EXTENDED SPECTRUM BETA-LACTAMASES- A COMPREHENSIVE REVIEW

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1. Beta-lactam antibiotics

1. 1 Discovery of beta-lactam antibiotics:

Globally bacterial infectious diseases have been responsible for a very large number of deaths and mankind has been in a constant state of conflict with bacteria since time immemorial for the diseases they produce.

The earlier attempts to cure bacterial infections involved the use of plant extracts or crude preparations of heavy metals. One of the earliest synthetic compounds with specific antibacterial activity was sulphonamide. Antibiotic was discovered in 1928 when Alexander Fleming published his famous discovery of Staphylococcal colonies being inhibited by a contaminating Penicillium mould. Upon subsequent experiments on selected bacterial cultures, he noted that the extract of fungal culture possessed "inhibitory, bactericidal and bacteriolytic properties" against most gram positive bacteria such *Staphylococcus aureus*, *Bacillus anthracis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Corynebacterium diphtheria* but not against Gram negative bacteria such *Escherichia coli, Klebsiella pneumoniae, Vibrio cholerae*, and *Hemophilus influenzae*. He coined the termed "penicillin" to refer to the "mould broth filtrate" that had antibacterial properties, since it was derived from *Penicillium sps*[1].

Although Fleming's discovery was a matter of providence, investigation into the antibacterial properties of fungi had begun much earlier. The observation that fungus can inhibit bacteria was first documented by a French physician, Ernest Duchesne in 1897. In his dissertation, he described the antibacterial properties of the fungus *Penicillium glaucum* [2, 3]. Unfortunately, he did not purse on his discovery and such a significant work went unnoticed for a very long time. In fact, more than 25 earlier to this discovery, Joseph Lister is believed to have noted antibacterial effects of

Penicillium glaucum on bacteria in 1871 and in 1884, he had successfully treated a case of abscess with this fungus [3].

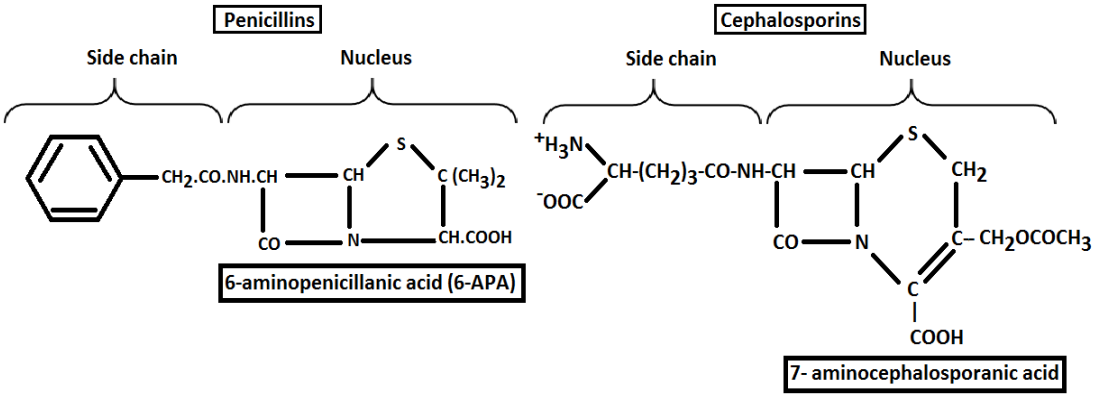
Fleming did not undertake any experiments to evaluate the clinical utility of his discovery. But, his work was continued by Howard W. Florey and Ernst Boris Chain in 1940, who successfully demonstrated the utility of purified penicillin by conducting in-vivo studies using mice deliberately infected with Streptococcus [4]. This was followed by successful clinical trials in 1941 and the drug was then made available for therapeutic use [5]. At the same time other biologically derived compounds with antibacterial property were being discovered from actinomycetes. In 1940, Selman A. Waksman, along with his students, discovered Streptomycin from *Streptomyces griseus* [6]. In order to differentiate such biologically derived compounds from other chemotherapeutic agents, he coined the term "antibiotic" [7].

These initial discoveries led to vigorous interest in searching for other antibiotics and one such attempt was made by Giuseppe Brotzu in 1948, who discovered antibacterial activity in crude extracts of the mould *Cephalosporium acremonium* (now known as *Acremonium chrysogenum*). Of the three compounds characterized in the culture extract, Cephalosporin C possessed most useful antibacterial property [8]. Thus, a new class of antibiotics called cephalosporins came into existence. Unlike penicillins, whose activities were restricted to Gram positive bacteria only, the cephalosporins had extended spectrum of activity to include Gram negative bacteria. In order to understand the mechanism of bacterial inhibition, studies were then undertaken to unravel their structure.

1.2 Structure of beta-lactam antibiotics

Following chemical and X-ray crystallographic studies by the pioneering effort of Chain and Hodgkin, the penicillin was shown to contain a unique structure; the beta-lactam nucleus. Beta (β)-lactam ring is a four-membered cyclic amide and 'β' represents the position of Nitrogen (N) atom relative to the carbonyl (C=O) group. In penicillin, the β-lactam ring is fused to a five membered thiazolidine ring. Thus, penicillin is structurally 6-amino-penicillanic acid 'nucleus' along with a side chain (Figure 1). Cephalosporins too contain a beta-lactam ring, which is fused to a six-membered dihydrothiazine ring. Chemically, it is 7-aminocephalosporanic acid nucleus with an attached side chain (Figure 1).

Figure 1: Structure of penicillin and cephalosporin molecules



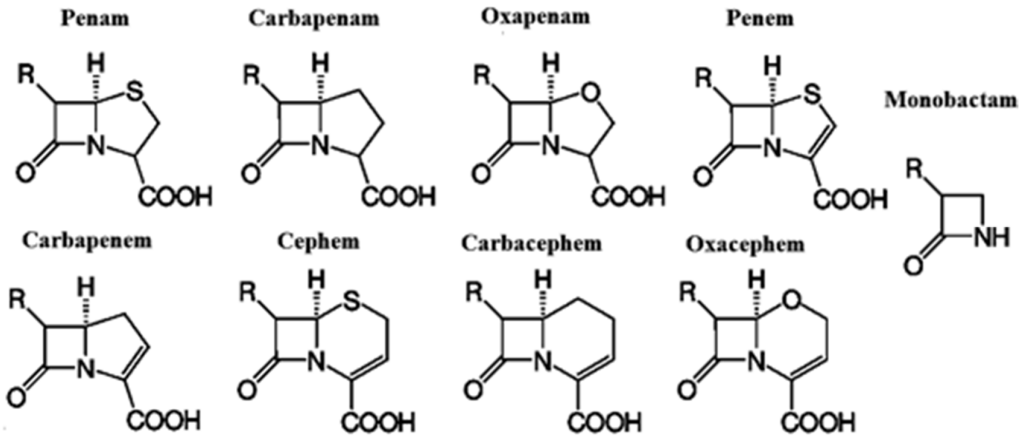
Between 1959 and 1970, a large numbers of semi-synthetic penicillins were created by modifying the side chains attached to the β-lactam ring [9]. Early penicillins were active only against Gram positive bacteria but subsequent generations had expanded spectrum of activity to include Gram negative bacteria. Unlike penicillins, most cephalosporins have good antibacterial activity against Gram negative bacteria. Several modifications in the side chain have made cephalosporin the largest group of beta-lactam antibiotics, comprising of five generations, each with distinct spectrum of

activity. Resistance exhibited by the bacteria to these antibiotics necessitated these modifications. Both, the naturally and semi-synthetically derived beta-lactam compounds are currently the largest family of antimicrobial drugs in use.

1.3 Classification of beta-lactam antibiotics

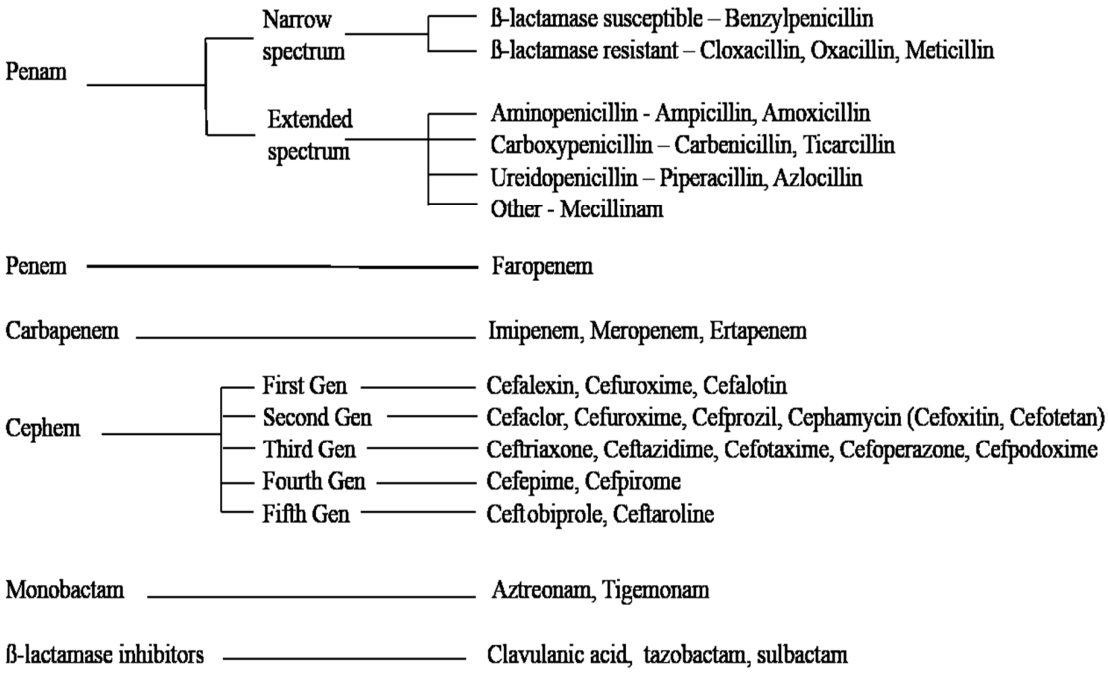
Beta-lactam groups are named after the type of rings that are fused to beta–lactam rings. The groups include penams, clavams (oxapenams), penems, carbapenems, cephems, carbacephems, oxacephems, monobactams and cephamycins. The core structure of various beta-lactam compounds are similar and is displayed in Figure 2.

Figure 2. Core structures of various beta–lactam compounds



A broad consensus on the classification of generations of cephalosporin and the position of cephamycins in that classification is yet to form. Figure 3 shows classification of beta-lactam antibiotics along with examples. The fifth generation cephalosporin such as ceftaroline and ceftobiprole are considered to be the latest generation of cephalosporins with enhanced activity against both Gram positive and Gram negative bacteria. Although beta-lactam antibiotics have slightly different pharmacologic properties and spectrum of activities, all of them act by inhibiting bacterial cell wall synthesis.

