactamase which shows phenotypic characteristic of ESBLs. Although TEM are presented mostly in *E. coli* and *K. pneumoniae*, this group of **β**-lactamases has also been reported in other gram-negative bacteria such as *Enterobacter aerogenes*, *Morganella morgannii*, *Proteus mirabilis, P. rettgeri,* and *Salmonella* spp. Phenotypic variation

of ESBLs is caused by alteration of amino acid sequence at the active site. This amino acid alteration makes the enzyme bind to oxyimino **β**-lactams. By opening the active site to bind to the **β**-lactam ring, the enzyme is more sensitive to **β**-lactamase inhibitors. Changing of amino acids at position 104, 164, 238, and 240 cause phenotypic variation of ESBLs. Most ESBLs have amino acid mutation more than one position. Until May 2011, 190 types of TEM group have been reported (15). The TEM-10, TEM-12, and TEM-26 are the most abundant in USA.

Table 2. Classification of **β**-lactamases according to Bush-Jacoby-Medieros system (9)

Group	Type	Examples
1	Cephalosporinases	AmpCs, CMY-2
2	All clavulanic acid susceptible	
2a	Penicillinases	PC-1 from *S.aureus*
2b	Broad-spectrum penicillinases	TEM-1, SHV-1
2be	ESBLs	SHV-2, TEM-10, CTX-Ms
2br	Inhibitor resistant	TEM, IRT
2c	Carbenicillin hydrolyzing	PSE-1
2d	Oxacillin hydrolyzing	OXA-10, OXA-1
2e	Cephalosporinase inhibited by clavulanate	FEC-1
2f	Carbapenemases	KPC-1, SME-1
3	Metallo-beta-lactamases	IMP-1, VIM-1
4	Miscellaneous	

Inhibitor-Resistant-β-lactamases

Although inhibitor-resistant-**β**-lactamases are not ESBLs, they are usually included in the ESBLs. It is because there are many TEM- and SHV- derivatives classified in this group. In the early of 1990s, the clavulanic acid-resistant **β**-lactamases have been reported. Although these enzymes are classified in TEM-**β**-lactamase, the nucleotide sequence is found to be different from TEM-1 or TEM-2-**β**-lactamase. These enzymes can be found in some species of *K. pneumoniae, K. oxytoca, P. mirabilis*, and *Citrobacter freundii*.

SHV β-lactamases

The SHV-1 has approximately 68% amino acid sequence homology to TEM-1. They also have structure similarly. The SHV-1 **β**-lactamase is often found in *K. pneumoniae* and is involved in ampicillin resistance. The ESBLs in this group also have amino acid alteration at the active site similar to TEM-1, which are usually at position 238 or 238 and 240. There have been reported for 141 types of SHV at the end of May 2011 (15). This group is usually found not only in Europe and USA, but also in other geographic regions. The most prevalence enzymes of this group are SHV-5 and SHV-12.

CTX-M β-lactamases (class A)

The name of this group is derived from the fact that it is resistant to cefotaxime more than other oxyimino **β**-lactams such as ceftazidime, ceftriaxone, and cefepime. The emergence of this enzyme is not from mutation but because of acquisition of plasmid-encoded **β**-lactamase derived from chromosome of non-pathogenic bacteria genus *Kluyvera* especially *K. ascorbata* and *K. georgiana* (16). This enzyme shows only 40% homology to TEM and SHV. At the end of May 2011, 120 types of enzymes have been reported (15) and can be classified by amino acid sequences into 5 subgroups;

CTX-M1, CTX-M2, CTX-M8, CTX-M9, and CTX-M25 (Table 3). This group is usually found in *Salmonella enterica, Salmonella Typhimurium*, and *E. coli*, sometimes in other Enterobacteriaceae. It is the most prevalence ESBLs in South Africa. At present, CTX-M15 is the most abundant CTX-M found in *E. coli* isolated from UK (17).

OXA β-lactamases (class D)

This enzyme can hydrolyze oxacillin and anti-staphylococcal penicillins. This group is classified in molecular class D and functional group 2d. It is resistant to ampicillin, cephalotin, oxacillin, and cloxacillin, but is not inhibited by clavulanic acid. This enzyme is normally found in Enterobacteriaceae, *K. pneumoniae*, and *E. coli*. It is also found in *Pseudomonas aeruginosa*, especially *P. aeruginosa* isolated from Turkey and France. Previously, this group was classified by phenotypic system, not genotypic, so the amino acid homology within this group was very low.

