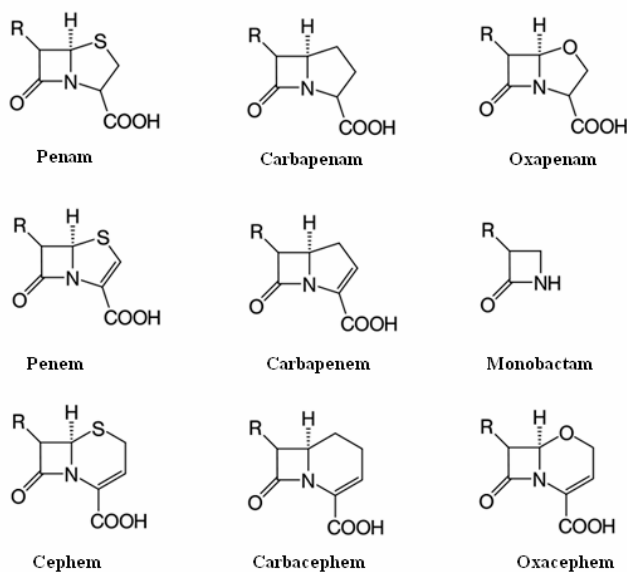


# Extended spectrum beta-lactamases

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## A. Beta-lactam antibiotics



A  $\beta$ -lactam (beta-lactam) ring is a four-membered cyclic amide consisting of three carbon atoms and one nitrogen atom. It is named so, because the nitrogen atom is attached to the  $\beta$ -carbon relative to the carbonyl (C=O). Antibiotics possessing this structure are called beta-lactam antibiotics.

Penams contain a  $\beta$ -lactam ring fused to a 5-membered ring, where one of the atoms in the ring is a sulfur and the ring is fully saturated. A carbapenam is a  $\beta$ -lactam compound that is a saturated carbapenam. They exist primarily as biosynthetic intermediates on the way to the carbapenam antibiotics. A clavam is a molecule similar to a penam, but with an oxygen atom substituted for the sulfur. Thus, they are also known as oxapenams. Carbapenems are very similar to the penams, but the sulfur atom of the unsaturated structure is replaced with a carbon atom. Penem is a type of unsaturated  $\beta$ -lactam, which is similar in structure to carbapenems but penems have a sulfur atom instead of carbon. Carbacephems are similar to cepheems but with a carbon substituted for the sulfur. An

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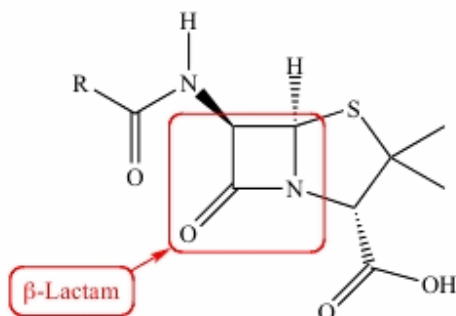
oxacephem is a molecule similar to a cephem, but with oxygen substituted for the sulfur. Monobactams are  $\beta$ -lactam compounds wherein the  $\beta$ -lactam ring is alone and not fused to another ring.

### $\beta$ -lactams are classified according to their core ring structures

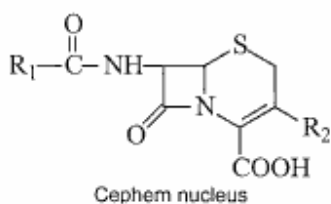
- $\beta$ -lactams fused to saturated five-membered rings:
  - $\beta$ -lactams containing thiazolidine rings are named penams (penicillins)
  - $\beta$ -Lactams containing pyrrolidine rings are named carbapenams (intermediates)
  - $\beta$ -lactams fused to oxazolidine rings are named oxapenams or clavams (clavulanic acid)
- $\beta$ -lactams fused to unsaturated five-membered rings:
  - $\beta$ -lactams containing 2,3-dihydro-1H-pyrrole rings are named carbapenems (imipenem)
  - $\beta$ -lactams containing 2,3-dihydrothiazole rings are named penems (faropemem)
- $\beta$ -lactams fused to unsaturated six-membered rings:
  - $\beta$ -lactams containing 3,6-dihydro-2H-1,3-thiazine rings are named cephems (cephalosporins)
  - $\beta$ -lactams containing 1,2,3,4-tetrahydropyridine rings are named carbacephems (loracarbef)
  - $\beta$ -lactams containing 3,6-dihydro-2H-1,3-oxazine rings are named oxacephems (moxalactam)
- $\beta$ -lactams not fused to any other ring are named monobactams (aztreonam)

Penicillin was inadvertently discovered by Alexander Fleming in 1928 when he observed that contaminating mould, *Penicillium notatum* inhibited growth of Staphylococcus on a culture plate. It took 12 years for the compound to be purified and used in treatment. It was due to the combined efforts of Ernst Boris Chain, Edward Abraham, Howard Florey and Norman Heatley that penicillin was obtained in pure and usable form.

#### a. Structure:



The molecular formula of **penicillin** is  $R-C_9H_{11}N_2O_4S$ , where R is the variable side chain. 6-amino penicillanic acid is the core of penicillins and is used as the main starting block for the preparation of numerous semisynthetic penicillins. The basic structure of penicillins consists of a thiazolidine ring connected to a  $\beta$ -lactam ring with an attached side chain (R). The R side chain determines many of the antibacterial and pharmacological characteristics of the penicillin type. Penicillin is available in various forms including benzylpenicillin (penicillin G), procaine benzylpenicillin, benzathine benzylpenicillin and phenoxymethylpenicillin (penicillin V). Only Penicillin V is administered orally. In order to overcome resistance to penicillin by the penicillinase enzyme, ampicillin was introduced in 1950s. The first semi-synthetic penicillin introduced in the clinical practice was phenethicillin. This was followed by ampicillin, amoxicillin, the  $\beta$ -lactamase-resistant isoxazolyl penicillins (methicillin, oxacillin, cloxacillin, dicloxacillin and flucloxacillin), and antipseudomonal penicillins (carbenicillin, piperacillin and ticarcillin). The active part of penicillins and related antibiotics (cephalosporins, monobactam and carbapenem) is the beta-lactam ring.



**Cephalosporin** was first isolated in 1948 from a fungus *Cephalosporium* (now Acremonium) *sps* by Italian scientist Giuseppe Brotzu. Cephalosporins are basically semi-synthetic derivatives of the cephalosporin C, which was isolated from the fungus. The first commercial preparation- cefalotin (cephalothin) was launched in 1964. The nucleus of cephalosporin antibiotics is 7-aminocephalosporanic acid

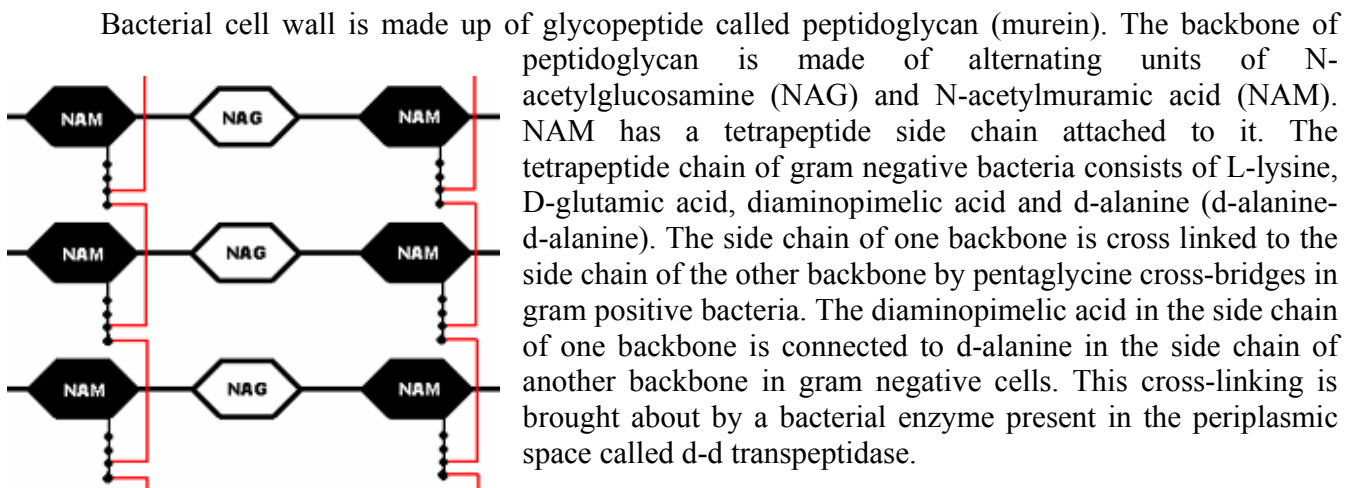
(a  $\beta$ -lactam ring). It is made of 4-membered  $\beta$ -lactam ring and 6-membered dihydrothiazolidine ring with two side chains R1 and R2. Modifications in the side chain have resulted in several types of cephalosporins, which are grouped into several generations. Cephalosporins are more effective against gram negative bacteria. The cephamycins are similar to the cephalosporins, but have a methoxy group at position 7 of the beta-lactam ring of the 7-aminocephalosporanic acid nucleus. Mechanism of action is similar to those of penicillins. First generation cephalosporins were introduced in the 1960s. In the late 1970s and early 1980s, penicillinase resistant oxyimino- aminothiazolyl (3<sup>rd</sup> generation) cephalosporins were introduced.

The initial cephalosporins were designated first-generation cephalosporins whereas, later cephalosporins, that had more extended-spectrum of activity were classified as second-generation. Each newer generation of cephalosporins has significantly greater Gram-negative antimicrobial properties than the preceding generation. Fourth-generation cephalosporins have true broad-spectrum activity. There are disagreements over “generations” and the placements of antibiotics in these generations.

## b. Types of Beta-lactam antibiotics:

Penicillins	Narrow spectrum	$\beta$ -lactamase susceptible	Benzylpenicillin, Benzathine benzylpenicillin, Procaine benzylpenicillin, Phenoxymethylpenicillin
		$\beta$ -lactamase resistant	Cloxacillin (Dicloxacillin, Flucloxacillin), Oxacillin, Meticillin, Nafcillin
	Broad spectrum	Aminopenicillins	Amoxicillin, Ampicillin, Epicillin
		Carboxypenicillins	Carbenicillin, Ticarcillin, Temocillin
		Ureidopenicillins	Azlocillin, Piperacillin, Mezlocillin
Other	Mecillinam, Sulbenicillin		
Cephalosporins	1 <sup>st</sup> generation	Cefazolin, Cefadroxil, Cefalexin, Cefaloridine, Cefalotin, Cefapirin, Cefatrizine, Cefazedone, Cefazaflur, Cefradine, Cefroxadine, Ceftezole	
	2 <sup>nd</sup> generation	Cefaclor, Cefamandole, Cefminox, Cefonicid, Ceforanide, Cefotiam, Cefprozil, Cefbuperazone, Cefuroxime, Cefuzonam	
	3 <sup>rd</sup> generation	Cefixime, Ceftriaxone, Cefoperazone, Cefotaxime, Cefpodoxime, Cefdinir, Cefsulodin, Cefcapene, Cefdaloxime, Cefditoren, Cefetamet, Cefmenoxime, Cefodizime, Cefpimizole, Cefpiramide, Cefteram, Ceftributen, Ceftiolene, Ceftizoxime	
	4 <sup>th</sup> generation	Cefepime, Cefpirome, Cefquinome, Cefozopran	
	5 <sup>th</sup> generation	Ceftobiprole, Ceftaroline fosamil	
Cephamycin	Cefoxitin, Cefotetan, Cefmetazole		
Carbacephem	Loracarbef		
Monobactam	Aztreonam, Tigemonam, Carumonam, Tabtoxin		
Carbapenem	Imipenem, Meropenem, Ertapenem, Doripenem, Biapenem, Panipenem		

### c. Action of beta-lactam antibiotics:



As the bacteria grow and multiply, they constantly remodel their cell walls by breaking down and building portions of cell wall. The  $\beta$ -lactam ring of the antibiotic is sterically identical to d-alanine-d-alanine. In the presence of  $\beta$ -lactam antibiotic in the periplasmic space, the transpeptidase enzyme mistakenly binds to the  $\beta$ -lactam antibiotic instead of its original substrate. Since the  $\beta$ -lactam antibiotic binds to the transpeptidase enzyme, the enzyme is also known as penicillin-binding-protein (PBP). The binding results in acylation of the enzyme leading to the production of an inactive penicilloyl-enzyme. As a result, further cross-linkages between the layers of peptidoglycan do not occur, which weakens the cell wall. Accumulation of cell-wall precursors are thought to initiate autolysis. The cell finally undergoes osmotic instability and lyses. Bacteria have multiple PBPs. *E. coli* has seven PBPs, each with a distinct role. PBP 1A and B are important in cell elongation, PBP 2 maintain rod shape of cell wall, PBP 3 forms septum between dividing cells. PBPs 4, 5, and 6 are thought to be non-essential. The sensitivity of individual PBP is known to vary with individual beta-lactam drug but at clinical doses most beta-lactam drugs bind to more than one PBP. Inhibition of different PBPs may have different effects; inhibition of PBP2 may cause delayed cell lysis, and inhibition of PBP3 may result in formation of long filamentous forms.

### d. Mechanisms of resistance to beta-lactam antibiotics

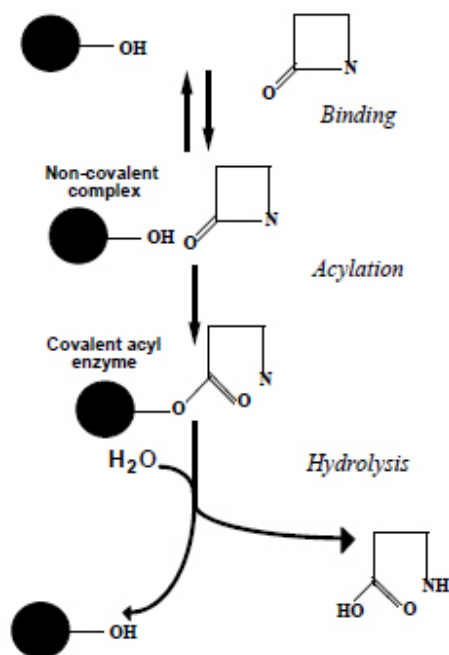
There are four known methods of resistance to  $\beta$ -lactams seen among bacteria. These are:

- Inactivation of the  $\beta$ -lactam antibiotic by an enzyme ( $\beta$ -lactamase)
- Production of newer penicillin binding protein with decreased affinity to the antibiotic
- Decreased permeability of the antibiotic through the cell envelope due to altered porin channels
- Active efflux pumps that throw the antibiotic out of the cell

### Resistance mediated through beta-lactamases:

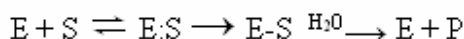
Production of an enzyme with penicillinase activity was first observed in *E. coli* by Abraham and Chain as early as 1940; even before the introduction of penicillin for therapeutics. These enzymes may have evolved for a possible physiological role in peptidoglycan assembly or to defend themselves against beta-lactams produced by environmental bacteria and fungi. First detection of penicillinase in gram positive bacteria (*Staphylococcus aureus*) was reported in 1944. Initially, the genes coding for beta-lactamases were found in bacterial chromosomes. These enzymes were inducible and constitutively expressed in low quantities. Apart from endogenous chromosomal beta-lactamases, many bacteria have acquired plasmid mediated beta-lactamases, which are being shared even across species. It is suspected

that most of the plasmid mediated beta-lactamases may have their origin in bacterial chromosomes. Genes coding for beta-lactamases are also present on transposons or insertion sequences, resulting in their dissemination among different plasmids.