Figure 3: Classification of beta-lactam antibiotics

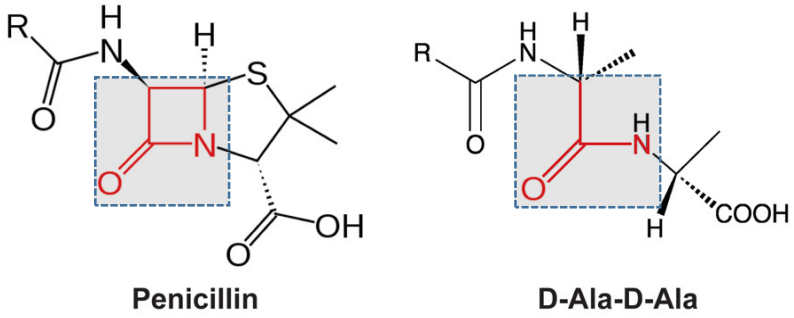


1.4 Mechanism of action of beta-lactam antibiotics

During the course of bacterial growth, the cell wall is continuously remodeled as the old ones are broken down and new ones are formed. Transglycosylase, transpeptidase, carboxypeptidase and endopeptidase are the bacterial enzymes that are involved in remodeling and synthesis of cell walls. These are a family of acyl serine transferases that catalyze the polymerization (transglycosylation) and cross-linking (transpeptidation) of the glycan strands of peptidoglycan. D-alanyl-D-alanine, a natural substrate of these enzymes is structurally similar to beta-lactam antibiotic (Figure 4). This similarity tricks the bacterial enzymes into binding with beta-lactam antibiotics in their active sites. It is for this reason that the transpeptidase enzymes are also known as Penicillin Binding Proteins (PBPs). By blocking the active site of these enzymes, beta-lactam antibiotics deprive them of their natural substrates. This prevents the cross linking of cell wall and ultimately the cell dies due to osmotic instability [10]. Bacteria

evolved quickly to counter the beta-lactam antibiotics and resistance appeared in *S. aureus* within a year of penicillin's use [11].

Figure 4: Structural similarity of beta-lactam ring and D-alanyl-D-alanine



1.5 Resistance to beta-lactam antibiotics:

Resistance to beta-lactam antibiotics can be intrinsic or acquired; most gram negative bacteria are intrinsically resistant to penicillin. Bacteria have evolved to counter the adverse effects of beta-lactam antibiotics in the following four diverse ways: a) mutations leading to loss or under-expression of porins that disallow entry of beta-lactams, b) production of new penicillin binding proteins that have low affinity to beta-lactams, c) expulsion of beta-lactams from periplasmic space mediated by efflux pumps and d) production of enzymes that hydrolyze beta-lactam rings.

Of all these methods, the enzymatic inactivation by beta-lactamases is the most common strategy adopted by the bacteria. The first evidence of enzymatic inactivation of penicillin came in 1940, even before the antibiotic was used in therapeutics. Abraham and Chain were able to demonstrate an enzyme in *E. coli* that hydrolyzed penicillin; they named it "penicillinase" [12]. Beta-lactamase is a broader name given to bacterial enzymes that hydrolyze various beta-lactam antibiotics. In case of Gram positive bacteria, these enzymes are excreted outside the cell whereas in Gram negative bacteria, they are present in the periplasmic space.

2. Beta-lactamases

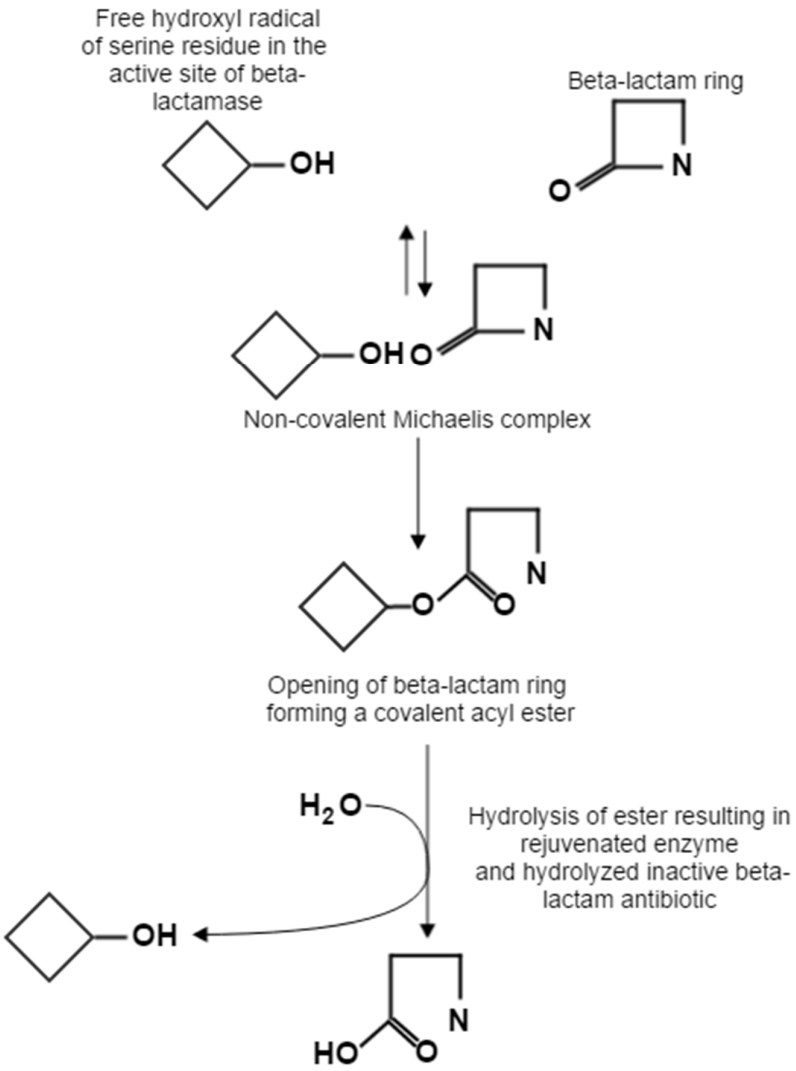
Beta-lactamases are classified as serine beta-lactamase when they have a serine radical or as metallo-beta-lactamases (MBLs) when they have zinc ion at the enzyme's active site. Typically, the inactivation of beta-lactam antibiotic involves acylation and deacylation steps. In the acylation step, the beta-lactam ring is opened forming an enzyme-acyl complex, which is then deacylated from serine following hydrolysis. While the acylation step requires nucleophilic serine, deacylation requires hydrolytic water molecule.

2.1. Mechanism of action of beta-lactamases

Following binding of the beta-lactam substrate in the active site of the beta-lactamase, a non-covalent (Henri-Michaelis) complex is formed. This step is reversible. The serine radical in the active site mounts a nucleophilic attack on the carbonyl leading to high-energy tetrahedral acylation intermediate. Protonation of the beta-lactam Nitrogen and cleavage of C-N bond results in opening up of the beta lactam ring and the intermediate then transitions into a lower-energy covalent acyl-enzyme complex.

An activated water molecule then attacks the covalent complex leading to high-energy tetrahedral deacylation intermediate. Hydrolysis of the bond between the beta-lactam carbonyl and the oxygen of the serine is then hydrolyzed, which regenerates the enzyme and releases the inactive beta-lactam molecule [13]. While the beta-lactam molecule gets destroyed, the enzyme is rejuvenated and becomes fully functional. Hydrolysis of beta-lactam molecule by serine beta-lactamase is depicted in Figure 5.

Figure 5: Mechanism of hydrolysis of beta-lactam molecule by beta-lactamase



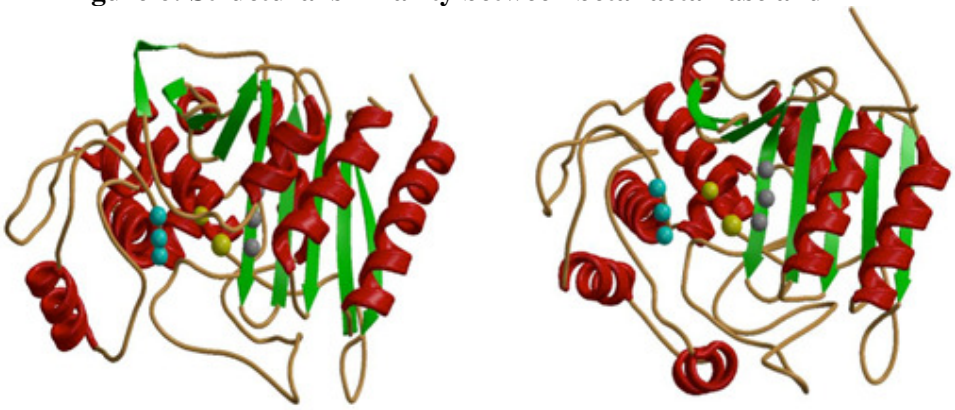
2.2 Molecular structure of beta-lactamases

Both the X-Ray crystallography and amino acid sequencing were pivotal in elucidating the molecular structure of beta-lactamases. In 1975, Ambler sequenced the entire beta-lactamase (*bla*) gene of penicillinase produced by *S. aureus* PC1 strain and that original numbering scheme of amino acids still remains the benchmark for numbering other serine beta-lactamases [14].

Beta-lactamases vary in several properties but the basic structure and amino-acid homology at the conserved regions suggests that they are phylogenetically related and that they have common evolutionary origin. Serine beta-lactamases and Penicillin Binding Proteins (PBPs) have similar tertiary folding, active-site topology and catalytic mechanism [15]. Hence, it has been proposed that serine beta-lactamases might have evolved from some ancestral D-D peptidases involved in synthesis and maintenance of peptidoglycan cell wall. Recently, a new family of PBPs with significant sequence similarity to serine beta-lactamases was discovered in a cyanobacterium, suggesting evolutionary relatedness between PBP and beta-lactamases [16]. The structural similarity between *E. cloacae* AmpC beta-lactamase P99 and PBP R61 of *Streptomyces sps* is shown in Figure 6.

X-ray crystallography have revealed the three-dimensional structure of beta-lactamases. The serine beta-lactamases are composed of two domains: α/β domains and α domain. The α/β domains is formed by five anti-parallel β-sheet surrounded by three α-helices on one face and one α-helix on the other. The α domain is formed entirely by α-helices. The catalytic activity of the active site is formed between these two domains [17]. A three dimensional structure of serine beta-lactamase is displayed in Figure 7. The characteristic arrangement of amino acids, which may also be found on other proteins is called a motif.