PER

PER ESBLs have about 25-27% amino acid homology to TEM and SHV. PER-1 **β**-lactamase hydrolyzes penicillins and cephalosporins efficiently, but is inhibited by clavulanic acid. In the past, PER was usually isolated from *P. aeruginosa*, but now from *Salmonella enterica* serovar *Typhimurium* and *Acinetobacter* sp. The PER-type ESBL can be found worldwide, but is most frequent in Europe.

Other types of ESBLs

Many types of ESBLs are not mentioned above such as VEB, GES, and IBC. The prevalence of other groups is rare compared with TEM, SHV, and CTX-M. They are mostly isolated from *P. aeruginosa* and are found in limited geographic region, for examples, VEB-1 and VEB-2 isolated from South East Asia or GES-1, GES-2 and IBC-2 isolated from South Africa, France and Greece. The rare type ESBLs such as BES-1, IBL-1, SFO-1, and TLA-1 are only found in Enterobacteriaceae family.

Table 3. Classification of CTX-M ESBLs.

CTX-M subgroup	Source	Examples
CTX-M1	*K. ascorbata*	CTX-M1 , CTX-M3, CTX-M15
CTX-M2	*K. ascorbata*	CTX-M2, CTX-M35, CTX-M42
CTX-M8	*K. georgiana*	CTX-M8, CTX-M40, CTX-M63
CTX-M9	*K. georgiana*	CTX-M9, CTX-M14, CTX-M16
CTX-M 25	*K. georgiana*	CTX-M 25, CTX-M26, CTX-M41

DETECTION OF ESBLs

Currently, the appropriate methods for ESBLs detection are seriously concerned because of the failure in treatment of the 3rd generation cephalosporins and aztreonam (18). The method for detection is complicated due to the varieties of enzymes. At present, the methods used to detect ESBLs can be classified into 2 groups: phenotypic detection and genotypic detection. The first detection method based on non-molecular method has been developed since 1980s. The Clinical and Laboratory Standard Institute (CLSI) of USA and the Health Protection Agency (HPA) of UK issued the guideline for ESBLs detection of Enterobacteriaceae especially *E. coli*, *Klebsiella* sp., and *P. mirabilis* (14, 19, 20). However, currently, there is no guideline for AmpC **β**-lactamase detection. The phenotypic detection methods usually examine the resistance of bacteria to the 3rd generation cephalosporins (cefotaxime, ceftazidime, and cefepime) together with **β**-lactamase inhibitor (clavulanic acid). The interpretation in primary susceptibility testing is by determination of MIC reduction when combined with clavulanic acid. After primary testing, the confirmation test should

be performed. There are many phenotypic detection methods used in both primary screening and confirmation test to detect ESBLs production in Enterobacteriaceae such as double disk synergy test, ESBL E-test, and combination disk method.

Double-disk synergy test is the first method developed by Jarlier and colleagues for detection of ESBLs in Enterobacteriaceae (21). At the beginning, this method was used to separate cefotaxime-resistant strain form others. This method is performed by applying cefotaxime disk (30μg) and amoxicillin/clavulanic acid disk (20/10 μg) on the bacterial plate. The distance between 2 disks is usually 30 mm. Positive result will be reported when the inhibition zone is increased at the side closed to clavulanic acid disk. The shape of inhibition zone looks like champagne cork or keyhole (Fig.1). This method was initially used to study ESBLs producing Enterobacteriaceae in French hospital (53, 54). The advantages of this method are cheap and easy to perform. It has been reported that the sensitivity and specificity of this method will be increased when the distance between 2 disks is reduced to 20 mm (55).

ESBL E-test was observed the synergy effect between extended-spectrum cephalosporins and clavulanate. This method is performed by using antibiotic strips containing cefotaxime, ceftazidime, or cefepime on one side and those drugs with clavulanic acid on the other side. The positive result will be reported when MIC value of the clavulanate side is more than 8 (3 doubling dilutions) compared with the antibiotic alone. This method is easy to perform and is more sensitive than the first method. However, the antibiotic strips are expensive and the result interpretation requires experience. The other limitation is that this method is not suitable when MIC of cephalosporin is less than the drug concentration on the strip.