The  $\Omega$ -loop, which is a conserved structural feature of most class A enzymes, consists of residues 164-179 and forms a portion of the enzyme's active site pocket. The active site contains a water molecule required for deacylation step of  $\beta$ -lactam hydrolysis. The ability of beta-lactamase enzymes to break the beta-lactam ring requires the presence of a nucleophile (a chemical group that can donate electrons) in the active site of the enzyme. In serine beta-lactamases, the nucleophile is attached to the amino-acid serine. Serine beta-lactamase enzyme (E) binds to its beta-lactam antibiotic substrate (S) and forms a reversible Michaelis complex (E:S). The free hydroxyl on the side chain of a serine residue at the active site of the enzyme performs a nucleophilic attack on the carbonyl carbon atom of the  $\beta$ -lactam ring resulting in an acylation intermediate. This intermediate transitions into covalent acyl-enzyme following protonation of the  $\beta$ -lactam nitrogen and cleavage of the C-N bond. Using strategically placed water molecule the acylated beta-lactam is hydrolyzed giving rise to inactivated product (P) and regenerated enzyme (E). Acylation requires the activation of the nucleophilic serine and deacylation

requires hydrolytic water. This enzymatic reaction may be represented by the equation:



A beta-lactamase is identified as a penicillinase or a cephalosporinase on the basis of rate of hydrolysis of the substrate. An enzyme is considered as a penicillinase if penicillin is hydrolyzed at rate  $>30\%$  that of cephalosporin. Cephalosporinases hydrolyze cephalosporins at a rate  $\geq 10\%$  of the hydrolysis rate of benzylpenicillin. Broad spectrum enzymes hydrolyze both the substrates at approximately equivalent rates.

### Location of beta-lactamase genes:

Beta-lactamase genes (*bla*) were initially discovered on chromosomes. Subsequently, they were found on plasmids. It is suspected that the chromosomal genes escaped into the plasmids. The *bla* genes are also found in and adjacent to insertion sequences as well as on integrons. *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes are often located on plasmids, *bla*<sub>PenA</sub> of *Burkholderia pseudomallei* is located on chromosome, and *bla*<sub>GES-1</sub> of *K. pneumoniae* and *bla*<sub>VEB-1</sub> of *P. aeruginosa* are located on integrons. VIM-metallo-beta-lactamases are also located on integrons.

### Factors affecting beta-lactamase function:

**Location:** Beta-lactamases produced by gram positive bacteria are generally extracellular whereas those produced by gram negative bacteria are present in the periplasmic space. Extracellular beta-lactamases of gram positive bacteria help to reduce the external antibiotic concentration so that the growth can commence when the concentration is reduced to an acceptable level. In case of gram negative bacteria periplasmic beta-lactamase works along with membrane permeability in contributing resistance.

**Enzyme quantity:** Constitutively expressed beta-lactamases generally confer higher resistance. Enzyme quantity can be more in cases where an isolate harbors multiple copies of the plasmid containing beta-

lactamase genes. Presence of a strong promoter near (upstream) the beta-lactamase gene can overexpress the beta-lactamase production; this is often seen with CTX-M ESBL producers. De-repressed strains often hyperproduce the enzyme; this is often observed among isolates producing AmpC type beta-lactamases. Among inducible beta-lactamases, expression of enzyme depends on the nature of inducing antibiotic as well as the ability of the enzyme to hydrolyze the antibiotic. An increase in the number of cells increases the amount of enzyme and number of drug molecules hydrolyzed. This explains the "inoculum effect", where the MIC rises several fold when the bacterial inoculum is raised to  $10^9$ /ml.

## B. Beta-lactamases:

### Chromosomal beta-lactamases:

Many gram negative bacteria carry *bla* genes on their chromosome. However, in these bacteria the phenotypic expression of ESBL phenotype is low because of presence of single *bla* gene copy coupled with a weak promoter. These bacteria are naturally resistant to amino-penicillins, variably resistant to carboxy-penicillins, first- and second-generation cephalosporins but susceptible to third-generation cephalosporins. The resistance is reversed by inhibitors of class A enzymes such as clavulanic acid. This phenotype is specific for a given species, such as *P. vulgaris* (CumA), *P. penneri* (HUGA), *Erwinia persicina* (ERP-1), *Citrobacter sedlakii* (SED-1), *Kluyvera spp.* (KLUA, KLUC, KLUG), *Serratia fonticola* (FONA), *Rahnella aquatilis* (RAHN-1), or *Klebsiella oxytoca* (K1 or Koxy). These enzymes are also seen among non-fermenters; *Stenotrophomonas maltophilia* (L2 enzyme), *Chryseobacterium meningosepticum* (CME-1 / 2), *Chryseobacterium gleum* (CGA-1) and *Burkholderia pseudomallei* (PENA) and strictly anaerobic bacteria (*Desulfovibrio desulfuricans* (DES-1), and *Prevotella intermedia* (CFXA2). It is believed that these chromosomally encoded enzymes may be the origin of plasmid-encoded enzymes.

### a. Classical beta-lactamases:

First instance of a plasmid-mediated beta-lactamase was reported from an ampicillin resistant *E. coli* isolate obtained from urine specimen of a woman named Temoniera in Greece in 1965. This enzyme was designated as TEM-1. Within a decade this enzyme had spread to several members of Enterobacteriaceae as well as to *Haemophilus influenzae* and *Neisseria gonorrhoeae*. In 1969, TEM-2, a closely related beta-lactamase was found in a *Pseudomonas aeruginosa* isolate in Great Britain. It differed from TEM-1 in only one amino acid but not in substrate profile. A similar chromosomal enzyme but with slightly better activity against ampicillin was described in 1972 by Pitton in a *Klebsiella pneumoniae* isolate. Originally described as Pit-2, it was later designated as SHV-1 (for sulphhydryl variable). The name was derived from the observation that p-chloromercuribenzoate binds to sulphhydryl groups of the enzyme and inhibits hydrolysis of cephaloridine but not benzylpenicillin. Since then, it has been revealed that a serine hydroxyl, not a sulphhydryl, is the active site residue of SHV-1 enzyme. Both these enzymes are narrow-spectrum beta-lactamases and share only 68% sequence homology. However, the active site of SHV-1 is 0.7 to 1.2 Å wider than TEM-1 enzyme. SHV-1 is a typical chromosomal beta-lactamase of *K. pneumoniae* but also occurs as a common plasmid-encoded enzyme. All SHV variants described to date are derived from *bla*<sub>SHV-1</sub> with one to seven amino acid substitutions. TEM-1, TEM-2 and SHV-1 beta-lactamases confer resistance to anti-gram negative penicillins (except temocillin), first generation cephalosporins and cefoperazone but are ineffective against oxyimino-aminothiazolyl cephalosporins, cephamycins, monobactams, and carbapenems. TEM and SHV enzymes give high-level resistance to ampicillin, amoxicillin, and ticarcillin whereas piperacillin and cefoperazone are weaker substrates. These enzymes are well inhibited by clavulanic acid and tazobactam than by sulbactam. Some authors refer them as expanded-spectrum beta-lactamases.

## **b. Extended spectrum beta-lactamases (ESBLs):**

In 1983, an isolate of *K. pneumoniae* producing a mutant of SHV-1 enzyme was reported from Germany. This enzyme could hydrolyze oxyimino-cephalosporins and was designated SHV-2. This enzyme differed from SHV-1 isolated from *E. coli* p453 by only one amino acid substitution, Gly→Ser, at position 213 of the mature protein. Compared to SHV-1, this enzyme showed a displacement in the b3  $\beta$ -strand containing residues 238 to 242, which created an expanded  $\beta$ -lactam binding site. Mutations in the TEM and SHV genes resulting in replacement of one to six amino acids gave more flexibility and accommodation in the enzyme's active site. These enzymes are capable of hydrolyzing the extended spectrum cephalosporins, which possess 2-amino-4-thiazolyl methoxyimino (ATMO) R1 side group. A mutant of TEM type beta-lactamase (TEM-3/CTX-1) was detected following an outbreak involving several *K. pneumoniae* isolates in Clermont-Ferrand region of France during 1985-87. TEM-3 enzyme had two amino-acid substitutions (Gly→Lys at position 103 and Gly→Ser at position 236) when compared with the parent enzyme. This enzyme also conferred resistance to amino-, carboxy-, ureidopenicillins, aztreonam and extended spectrum cephalosporins. SHV-2 and TEM-3 beta-lactamases were termed as extended spectrum beta-lactamases (ESBL). Most ESBLs are mutants of TEM-1, TEM-2, and SHV-1, with 1- to 6-amino-acid sequence substitutions. ESBLs have been observed in several members of Enterobacteriaceae (*Enterobacter spp*, *Salmonella spp*, *Proteus spp*, *Citrobacter spp*, *Morganella morganii*, *Serratia marcescens*, *Shigella dysenteriae*) as well as in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. There are currently 200 TEM types (~86 ESBLs) and 162 SHV types (~43 ESBLs) of beta-lactamases, many of which do not have ESBL phenotype. Newer beta-lactamases continue to be discovered across the world. An updated database of beta-lactamases maintained by George Jacoby is available at [www.lahey.org/studies](http://www.lahey.org/studies)

## **c. Non-TEM, non-SHV ESBLs:**

### **1. PER-type ESBLs:**

PER-1 (for *Pseudomonas* extended resistance) type beta-lactamase was reported in 1991 from France in a *P. aeruginosa* isolate obtained from a patient transferred from Turkey. Besides *P. aeruginosa*, PER-1 has also been detected in *Salmonella spp* and *Acinetobacter spp* in Turkey. It has also been reported in *P. vulgaris* and *P. stuartii* from Algeria. PER-2, which has 87% homology to PER-1, has been reported from Argentina. PER-3 was discovered in an isolate of *Aeromonas punctata* in France. PER-4 was described in *P. vulgaris*, PER-5 in *Acinetobacter baumannii*, PER-6 in an environmental species of *Aeromonas* and PER-7 in *A. baumannii*. A total of seven PER-types have been described. PER-type enzymes give high level resistance to ceftazidime but low level resistance to other cephalosporins and penicillins. Like TEM ESBLs, PER enzymes are ineffective against cephamycins and carbapenems. They are inhibited by clavulanic acid but not by tazobactam.

### **2. VEB-type ESBLs:**

VEB-1 (for Vietnamese extended spectrum beta-lactamase) was first described in an *E coli* strain isolated in a French hospital from the pus of a 4-month old Vietnamese boy in 1996. It was subsequently reported in many gram negative bacteria such as *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, *P. mirabilis*, *E. cloacae*, *C. freundii*, *P. stuartii* etc. VEB-1 ESBL is widespread in Southeast Asia but has also been reported from France, Belgium, the UK, Bulgaria, Iran, Kuwait, Algeria, China, Korea and India. It has very little amino acid homology with other known ESBLs; the maximum being a homology of 38% with PER-1 and PER-2. In most cases, *bla*<sub>VEB-1</sub> is located on a gene cassette, which is present on a class 1 integron. Being present on self-transmissible large plasmids, *bla*<sub>VEB-1</sub> has spread among the Enterobacteriaceae. *A. baumannii* producing VEB-1 ESBL has been implicated in hospital outbreaks in several countries including France, Korea and China. Currently, seven VEB-type ESBLs have been described.

### 3. OXA-type ESBLs:

These enzymes are named OXA because they preferentially hydrolyze oxacillin and cloxacillin. These enzymes confer resistance to ceftazidime and are poorly inhibited by clavulanic acid. They have been often observed in *P. aeruginosa* isolates. Not all OXA enzymes are ESBLs; in fact some of them (group 2d) are plain oxacillinases, some have ESBL phenotype (group 2de) and some are carbapenemases (group 2df). Of the 244 known OXA-type beta-lactamases, at least 16 are known to have an ESBL phenotype.

### 4. CTX-M type ESBLs:

This family of ESBLs has been derived from the chromosomal cephalosporinase of the environmental bacteria *Kluyvera* spp. The first clinical isolate was reported in an *E. coli* isolate from Munich, Germany in 1989. It is believed that CTX-M genes have been captured by mobile elements (such as *ISEcp1*) from the chromosomes of the *Kluyvera* spp. These are closely related to chromosomal K1 beta-lactamase of *K. oxytoca* but are distinct from TEM or SHV type beta lactamases with less than 40% amino acid homology. These enzymes preferentially hydrolyze cefotaxime over ceftazidime, hence the name CTX-M (cefotaximase, Munich). Isolates producing CTX-M type beta-lactamases typically have cefotaxime MICs in the resistant range (> 64µg/ml), while ceftazidime MICs are usually in the apparently susceptible range (2–8 µg/ml). CTX-M type beta-lactamases hydrolyze cefepime with higher efficiency than observed with other ESBL types, and the isolates producing them usually display high cefepime MIC levels (≥64 µg/ml).

These were initially classified into CTX-M groups I, II, III, and IV. These enzymes are presently phylogenetically subclassified into five groups based on amino acid sequence similarities. Members within the group share >94% similarity, whereas ≤90% similarity is observed between members of different groups. The five groups are CTX-M-1, -2, -8, -9, and -25. It is believed that *K. ascorbata* and *K. georgiana* are the progenitors of the CTX-M-2 and CTX-M-8 groups, respectively. Some enzymes that were designated as Toho have been renamed as CTX-M enzymes. CTX-M enzymes are predominantly found in members of Enterobacteriaceae family (mainly *E. coli*) but have also been reported from *P. aeruginosa* and *Stenotrophomonas maltophilia*. At least 128 different CTX-M enzymes have been characterized.

Various genetic elements are found associated with *bla*<sub>CTX-M</sub> genes. Among them *ISEcp1*-like insertion sequences are most commonly reported, which is found to be associated with four CTX-M groups (CTX-M-1, -2, -9, and -25). CTX-M genes may be present within transposons or within mobile gene cassettes, which helps it to disseminate rapidly. The genes coding for CTX-M beta-lactamases are encoded by plasmids belonging to both narrow host-range types (IncFI, IncFII, IncHI2 and IncI) as well as broad host-range types (IncN, IncP-1-a, IncL/M and IncA/C). IncF plasmids carrying the *bla*<sub>CTX-M-15</sub> gene have been detected in *E. coli* ST131, other *E. coli* sequence types (ST405, ST354, ST28, and ST695), *Shigella sonnei*, *Salmonella enterica* serovar enteritidis as well as in *K. pneumoniae*. CTX-M beta-lactamases are now endemic in several countries and are frequently encountered in both community acquired and nosocomial strains.

Amino acid positions 240 and 167 seem to be involved in the evolution of CTX-M enzymes. Although CTX-M enzymes are more active against cefotaxime and ceftriaxone than ceftazidime, point mutations around the active site of some enzymes belonging to the CTX-M-1 and CTX-M-9 groups have significantly increased hydrolytic activity against ceftazidime. CTX-M-15,-16,-19, -25, -27 and -32 have greater catalytic efficiencies against ceftazidime. The active sites of CTX-M beta-lactamases are not enlarged compared to those of the narrow-spectrum TEM-1 and SHV-1. Instead, extended substrate profiles in the CTX-M enzyme family appear to be specific amino acid (i.e., Ser237 and Asp104) interactions with the oxyimino-cephalosporins and increased mobility of the b3 β-strand.



CTX-M-15 was first detected in an *E. coli* isolate from India in 1999. It is derived from CTX-M-3 by one amino acid substitution at position 240 (Asp→Gly), which confers increased catalytic activity to ceftazidime. Isolates producing this enzyme are often resistant to ceftazidime. It belongs to the CTX-M-1 cluster. Located 49 bp upstream of the CTX-M gene is the insertion sequence *ISEcp1*, which plays a role in its mobilization and expression.