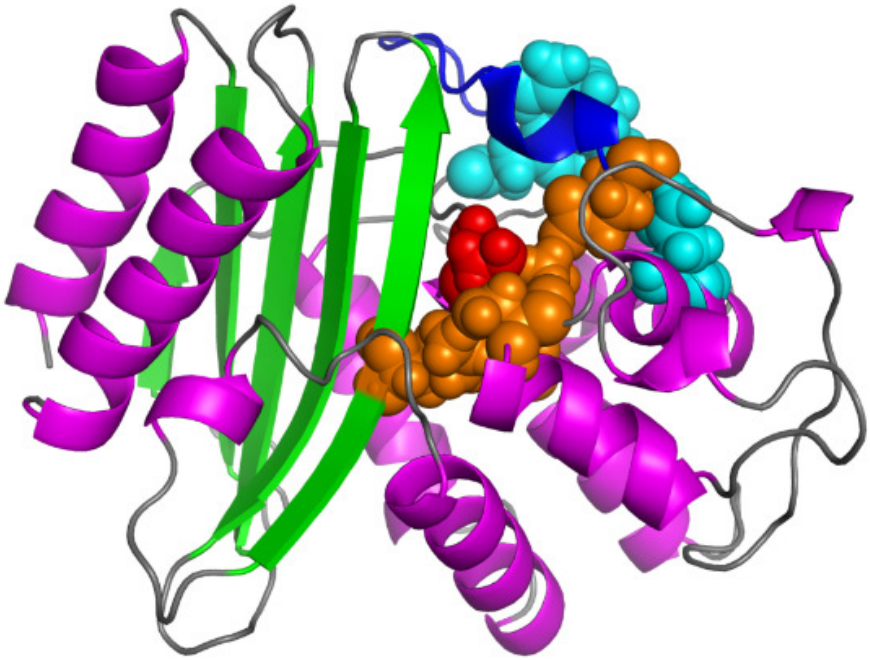
Figure 6: Structural similarity between beta-lactamase and PBP



beta-lactamase PBP

Similarity in tertiary folding and active-site topology is apparent. Coloured spheres represent amino acid residues that are involved in active sites.
(Image credit: Jean-Marie Frère. Nova Science Pub Incorporated)

Figure 7: Three dimensional structure of serine beta-lactamase



The catalytic residue Serine-70 is shown in red. Other catalytic residues are shown in orange, whereas the Ω-loop is shown in blue and the residues that maintain the structural integrity are shown in cyan. (Image credit: Verma D, *et al*. PLoS Comput Biol 9(7): e1003155.)

The highly conserved structural motifs in serine beta-lactamases include four polar regions:

1. Active-site pocket: Ser70 – X – X - Lys73 (where X can represent any amino acid)

2. "Ω-loop": residues 163 to 178

3. "SDN loop": Ser/Tyr130 - Asp131 - Asn132 (on a loop in the all α-domain)

4. Lys234 - Thr/Ser235 - Gly236 (on a β-sheet forming opposite wall of active site)

The "SDN loop" is believed to participate in maintaining the structure of the active site cavity and stability of the enzyme. The beta-lactam antibiotic in the active site cavity of beta-lactamase interacts with the dense hydrogen-bond network formed by the amino acid residues of the conserved structural motifs. The nucleophilic attack of the beta-lactam ring is initiated by the hydroxyl group of Serine at position 70. The strategically placed water molecule, which hydrolyses the acyl-enzyme bond is activated by glutamic acid, tyrosine or an unusual carbamylated lysine at position 166 located in the Ω-loop. [17]

Point mutations in the nucleotide sequences of *bla* gene leading to single amino acid substitution can have varying effect on the enzyme property. While silent mutations confer no change, other mutations may result in the transformation of the enzyme with enhanced hydrolytic capacity or even acquire resistance to inhibitors. By performing site-direct mutagenesis studies, scientists have been able to identify the significance of specific amino acids at specific locations.

The knowledge of molecular structure, especially the active site, is crucial in drug discovery and development. The various molecular interactions occurring between

the drug and the active site residues help scientists in the pharmaceutical industry to develop or modify drugs that can inhibit the activity of these enzymes.

2.3 Beta-lactamase inhibitors:

In order to counter the growing menace of enzyme mediated resistance to beta-lactam antibiotics, an earnest search was made to find suitable inhibitors. Beta-lactamases are inhibited by certain beta-lactam antibiotics as well as by beta-lactamase inhibitors, which also mimic beta-lactam structure. Reversible inhibitors (such as extended-spectrum cephalosporins, monobactams, and carbapenems) form acyl-enzymes and are able to bind to the active site of beta-lactamases with high affinity, but have the limitation of getting hydrolyzed very slowly. Irreversible inhibitors too act as substrates for beta-lactamases, but after hydrolysis, they persist in the active sites and inactivate the enzymes. Hence, irreversible inhibitors are also known as "suicide inhibitors" or "suicide inactivators". [18]

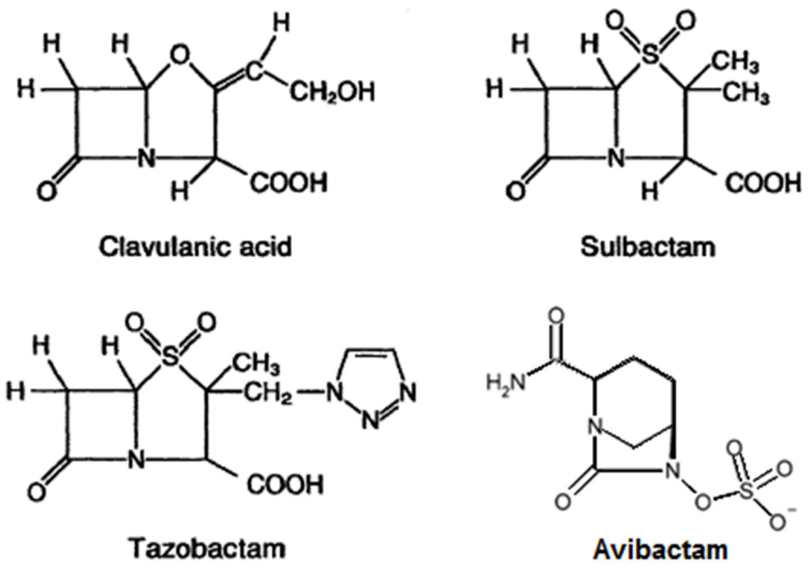


Figure 8: Structures of beta lactamase inhibitors in use

The first natural beta-lactamase inhibitor was obtained from a soil bacterium- *Streptomyces clavuligerus*. This organism was isolated from South American soil sample in 1971 while investigating potential beta-lactam antibiotic

producers. [19] Although it was found to produce a few antibacterial substances similar to cephalosporin, it was the discovery of a compound with anti-beta-lactamase property that caught the attention of researchers. This novel beta-lactamase inhibitor was named as clavulanic acid to indicate the source of this compound [20]. Subsequently, semi-synthetic beta-lactamase inhibitors such as sulbactam and tazobactam were introduced. Avibactam is a member of a new class of synthetic, non-beta-lactam inhibitors called the diazabicyclooctanes. Previously known as NXL104, avibactan is known to inhibit several beta-lactamases. It forms covalent enzyme-substrate complex in the enzyme's active site, leading to its deacylation but does not undergo hydrolysis and therefore, is a slow reversible inhibitor. It has been combined with ceftazidime and ceftaroline (a new fifth generation cephalosporin) [21]. The molecular structure of the common beta-lactamase inhibitors in use is displayed in Figure 8.

Like beta-lactam antibiotics, these inhibitors also bind to the active site of the beta-lactamase enzyme and undergo acylation. Following acylation, the beta-lactam ring is broken and forms a transient imine intermediate. The imine intermediate may then rearrange to form enamine intermediate in either cis- or trans- conformation. While tazobactam forms stable trans-enamine intermediates, clavulanic acid and sulbactam form a mixture of trans-enamine and the less stable cis-enamine intermediates. Prolonged inhibition of the enzyme occurs when this intermediate is stabilized. The sulphone and triazolyl moieties of tazobactam stabilizes the intermediate in the enzyme's active site. These properties make tazobactam a better inhibitor than the others. Depending on the nature of the enzyme and the inhibitor, the reaction may either proceed to deacylation or complete inhibition of the enzyme. In case of reversible inhibitors, the enamine intermediate undergoes decarboxylation and ester bond hydrolysis, which ultimately results in regeneration of the enzyme. However, this

reaction is very slow and may last beyond the generation time of the bacterium. Irreversible inhibitors permanently inactive the enzyme. [17]

Although these three inhibitors have similar mechanism of action, they have different pharmacological and inhibitory properties. Unlike sulbactam and tazobactam, clavulanic acid is a potent inducer of AmpC beta-lactamase. Compared to clavulanic acid, sulbactam is stable in solutions, lasts longer in body fluids and has intrinsic activity against *Bacteroides spp*, *Acinetobacter spp*, and *N. gonorrhoeae*. Tazobactam is more active against CTX-M type beta-lactamases than clavulanic acid or sulbactam. OXA-2 and OXA-32 beta-lactamases are inhibited by tazobactam but not by clavulanic acid or sulbactam. [17] The difference in the number of molecules required to inhibit different enzymes is displayed in Table 1. [17]

Table 1: Comparison of activities of three beta-lactamase inhibitors

	Number of molecules of inhibitor required to inactivate beta-lactamases		
	S. aureus PC1	TEM-1	SHV-1
Clavulanic acid	1	160	60
Sulbactam	Not Available	10,000	13,000
Tazobactam	1	140	5

The knowledge of these properties will enable physicians to select appropriate beta-lactamase inhibitor depending on the type of beta-lactamase produced by the infecting organism. Since beta-lactamases vary in their susceptibility to inhibition by beta-lactamase inhibitors, this property is a key factor used in the functional classification and characterization of beta-lactamases.

2.4 Characterization of beta-lactamases:

Characterization of beta-lactamases on various properties not only helps scientists in detecting new variants or types but also allow researchers to identify the existing types. Several methodologies have been used to characterize these enzymes, each with its own advantages and limitations. These methods include determination of substrate profile, inhibition of enzyme activity, determination of isoelectric point by isoelectric focusing, immunological reactivity to antisera, estimation of molecular weight, inducibility of enzyme, determination of MIC, determination of gene location, detection of specific sequences by labeled probes, and determination of nucleotide and amino acid sequences by molecular techniques.

Substrate profile indicates the hydrolytic activity of a beta-lactamase against a number of different beta-lactam substrates. An enzyme is considered as a penicillinase if benzylpenicillin is hydrolyzed at rate >30% that of cephalosporin. An enzyme is considered as a cephalosporinase if it hydrolyzes cephaloridine at a rate of >30% than that of benzylpenicillin. For penicillinases, benzylpenicillin has been used as the historical reference for hydrolysis, whereas cephaloridine was the chosen reference for cephalosporinases. For complete characterization, the enzymes are often tested against a panel consisting of benzylpenicillin, ampicillin, carbenicillin, cloxacillin, cefotaxime, ceftazidime, aztreonam and imipenem. Early studies used ratio of hydrolysis of the substrate against a chosen standard, such as penicillin G, but in in subsequent studies, rate of hydrolysis (*V*max) and binding affinity (*K*m) for a number of substrates were measured [22]. Substrate profile provides a valuable functional property of the enzymes and since not many laboratories are not equipped undertake these studies, most of the recently discovered enzymes remain uncharacterized.