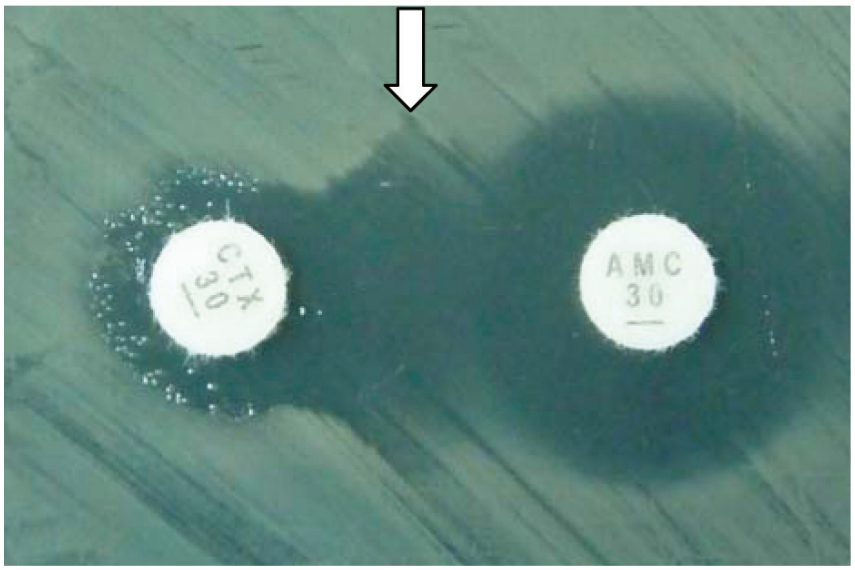

Figure 1. A positive result of double disk synergy test. The inhibition zone is enhanced between cefotaxime (CTX) and amoxicillin-clavulanate (AMC) disks.

Combination disk method is another method developed for screening ESBLs. The principle of this method is by comparison of the inhibition zone around cephalosporin disk with cephalosporin plus clavulanic acid. It will be positive when the inhibition zone difference is more than 5 mm or more than 50% increase in the presence of clavulanic acid (25, 26). This method is easy to perform and the result can be read out directly. The sensitivity and specificity of this method are 96% and 100%, respectively.

Phenotypic detection is usually performed routinely in clinical laboratory because it is easy, cheap, and can be used with automatic machine such as VITEK-2,

ESBL test, or Phoenix ESBL test. However, this method cannot verify the exact type of ESBLs (SHV, TEM, or CTX-M).

In addition, there are many developed and adapted methods to increase the sensitivity of phenotypic detection including double disk synergy test on Muller Hinton agar containing cloxacillin, and combination disk method on Muller Hinton agar containing cloxacillin. The other method is Cica-Beta test, which observes the hydrolysis of cephalosporin dye, HMRZ-86, on paper strip. This method is easy, fast and the result can be read within 15 min (27). Gerrac and colleagues compared 9 phenotypic detection methods used for detection of ESBLs and found that combination of the method in routine laboratory with specific confirmation test gives almost 100% specificity in detection of ESBL producing *Enterobacteriaceae* (24).

The genotypic detections use molecular method to detect the ESBL-encoded gene presented in bacteria. The exact types of ESBLs can be reported by this method. The genotypic methods usually use the polymerase chain reaction (PCR) technique in amplification the ESBL gene. The PCR product will be subsequently sequenced for confirmation and classification of ESBL (1). This method can detect the gene even in small amount, which cannot be detected by phenotypic detection (28). The advantage is that it can detect directly from specimen, not necessary to isolate pure culture (1). There are many attempts to accelerate the process by skipping the sequencing step. The examples of these methods are PCR followed by restriction fragment length polymorphism (RFLP), PCR with single strand conformation polymorphism, ligase chain reaction, restriction-site insertion PCR, and real-time PCR. However, these methods require high technology and expensive instruments which cannot be used in routine clinical laboratory. These methods are usually used only in research laboratory or reference laboratory.

CONCLUSIONS

During the past 20 years, the increase of antibiotic resistant bacteria especially ESBLs is becoming the major public health problem. Treatment with the 3rd generation cephalosporins often fails when the ESBLs producing bacteria are present. The best way to reduce the risk of failure is the rational use of antibiotics and ESBLs testing should be performed before antibiotics selection.

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