**5) Other minor ESBLs** include SFO-1 (*Serratia fonticola*), TLA-1 (TLAhuicas (indian tribe)), BES-1 (Brazilian Extended-Spectrum  $\beta$ -lactamase), GES-1 (Guyana Extended-Spectrum  $\beta$ -lactamases), and BEL-1 (Belgium Extended-spectrum  $\beta$ -Lactamase). SFO-1 was found in a single clinical *Enterobacter cloacae* isolate from Japan in 1988. On the basis of the high degree of homology to the  $\beta$ -lactamase of *S. fonticola*, the enzyme was named SFO-1. It hydrolyses cefotaxime very efficiently, ceftazidime poorly, but not cephamycins and carbapenems. Its activity is inhibited by clavulanic acid and imipenem. Unlike most other ESBLs, SFO-1 has *AmpR* gene and is strongly induced by imipenem. BES-1 was isolated from a *S. marcescens* strain from a hospital in Brazil in 1996. It confers a high level of resistance to aztreonam and a higher level of resistance to cefotaxime than to ceftazidime. This is plasmid-encoded enzyme is well-inhibited by clavulanate than by tazobactam. BEL-1 was identified in a *P. aeruginosa* strain isolated in Belgium in 2004. It significantly hydrolyses most expanded-spectrum cephalosporins and aztreonam, but not cephamycin or carbapenem. Its activity is well-inhibited by clavulanate but poorly so by tazobactam. The chromosomal gene *bla*<sub>BEL-1</sub> is embedded in a class 1 integron. TLA-1 was identified in an *E. coli* isolate from a patient in Mexico in 1993. This plasmid mediated enzyme is strongly inhibited by tazobactam and, to a lesser extent, by clavulanate and sulbactam. These minor ESBLs are not simple point-mutant derivatives of any known  $\beta$ -lactamases and, except for SFO-1, their progenitor genes remain unknown.

#### **d. Inhibitor Resistant TEMs (IRT):**

By 1987, new TEM-1 mutants which conferred resistance to beta-lactamase inhibitors started to surface. By introducing a series of random substitutions into DNA segment that coded for active site of TEM-1 enzyme, it was found in 1989 that enzymes with Ile, Leu or Val substitution at Met69 had increased resistance to ampicillin-clavulanate and ampicillin-sulbactam. Further studies revealed that Arg-to-Cys or -Ser substitution at residue 244 was significant. The first inhibitor resistant beta-lactamase was isolated from a clinical isolate of *E. coli*. Since the enzyme was related to TEM-1 or TEM-2, it was designated as inhibitor resistant TEM-1. IRTs have been identified in *Klebsiella spp.*, *Proteus spp.*, *Shigella spp.*, and *Citrobacter spp.* IRTs are placed in Bush's subgroup 2br.

IRT confers resistance to clavulanic acid, sulbactam and tazobactam. However, these enzymes seem to hydrolyze the cephalosporins with lesser efficiency than classical TEM enzymes and remain fully susceptible to extended-spectrum cephalosporins. Some authors use the term IRT to refer resistance to amoxicillin-clavulanate and not necessarily include resistance to sulbactam and tazobactam. Isolates producing IRTs typically have amoxicillin/clavulanate MIC of >16/2  $\mu$ g/ml.

IRTs that are derived from TEM-type enzymes have been renamed as TEM- enzymes. Thirty six TEM-derived IRTs have been described so far and all of them have been assigned TEM numbers. Inhibitor resistant enzymes have also been found among SHV mutants and have been observed in *K. pneumoniae* isolates. Seven SHV enzymes (SHV-10, -26, -49, -56, -72, -84 and -107) with IRT phenotypes are known. Identification of inhibitor resistant beta-lactamases is difficult and often requires specific enzyme kinetic testing, determination of isoelectric points, and molecular characterization. In general, the MICs for organisms expressing IRT enzymes are lowered for penicillins and increased for clavulanate and sulbactam.

The amino acid substitutions that contribute to clavulanate resistance in IRTs are different from those that contribute to ESBL phenotype. Sometimes, these substitutions occur in pairs or these substitutions may even increase the ability to hydrolyze oxyimino-cephalosporins. These variants are called complex mutants of TEM (CMTs). CMTs are placed in Bush's subgroup 2ber. A total of 11 of them have been described so far.

## C. Definition, properties and classification of ESBLs

### a. Definition

ESBLs confer resistance to penicillins, oxyimino-cephalosporins as well as to monobactams. They have very little activity against cephamycins, carbapenems and beta-lactamase inhibitors.

Livermore in 2008 had broadened the classical definition of ESBL to include (1) beta-lactamases with spectra similar to those of TEM and SHV variants, but derived from other sources (CTX-M ESBLs, GES-ESBLs: GES-types without carbapenemase activity; and VEB-ESBLs) (2) TEM and SHV variants with borderline ESBL activity; for example, TEM-12 that have only slightly increased activity against oxyimino-cephalosporins, with  $V_{\max}$  rates much lower than 10% of that for benzylpenicillin (3) various beta-lactamases conferring wider resistance than their parent types but not meeting the definition for group 2be; for example, OXA-types and mutant AmpC-types (designated as extended-spectrum AmpC [AmpC-ESBLs]) with increased activity against oxyimino-cephalosporins and with resistance to clavulanic acid.

The following functional definition of ESBL was put forth by Giske and co-workers in 2009: "ESBLs is functionally defined as beta-lactamases (belonging to Bush's functional group 2be and Ambler molecular class A) able to hydrolyze extended-spectrum cephalosporins (ceftazidime or cefotaxime) and monobactams (aztreonam) at a rate that is equal to or higher than 10% of that for benzylpenicillin and to be inhibited by beta-lactamase inhibitors, such as clavulanic acid, whereas ESBLs cannot hydrolyze cephamycins (cefoxitin or cefotetan) or carbapenems (imipenem or meropenem) efficiently."

In 2009, Giske and others proposed an all-inclusive definition of ESBL for easier communication between various groups of healthcare professionals. This definition includes three classes of beta-lactamases; 1)  $ESBL_A$  (inclusive of TEM-, SHV-, CTX-M-, GES-, and VEB-ESBLs). This is subdivided into high prevalent  $ESBL_A$  and low prevalent  $ESBL_A$ . 2.  $ESBL_M$  (named for miscellaneous ESBLs), which has been subdivided into  $ESBL_{M-C}$  (class C; plasmid-mediated AmpC) and  $ESBL_{M-D}$  (class D; OXA-ESBLs). 3.  $ESBL_{CARBA}$  (named for ESBLs with hydrolytic activity against carbapenems), which has been subdivided into  $ESBL_{CARBA-A}$  (class A carbapenemases),  $ESBL_{CARBA-B}$  (class B carbapenemases), and  $ESBL_{CARBA-D}$  (class D carbapenemases).

In 2010, Jung Hune Lee and others proposed a broadened definition of ESBLs. The Ambler Class A ESBLs were named aESBLs, class C enzymes with extended spectrum as cESBLs, and class D extended-spectrum variant of OXA enzymes as dESBLs. As per this definition aESBLs include TEM-ESBLs, SHV-ESBLs, CTX-M-ESBLs, GES-ESBLs, and VEB-ESBLs; cESBLs include AmpC-ESBLs and dESBLs include OXA-ESBLs.

### b. Properties:

Although ESBLs generally confer resistance to oxyimino cephalosporins, the substrate profile of individual ESBL type may vary. While TEM-3 and -4 confer resistance to all 3<sup>rd</sup> generation

cephalosporins TEM-5, -6, -7, -9, -10, -16 and -26 confer high level resistance to ceftazidime and low level resistance to cefotaxime, ceftriaxone and fourth generation cephalosporins. TEM-13 has a classical TEM-1 like phenotype and TEM-22 and -25 preferentially hydrolyze aztreonam. They are inhibited by beta-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam.

The main contact residues in the active site of TEM-1 beta-lactamase involve the b3  $\beta$ -strand residues 234–240. The presence of Lys and Arg residues at position 240 results in the increased hydrolysis of ceftazidime by the TEM and SHV ESBLs. This activity decreases when Gly is present in the same location. The Lys and Arg residues are positively charged and therefore they can form electrostatic interaction with the negatively charged carboxylic acid group on oxyimino substituents of ceftazidime. Since Gly is neutral, it can not electrostatically interact the same way as Lys or Arg; instead favors the accommodation of ceftazidime side chain. Gly at position 240 is present in PER, VEB-1 and BES-1 ESBLs.

The transformation of a beta-lactamase enzyme to extended spectrum beta-lactamases usually follows point mutations in the *bla* gene resulting in changes in amino acid sequences near the enzyme's active site and therefore altered substrate specificity. The active site gets modified or remodeled to accommodate the side chains of cephalosporins. In TEM, changes at positions Arg164 (-His, -Ser), Gly238 (-Ser,-Ala), and Glu240 (-Lys) result in variants with ESBL phenotype.

Resistance in ESBL producers is not just because of point mutations in the *bla* gene, alterations in enzyme regulation leading to increased enzyme production or alteration in outer membrane protein porin channels can also contribute to increased resistance.

### **c. Classification of ESBLs:**

Earlier classifications of beta-lactamases were based on several phenotypic (physiological) parameters such as hydrolytic spectrum, rate of hydrolysis, susceptibility to inhibitors and whether they were chromosomal or plasmid mediated. Susceptibility to cloxacillin, clavulanic acid, aztreonam or p-chloromercuribenzoate was also taken into consideration.

1. Sawai T: One of the earliest classifications of beta-lactamases was attempted by Sawai T et al. in 1968. They described penicillinases and cephalosporinases by using antisera as an additional way of separation.
2. Richmond & Sykes: Originally proposed by Jack and Richmond in 1970, it was subsequently expanded by Richmond and Sykes in 1973. Beta-lactamases were classified into five groups based on substrate profile. It was subsequently extended in 1976 by Sykes and Mattew, where plasmid mediated beta-lactamases were differentiated by isoelectric focusing.
3. Mitsuhashi and Inoue proposed another scheme in 1981, which included "cefuroxime hydrolyzing beta-lactamases" apart from penicillinase and cephalosporinase.
4. Bush, Jacoby & Medeiros phenotypic classification: Karen Bush proposed another classification in 1989 based on action of the enzymes on the substrates (penicillin, oxacillin, carbenicillin, cephaloridine, expanded-spectrum cephalosporins, and imipenem) as well as susceptibility to inhibition by clavulanic acid. The classification was further updated in 1995 by Karen Bush, George Jacoby and Antone Medeiros and revised again in 2009. It consists of 3 groups and several subgroups within each group.

**Group 1:** Group 1 enzymes are basically cephalosporinases that corresponds to Ambler's molecular class C enzymes. These are inhibited by cloxacillin and aztreonam but not by clavulanic acid. Plasmid-mediated group 1 enzymes are CMY, ACT, DHA, FOX, and MIR. They are active on cephamycins, such as cefoxitin and have a high affinity for aztreonam. When produced in large amounts, group 1 enzymes can provide resistance to carbapenems, especially ertapenem. Group 1e consists of enzymes that are basically variants of group 1 as a result of amino acid substitutions, insertions, or deletions. These enzymes have greater activity against ceftazidime and other oxyimino-cephalosporins. They have been termed extended-spectrum AmpC (ESAC) beta-lactamases and include GC1 in *E. cloacae*, plasmid-mediated CMY-10, CMY-19, and CMY-37. AmpC-type  $\beta$ -lactamases conferring resistances to expanded-spectrum cephalosporins that are produced by *Acinetobacter spp* have been named ADC, for Acinetobacter-derived cephalosporinases.

**Group 2:** Group 2 consists of penicillinases, cephalosporinases, broad-spectrum beta-lactamases and serine carbapenemases. These enzymes are generally inhibited by clavulanic acid. Classical beta-lactamases, OXA-type, KPC-type as well as ESBLs belong here.

**Subgroup 2a** enzymes are penicillinases that are often seen in Gram-positive cocci, including the staphylococci and occasionally enterococci. They preferentially hydrolyze benzylpenicillin and hydrolyze cephalosporins at rates  $\leq 10\%$  those for benzylpenicillin or ampicillin. However, they can hydrolyze nitrocefin. They are inhibited by clavulanic acid and tazobactam. E.g., PC1

**Subgroup 2b** beta-lactamases readily hydrolyze penicillins and early cephalosporins, such as cephaloridine and cephalotin, and are strongly inhibited by clavulanic acid and tazobactam. They include the TEM-1, TEM-2, and SHV-1 enzymes. This group contains at least 13 TEM and 33 SHV enzymes.

**Subgroup 2be:** Since ESBLs are derived from 2b enzymes, they are placed in subgroup 2be. In addition to the hydrolytic activity of 2b group, these enzymes hydrolyze one or more oxyimino-beta-lactams, such as cefotaxime, ceftazidime, and aztreonam, at a rate generally  $>10\%$  that of benzylpenicillin. Apart from TEM and SHV derived ESBLs, the group also consist of CTX-M enzymes. The group also comprises of less common ESBLs unrelated to TEM, SHV, or CTX-M, including BEL-1, BES-1, SFO-1, TLA-1, TLA-2, and members of the PER and VEB enzyme families. Members of the subgroup 2be beta-lactamases remain susceptible to inhibition by clavulanic acid.

**Subgroup 2br** Beta-lactamases that are structurally related to 2b enzymes but are poorly inhibited by the beta-lactamase inhibitors are placed in subgroup 2br. The subgroup comprises of the enzymes previously designated as Inhibitor Resistant TEM (IRT). It includes 36 TEM-type and seven SHV-type enzymes. E.g., TEM-30, SHV-10

**Subgroup 2ber** includes TEM derived enzymes that are resistant to inhibition by clavulanic acid and possesses hydrolytic activity against oxyimino-cephalosporins. These enzymes are termed Complex Mutant TEM (CMT). A total of 11 CMTs have been described E.g, TEM-50, -68, -89.

**Subgroup 2c** enzymes are penicillinases, which are characterized functionally by their ability to hydrolyze carbenicillin or ticarcillin at rate  $\geq 60\%$  that of benzyl penicillin, but hydrolyze cloxacillin or oxacillin at rates  $<50\%$  that of benzylpenicillin. These are generally easily inhibited by clavulanic acid or tazobactam. Examples include PSE-1, and CARB-3 enzymes.

**Subgroup 2d** consists of beta-lactamases that have ability to hydrolyze cloxacillin or oxacillin at a rate of  $>50\%$  that for benzylpenicillin and hence are known as OXA enzymes. E.g., OXA-1, -2, -10

**Subgroup 2de** comprises of cloxacillin- or oxacillin-hydrolyzing enzymes with an extended spectrum that includes oxyimino-beta-lactams but not carbapenems. Many among them are derived from OXA-2 and OXA-10 by amino acid substitutions. Resistance to ceftazidime is usually more pronounced than resistance to cefotaxime or aztreonam. Many  $\beta$ -lactamases in this subgroup are inhibited by NaCl and weakly inhibited by clavulanic acid. Currently 16 OXA-ESBLs are known. Eg., OXA-11, OXA-15.

**Subgroup 2df:** Serine carbapenemases are placed in two subgroups: subgroup 2df enzymes that hydrolyze oxacillin and carbapenems (OXA-23,-48), and subgroup 2f that hydrolyze carbapenems, oxyimino-cephalosporins and cephamycin (KPC-2, IMI-1, SME-1). Subgroup 2df enzymes are most frequently observed in *Acinetobacter baumannii* and are usually produced by genes that are located on the chromosome. OXA-23 and OXA-48 enzymes are plasmid mediated enzymes encountered in the Enterobacteriaceae. These enzymes are not inhibited by clavulanic acid.