Inhibition profiles were built using susceptibility of enzymes to inhibition by oxacillin, methicillin, aztreonam or non-beta-lactam compounds such as chloride ions and p- chloromercuribenzoate. Since the discovery of beta-lactamase inhibitors, clavulanic acid and sulbactam are used for comparing inhibition profiles of these enzymes [22]. The results of these methods are often unreliable when isolates express multiple beta-lactamase types. In order to obtain the inhibition profile of the enzyme, it must be made available in pure form, which is beyond the scope of many laboratories.

Isoelectric focusing is used to characterize and identify beta-lactamases based on their isoelectric points (pI). This is based on the principle that positively or negatively charged proteins move accordingly under electric field during electrophoresis. In this technique, the proteins are made to move in a gradient of pH and the pH at which the protein has no net charge is identified as its isoelectric point (pI). At its isoelectric point, the protein is focused as a narrow band, which can be visualized by detecting its enzymatic activity using chromogenic nitrocefin as the substrate. Enzymes, which are identical in their substrate profiles can been differentiated based on their pI values [23]. This technique is useful in differentiating multiple beta-lactamases produced by an isolate and also distinguishes chromosomal enzymes from plasmid mediated enzymes. Isoelectric points has been shown to vary from one laboratory to another and unrelated beta-lactamases may have similar pI values [22, 24]. Despite the advantages, not many laboratories are equipped to perform isoelectric focusing.

Immunological reactivity by specific antisera raised against beta-lactamase types were used to characterize beta-lactamases in early studies [25]. Beta-lactamases were identified by gel precipitation methods or by inhibition of substrate hydrolysis using specific antisera. As large number of new variants were being frequently reported,

it became impossible to raise antisera against all the types. High costs and frequent cross-reactions rendered this method obsolete.

Initially, the determination of molecular weight was a popular method of characterizing beta-lactamases. Molecular weight were determined by various techniques such as gel filtration, dodecyl sulphate-polyacrylamide gel electrophoresis, equilibrium centrifugation, or amino acid analysis. Estimation involved laborious process of purification as a preliminary step. Molecular weight estimation varied according to the method employed. Sometimes, beta-lactamases that differed in many aspects had identical molecular weight and molecular weights of different beta-lactamases were found to be similar when estimated by amino acid composition [22]. Currently, amino acid composition is used to deduce the molecular weight of a protein.

Mating assays for conjugal transfer of plasmids were used to determine the location of *bla* gene. Successful transfer of *bla* gene to the standard recipient strain indicated that the gene was located on a plasmid. This method is not always reliable as some *bla* genes may be present on non-conjugative plasmids. In some cases multiple attempts may have to be performed for successful conjugation. In order to avoid these potential problems, the plasmids are separated, cut with restriction enzyme, cloned into a vector and then transformed into a recipient strain. Expression of beta-lactamase in the recipient strain confirms the presence of *bla* gene on the plasmid [26]. These methods are labour intensive and expensive.

Induction tests are useful to determine if the enzyme is produced constitutively or can be induced by any other beta-lactam antibiotic. AmpC beta-lactamases are often inducible in gram negative bacilli such as *Enterobacter sps, Citrobacter sps, Proteus sps* and *Morganella sps*. Broth culture of beta-lactamase producing isolate is exposed

to the potential inducer at varied concentrations for two hours. After the protein synthesis is arrested by the addition of 8-hydroxyquinoline, the cells are harvested by centrifugation, washed in buffer and the pellet is sonicated to extract the enzymes. Beta-lactamase activity contained in the sonicate is determined against a suitable substrate such as nitrocefin or cephalothin. A positive result indicates that the enzyme is inducible [27].

Estimation of MIC levels of various beta-lactam antibiotics against beta-lactamase producing bacteria is a useful marker of the enzyme activity. MICs can be estimated by microbroth dilution, agar dilution, E-test strips or by automated systems. MIC values are affected by several factors such as inoculum density, co-production of other beta-lactamases, loss of porins and the nature of beta-lactamase expression. In order to avoid these pitfalls, the plasmid encoding *bla* are cloned in standard recipient strains and then the MICs are estimated.

DNA hybridization with labelled probes were used in early studies to detect specific beta-lactamases. This method could also detect chromosomal location of *bla* gene. The procedure involves extraction of plasmids, digestion by restriction endonulcease enzymes, electrophoresis on gel, Southern blot transfer to nylon/nitrocellulose membranes, hybridization by labeled probes followed by visualization [28]. As more and more mutant forms of these enzymes were detected it was not possible to design probes for all of them. In order to attain accurate hybridization, high levels of stringency are required to be maintained. Since silent mutations can code for the same amino acid, mere nucleotide substitutions are not helpful in characterizing a beta-lactamase. After the advent of PCR, probe hybridization has fallen into disuse.

Currently, many laboratories clone the entire *bla* gene by PCR and obtain its complete nucleotide sequence. Both the nucleotide sequences and their translated amino acid sequences are maintained in public databases (GenBank). Using appropriate bioinformatics tools, researchers can now easily identify and characterize beta-lactamases. A big disadvantage of this method is that it provides no clue on the functional properties of the enzyme.

The methods to characterize the beta-lactamases are many; but it is beyond the means of most laboratories to undertake all of them and are thus restricted only to research laboratories. As more and more beta-lactamases were discovered and characterized by the aforementioned methods, it is only logical that these enzymes had be grouped or classified.

2.5 Classification of beta-lactamases:

In 1968, Sawai and Mitshashi attempted classification of beta-lactamases for the first time. Thereafter, few other schemes were proposed by Jack and Richmond, Richmond and Sykes and Sykes and Matthew [29-32]. These schemes were based on physiological properties of the enzymes such as inducibility, hydrolytic prolife, inhibitory profile, isoelectric point and molecular weight. The key features of these initial classification schemes is summarized in the Table 2.

Presently, the two popular classification schemes are derived from the works of Ambler RP and Karen Bush. In 1980, Ambler proposed the "phylogenetic" or "molecular" classification based on the amino-acid sequences of the beta-lactamases. In his classification, he divided beta-lactamases into two groups: Class A (serine beta-lactamases) and Class B (metallo-beta-lactamases) [33]. Class C, consisting of AmpC beta-lactamases was added subsequently by Jaurin and Grundstrom in 1981 [34]. In

1988, Huovinen P *et al* expanded this classification by including Class D, which encompasses oxacillinases (OXA-type) [35].

Table 2: Key features of the initial classifications of beta-lactamases

Year	Authors	Key features
1968	Sawai T & Mitsuhashi S	a) inducible cephalosporinase that hydrolyzes cehaloridine efficiently but not penicillins b) inducible cephalosporinase that also had penicillinase property c) Constitutive penicillinase
1970	Jack GW & Richmond MH	Enzymes were grouped into eight types (Enzyme types 1 to 8) on the basis of hydrolysis to benzylpenicillin, ampicillin, cephaloridine and susceptibility to anti-TEM serum, p-chloromercuribenzoate and cloxacillin.
1973	Richmond MH & Sykes RB	Enzymes were grouped into classes I to V based on substrate profile and inhibition of beta-lactamases; classified as penicillinases, cephalosporinases or broad-spectrum; location of *bla* gene on chromosome or plasmid was also considered
1976	Sykes RB & Matthew M	Additional parameters such as isoelectric point (pI values) and molecular weight of beta-lactamases were included

Building on the foundations laid by Richmond and Sykes, Karen Bush proposed another classification based on physiological properties of beta-lactamases in 1988 [36]. After a minor update in 1989, it was re-launched as the "functional" classification by the team of Bush K, Jacoby GA and Medeiros AA [37- 39]. It was updated again in 2010 [40]. Based on the substrate and inhibitory profiles, beta-lactamases were divided into three groups: 1, 2 and 3. Based on the differences among the enzymes in these

groups, they were further divided into several subgroups. Among the three groups, group 2 has the most number of subgroups.

Despite being based on physiological properties, this classification is in good agreement with Ambler's molecular classification. AmpC beta-lactamases fall under Bush's group 1 and Ambler's class C, whereas metallo-beta-lactamases fall under Bush's group 3 and Ambler's class B. Rest of the serine beta-lactamases were included in Ambler class A, whereas Bush divided them into 11 subgroups under group 2. Bush's functional classification in relation to Ambler's molecular classification is displayed in Table 3.

Among the various subgroups listed in Bush's classification, most enzymes that are encountered in clinical isolates of Gram negative bacilli belong to the subgroups of group 2. In the functional classification, the letter "e" in the subgroup names is used to denote extended spectrum of activity. The subgroup 2b comprises of enzymes that hydrolyze penicillins and early cephalosporins and are inhibited by clavulanic acid. These enzymes are sometimes termed as "broad-spectrum" or "classical" beta-lactamases as they exhibit better activity against first and second generation cephalosporins.

Subgroup 2be includes enzymes that are able to hydrolyze penicillins, early cephalosporins and one or more of the third generation cephalosporins or monobactams. Because of their extend activity against third generation cephalosporins, they are called Extended Spectrum Beta-Lactamases (ESBLs).

Table 3. Functional classification by Bush, Jacoby and Medeiros

Group and subgroup	Molecular class (Ambler)	Preferred substrate	Inhibited by		Representative enzymes
			CA/TZB	EDTA	
1	C	Cephalosporins	-	-	MIR-1, ACT-1, CMY-2, FOX-1
1e	C	Cephalosporins	-	-	GC-1, CMY-37
2a	A	Penicillins	+	-	PC1
2b	A	Penicillins, early cephalosporins	+	-	TEM-1, TEM-2, SHV-1
2be	A	ESC, monobactams	+	-	TEM-3, SHV-2, CTX-M-15, K1, PER-1, VEB-1
2br	A	Penicillins	-	-	TEM-30, SHV-10
2ber	A	ESC, monobactams	-	-	TEM-50
2c	A	Carbenicillin	+	-	PSE-1, CARB-3
2d	D	Cloxacillin	±	-	OX-1, OXA-10
2de	D	ESC	±	-	OXA-11, OXA-15
2df	D	Carbapenems	±	-	OXA-23, OXA-48
2e	A	ESC	+	-	CepA
2f	A	Carbapenems	±	-	KPC-2, IMI-1, SME-1
3a	B	Carbapenems	-	+	IMP-1, L1, NDM-1, VIM-1
3b	B	Carbapenems	-	+	CphA, Sfh-1

CA: clavulanic acid, TZB: tazobactam, ESC: Extended spectrum cephalosporin

Inhibition by clavulanic acid is a characteristic property of ESBLs. Subgroup 2br includes broad-spectrum beta-lactamases having hydrolytic properties similar to those

in group 2b, but are characteristically resistant to inhibition by clavulanic acid. These enzymes are also referred to as Inhibitor-Resistant TEMs (IRTs), as they were initially recognized among TEM types. Subgroup 2ber includes mutant forms of enzymes that have inhibitor resistant property of group 2br as well as extended activity against third generation cephalosporins. These are sometimes referred to as Complex Mutant Types (CMT) [40].

The Ambler's classification is convenient to group the enzymes based on molecular relatedness but Bush's classification seems to be more practical as the subgroups correlate well with their functional properties. Bush is of the opinion that functional properties of the enzymes will allow the clinicians to make informed selections of appropriate antibiotics for therapy [41]. The system of classification is essential to group the related members together and at the same time, also brings an order in their nomenclature.

2.6 Nomenclature of beta-lactamases

The absence of any naming convention in the past led the discoverers of beta-lactamases to name the enzymes in a variety of ways. Beta-lactamases were named after the places of discovery, names of the bacterial species or strain, gene, plasmid, patient, hospital, the discoverer or some property of the enzyme [42]. Nomenclature of some beta-lactamases has since, undergone some revisions. Some enzymes were renamed, as they turned out to be identical to the enzymes previously described. The names of a few variants have been withdrawn for the same reason. As per the present naming convention, once the name of an enzyme gets accepted, subsequent new variants are named by incrementing the number. (TEM-1, TEM-2, TEM-3 etc.). The disadvantage of this naming scheme is that even if a new variant possess a considerably different

property, its name doesn't does not give any clue about the new property. Of all the names that have been given to a class or a family of beta-lactamases, none gives more significance to its property than the name "Extended Spectrum Beta-Lactamases".

3. Extended Spectrum Beta-Lactamases (ESBL):

In 1988 Jarlier *et al* used the term "extended broad-spectrum beta-lactamases" to refer those enzymes which had 'extended' activity compared to the 'broad spectrum activity' of classical TEM or SHV enzymes [43]. In subsequent publications, the term 'broad' was dropped and the name "Extended-Spectrum Beta-Lactamase" became established [44].

3.1 Definition and properties

ESBLs are enzymes whose rates of hydrolysis of the extended-spectrum beta-lactam antibiotics such as ceftazidime, cefotaxime, or aztreonam are >10 % than that for benzylpenicillin. These are susceptible to inhibition by beta-lactam inhibitors such as clavulanic acid, tazobactam, or sulbactam but have no hydrolytic activity against cephamycins and carbapenems [41]. In the light of discovery of newer beta-lactamases and their hydrolytic profiles, the existing definition of ESBL is being strongly contested. Livermore insisted that all beta-lactamases conferring resistance to extended-spectrum cephalosporins should be considered as ESBLs without taking into account an arbitrary cut-off value of >10% hydrolysis rate [45]. Giske *et al* proposed that the definition of ESBLs should include AmpC beta-lactamases and carbapenemases [46]. The debate over the two alternate proposals by Livermore and Giske, respectively, shows that the definition and classification of ESBLs might undergo some changes in future [47]. Despite the ongoing debate, there is global acceptance of the current definition.

Using the current definition, ESBLs have been detected among several bacteria, although members of Enterobacteriaceae remain their chief hosts. They have also been encountered among a few non-Enterobacteriaceae bacteria such as *Pseudomonas spp*, *Stenotrophomonas spp, Acinetobacter spp, Vibrio spp,* and *Haemophilus spp*, among others [48-50]. Despite the diversity in their hosts, the ESBLs predominantly belong to a few limited types.

Depending on the general prevalence, ESBLs are broadly grouped into major and minor ESBL types. Major ESBL are commonly expressed by many clinical isolates and are detected in many parts of the world whereas the minor types are rarely encountered or are restricted to certain geographical locations only. Currently, the three major ESBL types are TEM, SHV and CTX-M.

3.2 TEM type ESBLs:

All the beta-lactamases among Gram negative bacteria known to scientists until 1965, were chromosomally encoded. The first report of a plasmid encoded beta-lactamase in Gram negative bacilli came in 1965 from Athens, Greece [51]. The transferable plasmid, named RTEM1, encoding resistance to ampicillin and streptomycin, was detected in an *E. coli* TEM strain cultured from the blood of a female patient by the name Temoniera. In the subsequent literature, this beta-lactamase came to be known as TEM-1. TEM-2, a variant of TEM-1 that differed by one amino-acid substitution was detected in 1979, but its hydrolytic properties were similar to that of TEM-1 enzyme [52].

In 1985, strains of *K. pneumoniae* with transferable resistance to higher cephalosporins were reported from French hospitals. The beta-lactamase detected in those strains was named CTX-1 to denote its hydrolytic activity against cefotaxime

[53]. Amino acid sequencing revealed that it differed from TEM-2 beta-lactamase by two amino acid substitutions; Lys104Glu and Ser238Gly, which conferred it the ability to hydrolyze extended spectrum cephalosporins [54]. It was ultimately renamed as TEM-3 and is the first ESBL of the TEM type to be discovered.

The *bla*TEM gene codes for a peptide of 286 amino acids, but first 23 amino acids form the signal sequence at the N-terminal, and is cleaved to form the mature enzyme. ESBL phenotype among TEM enzymes is associated with amino acid substitutions at positions 104, 164, 238, or 240. The prominent substitutions include Glu104Lys, Arg164Ser, Arg164His, Gly238Ser, and Glu240Lys. Substitutions occurring in the Ω-loop (positions 164, 179) and β3 sheet (position 238) are critical for change in the hydrolytic spectrum of these enzymes [55]. Random mutations in the nucleotide sequences of *bla* gene are responsible for these substitutions, but further selections of these mutants result from the selective pressures exerted by exposure to antibiotics. At least 90 mutations occurring at different locations are documented among *bla*TEM genes (http://www.lahey.org/studies/temtable.asp). Saturation mutagenesis study reveal that 220 out of 263 amino-acid positions of the mature TEM-1 protein can tolerate substitutions [56]. This suggests that further evolution of TEM beta-lactamases is possible.

Currently there are 219 TEM type beta-lactamases documented in the website-http://www.lahey.org/studies/temtable.asp. Of these, only 92 (42%) are ESBLs belonging to 2be subgroup, 16 (7.3%) are non-ESBLs belonging to 2b subgroup, 38 (17.4%) are inhibitor resistant type belonging to 2br subgroup, 11 (5%) are beta-lactamases with both extended-spectrum activity and inhibitor resistant property belonging to 2ber subgroup (CMT) and the rest 62 (28.3%) are currently unclassified.

Globally, the most commonly reported TEM type ESBLs include TEM-3, TEM-5, TEM-10, TEM-12, TEM-26 and TEM-52. Prevalence of some of the TEM ESBLs appear to be restricted to certain areas whereas few others are globally distributed. Reports of TEM-10 from USA and Europe, TEM-3 from France, TEM-47 from Poland and TEM-52 from South Korea indicate their restrictive distributions [24].

3.3 SHV type ESBLs:

First account of a plasmid mediated beta-lactamase in *Klebsiella sps* was made by Pitton in 1972 [57]. O'Callaghan *et al* named the enzyme as Pit-2 to honor Pitton. In 1979, Matthew M renamed it as SHV-1 (for sulfhydryl variable) as he believed that the sulphydryl group of the enzyme was inhibited by p-mercuricholorobenzoate [58]. Despite the fact that the active site contained hydroxyl group instead of the assumed sulphydryl group, the name SHV gained global acceptance. Complete amino acid sequence of SHV-1 revealed that it shared only 68% amino acid homology with TEM-1 enzyme [59]. SHV type beta-lactamases are often chromosomally encoded in *K. pneumoniae*, which have since escaped on to the plasmids.

In 1983, plasmid mediated resistance to cefotaxime in clinical isolates of *K. pneumoniae* and *S. marcescens* was described from Germany [60]. Kliebe *et al* took one strain of *Klebsiella ozaenae* from that collection for further studies and after confirming the ESBL phenotype, it was designated as SHV-2 [61]. This became the first ESBL to be discovered. Amino acid sequencing of SHV-2 revealed that it differed from SHV-1 by a single amino acid substitution- Gly213Ser [62]. As per Ambler's present numbering scheme, that position is now identified as 238. Substitutions of amino acids at positions 146, 156, 169, 179, 205, 238 or 240 are responsible for ESBL

phenotype in SHV enzymes [55]. Globally SHV-2, SHV-2a, SHV-5, and SHV-12 are the common SHV type ESBLs.

Currently there are 185 SHV types documented in the website- http://www.lahey.org/studies/. Of these, only 47 (25.4%) are ESBLs belonging to 2be subgroup, 37(20%) are non-ESBL belonging to 2b subgroup, seven (3.8%) are non-ESBL inhibitor resistant types belonging to 2br subgroup and the rest 94 (50.8%) are yet to be classified.

3.4 CTX-M type ESBLs

In 1988, a new type of non-TEM, non-SHV plasmid mediated ESBL was reported in fecal *E. coli* obtained from laboratory dogs from Japan [63]. Named FEC-1, this ESBL had prominent cefotaximase activity as it conferred high-level resistance to cefotaxime and ceftriaxone but not to ceftazidime. Although this was the first description of a new class of ESBL, it remained obscure for several years. In 1990, a similar type of ESBL was discovered in an *E. coli* strain isolated from the ear exudate of a neonate in Munich, Germany [64]. Because of its predominant cefotaximase activity and the location of its isolation (Munich), the enzyme was named CTX-M. Around the same time, an *E. coli* MEN strain producing a similar enzyme, designated as MEN-1, was reported from France [65] Amino acid sequencing revealed that both CTX-M and MEN-1 enzymes were identical and the name CTX-M-1 gained acceptance.

Thereafter, several variants of CTX-M enzymes were discovered from the various parts of the world; those with amino acid homology of ≤90% formed separate groups and those with >94% homology were grouped together as members of a group. Presently, CTX-M ESBLs are divided into five clusters or groups where members of

each group share >94% amino acid homology and members across the groups have ≤90% homology [66]. The five groups of CTX-M are CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. By convention, each group is named after the first member of the group that was described. Recent studies suggest that CTX-M-45 might represent a new group and that CTX-M group 1 can be subdivided into two subgroups, A and B [67, 68]. The discoveries of major CTX-M groups and their members is displayed in Table 4. As of now, 158 allelic variants of CTX-M enzymes have been catalogued. Among the five CTX-M groups, CTX-M-1 and CTX-M-9 groups are the most diverse. While CTX-M-8 group is the smallest with only three members, CTX-M-9 group is the largest group with over 40 variants.

In 2013, Zhao and Hu used the aligned amino acid sequences of 124 CTX-M ESBLs to derive a phylogenetic tree [69]. By comparing the amino acid homology, they identified seven clusters, namely cluster 2, cluster 3, cluster 8, cluster 14, cluster 25, cluster 45 and cluster 64 (Figure 9). Based on the central positions in the tree, CTX-M-2, CTX-M-3, CTX-M-8, CTX-M-14, CTX-M-25, CTX-M-45 and CTX-M-64 were chosen as the representative enzymes in each cluster.

Phylogenetic studies suggest that *bla*CTX-M genes have evolved from chromosomal *bla* genes of *Kluyvera sps.* The *bla*CTX-M gene encodes a 291 amino acid enzyme although insertions and deletions in a few variants have sequences ranging from 282 to 292 amino acids. Even a single amino acid change in the gene constitutes a new CTXM type. After the first 28 amino acids at the N-terminal are cleaved, the mature enzyme contains 263 amino acids.