**Subgroup 2e** consists of cephalosporinases that hydrolyze extended-spectrum cephalosporins well but that lack good penicillin-hydrolyzing activity and are inhibited by clavulanic acid or tazobactam but not by aztreonam. The inducible, chromosomal cephalosporinases in the Proteae often belong to this subgroup. These enzymes can be differentiated from AmpC enzymes by their poor affinity for aztreonam. E.g., CepA

**Subgroup 2f** consists of serine carbapenemases that correlates with Ambler class A serine carbapenemases. Extended-spectrum cephalosporins such as ceftazidime are not well hydrolyzed by the SME and IMI-1 enzymes, but aztreonam can be degraded by most of them, except for GES-3 and GES-4.

**Group 3:** These include metallo-beta-lactamases (MBL), which have requirement for a zinc ion at the active site. In contrast to the serine beta-lactamases, the MBLs have poor hydrolytic capability for monobactams and are not inhibited by clavulanic acid or tazobactam. They are inhibited by metal ion chelators such as EDTA, dipicolinic acid, or 1,10-o-phenanthroline. These metalloenzymes have been subdivided, based on either structure (subclasses B1, B2, and B3) or function (subgroups 3a, and 3b). **Subgroup 3a** includes the major plasmid-encoded MBL families, such as the IMP and VIM enzymes. These enzymes belong to molecular subclass B1, which requires two zinc atoms for their hydrolytic activity. It also includes enzymes of subclass B3 MBLs such as L1, CAU-1, GOB-1, and FEZ-1. **Subgroup 3b** contains a smaller group of MBLs that preferentially hydrolyze carbapenems in contrast to penicillins and cephalosporins. They contain only one zinc atom in their active sites. They correspond to molecular subgroup B2. Eg., CphA, Sfa-1.

Since point mutations in the ESBL genes can result in a change in substrate profile or susceptibility to beta-lactamase inhibitors, the enzymes have to be re-classified into a new group. The advantage of functional classification is that it provides an opportunity to relate the various enzymes to their clinical role. The selective resistance to various classes of beta-lactam drugs can be made out from this classification.

**5. Ambler's Molecular classification:** Classification of beta-lactamases has traditionally been based on either the functional characteristics of the enzymes or their primary structure. A molecular classification was proposed by RP Ambler in 1980, which took into consideration the amino acid sequence of the beta-lactamases. Initially only two classes (A and B) were described to account for serine and metallo-beta lactamases respectively. The class C cephalosporinases were described by Jaurin and Grundstrom in 1981. In the late 1980s, class D oxacillin-hydrolyzing enzymes were included in the classification. Presently, the beta-lactamases are divided into four classes- A, B, C and D. Molecular phylogeny of the enzymes is obtained by the amino-acid sequences. Based on sequence similarity, subdivisions within the class have been made by illustrating a dendrogram.

### **Class A beta-lactamases:**

This class consists of chromosomal and plasmid-mediated penicillinases, cephalosporinases and carbapenemases that have serine in their active site. It includes early and classical beta-lactamases as well as extended-spectrum beta-lactamases. Present in this group are following enzymes:

- Penicillinases (PC1), which hydrolyzes benzylpenicillin more efficiently than cephalosporins. These are inhibited by clavulanic acid.
- Penicillinases and early cephalosporinases (TEM-1, TEM-2, SHV-1), which hydrolyzes penicillinases and early cephalosporinases equally. These are inhibited by clavulanic acid.
- Extended-spectrum beta-lactamases (SHV-2, TEM-3, CTX-M-15), which hydrolyzes cephalosporins at a rate 10% more than penicillinases. These are able to hydrolyze oxyimino-cephalosporins and monobactam, and are susceptible to inhibition by clavulanic acid.
- Penicillinases (TEM-30, SHV-10), which are resistant to inhibition by clavulanic acid, tazobactam and sulbactam.
- Extended-spectrum cephalosporinases (TEM-50), which hydrolyzes extended spectrum cephalosporins and monobactams but are resistant to clavulanic acid, tazobactam and sulbactam.
- Extended spectrum cephalosporinases (CepA), which can hydrolyze cephalosporins but not aztreonam. These are inhibited by clavulanic acid.
- Carbenicillinases (PSE-1, CARB-3), which preferentially hydrolyze carbenicillin and remain susceptible to clavulanic acid.
- Carbenicillinases (RTG-4), which also hydrolyze cefepime and ceftiofime, but are inhibited by clavulanic acid.
- Carbapenemases (KPC-2, IMI-1, SME-1), which hydrolyze oxyimino-cephalosporins, cephamycins and carbapenemases. Their susceptibility to clavulanic acid is variable. These enzymes are often seen in *Enterobacter cloacae*, *Serratia marcescens*, and *K. pneumoniae*. The genes for these enzymes are chromosomally encoded in *E. cloacae* and *S. marcescens*, whereas it is plasmid encoded in *K. pneumoniae*.

### **Class B beta-lactamases:**

These are carbapenemases with Zinc<sup>2+</sup> ion present at their active site. These are also known as metallo-beta-lactamases. They are able to hydrolyze penicillins, cephalosporins, carbapenems, and are resistant to inhibition by the common beta-lactamase inhibitors. However, their hydrolytic activity does not extend to aztreonam. The *bla*<sub>MBL</sub> genes are located on the chromosome, plasmid, and integrons. In *Bacillus spp.*, *Chryseobacterium spp.*, and *Stenotrophomonas maltophilia* MBL is chromosomally encoded. In *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*, MBL is encoded by mobile genetic elements.

### **Class C beta-lactamases:**

These are cephalosporinases with serine in their active sites. These are usually encoded by the genes located on the bacterial chromosome, but plasmid encoded enzymes are becoming common. It is chromosomally encoded in *Enterobacter spp* and *Citrobacter spp* but in *Klebsiella spp*, *Salmonella spp* and *Proteus spp* it is often plasmid-mediated. Isolates producing these enzymes are resistant to aminopenicillins, oxyimino-cephalosporins, cephamycins and beta-lactamase inhibitors. Overexpression confers resistance to broad-spectrum cephalosporins including cefotaxime, ceftazidime, and ceftriaxone and is a problem especially in infections due to *E. aerogenes* and *E. cloacae*. AmpC enzymes poorly hydrolyze cefepime and are inhibited by cloxacillin, oxacillin, and aztreonam. Chromosomal AmpCs in Gram-negative bacteria are normally expressed at a low level (“repressed”) but can be “derepressed” by mutations in *ampR* and *ampD* gene or by induction with certain beta-lactams, particularly ceftiofime.

## **Class D beta-lactamases:**

These enzymes were initially categorized as “oxacillinases” because of their ability to hydrolyze oxacillin at a rate of at least 50% that of benzyl penicillin. Therefore, these enzymes are named OXA-type beta lactamases. Reported first from Turkey in 1991, these enzymes also confer resistance to penicillins and cephalosporins. OXA-type ESBLs (OXA-11, -15) confer resistance to oxyimino-cephalosporins. OXA-type carbapenemases (OXA-23, -48) can hydrolyze carbapenems. Most OXA enzymes are resistant to inhibition by clavulanate, sulbactam, and tazobactam. However, there are some exceptions- OXA-2 and OXA-32 are inhibited by tazobactam but not sulbactam and clavulanate, and OXA-53 is inhibited by clavulanate. Approximately 240 OXA-type beta-lactamases have been described so far.

Ambler’s molecular classification appears to be widely accepted than the Bush’s phenotypic classification due to its simplicity and phylogenetic relationships among the enzymes.

## **6. Ogawara Hiroshi’s Phylogenetic tree:**

Based on the amino acid sequences of 47 beta-lactamases and the computer-aided analysis, a phylogenetic tree was proposed by Ogawara Hiroshi in 1993. According to the tree, beta-lactamases were classified into six groups. Group 1 beta-lactamases included plasmid-mediated enzymes from gram-negative bacteria and chromosomal beta-lactamases from *K. pneumoniae* and *Rhodopseudomonas capsulata*. Group 2 enzymes included chromosome-mediated beta-lactamases from *Streptomyces*, *Yersinia enterocolitica*, *Citrobacter diversus*, and *K. oxytoca*. Group 3 enzymes included chromosome-mediated beta-lactamases from other gram negative bacteria. Group 4 beta-lactamases was composed of metallo-beta-lactamases. Group 5 was composed of OXA-type beta-lactamases. Group 6 was composed of chromosome-encoded beta-lactamases from gram-positive bacteria.

## **Nature of expression:**

Expression of beta-lactamases may be low-level constitutive, high-level constitutive, inducible or de-repressed. *Bacteroides fragilis* group and *Klebsiella spp* possess constitutive chromosomal class A enzymes. *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Morganella morganii*, *Serratia spp.*, *Providencia spp.*, *Pseudomonas aeruginosa* and other fluorescent pseudomonads have inducible chromosomal class C enzymes. *Citrobacter diversus*, *Proteus vulgaris*, and *Burkholderia cepacia* possess inducible chromosomal class A enzymes.

## **Role of Outer Membrane Proteins in antibiotic resistance:**

Many studies on the role of outer membrane protein in antibiotic resistance have been conducted. They indicate that the loss of porins in *K. pneumoniae* isolates, which don't have any known mechanism of antibiotic resistance, do not lead to significant resistance. Most *K. pneumoniae* isolates that do not produce ESBLs express both OmpK35 and OmpK36 porins. However, most ESBL producing *K. pneumoniae* isolates express only Ompk35 suggesting that the loss of OmpK36 could be an additional factor contributing to resistance.

ESBLs have no hydrolytic activity against cephamycins (cefoxitin, cefotetan), but several isolates are found to be resistant to them with high levels of MIC. Studies have revealed that MICs of cefoxitin and oxyimino-cephalosporins such as cefotaxime are increased for porin-deficient mutants that produce ESBLs. Several studies have demonstrated that cefoxitin is readily able to select resistant *K. pneumoniae* mutants, which are deficient in both the porins, both in vivo and in vitro. It is believed that presence at least one of the two major porin proteins is required for the entry of beta-lactam drug in *K. pneumoniae* isolates. However, loss of both the porins is associated with resistance to cefoxitin. OmpF and OmpC are

the major porins of *E. coli*. It is suggested that the loss of both OmpF and OmpC causes resistance to beta-lactams in ESBL producing *E. coli*.

## D. Epidemiology and risk factors

Phylogenetic analyses of *E. coli* have shown that isolates can be divided into four main phylogenetic groups (A, B1, B2 and D) and seven subgroups (A0, A1, B1, B2<sub>2</sub>, B2<sub>3</sub>, D1 and D2). Virulent isolates causing extraintestinal infections belong mainly to group B2 and, to a lesser extent, to group D, whereas most commensal strains belong to groups A and B1. Prevalence of CTX-M-15 ESBL is particularly more among isolates belonging to the B2 phylogenetic group. ESBL producing *K. pneumoniae* strains have been responsible for outbreaks of infection in several hospitals. This is probably due to the fact that *K. pneumoniae* survive better than most other Enterobacteriaceae members on skin and inanimate surfaces. It is also suspected that Klebsiellae are particularly good hosts for plasmids or allow evolution of ESBL genes more readily than other members of Enterobacteriaceae.

Hospital outbreaks have been known to occur by a single strain, multiple strains or strains with multiple beta-lactamases. In certain outbreaks that were caused by a single epidemic strain, variations have been observed in antibiogram and plasmid profile of the strains. Such a situation could have arisen due to acquisition or loss of plasmids; or mutations in the ESBL genes resulting in conversion of one ESBL type to another. While TEM and SHV derived ESBLs are commonly observed among hospital strains, CTX-M enzymes are often observed in community strains.

Beta-lactamase production among nosocomial strains is influenced by factors such as country, unit type or patient type. Resistance rates are known to be more in developing countries, units such as intensive care unit, burns unit as well as in patients who require long term antibiotic therapy. High levels of resistances are often observed among community acquired infection in countries where antibiotic usage is high and antibiotics are available for purchase over the counter.

Distribution of beta-lactamase type may be widely disseminated across the globe or a particular type may be more prevalent in one geographical area. TEM- type beta-lactamases were a major problem in France. PER-1 class A cephalosporinase is reported in salmonellae and *P. aeruginosa* from Turkey. CTX-M-9 and CTX-M-14 are mostly reported from Spain, CTX-M-14 from Canada and China, CTX-M-1 from Italy, CTX-M-3 from Poland, and CTX-M-2 from several South American countries, Japan and Israel. CTX-M-15 has been reported from all continents, which is also the major ESBL type found in India. Studies have shown that CTX-M-15-producing *E. coli* are more likely to be multi-drug resistant when compared with other CTX-M-producing isolates. Pulsed field gel electrophoresis typing of CTX-M-15 producing *E. coli* from the Indian, Austrian and US studies demonstrated great diversity among the different isolates.

Multilocus sequencing typing (MLST) is used to type the isolates into 'sequence types' (ST) based on polymorphisms within strongly conserved 'housekeeping' genes. Outbreaks of infections by *E. coli* clones identified as 'Clonal Group A' (CGA) in North America and O15:K52:H1 in multiple nations were observed in the past. A new CTX-M-15-producing *E. coli* clone named ST131 was observed from several countries simultaneously by two research groups in 2008. This clone belongs to serogroup O25 and to the highly virulent phylogenetic group B2. The strain is designated as O25:H4-B2-ST131. The clone is known to harbour IncFII plasmids that are responsible for multi-drug resistance. These were isolated among antibiotic resistant community and hospital *E. coli* strains in UK between 2003 and 2004, but were identified as ST131 only much later. Studies have demonstrated that ST131 clones have been detected in *E. coli* from stools of healthy volunteers in France. Some studies have revealed a high degree of fluoroquinolone resistance among ST131 *E. coli* clones with little resistance to cephalosporins. It is



believed that the spread of CTX-M-15 in *E. coli* is mostly due to clone ST131 but plasmid transfer also seems to be important in certain places. Although MLST is the most reliable method for the identification of clone ST131, it is expensive and time consuming. Repetitive-element polymerase chain reaction (PCR) typing schemes can detect this clone more easily and rapidly.

Property	<i>E. coli</i>	<i>K. pneumoniae</i>
Origin	Often community acquired	Often nosocomial
ESBL type	Class A enzymes, mostly CTX-M type	Class A enzymes including both TEM (-3, -26, -51) and SHV (-2, -5, -12) types
Infection type	UTI, intra-abdominal infection and primary bacteremia	Primary bacteremia, respiratory tract infections, intra-abdominal infections, skin & soft tissue infections, UTI
Co-resistance	Often to fluoroquinolones and cotrimoxazole	Often to aminoglycosides and cotrimoxazole
Molecular epidemiology	Worldwide spread of clone ST131 that produce CTX-M-15 and associated with IncFII plasmids	Most often clonal spread within a ward/hospital; plasmid exchange known to occur
Risk factors	Repeated UTI with underlying renal pathology, previous cephalosporin & fluoroquinolone antibiotic treatment, previous hospitalization, residence in nursing home, older age, diabetes mellitus, underlying liver pathology, international travel	Longer stay in hospital/ICU, severity of infection, intubation & mechanical ventilation, urinary or arterial catheterization, previous exposure to antibiotics (especially cephalosporins)

*K. pneumoniae* clones producing SHV-5 ESBL have been responsible for several hospital outbreaks across Europe. *K. pneumoniae* ST11, ST15 and ST147 that are ciprofloxacin resistant and CTX-M-15 producing clones have been isolated in hospitals of Hungary. ST15 *K. pneumoniae* have been observed in France, Poland and Portugal.