Figure 9: Phylogenetic tree of CTX-M created by Zhao and Hu

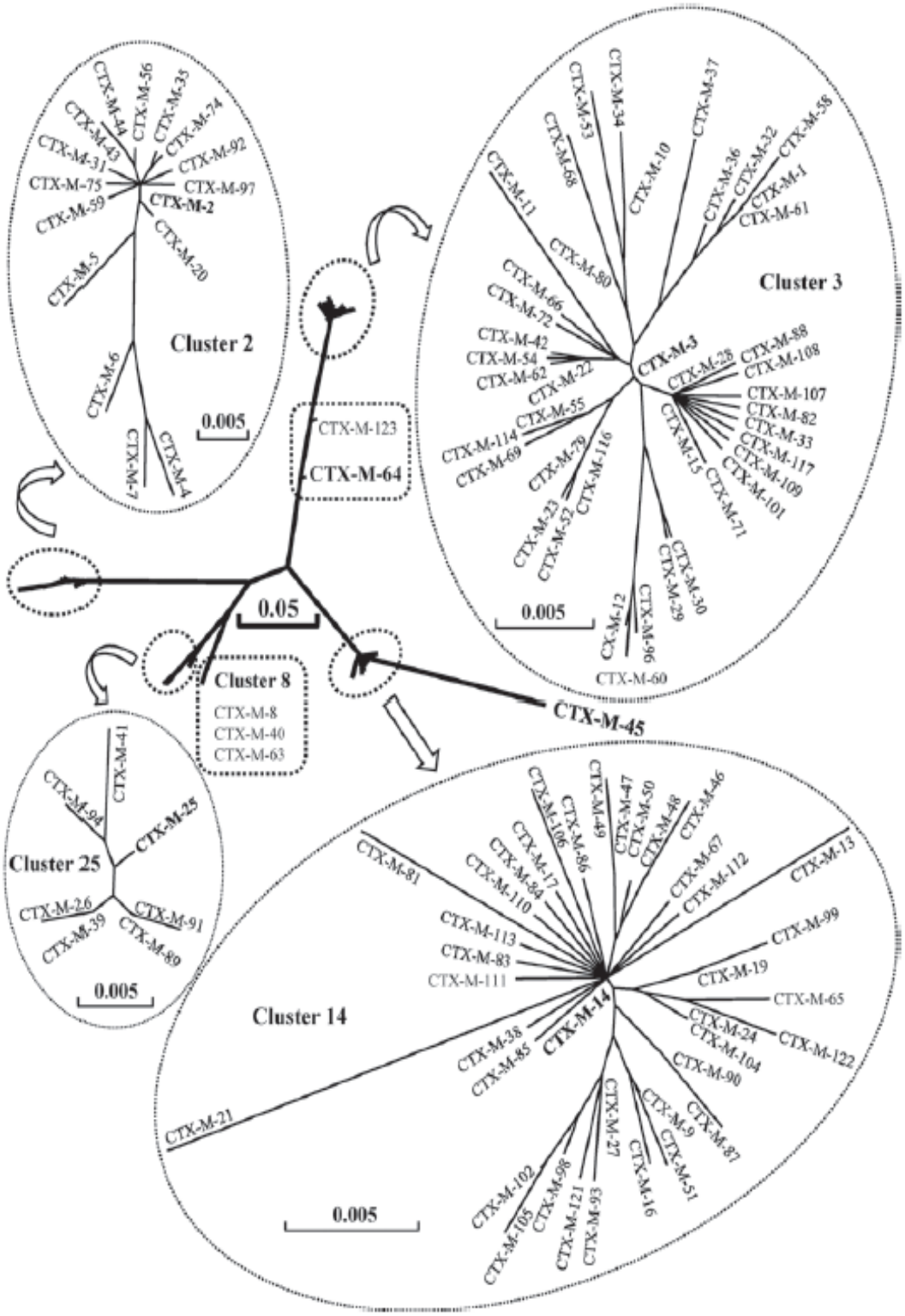


Table 4: CTX-M groups along with their year and place of detection

Year	Country	Source	Old/present name	Group	Reference
1998	Japan	*E. coli*	FEC-1	CTX-M-1	[63]
1990	Germany	*E. coli*	CTX-M/ CTX-M-1	CTX-M-1	[64]
1992	France	*E. coli*	MEN-1/ CTX-M-1	CTX-M-1	[65]
1992	Argentina	*S. typhimurium*	CTX-M-2	CTX-M-2	[70]
1995	Japan	*E. coli*	Toho-1CTX-M-44	CTX-M-2	[71]
1998	Japan	*E. coli*	Toho-2/CTX-M-45	?CTX-M-45	[72]
1998	Poland	*C. freundii, E. coli*	CTX-M-3	CTX-M-1	[73]
2000	Brazil	*E. cloacae, E. aerogenes, C. amalonaticus*	CTX-M-8	CTX-M-8	[74]
2000	Spain	*E. coli*	CTX-M-9	CTX-M-9	[75]
2001	India	*E. coli, K. pneumoniae, E. aerogenes*	CTX-M-15	CTX-M-1	[76]
2004	Canada	*E. coli*	CTX-M-25	CTX-M-25	[77]

The ribbon picture of TEM-1 beta-lactamase showing locations of key amino acids and the comparative sequences of TEM, SHV and CTX-M ESBLs in relation to beta-lactamase of *S. aureus* PC1 strain is shown in Figure 10 and 11, respectively.

CTX-M beta-lactamases share less than 40% identity with TEM and SHV-types. While only some TEM and SHV types are ESBLs, all CTX-M enzymes express ESBL phenotype. In TEM and SHV ESBLs, the increased activity towards oxyimino-cephalosporins is due to increased active site volume, which serves to accommodate bulkier side chains. In case of CTX-M enzymes, the flexibility of the β3 strand and Ω loop allows it to accommodate the bulky cephalosporins [78, 79]. CTX-M-9 and CTX-M14, which are characteristic of the overall family, possess extraordinarily high hydrolytic activity (>1000 folds) against cefotaxime than ceftazidime [79]. The CTX-M ESBLs discovered initially were chiefly cefotaximases and the first CTX-M ESBL to have significant hydrolytic activity against ceftazidime was CTX-M-15, which was reported from India [76]. Besides CTX-M-15, CTX-M-16, CTX-M-19 and CTX-M-27 too have significant activity against ceftazidime [78]. In fact, CTX-M-16 and CTX-M-27 are at least four times more active against ceftazidime than cefotaxime. The substitution Asp240Gly is responsible for the enzymes' enhanced activity against ceftazidime molecule in CTX-M-15, CTX-M-16, and CTX-M-27, whereas the substitution Pro167Ser serves the same purpose in CTX-M-19.

While some CTX-M enzymes have remained endemic in the region where they originated, few others have spread all over the world. CTX-M-1 in Italy, CTX-M-2 in Argentina, CTX-M-3 in Poland, CTX-M-9 Spain, and CTX-M-14 in Spain, Canada, and China are examples of endemicity. CTX-M-15, which is endemic in India is now the most dominant CTX-M type worldwide. [68, 80, 81] In almost every country, CTX-M ESBLs are being reported and therefore the term "CTX-M β-lactamase pandemic" coined by Canton *et al*. seems more befitting [81].

Figure 10. Key positions of amino acids of TEM-1 beta-lactamase

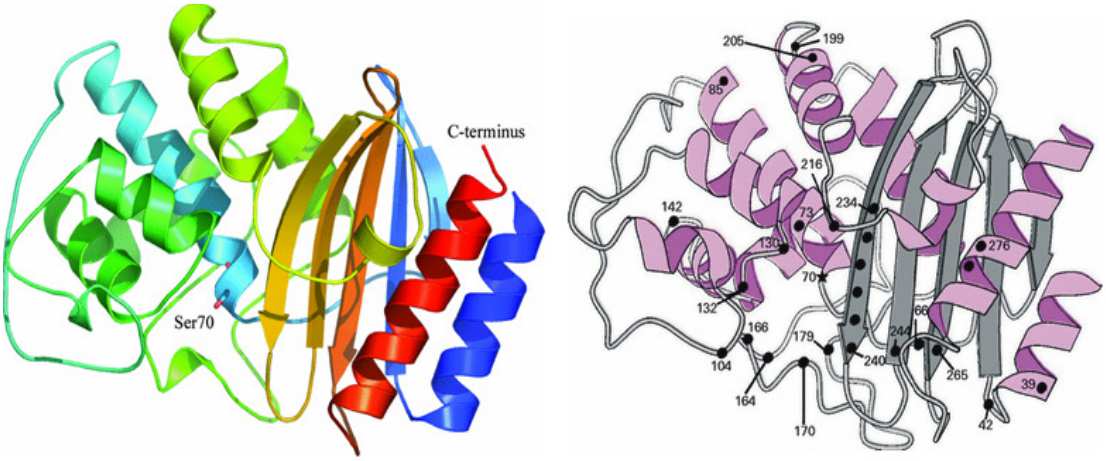


Figure 11: Amino acid sequences of PC1, TEM-3, SHV-12 and CTX-M-15

```
                10        20        30        40        50        60        70        80
                 |         |         |         |         |         |         |         |
PC1       ----------MKKLIFLIVIALVLSACNSNSSHAKELNDLEKKYNAHIGVYALDTKSGKEV-KFNSDKRFAYASTSKAIN
TEM_3     ---MSIQHFRVALIPFFAAFCLPVFAHPETL---VKVKDAEDKLGARVGYIELDLNSGKILESFRPEERFPMMSTFKVLL
SHV_12    -----MRYIRLCIISLLATLPLAVHASPQPL---EQIKQSESQLSGRVGMIEMDLASGRTLTAWRADERFPMMSTFKVVL
CTX_M_15  MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQI-LYRADERFAMCSTSKVMA
                90       100       110       120       130       140       150       160
                 |         |         |         |         |         |         |         |
PC1       SAILLEQVPY--NKLNKKVHINKDDIVAYSPILEKYVGKDITLKALIEASMTYSDNTANNKIIKEIGGIKKVKQRLKELG
TEM_3     CGAVLSRVDAGQEQLGRRIHYSQNDLVKYSPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGPKELTAFLHNMG
SHV_12    CGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCAAAITMSDNSAANLLLATVGGPAGLTAFLRQIG
CTX_M_15  AAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTMSLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLG
               170       180       190       200       210       220       230       240
                 |         |         |         |         |         |         |         |
PC1       DKVTNPVRYEIELNYYSPKSKKDTSTPAAFGKTLNKLIANGKLSKENKKFLLDLMLNNKSGDTLIKDGVPKDYKVADKSG
TEM_3     DHVTRLDRWEPELNEAIPNDERDTTMPAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSG
SHV_12    DNVTRLDRWETELNEALPGDARDTTTPASMAATLRKLLTSQRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTG
CTX_M_15  DETFRLDRTEPTLNTAIPGDPRDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTG
               250       260       270       280       290
                 |         |         |         |         |
PC1       QAITYASRNDVAFVYPKGQSEPIVLVIFTNKDNKSDKPNDKLISETAKSVMKEF--  281
TEM_3     AS-ERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQ-IAEIGASLIKHW--  286
SHV_12    AS-KRGARGIVALLGPNNKAERIVVIYLRDTPASMAERNQQ-IAGIGAALIEHWQR  286
CTX_M_15  SG-GYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDV-LASAAKIVTDGL--  291
```

Note: numbering is not as per Ambler's scheme. Red colour indicates similarity and likely indicates conserved region.

Key: G-Glycine-Gly, P-Proline-Pro, A-Alanine-Ala, V-Valine-Val, L-Leucine-Leu, I-Isoleucine-Ile, M-Methionine-Met, C-Cysteine-Cys, F-Phenylalanine-Phe, Y-Tyrosine-Tyr, W-Tryptophan-Trp, H-Histidine-His, K-Lysine-Lys, R-Arginine-Arg, Q-Glutamine-Gln, N-Asparagine-Asn, E-Glutamic Acid-Glu, D-Aspartic Acid-Asp, S-Serine-Ser, T-Threonine-Thr

Until the 1990s, TEM and SHV types were the predominant ESBLs but now, CTX-M ESBL seems to have displaced them. Once regarded as minor ESBLs, CTX-

M are now the major ESBLs [82]. At this rate, it can be speculated that in future the TEM and SHV ESBLs might become one among the minor ESBLs.

3.5 Minor ESBLs

The minor ESBLs are rarely encountered types that are not related to any of the three major ESBL types. Currently these include OXA, PER, VEB, SFO, BES, BEL, TLA, and GES types. The origins of many of these enzymes are currently unknown and many of them are currently classified under class A or D of Ambler's classification. These ESBLs can be either chromosomal or plasmid encoded. Chromosomal ESBLs have been observed in bacteria like *K. oxytoca* (K1/KOXY), *Proteus vulgaris* (CumA), *Proteus penneri* (HUGA), *Erwinia persicina* (ERP-1) , *Citrobacter sedlakii* (SED-1), *Kluyvera spp* (KLUA, KLUC, KLUG), *Serratia fonticola* (FONA), and *Rahnella aquatilis* (RAHN-1) [83].

Presence of at least 430 allelic variants makes OXA type, the most common plasmid mediated minor ESBL. These are basically oxacillinases, some of which have extended spectrum of activity against oxyimino-cephalosporins or carbapenems. Derivatives of OXA-2, such as OXA-15 and OXA-18 and the derivatives of OXA-10 such as OXA-11, OXA-14, OXA-16, and OXA-19 show ESBL like property, although they are not well inhibited by clavulanic acid. Initially detected among *P. aeruginosa* in Turkey, these enzymes are now also being detected among Enterobacteriaceae members. The other two frequently reported minor ESBLs include VEB and PER types, which too are reported more commonly among *Pseudomonas spp* than Enterobacteriaceae members [48]. Other types such as SFO, GES, BES, BEL and TLA types are rarely encountered or reported only in countries where they are endemic [83].

In the last two decades, the world has witnessed an explosive increase in the numbers and types of beta-lactamases. In this period, one minor ESBL has transformed into major type and few major types are on the verge of becoming minor types. It is intriguing to note that the combined effects of selective pressure from the antibiotic abuse and the roles played by the mobile genetic elements have contributed immensely in the evolution of beta-lactamases.

3.6 Evolution of beta-lactamases

Since the discovery of plasmid (R factor) mediated antibiotic resistance in the 1940s, the scientific community has witnessed different strategies adopted by the bacteria to acquire and disseminate antibiotic resistance. The SHV type ESBLs have originated from the chromosomal *bla*SHV of *K. pneumoniae*. The fact that first *bla* gene in *Klebsiella sps* was found on a transposon suggests that this mobile genetic element has played a role in the escape of genes from the chromosome into the plasmid [57]. The origin of TEM enzymes is less certain, but they are almost always found encoded on plasmids with exceptions of TEM-12 and TEM-134, whose genes were found on chromosomes [84]. The origin of CTX-M genes have been traced to the chromosomal *bla* genes of *Kluyvera spp*. Phylogenetic studies suggest that *bla*CTX-M genes of CTX-M-1 cluster has evolved from chromosomal *bla* genes of *K. cryocrescens*, CTX-M-2 cluster from K. *ascorbata* whereas CTX-M-8, CTX-M-9 and CTX-M-25 clusters have evolved from *K georgiana* [80]. Mobile genetic elements such as transposon (Tn*1*, Tn*2*, Tn*3,* Tn*21*), integrons (Class 1, 2), insertion sequences (IS*Ecp1*, IS*26,* IS*CR1*) and even bacteriophages are believed to have helped these genes cross the genus barrier [85]. While mutations are the primary reason for the large numbers of allelic variants, recombination events too have played a role [86].

4. Epidemiology of ESBL producers

4.1 Dissemination of ESBL producers

ESBL producing organisms have been detected from almost all the continents of this planet, including Antarctica [87]. CTX-M type ESBLs have been isolated from the tribal population in an Amazonian village, which had very little exposure to the modern world [88]. In that study, TEM-52 was the only ESBL detected when samples were taken in 2001 but the scene had changed completely when the study team revisited the place in 2006. By then, the intestinal carriage rates had jumped from 3.2% to 8% and CTX-M-2 and SHV-2 were the only ESBL types found among them. It is not clear how the change happened, but is likely that these bacteria acquired some resistance plasmids from non-resident visitors.

Antibiotic resistance genes are transferred horizontally among bacteria mainly by conjugation. While plasmids of narrow host range such as (IncFI, IncFII, IncHI2 and IncI) serve to disseminate gene within the species or very closely related species, the broad host-range (IncN, IncP-1-α, IncL/M and IncA/C) plasmids disseminate genes across different species. Dissemination of *bla*CTX-M-15 in bacteria across the world seems to be associated with IncFII plasmids [89]. Transfer of promiscuous plasmids alone does not explain the spread of ESBL producers. In 2008, simultaneous emergence of *E. coli* O25:H4-ST131 clone in samples collected across three continents was reported [90]. This clone was multi-drug resistant, possessed IncFII plasmid encoding multiple drug resistance (including *bla*CTX-M) and belonged to the virulent phylogenetic group B2. This clone is now being increasingly reported from many parts of the world, including India [91]. This pandemic clone has been isolated from healthy persons, patients, companion animals, food as well as the environment. Travel to

countries such as India and Pakistan has been shown to be associated with infection by *E. coli* ST 131 strains producing CTX-M-15 ESBLs. Hence, the clone *E. coli* ST131 is also believed to have played a role in worldwide dissemination of CTX-M-15 ESBLs [92].

4.2 Risk factor for acquisition of ESBL producers:

Several studies have been undertaken to find the association between several well characterized risk factors and colonization or infection by the ESBL producers. Many studies have reported isolation of ESBL producing organisms from raw vegetables, fruits as well as food of animal origins [93-95]. It is most likely that these animals might have acquired the ESBL producers from human contacts, at the same time, origin among themselves cannot be entirely ruled out. Although there are not many reports indicting human acquisition of ESBL producers following consumption of contaminated animal food, the risk is always there [96].

Some of the risk factors that have been positively associated with increased colonization or infection by ESBL producers include Asian/African country of birth, travel to Asian/Middle-East countries, hospital stay of more than seven days, nursing home residence, transfer from another health care facility, stay in surgical ward, low birth weight, intra-abdominal surgery, invasive procedures, presence of central venous catheter, hemodialysis, diabetes mellitus, and antibiotic use (especially third-generation cephalosporins and quinolones) for more than a week [97-102].

4.3 Global distribution of ESBL producers:

Several national and international collaborative studies such as SENTRY Antimicrobial Surveillance Program, Meropenem Yearly Susceptibility Test Information Collection (MYSTIC), and Study for Monitoring Antimicrobial Resistance

Trends (SMART) among others are monitoring the trends and emergence of antibiotic resistance in many parts of the world [103]. The compiled data from the several international collaborative studies from 1994 to 2013 along with the references is presented in Annexure 1.

Although the studies differed in time period, geography and sample sizes, they give a broad picture on the changing pattern of prevalence of ESBL producers across time and place. By compiling data from several studies, the bar charts for ESBL producing *E. coli* (ESBL-EC) and ESBL producing *K. pneumoniae* (ESBL-KP) is presented in Figure 12 and 13, respectively. In the studies reviewed here, the prevalence of ESBLs was notably higher among *K. pneumoniae* than *E.coli* until 2008-09, thereafter the prevalence among *E. coli* increased significantly and in many cases, exceeded that of *K. pneumoniae*.

The prevalence of ESBL-EC in North America (Canada and United States of America) had generally remained consistently low at the range of 2-4% until 2005. Thereafter, it rose from 4.8% in 2007 to 18.4% in 2012. The prevalence of ESBL-KP in North America had been stably low until 2004, where it ranged between 4.9% and 7%. Since 2005, the rates have risen from 8% to 12.7% with a peak of 17.2% in the year 2011. The reason for their comparatively low prevalence of ESBL producers appears to be due to strict antibiotic usage and robust infection control measures.