CTX-M type ESBLs, which were first reported from Japan in 1986 are now the most prevalent ESBL type worldwide. Outbreaks involving CTX-M-2-producing Enterobacteriaceae were reported from Argentina during 1990s. Since 2000 CTX-M producing *E. coli* has emerged worldwide as important causes of community-acquired UTI. This is now being labeled as 'the CTX-M pandemic'.

Other methods used to type the ESBL producing strains include genomic DNA RFLP and plasmid fingerprinting. Genomic DNA RFLP: For restriction fragment length polymorphism (RFLP) analysis total DNA preparations are digested with the *XbaI* restriction enzyme and are separated in a 1% agarose gel using commercial pulsed-field gel electrophoresis system. Plasmid fingerprinting: Approximately 5 µg of plasmid DNA is digested with 10 U of the *EcoRI* or *PstI* restriction enzyme for two hours at 37°C. The resulting DNA fragments are electrophoresed in 1% agarose gels.

#### **Risk factors for acquisition of ESBL producing bacteria:**

Various studies have been conducted on epidemiology and risk factors associated with infections by ESBL producing *K. pneumoniae* and *E. coli*. The following are the prominent risk factors:

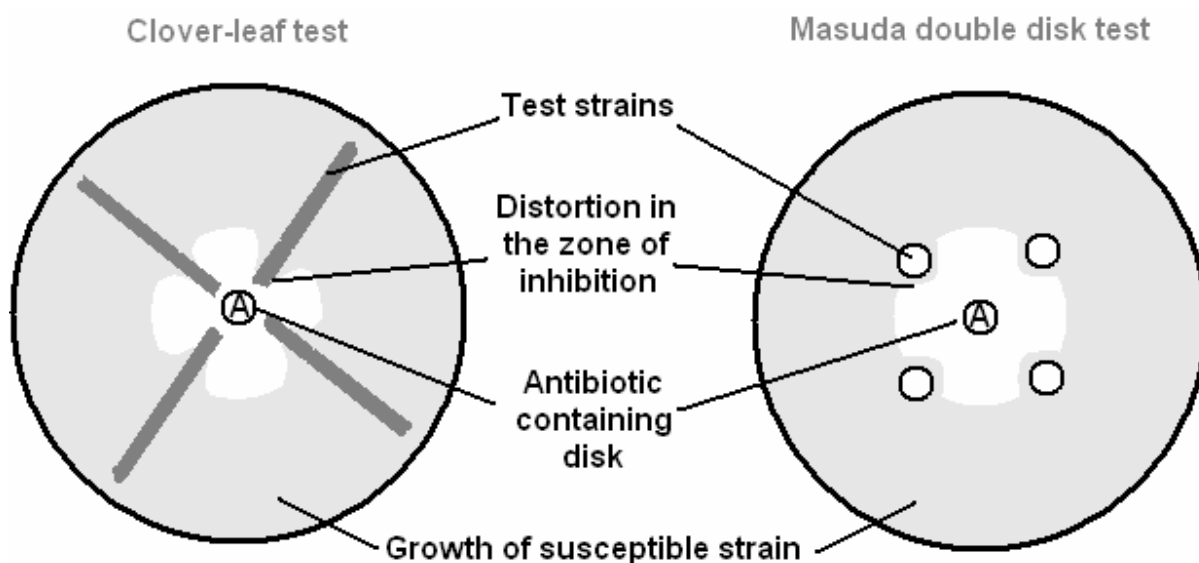
- hospital stay of more than seven days
- exposure to nosocomial isolates
- transfer from another health care facility
- central venous catheterization
- hemodialysis
- surgical intervention
- antibiotic use (third-generation cephalosporins and quinolones) for more than a week
- travel to endemic countries

## E. LABORATORY DETECTION & IDENTIFICATION OF ESBLs:

### Detection of beta-lactamases:

Beta-lactamases can be readily detected by iodometric, colorimetric and chromogenic cephalosporin methods. Iodometric method relied on the principle that beta-lactamase enzyme, whenever present would hydrolyze penicillin, which in turn would render the violet color of starch-iodine mixture colorless by reducing iodine to iodide. Acidimetric method relies on lowered pH when penicillin is hydrolyzed to penicilloic acid, which is then detected by an indicator system (bromocresol purple turns to yellow). Chromogenic cephalosporins are specially designed cephalosporins, which when hydrolyzed generate colored product. Nitrocefin changes from yellow to pink on hydrolysis, and 7-(thienyl-2-acetamido)-3-[2-(4-N,N-dimethylaminophenylazo) pyridinium methyl]-3-cephem-4-carboxylic acid (PADAC) changes from violet to yellow. All the three detection methods can be performed on bacterial suspension or on paper disks impregnated with the suitable reagents. Nitrocefin is an expensive reagent. False positives can occur with acidometric methods if the pH of the distilled water is slightly acidic. False positive can also occur with iodometric method due to nonspecific reaction of iodine with bacterial proteins. HMRZ-86, which changes colour from yellow to red, is another chromogenic cephalosporin that is used in the detection of ESBLs and MBLs

Beta-lactamases have also been detected by biological methods such as clover-leaf plate test and Masuda double-disk test. In the clover-leaf plate method, a known beta-lactam susceptible isolate is swabbed on a test plate. The antibiotic disk (e.g penicillin) is placed in the center and the test isolate is inoculated as crossing lines and the plate is incubated. Distortion of the zone of inhibition around the centrally placed disk by the lines of growth indicates positive beta-lactamase production. In the Masuda double-disk test, a known susceptible isolate is swabbed on the plate and an antibiotic disk is placed in the center. Filter paper disks containing test isolates (or growth filtrate) are placed few mm away from the central disk. Distortion of the zone of inhibition around the centrally placed disk by the discs containing test isolates indicates positive beta-lactamase production.



## Identification of beta-lactamase classes:

An attempt to identify the beta-lactamase class was made in 1993 by testing the hydrolysis of nitrocefin in the presence and absence of 0.1 mM clavulanic acid and 0.1 mM cloxacillin. Most class A enzymes are inhibited by clavulanic acid but not by cloxacillin. Class C enzymes are inhibited by cloxacillin but not by clavulanic acid. Class B and most Class D enzymes are not inhibited by either of these. Since many isolates now produce multiple beta-lactamase types, this system may not give accurate results.

### a. Screening, phenotypic and genotypic methods:

Extended spectrum beta-lactamases enzymes in isolates such as *E. coli*, *K. pneumoniae*, *K. oxytoca* and *P. mirabilis* can be identified by phenotypic and genotypic methods. These methods are often unreliable in isolates that are known to produce chromosomal AmpC beta-lactamases (e.g., *Enterobacter sps*). Some of the inhibitor based phenotypic systems are not suitable for detection in *Acinetobacter sps* because of antibacterial activity of clavulanic acid against the bacterium.

**Phenotypic methods:** These include both screening and confirmatory methods. Some laboratories conduct confirmatory tests on isolates that are positive in screening tests. Such an approach may cause some delay in issuing susceptibility results.

**Screening tests:** As per the guidelines issued by the Clinical Laboratory Standards Institute (CLSI) published in document S100-21 (2011), *K. pneumoniae*, *K. oxytoca*, *E. coli*, and *P. mirabilis* isolates may be regarded as positive for screening test under the following conditions.

Screening by disk diffusion method:

Antibiotic	Zone diameters when testing	
	<i>K. pneumoniae</i> , <i>K. oxytoca</i> , <i>E. coli</i>	<i>P. mirabilis</i>
Cefpodoxime 10 µg	≤ 17 mm	≤ 22 mm
Ceftazidime 30 µg	≤ 22 mm	≤ 22 mm
Cefotaxime 30 µg	≤ 27 mm	≤ 27 mm
Ceftriaxone 30 µg	≤ 25 mm	-
Aztreonam 30 µg	≤ 27 mm	-

The screening test must be performed on Mueller Hinton agar on standardized inoculum (0.5 McFarland turbidity) and diameter of the zone of inhibition must be carefully measured after 16-18 hours of incubation at 35±2 °C.

### Screening by microbroth dilution method:

Broth dilution tests must be performed on cation adjusted Mueller Hinton broth after standardizing the inoculum. MIC must be observed after 16-18 hours of incubation at 35±2 °C. For *E. coli*, *K. pneumoniae*, and *K. oxytoca*, MIC ≥ 8 µg/ml for cefpodoxime or MIC ≥ 2 µg/ml for ceftazidime, aztreonam, cefotaxime, or ceftriaxone is suggestive of ESBL production. For *P. mirabilis*, MIC ≥ 2 µg/ml for cefpodoxime, ceftazidime, or cefotaxime is suggestive of ESBL production.

Screening of *P. mirabilis* may be performed only if it is considered clinically relevant; such as in bacteremic isolates.

Quality control: *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 must be used as negative and positive controls, respectively. Following are the zone diameters by disk diffusion testing and the MIC by microbroth dilution testing observed with *K. pneumoniae* ATCC 700603 strain:

Disc diffusion breakpoints	MIC breakpoints
Cefpodoxime zone 9–16 mm	Cefpodoxime MIC $\geq$ 8 $\mu$ g/ml
Ceftazidime zone 10–18 mm	Ceftazidime MIC $\geq$ 2 $\mu$ g/ml
Aztreonam zone 9–17 mm	Aztreonam MIC $\geq$ 2 $\mu$ g/ml
Cefotaxime zone 17–25 mm	Cefotaxime MIC $\geq$ 2 $\mu$ g/ml
Ceftriaxone zone 16–24 mm	Ceftriaxone MIC $\geq$ 2 $\mu$ g/ml

Since ESBLs vary in their substrate profiles, CLSI recommends that the use of more than one antibiotic increases the sensitivity of detection. If only one antibiotic is used, there are chances of reporting false susceptibility. A positive screening test does not necessarily indicate ESBL production since resistance can also be due to production of other beta-lactamases or lack of certain porin proteins. Therefore, positive screens should be followed by confirmatory tests.

CLSI (2010) guidelines considers an isolate to be susceptible to cefepime if MIC is  $\leq$ 8  $\mu$ g/ml and resistant if its MIC is  $\geq$ 32  $\mu$ g/ml. European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommends that an isolate should be considered susceptible to cefepime if its MIC is  $\leq$ 1  $\mu$ g/ml and resistant if MIC is  $>$ 4  $\mu$ g/ml. EUCAST further recommends that if the MIC for all cephalosporins (including cefepime) is  $\leq$  1  $\mu$ g/ml, the isolates should be reported as susceptible, regardless of ESBL production.

#### Screening using chromogenic selective medium:

Commercially available chromogenic media such as Brilliance ESBL agar (Oxoid, UK) and ChromID ESBL agar (bioMe'rieux, France) have been used to detect ESBL production in Enterobacteriaceae members. *E. coli* isolates produce red-burgundy colonies on Brilliance ESBL agar and blue-violet colonies on ChromID ESBL agar. Klebsiella-Enterobacter-Serratia-Citrobacter group grow as green colonies on both these media. Proteus-Morganella-Providencia group grow as tan or light brown colonies on both the media. According to the instructions of both the manufacturers, any colored and oxidase-negative colonies growing on either of the two chromogenic media should be regarded as presumptive ESBL-producing Enterobacteriaceae isolates. Studies evaluating these media have reported that while ESBL detection rates were good on both media, isolates with identical resistance mechanisms (e.g., AmpC and K1-OXY penicillinase overproduction) were not discriminated.

### ESBL Confirmatory methods

#### 1. Double Disk Synergy Test (DDST):

This test was described in Jarlier V and others in 1988 for detection of ESBL enzymes in *E. coli* and *K. pneumoniae* isolates. The test has also been described by few authors as Double Disk Diffusion Test (DDDT). In this method, a standardized inoculum of the test isolate is swabbed on the surface of a Mueller Hinton agar. An amoxyclav (amoxicillin/clavulanic acid 30/10  $\mu$ g) is placed at the center of the plate. Disks containing 30  $\mu$ g ceftazidime, cefotaxime, ceftriaxone, aztreonam or 10  $\mu$ g cefpodoxime are placed 20-30 mm away from the central disk. The distance of disk placements have varied among different researchers. While some measure the distance from center-to-center, others measure it from edge-to-edge. Optimal distance of disk spacing is critical for successful result. An extension in the zone of inhibition around the peripheral disks towards the centrally placed amoxyclav disk indicates ESBL production. This extension of the inhibition has been variously labeled as "keyhole effect" or "lens of inhibition". Incorrect disk placement may give rise to false-negative or indeterminate results. Since the results are subjective, the test may have to be repeated for better interpretation.

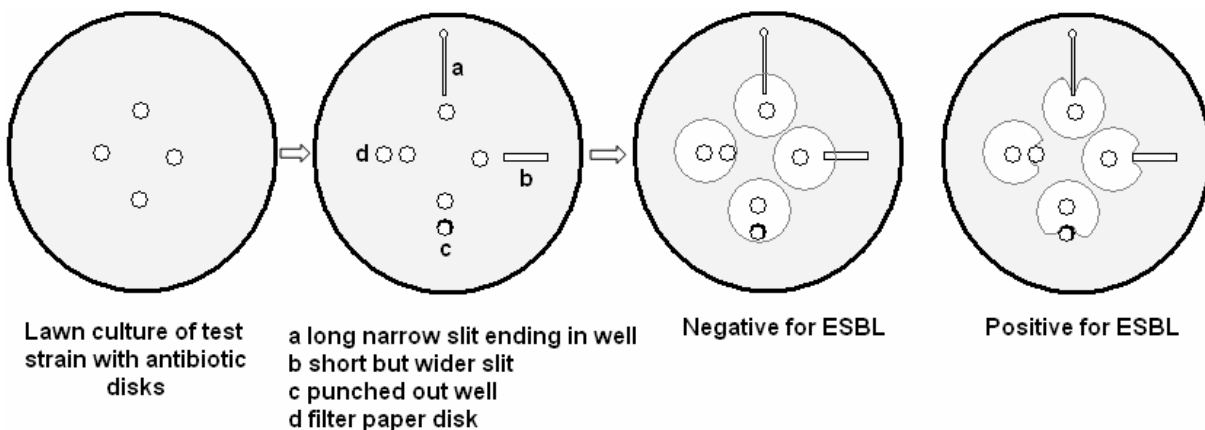
## 2. CLSI Phenotypic Confirmatory Test (PCT):

The method recommended by CLSI can be performed as a disk diffusion or microbroth dilution. In disk diffusion method (also referred as Combined Disk Method by some authors), a standardized inoculum of the test isolate is swabbed on the surface of a Mueller Hinton agar. Ceftazidime (30 µg), ceftazidime/clavulanic acid (30/10 µg), cefotaxime (30 µg), cefotaxime/clavulanic acid (30/10µg) disks are placed on the plate and incubated in ambient air for 16-18 hours of incubation at 35±2 °C. Confirmatory testing requires use of both cefotaxime and ceftazidime, alone and in combination with clavulanic acid. An increase in the zone diameter by ≥5 mm around the disks with clavulanic acid over the disks with cephalosporins alone confirms ESBL production. It should be noted that *K. pneumoniae* ATCC 700603 displays ≥ 5 mm increase in ceftazidime/clavulanic acid zone diameter but only ≥ 3 mm increase in cefotaxime/clavulanic acid zone diameter. In PCT by microbroth dilution, the organism is tested against different dilutions of cefotaxime and ceftazidime alone as well as in combination with 4 µg/ml of clavulanic acid. The dilutions are- ceftazidime 0.25–128 µg/ml, ceftazidime-clavulanic acid 0.25/4–128/4 µg/ml, cefotaxime 0.25–64 µg/ml and cefotaxime-clavulanic acid 0.25/4–64/4 µg/ml. MIC is observed after incubation for 16-20 hours at 35±2 °C. A reduction in MIC by ≥ 3 twofold dilutions for cephalosporin with clavulanic acid as against the MIC of cephalosporins alone confirms ESBL production.

Since CLSI PCT method is quantitative, it eliminates the subjectivity in interpretations. False positive results may occur with isolates producing KPC-beta-lactamases or hyperproduction of K1 beta-lactamases. A positive ESBL-PCT in *K. oxytoca* isolates could be attributed to K1 hyperproduction if its ceftriaxone MIC is ≥8-fold higher than the cefotaxime MIC. The MICs of ceftriaxone and cefotaxime are the same or similar if the isolate is an ESBL producer. False negative results may occur in isolates that also produce high levels of AmpC enzymes. Clavulanic acid based tests give false positive results when tested for *Acinetobacter* spp. CLSI-PCT recommendations are only for *K. pneumoniae*, *K. oxytoca*, *E. coli*, and *P. mirabilis* isolates. There are no guidelines for detection of ESBL in other isolates. Some researchers believe that CLSI guidelines may also be extended to other organisms such as *Salmonella* spp, *Shigella* spp, and *Citrobacter koseri* as long as they are not producing AmpC beta-lactamases.

## 3. Three dimensional (3D) tests:

These tests are of two types; direct and indirect. In the direct test, enzyme containing extracts are tested against the same isolate whereas in indirect method, the extracts are tested against a standard strain. The 3D tests have been variously modified by different researchers. The extract of the test isolates believed to contain ESBLs hydrolyze the antibiotic diffusing from the disk therefore distorting the (otherwise perfect) zone of inhibition.



In direct 3D test, the extracts of the test isolates are obtained by various methods (enzymatic, repeated freezing & thawing, sonication or using glass beads). The test isolate is inoculated on surface of Mueller Hinton agar as for disk diffusion. Cefotaxime or ceftazidime disk is placed on the agar surface. A narrow circular slit ~3 mm from the disk is made on the agar using the services of a turntable. Since this procedure is difficult, a narrow slit (20-30 mm long) is made or a well is punched 2-5 mm close to the disk, which is then carefully filled with the extract. Some researchers have placed a plain filter paper disk saturated with the cell extracts. Instead of the extracts, few workers have used a heavy inoculum ( $10^9$  cfu/ml or McFarland No. 5 turbidity standard) of test isolates to fill the slit. The plate is incubated in air at 37°C for 18-24 hours. A distortion on the zone of inhibition around the disk, which is observed as inward growth of the isolate is interpreted as positive for ESBL production.

The test is useful as long as some zone of inhibition is formed around the antibiotic disc. In indirect 3D test, lawn culture of *E. coli* ATCC 25922, which is susceptible to oxyimino-cephalosporins, is made. Rest of the procedure and its interpretation are the same. The results of indirect 3D tests are better than direct 3D test as zone of inhibitions are always seen around the antibiotic disks. The result of 3D tests depends on success of enzyme extraction from the cells.

#### 4. ESBL E-test:

The Etest ESBL confirmatory test strips (AB Biodisk, Solna, Sweden) are based on the CLSI dilution method. The strips are thin, inert and non-porous plastic carriers, which measures 5 x 60 mm.



Cefotaxime +  
clavulanic acid

Cefotaxime



Ceftazidime +  
clavulanic acid

Ceftazidime

One side of the strip is calibrated with MIC reading scales in µg/ml while the reverse surface carries two predefined antibiotic gradients. There are two strips; one strip (CT/CTL) contains cefotaxime gradient at one end and cefotaxime/clavulanic acid gradient at the other, and the other strip (TZ/TZL) contains ceftazidime gradient at one end and ceftazidime/clavulanic acid at the other end. Testing

must be performed with both the strips. The MIC is interpreted as the point of intersection of the inhibition ellipse with the Etest strip edge.

Following concentration gradients are used with these strips:

- Cefotaxime (0.25 to 16 µg/ml) AND cefotaxime/clavulanic acid (0.016 to 1 µg/ml) plus 4 µg/ml of clavulanic acid
- Ceftazidime (0.5 to 32 µg/ml) AND ceftazidime/clavulanic acid (0.064 to 4 µg/ml) plus 4 µg/ml clavulanic acid

The presence of an ESBL is confirmed by the appearance of a phantom zone or deformation of the cefotaxime or ceftazidime ellipse or when either the MIC of cefotaxime or ceftazidime is reduced by  $\geq 3$  log<sub>2</sub> dilutions in the presence of clavulanic acid. A “rounded” zone (also known as phantom zone) may be seen below the CTL or TZL gradients and an ellipse may/may not be seen around the CT or TZ ends. The CT or TZ inhibition ellipse may also be deformed at the tapering end. The presence of a phantom zone or ellipse deformation indicates ESBL detected at unusual ratios of synergy between the CT or TZ and the clavulanic acid. Etest confirmatory strips are convenient but expensive. Since the concentration gradient is not as extensive as recommended by CLSI, they yield more inconclusive results than CLSI phenotypic confirmatory test. When MICs of cefotaxime/clavulanate or ceftazidime/clavulanate are higher than cefotaxime or ceftazidime respectively, it may reflect the induction of beta-lactamase production by clavulanic acid. When both MIC values are above the test ranges, the result is non-determinable. This may

suggest the presence of inhibitor resistant TEM or AmpC enzymes or that MIC values are outside the test device range. When one result is ESBL negative and the other non-determinable, the interpretation for the strain should be non-determinable.

### 5. Automated methods:

Automated ESBL tests such as Vitek Legacy and Vitek 2 (BioMérieux), MicroScan (Siemens Medical Solutions Diagnostics), Sensititre (TREK Diagnostic Systems), and Phoenix (BD Diagnostic Systems) offer the potential for rapid detection of ESBLs in clinical isolates. The Vitek 2 and Phoenix confirmatory tests have shown to be both sensitive and specific. Automated microdilution-based tests, such as MicroScan and Sensititre, have also displayed the same level of accuracy as the CLSI microdilution confirmatory tests. These automated systems are programmed to screen as well as confirm the presence of ESBLs.

### Characterization of ESBLs:

#### Isoelectric focusing:

ESBLs vary in their isoelectric points (pI value) and pI values give relative identity of the ESBL type. The cells of the test isolate are lysed to extract the enzymes. Lysozyme based enzyme extract is commonly used. An ampholine gel (pH 3.0 to 10.0) is laid on the isoelectric focusing apparatus and the nitrocefin is spread on the gel surface. Extracts from the test strains and known strains (used as controls) are electrophoresed on the gel. Depending on the isoelectric point, enzymes position themselves differently. ESBL activity of the extracts are detected by the substrate gel overlay method using cefotaxime at 1 µg/ml and *E. coli* ATCC 25922 as an indicator.

ESBL type	pI range
TEM	5.2 to 6.5
SHV	7 to 8.2
CTX-M	7.4 to 9

Since there are several ESBL types which have identical isoelectric points, determination of ESBL type by isoelectric point is no longer possible. However, it is still useful in characterization of a newly discovered beta-lactamase enzyme.

#### Plasmid transfer by conjugation experiments:

ESBL genes are located on plasmids, most of which are self-transmissible. In order to demonstrate this property conjugation experiment can be performed. *E. coli* J53Azr<sup>+</sup>, which is resistant to sodium azide is often used as a recipient stain in conjugation experiment. The test isolate (donor) and the recipient strains are incubated at 37°C separately in nutrient broth or Luria-Bertani broth with intermittent shaking for 3-4 hours. The donor strain is then mixed with the recipient strain at 1:10 ratio and incubated at 37°C without shaking for another 18-24 hours allowing for the conjugation to occur. Approximately 30 µl of the conjugate is transferred to McConkey agar containing 2 µg/ml cefotaxime and 100-200 µg/ml sodium azide. The plates are incubated at 37°C for 48 hours. Donor strains, which are susceptible to sodium azide and recipient cells, which are susceptible to cefotaxime do not grow on the medium. Only those recipient strains that have successfully received the plasmid following conjugation from the donor cells grow on the medium. These conjugates are subjected to CLSI phenotypic confirmatory test to confirm the presence of ESBL in them. PCR, followed by sequencing may also be performed to characterize the ESBL type.

Conjugation experiments are not always successful. Some plasmids are readily transferable than others. In some cases several attempts may be required for successful conjugation. If conjugation (mating) experiments fail, transformation of *E. coli* TOP10 or DH5α strain by electroporation of whole-plasmid DNA can also be done. The transformants can be selected on Drigalski agar supplemented with 1 µg/ml cefotaxime.

## Genotypic methods:

### 1. Hybridization with DNA Probes:

Early detection of beta-lactamase genes were performed using DNA probes that were specific for TEM and SHV enzymes. One of the first probe based detection system was developed in 1988 to differentiate between TEM-1 and TEM-2. Radioisotope or biotin labeled oligonucleotide probe was designed to detect point mutations under stringent hybridization conditions. Subsequently, additional probes were designed to detect mutations at six positions within *bla*<sub>TEM</sub> gene, which was helpful in identifying several new TEM variants.

### 2. PCR followed by gene sequencing

Several modifications or variations of polymerase chain reaction (PCR) have been described by several researchers. It offers a convenient and a quicker method to identify the ESBL type. The procedure of a typical PCR based detection system involves DNA extraction and amplification of the ESBL genes by using specific primer pairs. Oligonucleotide primers can be chosen from sequences available in public databases such as Genbank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>). The primers are designed to anneal at regions where point mutations are not known to occur. The region spanning the primers may have mutations; hence PCR alone can not identify the ESBL type. DNA sequencing following the PCR is necessary to identify the ESBL type.

#### 2a. Conventional PCR:

In a typical setup the reaction mixture consists of 30-50 µl DNA template, 0.4 µM of each oligonucleotide primer in 1x PCR buffer, 200 µM each of the four deoxynucleoside triphosphates, 2.0 µM MgCl<sub>2</sub> and 1.5 U Taq polymerase enzyme in buffer. The master mixture is then overlaid with mineral oil. The amplification parameter includes 30 cycles of denaturation (1 minute at 95°C), primer annealing (45s at 55°C) and primer extension (45s at 72°C). A final elongation is carried out at 72°C for 10 minutes. Following PCR, the generated amplicons may be subjected to electrophoresis on 2% agarose gel with ethidium bromide for determination of amplicon size. The amplicons are purified and subjected to sequencing. Sequencing is essential to discriminate between the non-ESBL parent enzymes and different variants of ESBLs.

Primer pairs can be designed differently depending on the requirements and PCR conditions. Examples of primer pairs used are given below.

#### Primers for TEM gene:

Forward: 5'-TTC TTG AAG ACG AAA GGG C-3'

Reverse: 5'-ACG CTC AGT GGA ACG AAA AC-3'

#### Primes for SHV gene:

Forward: 5'-GCC CGG GTT ATT CTT ATT TGT CGC-3'

Reverse: 5'-TCT TTC CGA TGC CGC CGC CAG TCA-3'

#### CTX-M consensus primer:

Forward: 5'-SCV ATG TGC AGY ACC AGT AA-3'

Reverse: 5'-CCG CRA TAT GRT TGG TGG TR-3'

#### Primers CTX-M-1 group

Forward: 5'-GGT TAA AAA ATC ACT GCG TC-3'

Reverse: 5'-TTG GTG ACG ATT TTA GCC GC-3'



**Primes CTX-M-2 group**

Forward: 5'-ATG ATG ACT CAG AGC ATT CG-3'

Reverse: 5'-TGG GTT ACG ATT TTC GCC GC-3'

**Primers CTX-M-9 group:**

Forward: 5'-ATG GTG ACA AAG AGA GTG CA-3'

Reverse: 5'-CCC TTC GGC GAT GAT TCT C-3'

PCR amplification of the ESBL gene followed by nucleotide sequencing is the golden standard for identifying specific ESBL genes. Presence of multiple ESBL types and presence of multiple copies of ESBL genes as well as the diversity of point mutations that confer ESBL phenotype makes the characterization even more complex. While PCR amplification of CTX-M genes alone indicates ESBL phenotype, DNA sequencing is must to identify ESBL phenotype for TEM and SHV gene types.

**2b. Multiplex PCR:**

In order to simplify the process of identification of ESBL type, multiplex PCRs have been designed using primer pairs against different ESBL genes (*bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>). Following amplifications, the amplicons are electrophoresed and the bands are identified by comparing the bands obtained from standard strains. Further identification is possible by DNA sequencing.

**2c. PCR-restriction fragment length polymorphism (PCR-RFLP):**

The amplicons generated following PCR are digested by specific restriction endonucleases and the fragments that are generated are separated by gel electrophoresis. The size of the fragments indicates point mutations within the structural gene. Although PCR-RFLP technique is a simple and rapid alternative, it cannot identify all known mutations.

**2d. PCR with single-strand conformational polymorphism (PCR-SSCP)**

It has been observed that point mutations (involving change in single bases) can bring about conformational change in the single stranded DNA molecule. Point mutations cause the short sequences to migrate at a different and characteristic rate from the parent DNA. It thus permits detection of single base mutations without the requirement of sequencing. For successful detection of point mutation, the amplimer must not be longer than 200bp. A pattern of electrophoretic mobilities of gene fragments from various standard strains with known mutations are made. Mutations in a strain can be identified by comparing its electrophoretic mobility with those of reference strains. This method allows for a rapid screening of strains to identify known mutations as well as to indicate any novel mutant, which can then be identified by full nucleotide sequencing.

In this procedure, the amplicon obtained after PCR amplification is digested by endonuclease (e.g., *Pst* I). The digested amplimer is then denatured to yield single-stranded DNA fragments by mixing 2  $\mu$ L of the digestion product with an equal volume of denaturation solution (97% deionised formamide, 4.6 M urea, 0.3% bromophenol blue, 0.3% xylene cyanol and 10 mM EDTA). The mixture is heated again at 95°C for five minutes and then separated on gel by electrophoresis, stained and observed. This method was applied by few researchers in the detection of SHV ESBLs. It provides opportunity to screen a large array of strains for both known and unexpected mutations occurring within the amplimer. PCR-SSCP patterns are sometimes difficult to differentiate, and the technique relies upon relatively expensive instrumentation.

**2e. Restriction site insertion-PCR (RSI-PCR):**

It was developed to detect point mutations between closely related DNA sequences. Primers with one to three base mismatches near the 3' end are designed to obtain a fragment harbouring an artificial

restriction site specific for a given strain. The 3' end nucleotide is usually unmodified in order to minimize the effects of the mismatches during annealing with the template. The primers are designed to identify mutations affecting the amino acids at specific positions. The modified forward primer coupled with a specific reverse primer allows for the amplification of DNA fragments. The fragments can be digested with the specific endonuclease only in those strains where the restriction site is inserted by the DNA polymerase. The artificial restriction site which is included in the amplified product is designed for enzymes which do not cut the native sequence in any point between the primers. The PCR products are digested with restriction endonucleases and the products are analyzed by gel electrophoresis using 3% low-melting-point agarose. A 100-bp ladder is used as a DNA size marker. The RSI-PCR technique has been developed to extend the identification of SHV beta-lactamases by PCR-RFLP analysis. By a combination of both these techniques, it has been made possible to differentiate genes encoding the 27 SHV variants (*bla<sub>SHV-2</sub>* to *bla<sub>SHV-27</sub>*).

The RSI-PCR and RCR-RFLP techniques can be used for screening several strains and in conditions when nucleotide sequencing is not available. Since RSI-PCR has the flexibility of creating or removing restriction sites, it is a good method for characterizing newly described variants of *bla<sub>SHV</sub>*. The limitation of this technique is that it can detect mutations only at sites where primers create restriction site. It will fail to detect *bla<sub>SHV</sub>* variants with mutations in previously undescribed positions.

## **2f. Real-Time PCR and Melting Curve Analysis:**

Another modification for the detection of mutations in SHV genes is the SHV melting-curve mutation detection (MCMD). This can detect mutations in the three crucial codons at positions 179, 238 and 240 of the *bla<sub>SHV</sub>* in a single reaction. This is a PCR where special fluorescent labeled oligonucleotide hybridization probes are used. This method allows detection of SHV genes, differentiate non-ESBL from ESBL genes and categorize the SHV ESBL producers into three phenotypically relevant subgroups within one hour.

This system uses Fluorescence Emitting Resonance Technology, where two differently labeled probes are used. One probe, which is labeled with donor fluorophore (FITC) acts as anchor probe whereas the other probe, which is labeled with acceptor fluorophore (LightCycler Red 640 or Red 705) acts as detection probe. The probes are designed in such a way that they bind to the template at a distance of 4-5 nucleotides of each other. The probes are also designed to bind at locations where mutations are expected. When exposed to light the FITC emits light with a wavelength of 640 or 710 nm, which in turn excites the acceptor fluorophore present on detection probe. The acceptor fluorophore emits light of a greater wavelength, which is measured. The 3' ends of the probes are phosphorylated to prevent extension during PCR cycles. Fluorescence is measured at the end of the annealing period of each cycle to monitor the progress of amplification. The anchor probes are designed to be longer than the detection probes, hence anchor probes have higher  $T_m$  melting temperature (e.g., 67°C) than those of detection probes (e.g., 62°C). Since mutations destabilize the effective hybridization of the detection probes, a decrease in melting temperature is characteristically observed. After the completion of PCR cycles, the reaction mixture is cooled to 35°C and then slowly heated to 85°C at 0.2°C/second. During this period, the fluorescence is continuously measured to monitor the disassociation of detection probes. The signals are plotted in real time versus temperature to produce melting curves for mutations, which are then converted into melting peaks.

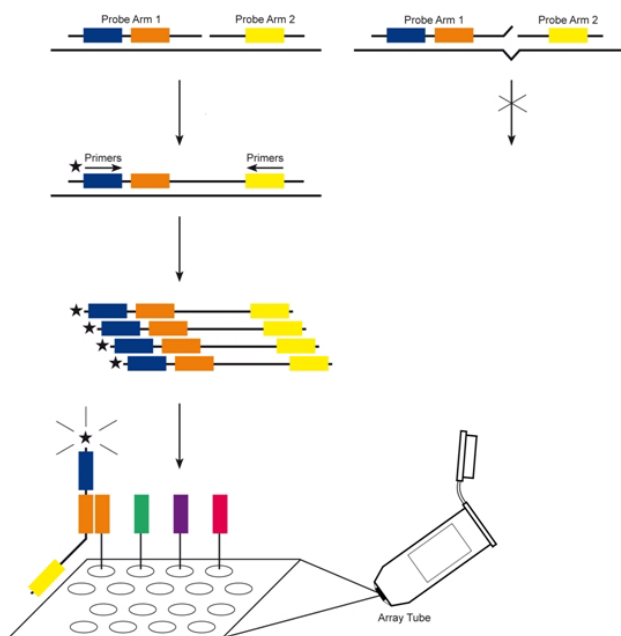
## **3. Ligase chain reaction (LCR)**

LCR is a probe amplification system that has been adapted to characterize point mutations and differentiation of *bla<sub>SHV</sub>* genes. Using four different sets of biotinylated LCR primers, seven SHV genes can be distinguished according to their point mutations. Here, the target DNA sequence is denatured at 94°C and the four primers hybridize to their complementary strands at 60°C. The thermostable ligase

enzyme ligates only the primers that are perfectly complementary to their target sequence and have hybridized directly adjacent to each other. This generates a short oligonucleotide sequence, which get amplified during the amplification rounds. Even a single base mismatch at the junction between primers will not get amplified. Thus, LCR allows for the detection and discrimination of parental and mutated nucleotide sequences of SHV enzymes.

#### 4. DNA Microarray systems:

A commercially available Check-Points ESBL/KPC array system uses a methodology called multiplex ligation detection reaction (LDR). Two specially designed probes are used in this system. The first probe, which is short, contains a region complementary to the target and also acts as a PCR primer. The second probe, which is longer, contains two parts; the first part has a region complementary to the target and also acts as a PCR primer and the second part contains a unique ZIP code that is complementary to a unique oligonucleotide (cZIP) immobilized on the microarray. The probes are allowed to hybridize with the target nucleic acid and the two probes are then ligated.



The probe arms will be ligated together only if they match exactly to the template and a single nucleotide mismatch will not result in the ligation of probes. It is subsequently amplified by PCR using single primer pair. One of the primer pair is biotin labeled. The PCR products are detected by hybridization to a low-density DNA microarray. The different probes will hybridize to different positions on the microarray, which are subsequently detected by colorimetric avidin based systems. Series of different probes have been developed to detect TEM, SHV as well as five CTX-M groups.

The system can simultaneously identify the most prevalent carbapenemases, AmpC and ESBL genes. The advantage of this system is detection of over 100 specific DNA markers in one single test.

## Epidemiological typing of ESBL producing bacteria

### Phenotypic methods:

**Phene Plate system** is a novel automated system used for simple yet rapid subtyping of bacteria. It is based on the evaluation of the kinetics of biochemical reactions of isolates to be typed. The Phene Plate system consists of 96-well microtitre plates, each with four sets of 22 different dehydrated reagents and two sets of 46 different reagents, respectively. The absorbance value for each reaction is measured after 8, 24 and 48 hours at 620 nm. The results for all isolates are compared pairwise and the similarities are calculated by computer software to produce a dendrogram. Isolates with identical or very similar biochemical fingerprints are considered to be of the same PhP type.

**Serotyping:** Determination of O and H antigens is performed using all available O (O1 to O181) and H (H1 to H56) antisera. Isolates that do not react with O antisera are classified as non-typeable (ONT), and non-motile isolates are denoted as HNM.

**Raman spectroscopy analysis (RA)** is a new tool for bacterial typing. This technology is based on the scattering of light by molecules. Since bacterial strains differ from each other in their molecular make-up, the differences get reflected in their spectroscopic fingerprints. This enables accurate characterization of the strains. RA generates results that are reproducible and comparable to that of PFGE. The technology is able to discriminate isolates that are part of the outbreak from those that are not. Besides discriminating strains, RA can recognize clones, irrespective of the ESBL type.

#### **Genotypic methods:**

**Plasmid profiling:** Plasmid typing was the first molecular method to be used as a bacterial typing tool. Typing is performed through the isolation of plasmid DNA and comparison of the numbers and sizes of the plasmids by agarose gel electrophoresis. For bacteria having large plasmids (100 to 150 kb), separation becomes difficult. In such situations, the plasmids are digested by restriction endonuclease (S1) enzyme and then subjected to electrophoresis. Further discrimination can be achieved by using PFGE. Plasmid profiles are useful when isolates harbour several plasmids. A limitation of plasmid profiling is that plasmids can be readily acquired and lost. It is possible that epidemiologically related isolates can exhibit different plasmid profiles. Frequently, the DNA composition of the plasmid can be altered by the movement of transposons in and out of the plasmid.

**Replicon typing:** Incompatibility (Inc) typing is based on the fact that two plasmids sharing common replication and partitioning elements are unable to proliferate stably in the same cell. Initially, Inc typing relied on introduction of a plasmid into a strain carrying another plasmid and determining whether both plasmids were stably maintained in the progeny. However, physical Inc typing is time consuming and not easily used in large-scale applications. Since 2005, plasmids are typed using PCR based replicon typing (PBRT), which target the replicons of major plasmid families. The PCR-based replicon typing protocol to detect plasmid replicons found among the Enterobacteriaceae requires several steps, including five multiplex and three simplex PCR procedures.

**Multilocus Sequence Typing:** MLST is performed by gene amplification and sequencing of the seven housekeeping genes (*adhA*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*). Approximately 450-500 bp internal fragments of each gene is amplified and sequenced. The different sequences present for each house-keeping gene within a bacterial species are assigned as distinct alleles. The alleles at each of the seven loci define the allelic profile or sequence type (ST) of an isolate. Each isolate of a species is therefore unambiguously characterized by a series of seven integers which correspond to the alleles at the seven house-keeping loci. Most bacterial species have sufficient variation within house-keeping genes to provide many alleles per locus, allowing billions of distinct allelic profiles to be distinguished using seven house-keeping loci. There are 985 STs in *E. coli* and 960 STs in *K. pneumoniae*.

**Ribotyping:** Analysis of DNA polymorphism in the chromosomal regions containing the highly conserved rRNA genes (*rrn*), and referred to as ribotyping. Once DNA has been cleaved with a restriction enzyme, the size of the generated fragments is analyzed by electrophoresis on agarose gels. Inter-individual strain-to-strain variations in fragment sizes are referred to as restriction fragment length polymorphisms (RFLPs). RFLPs result from single-base-pair variations that account for the presence or absence of a restriction site at given positions in the genome. Restriction fragments resulting from digestion with enzymes recognizing 6-bp sites commonly vary in size from 1,000 to 20,000 bp. The discriminatory power of this method is related to the copy numbers of rRNA genes in the bacterial genome and their distribution among the restriction fragments following electrophoresis. Variations in the number and sizes of fragments detected are used to type the microorganisms.

**Rep-PCR (Repetitive element polymorphism):** Most bacteria have several repeating DNA sequences throughout their genome. DNA primers are designed to bind to two such repetitive sequence

closely present to each other, thereby amplifying the DNA present between the two repetitive sequences. The regions located between the repeated elements often vary in size due to difference among separate strains, and thus fragments of different sizes will be amplified. The bands are compared to one another to genotype the organism.

**Phylogenetic grouping:** The phylogenetic group of the *E. coli* strains (A, B1, B2 and D) can be determined by the multiplex PCR, multi-locus enzyme electrophoresis (MLEE) or ribotyping. A triplex PCR, which uses combination of two genes *chuA* and *yjaA* along with an anonymous 14.9kb fragment (TSPE4.C2), shows excellent correlation.

### **ESBL detection in AmpC co-producers:**

Due to increased selective pressure by antibiotic usage, microorganisms are evolving by producing multiple beta-lactamase types. Several different beta-lactamase types including classical TEM type, ESBLs, AmpC as well as carbapenemases are being reported occurring in single strains, especially those isolated from the hospitals. Overproduction of classical TEM enzymes can also mask the inhibitor activity. AmpC beta-lactamases are not affected by clavulanic acid thereby give false-negative ESBL detection results in clavulanic acid based detection system in isolates that produce both ESBL and AmpC beta-lactamases. ESBLs can be detected in such isolates by incorporating agents that selectively inhibit AmpC beta-lactamase.

#### **a) Modified double disk diffusion test:**

The original double disk diffusion was modified by including a cephalosporin (cefepime) that is resistant to hydrolysis by derepressed AmpC enzymes. Enhanced area of inhibition between cefepime and amoxyclav disks indicates the co-production of ESBL and AmpC enzymes. The test can appear false negative if ESBLs capable of hydrolyzing cefepime are produced or the isolate produces multiple types of beta-lactamases.

#### **c) Using cefepime and cefepime/clavulanic acid combination**

This is a modification of the CLSI recommended confirmatory test. Here, cefepime disc (30 µg) and a cefepime/clavulanic acid disc (30/10 µg) are used. An increase in zone diameter by  $\geq 5$ mm for the cefepime/clavulanic acid disc versus the cefepime disc is interpreted as production of ESBL. The test can appear false negative if ESBLs capable of hydrolyzing cefepime are produced or the isolate produces multiple types of beta-lactamases.

#### **a) Inhibition of AmpC by cloxacillin**

Cloxacillin is resistant to hydrolysis by AmpC beta-lactamases. ESBL production in isolates that also produce AmpC can be detected by performing CLSI PCT on Mueller Hinton agar supplemented with 200 µg/ml of cloxacillin.

In a modified indirect 3D test, the extracts of test isolates are mixed with 1 mM cloxacillin. Cloxacillin inhibits the AmpC enzyme and the distortion of zone of inhibition represents ESBL production

#### **b) Inhibition of AmpC by phenyl boronic acid**

Boronic acid [benzo(b)thiophene-2-boronic acid] is a potent inhibitor of class C beta lactamases. Discs supplemented with 400 µg/ml phenylboronic acid can be used to suppress AmpC production in disk based tests. A stock solution of phenylboronic acid can be prepared by dissolving 120 mg of phenylboronic acid in 3ml of dimethyl sulfoxide. Three milliliters of sterile distilled water is then added to this solution. Twenty microliters of the stock solution is dispensed onto disks containing 30 µg of cefotaxime and cefotaxime/clavulanic acid. Disks are allowed to dry for 30 min and stored in airtight vials

with desiccant at 4°C. An increase in  $\geq 5$  mm around the disc containing cefotaxime/clavulanic acid/phenyl boronic acid versus cefotaxime/clavulanic acid indicates co-production of ESBL and AmpC in an isolate.

CTX	CTX + CLA	CTX + CLA+ PBA	CTX+ PBA	Interpretation
R	+	NA	NA	ESBL only
R	-	+	+	AmpC only
R	-	+	-	ESBL & AmpC

CTX = cefotaxime, CLA = clavulanic acid, PBA = phenyl boronic acid, R = resistant, NA= Not applicable, (+) indicate  $\geq 5$  mm, (-) indicate  $< 5$  mm

Boronic acid-based tests are sometimes less sensitive in detecting ESBL producers if the isolate co-produces plasmid-mediated DHA-1 AmpC enzyme. Boronic acid is also known to inhibit KPC enzymes, OXA-12 and sometimes certain ESBLs as well.

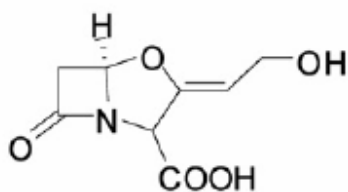
## F. Beta-lactamase Inhibitors:

Resistance to the hydrolytic activity of the beta-lactamases can be achieved by preventing the binding of the drug to the active site or by displacing the water molecule in the enzyme's active site. Access to the binding site can be prevented by attaching a bulky acyl group to the 6-aminopenicillanic acid or 7-aminocephalosporanic acid. Replacing the hydrogen atom on carbon 6 or 7 of penicillin and cephalosporin, respectively, with an alpha-methoxy group displaces the water molecule required for hydrolysis. Inhibitors of beta-lactamases can be classified as reversible and irreversible. Most of the beta-lactamase inhibitors contain beta-lactam ring. When the inhibitors bind at or near the enzyme's active site, they can also be hydrolyzed as substrates. Reversible inhibitors bind to the enzyme with high affinity but are hydrolyzed poorly as they act as poor substrates. Irreversible inhibitors are more effective than reversible inhibitors as they completely inactivate the enzyme. Molecules that initially bind to the enzyme's active site and gets hydrolyzed to a form, which in turn inactivates the enzyme are termed "suicide inhibitors".

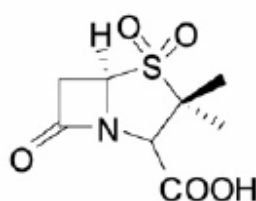
The three common beta-lactamase inhibitors currently in clinical use are clavulanate, sulbactam and tazobactam. All three beta-lactamase inhibitor compounds share structural similarity with penicillin. These are effective against most Class A beta-lactamases. These inhibitors have no inhibitory activity against PBPs, however the following exceptions occur:

- Sulbactam is active against *Bacteroides spp.*, *Acinetobacter spp.*, and *N. gonorrhoeae*
- Clavulanate is active against *Haemophilus influenzae*, *Acinetobacter spp.*, and *N. gonorrhoeae*
- Tazobactam is active against *Borrelia burgdorferi*

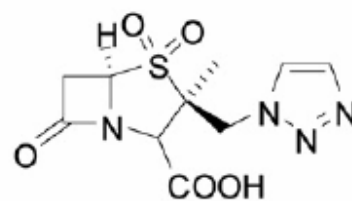
Currently, the following beta-lactam– beta-lactamase inhibitor formulations are available for clinical use: amoxicillin-clavulanate, ticarcillin-clavulanate, ampicillin-sulbactam, cefoperazone-sulbactam and piperacillin-tazobactam.



Clavulanic acid



Sulbactam



Tazobactam

The turnover number (tn) is defined as the number of inhibitor molecules that are hydrolyzed per unit time before one enzyme molecule is irreversibly inactivated. One clavulanate molecule is sufficient to irreversibly inactivate one PC1 beta-lactamase of *S. aureus*. While it requires only 60 clavulanate molecules to inactivate SHV-1 enzyme, 160 molecules are required to inactivate TEM-1 enzyme. Presence of a leaving group at position C-1 of the 5-membered ring makes these inhibitors (clavulanate, tazobactam and sulbactam) different from penicillins. Better leaving group facilitates secondary ring opening and modification of beta-lactamase. While clavulanate possesses enol ether oxygen at this position, sulbactam and tazobactam have sulfones. Sulbactam is not as efficient as clavulanate because of the relatively poor leaving group present in it.

Binding of the beta-lactamase inhibitor with the beta-lactamase forms acyl-enzyme complex. After acylation, the five-membered ring is opened leading to formation of a transient imine intermediate, which then rearranges to form enamine intermediates. The enamine intermediate may occur in either the trans or cis conformation. Depending on the nature of the enzyme and the inhibitor, the reaction may proceed to deacylation of the enamine intermediate or irreversible inactivation of the enzyme. If deacylation takes place, the acyl-enzyme undergoes decarboxylation and ester bond hydrolysis resulting in regenerated beta-lactamase. The stabilization of the enamine intermediate is a significant factor in prolonged enzyme inhibition. Studies with SHV-1 enzyme have revealed that tazobactam forms a predominant population of trans-enamine, whereas clavulanate and sulbactam form a mixture of trans-enamine and more chemically labile cis-enamine and imine intermediates.

#### **a) Clavulanic acid:**

This “suicide inhibitor” was first isolated in 1970 from a soil bacterium *Streptomyces clavuligerus*. It is also the first beta-lactamase inhibitor to be used in clinical practice. Alone, it has poor antibacterial activity. The MIC of amoxicillin was significantly reduced when it was combined with the salt form of clavulanic acid (potassium clavulanate). Compared to beta-lactam antibiotics, clavulanate occupies the enzyme's active site longer and fail to be hydrolyzed efficiently.

Amoxicillin-clavulanate was the first beta-lactam-beta-lactamase inhibitor combination approved for use in clinical practice. It is predominantly used as an oral preparation and is effective against penicillinase-producing *S. aureus*, *H. influenzae*, *Moraxella catarrhalis*, *Bacteroides spp.*, *N. gonorrhoeae*, *E. coli*, *Klebsiella spp.*, and *P. mirabilis*. Its parenteral usage has been mainly in treating ESBL producing *E. coli* bacteremia. Ticarcillin-clavulanate was introduced in 1985 as a parenteral preparation against infections caused by beta-lactamase-producing staphylococci, *E. coli*, *H. influenzae*, *Klebsiella spp.*, *Proteus spp.*, *Pseudomonas spp.*, *Providencia spp.*, *N. gonorrhoeae*, *Moraxella catarrhalis*, and *Bacteroides spp.*

Clavulanate is a potent inducer of chromosomally mediated AmpC beta-lactamase in isolates such as *Enterobacter spp.* and *Morganella morganii*. It has been suggested to use combination of clavulanate with fourth generation cephalosporin such as cefepime and ceftazidime since these cephalosporins are more stable against AmpC enzymes. The advantage of using cefepime clavulanate is that cefepime is poorly hydrolyzed by AmpC enzymes and clavulanate inhibits ESBLs.

#### **b) Sulbactam:**

Sulbactam is a penicillinate sulfone that was developed in 1978 as synthetic compound by the pharmaceutical industry. Ten thousand molecules of sulbactam are required to inactivate one TEM-1 enzyme and 13,000 molecules to inactivate one SHV-1 enzyme. Sulbactam are better inhibitors of penicillinases and ESBLs than cephalosporinases, but are slightly more active against cephalosporinases than clavulanic acid. Sulbactam is not well absorbed orally and must be administered parenterally. Ampicillin-sulbactam is useful against beta-lactamase-containing *S. aureus*, *H. influenzae*, *M. catarrhalis*,

*E. coli*, *Proteus spp.*, *Klebsiella spp.*, and anaerobes. Cefaperzone-sulbactam is another popular combination. One of the particular advantages of using sulbactam-containing combinations is that sulbactam itself has inherent activity against some *Acinetobacter baumannii*. Compared to clavulanate, sulbactam is not a potent inducer of AmpC chromosomal beta-lactamases in Enterobacteriaceae.

**c) Tazobactam:**

This is also a penicillinate sulfone that was developed in 1980 as synthetic compound by the pharmaceutical industry. Piperacillin-tazobactam was introduced for therapeutics in United States in 1993. Few studies have revealed that TEM-type ESBLs are more susceptible to piperacillin-tazobactam than are SHV-type ESBLs. It has almost 10-folds higher inhibitory activity than clavulanic acid against CTX-M type beta-lactamases. Although piperacillin-tazobactam combination has fared better than other beta-lactam-beta-lactamase inhibitor combinations, there is no assurance that it will give good patient treatment outcomes. Tazobactam has been reported to have a turnover number of 2–125 against the TEM-1 and TEM-2 beta-lactamases.

**d) Monobactam derivatives:**

Syn 2190 (1,5-dihydroxy-4-pyridone monobactam) is modified product of aztreonam. It possesses a novel C-3 side chain that may utilize the iron uptake pathway to gain entry into Gram negative bacteria. It has better inhibitory activity towards class C enzymes than class A enzymes. Another modified monobactam, Ro 48-1256 too displayed good activity when combined with ceftazidime or piperacillin against derepressed AmpC producers but failed to protect them against class A, B or D beta-lactamases. BAL29880, which is also a monobactam derivative, is an inhibitor of AmpC enzymes. BAL19764 and BAL30072 are monobactams with a siderophore side chain which can enhance cell entry through bacterial iron uptake systems. BAL19764 is stable to hydrolysis by MBLs. BAL30072 is an inhibitor of TEM-3 and AmpC enzymes.

**e) Methyldene penems** possess double bond at C-6 and a sulfide at penem position 1, which have potent inhibitory properties. These compounds have a larger R1 side chain that aids in cell permeability and contribute to active-site affinity. BRL 42715 C-6-(N1-methyl-1,2,3-triazolylmethylene) penem, is an effective inhibitor of class A, C, and D beta-lactamases. BLI-489, another novel penem derived compound has shown activities against molecular class A, C and D beta-lactamases.

**f) NXL104** is a bridged diazabicyclo octanone and is a beta-lactamase inhibitor able to inhibit both class A (TEM-1, CTX-M-types and KPC beta-lactamases) and class C beta-lactamases. It is currently being developed in combination with ceftazidime or ceftaroline.

**g) BLI-489** is a potent inhibitor of all serine beta-lactamase classes with low propensity towards development of resistance. Mouse experiments have suggested that Piperacillin-BLI-489 is effective against infections caused by class A, class C, and class D  $\beta$ -lactamase-expressing pathogens.

**h) Oxapenem** is a five-membered oxygen-containing ring that is fused to beta-lactam ring with a double bond between C2 and C3. These compounds were found to be potent inhibitors of beta-lactamases, but had poor stability. Compounds AM-113 and its stereoisomer AM-114 were found to reduce the ceftazidime MIC against TEM, and SHV derived ESBL producing strains. Different oxapenem molecules have varied inhibitory properties: AM-112 (class D enzymes), AM-113 (class C and D enzymes), AM-114 (class A and C enzymes), and AM-115 (class A enzymes).

**i) BAL30376**, three-component mixture containing N-sulfonic acid of a bicyclic  $\beta$ -lactam, clavulanate and a siderophore-substituted monobactam, has shown activity against many ESBL-, AmpC-, and MBL-expressing gram negative bacteria.



## Resistance to beta-lactam/beta-lactam inhibitor combinations:

Acquired resistance to such combinations in *E. coli* is due to:

- (i) penicillinase hyperproduction due to the presence of the *bla*<sub>TEM-1</sub> gene in small multicopy plasmids or strong promoters;
- (ii) overproduction of constitutive AmpC cephalosporinase; and
- (iii) OXA-type beta-lactamases and
- (iv) inhibitor-resistant TEM (IRT) beta-lactamases. IRT enzymes may evolve into complex mutants (CMT enzymes), which also confer resistance to extended-spectrum cephalosporins.

## G. Multiple drug resistance:

ESBL genes are often located on large plasmids that also harbour genes for resistance to other antimicrobials (fluoroquinolones, aminoglycosides and cotrimoxazole) and, thus, will often exhibit multidrug-resistant phenotypes. Isolates producing CTX-M-15 ESBLs are often also resistant to aminoglycosides and fluoroquinolones (such as norfloxacin and ciprofloxacin). These isolates often harbour epidemic narrow host range IncFII plasmids, which also codes for other beta-lactamases such as TEM-1, OXA-1 and the aminoglycoside modifying enzyme, *aac(6')-Ib-cr*. The aminoglycoside modifying enzyme has an additional property of acetylating fluoroquinolones such as ciprofloxacin and norfloxacin. Genes encoding resistance to macrolides, tetracyclines, sulphonamides, trimethoprim, and chloramphenicol have all been associated with *bla*<sub>CTX-M</sub> containing plasmids. It is believed that fluoroquinolone or multi-drug resistance gives advantage to CTX-M for maintenance due to co-selection process. Some of the CTX-M-15 producers have been found to be resistant to carbapenem, but no mutations are involved in this resistance. It is often due to co-production of other beta-lactamases or loss of porins. Carbapenem resistance in *K. pneumoniae* is often due to loss of OmpK35 and OmpK36 whereas decreased expression of OmpF and OmpC is associated with carbapenem resistance in *E. coli*. Some CTX-M producers have been found to co-produce VIM-1, NDM-1 or OXA-48 carbapenemases. The resistant bacteria tend to become even more resistant by acquiring multi-drug resistant phenotypes; this has been described as 'genetic capitalism of resistant bacteria'.

## H. Treatment options against ESBL producers:

Treatment of serious infections caused by ESBL producing isolates is challenging because most often the isolates are multi-drug resistant (especially CTX-M-15 producers). Individual ESBL type also has variability in substrate profiles. Isolates producing ESBLs may also produce other beta-lactamases and mutations in their porin protein genes can also lead to decreased susceptibility. An in-vitro susceptibility to a cephalosporin may not necessarily correlate with in-vivo efficacy. There have been cases of treatment failures associated with cephalosporin antibiotics despite having very low MIC values. At the same time, there are reports of successful treatment outcomes using cephalosporins despite confirmed ESBL production by the isolates. The reduced efficacy of the antibiotics in-vivo despite being susceptible in-vitro tests is possibly due to the so-called "inoculum effect", which occurs when the MIC of the drug rise with the increase in inoculum size.

The CLSI had recommended in the past that ESBL producing *E. coli*, *K. pneumoniae*, *K. oxytoca* and *P. mirabilis* should be reported as resistant to penicillins, cephalosporins and monobactams regardless of the in vitro susceptibility result. These guidelines included the fourth-generation cephalosporin such as cefepime, which also possesses oxyimino side group and often appear sensitive against ESBL-producing bacteria. Since 2010, this recommendation has been dropped.

Some studies have suggested that CTX-M-producing *E. coli* sensitive to ceftazidime can be successfully treated with this agent. More studies are needed to assert this suggestion. Even though many ESBL producing isolates appear susceptible to the fourth generation cephalosporin such as cefepime, clinical studies using this drug have not been very favorable. Although active against most extended-spectrum beta-lactamases, cefepime is reported to show a marked inoculum effect. There have been success and failures associated with cefepime.

Even though ESBLs are inhibited by beta-lactamase inhibitors, they don't fully restore the activity of beta-lactam drug in combination. However, few studies have shown good clinical outcome with beta-lactam/beta-lactamase inhibitor combinations. Combinations such as amoxicillin/clavulanic acid, cefoperazone/sulbactam, piperacillin/tazobactam are more popular. Co-amoxycylav has moderate in vitro antimicrobial activity against Enterobacteriaceae that produce ESBL. Piperacillin/tazobactam appears to be better combination especially against TEM-type ESBLs.

Most ESBLs have no hydrolytic activity against carbapenems. Carbapenems such as meropenem, imipenem, ertapenem, and doripenem have broad-spectrum antibacterial activity and are resistant to inactivation by most serine beta-lactamases. However, isolates producing KPC-2, NMC-A, SME-1, and IMI-1 serine carbapenemases are resistant to carbapenems. Imipenem, meropenem, doripenem and ertapenem, have widely become the first choice for the treatment of serious infections due to ESBL producing Enterobacteriaceae. Several studies have reported decreased mortality when treated with imipenem/cilastatin. Cilastatin inhibits the degradation of imipenem by a renal tubular dehydropeptidase. There are also few reports associated with treatment failures with imipenem in patients with ESBL producing isolates. The resistances to ertapenem in *Klebsiella spp.* and *Enterobacter spp.* have been attributed to the production of various types of ESBLs and AmpC beta-lactamases as well as the loss of porins.

Temocillin, a derivative of ticarcillin and a broad-spectrum carboxypenicillin is stable to ESBLs and could be used as an alternative. It is resistant to hydrolysis by most ESBLs and AmpC beta-lactamases.

Since ESBLs do not hydrolyze cephamycins, drugs such as ceftiofur and cefotetan may also be used. Cephamycins are stable to ESBLs but their usages have resulted in selection in porin deficient mutants. Some isolates may become resistant during the course of therapy due to decreased expression of the outer membrane proteins resulting in treatment failures. Flomoxef, a new cephamycin, was found to almost as effective as the carbapenems in a clinical study in Taiwan. Further studies are required to determine reliability of using this drug.

Tigecycline, which is a derivative of minocycline, is the first marketed glycylcycline-class antibiotic. It has high antimicrobial activity against *E. coli* (especially those producing CTX-M enzymes) and *K. pneumoniae* isolates that produce ESBLs or have a multidrug-resistance phenotype. However, the concentration of the drug in urine is not sufficient to treat UTIs.

Fosfomycin inhibits UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), an enzyme that catalyses the first step in bacterial cell-wall synthesis. It has a broad spectrum of antimicrobial activity, including activity against several Gram-negative and Gram-positive aerobic bacteria. It has good level of antimicrobial activity against clinical isolates of Enterobacteriaceae (especially *E. coli*) that produce ESBL. It is considered as a valuable option for the treatment of lower urinary tract infections caused by ESBL producing *E. coli*.

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