Figure 12. Patten of global prevalence of ESBL-EC

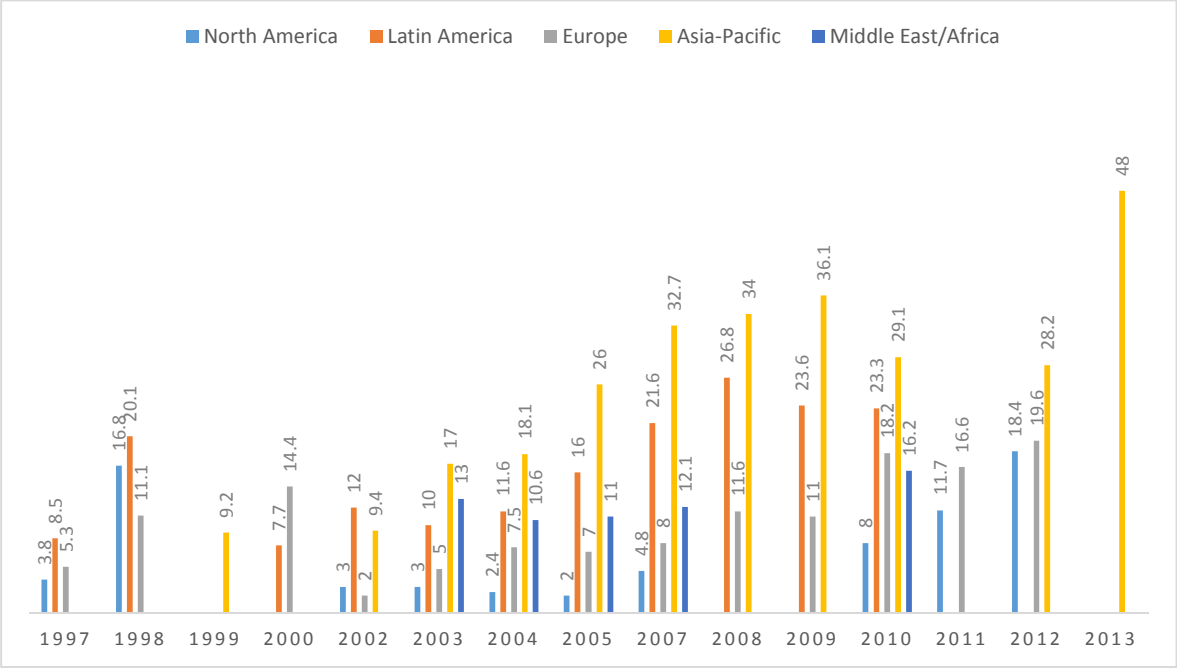
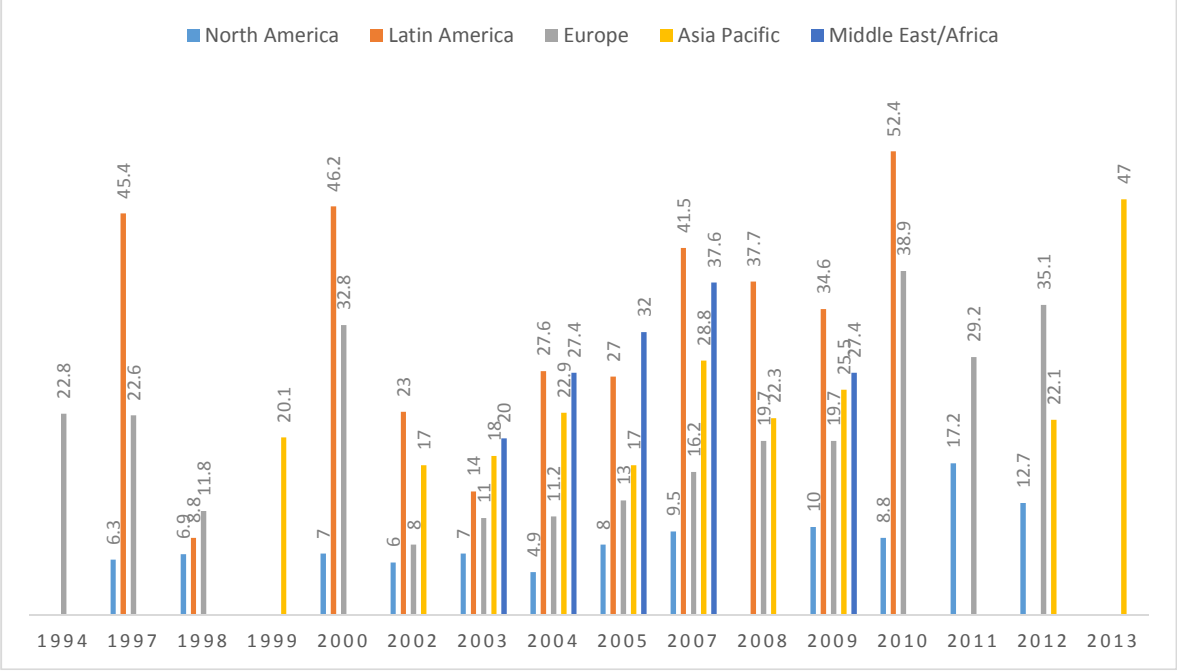


Figure 13. Patten of global prevalence of ESBL-KP



In Latin America, the prevalence of ESBL-EC and ESBL-KP seems to be much higher than North America. The prevalence of ESBL-EC remained more or less stable

at an average of 10.3% between 2000 and 2004. Since then, the rates peaked to 26.8% in 2008 and remained stable at an average of 23.3% for the next two years. The prevalence of ESBL-KP in Latin America were considerably high during 1997 and 2000, with an average of 45.8%. The rates declined by half to 23% in 2002 and thereafter rose steadily to 41.5% in 2007. After a minor drop to 34.6% in 2009, it rose spectacularly to 52.4% in 2010. Compared to ESBL-EC, the prevalence rates of ESBL-KP fluctuated in these regions.

The prevalence of ESBL producers were higher in the European countries compared to North America. While Scandinavian countries have the least prevalence rates (2-8%), those on Eastern bloc such as Poland, Bulgaria, Turkey have very high prevalence rates (40%) [104, 105]. The prevalence rates of ESBL-EC in Europe rose briefly from 5.3% in 1997 to 14.4% in 2000 and then fell sharply to 2% in 2002. Thereafter, the rates gradually increased from 5% in 2003 to 19.6% in 2012. As with Latin America, fluctuations in the prevalence rates of ESBL-KP were noted. The rates averaged 22.7% during 1994-96. After a brief fall to 11.8% in 1998, it rose sharply to 32.8% in 2000. Thereafter, the rate plummeted to 8% in 2002 and then witnessed a gradual increase over the next six years to 19.7% in 2008. In the 2009, the rate almost doubled to 38.9% and in the next two years, it averaged at 32.2%.

Asia-Pacific is a very diverse region and therefore the prevalence rates are bound to contrast across regions. The prevalence rates of ESBL-EC rose sharply from 9.2% to 36.1% in a decade (1999-2009). The rates dipped to an average of 28.7% for the next 2-3 years and then rose sharply to 48% in 2013. The prevalence rates of ESBL-KP in Asia Pacific region fluctuated without major spikes at a rate between 17% and 28.8% during the period 1999-2012. In the recent 2013 study, the rate has almost doubled to 47%. In contrast to the Asian regions, the prevalence rates in the Pacific

countries (Australia and New Zealand) are disproportionately low. The prevalence rates in Australia rose from 0.2-12% among ESBL-EC and 5.9-15% among ESBL-KP between 1998-99 and 2013.

Data from surveillance of the Middle East and African countries are scarce and appear in the literature from the year 2003 onwards. The prevalence rates of ESBL-EC have risen gradually from 13% in 2003 to 16.2% in 2010. With respect to ESBL-KP, the rates rose gradually from 18% in 2003 to 37.6% in 2007, thereafter the rate fell to 25.5% in 2009.

4.5 Global distribution of ESBL types

The distribution of ESBL types among the ESBL producers vary considerably. While some ESBL types have remained endemic in the region of their origin, few others have spread across continents. At the same time, some ESBL types such as CTX-M are found to be replacing the TEM or SHV type ESBLs.

In the North American countries of Canada and USA, CTX-M-15 and CTX-M-14 are the predominant ESBL types among Enterobacteriaceae members. In recent studies from USA majority of ESBLs were found to be of CTX-M type [106, 107]. Other CTX-M types that are common to both these countries include CTX-M-2, CTX-M-3 and CTX-M-27. CTX-M-9 and CTX-M-24 have been reported additionally, from Canada [108]. SHV-12 and SHV-2 are commonly reported from both these countries. Apart from these two SHV types, SHV-2a occur in Canada, whereas SHV-5 and SHV-7 are reported from USA. Among the TEM type ESBLs, TEM-10 and TEM-12 were predominantly seen in USA, followed infrequently by TEM-26 and TEM-155. TEM-11, TEM-12 and TEM-52 types have been reported from Canada [106-108].

The Latin American countries too are dominated by the CTX-M ESBLs; CTX-M-2 is frequently reported from Argentina, Brazil, Peru and Bolivia. Apart from this, CTX-M-8 has been observed in isolates from Brazil and Bolivia whereas CTX-M-14 is reported both from Bolivia and Peru. CTX-M-15, which is the predominant type in Mexico has also been reported from Brazil, Peru and Bolivia. Among the SHV type ESBLs, SHV-2 and SHV-5 are the ones that are reported from these countries. In addition to these major ESBL types, PER-2 from Argentina and TLA-1 from Mexico are the minor ESBLs that too are often reported [109-111].

Presently, TEM type ESBLs are infrequently reported from Europe, but earlier studies reported presence of TEM-3, TEM-5, TEM-7, TEM-15, TEM-24, TEM-26, TEM-47 and TEM-52. Among the SHV type ESBLs, SHV-2, SHV-3, SHV-5 and SHV-12 have been reported from several parts of Europe.CTX-M-15 is now reported from almost every part of Europe. Among the Scandinavian countries, CTX-M-1, CTX-M-2 and CTX-M-9 are often reported. In the Eastern countries of Europe, such as Russia and Poland CTX-M-1, and CTX-M-3 are endemic. In the western European countries such as Spain and Portugal, CTX-M-9 and its derivative, CTX-M-14 are endemic. Nine different types of CTX-M enzymes have been reported from studies conducted at United Kingdom. CTX-M-2 is reported from Belgium, Italy, France, Spain and Russia. CTX-M-32 has been reported from Spain, Portugal, Italy and UK [105, 112, 113].

Among the Asian countries, China seems to have maximum variants of CTX-M enzymes among the Enterobacteriaceae members. While CTX-M-3, CTX-M-14, CTX-M-15 and CTX-M-9 are the most commonly reported types, CTX-M-22, CTX-M-24, CTX-M-27, CTX-M-55 and CTX-M-79 are found less commonly. SHV-12, SHV-5, and SHV-13 are the predominant SHV types reported from China [114, 115].

Studies from Japan too reveal predominant presence of CTX-M enzymes among their isolates: CTX-M-2, CTX-M-9, CTX-M-1, CTX-M-15 and CTX-M-14 are the commonly reported types. Apart from these, CTX-M-8, CTX-M-27, CTX-M-3, and CTX-M-25 are also reported from different parts of Japan. Reports of TEM and SHV type ESBLs are scarce from Japan; only SHV-2, SHV-12, SHV-24, SHV-27 and TEM-28 have been reported [116, 117]. Among the South Korean isolates, CTX-M-14 is clearly the dominant type, although the prevalence of CTX-M-15 producers is rapidly increasing. Besides these two types, CTX-M-3, CTX-M-27, CTX-M-57, CTX-M-9, CTX-M-12, CTX-M-22 and CTX-M-24 are also reported in the literature. Among the SHV type ESBLs, SHV-12 and SHV-2a are the only types that are frequently reported. Among the TEM type ESBLs, TEM-52 is widely reported among Enterobacteriaceae isolates [118, 119]. In the rest of South Asian countries, CTX-M-14, CTX-M-15 and CTX-M-3 are the commonly reported ESBL types. Besides these, the less commonly reported types include CTX-M-9, CTX-M-22, CTX-M-24, CTX-M-27 and CTX-M-55. Among the SHV types, SHV-2, SHV-2a, SHV-5, SHV-12 and SHV-27 ESBLs are often encountered among Enterobacteriaceae isolates. VEB-1 type minor ESBLs have been reported in isolates from Thailand and Vietnam [120].

Enterobacteriaceae isolates from Australia are dominated by CTX-M-1 group enzymes, mainly belonging to the CTX-M-15 type. Other infrequently detected CTX-M types include CTX-M-3 and CTX-M-14. Among the SHV type ESBLs, SHV-12 is the most commonly reported type among the isolates [120]. Presence of various ESBL types across the continents is depicted in Figure 14.

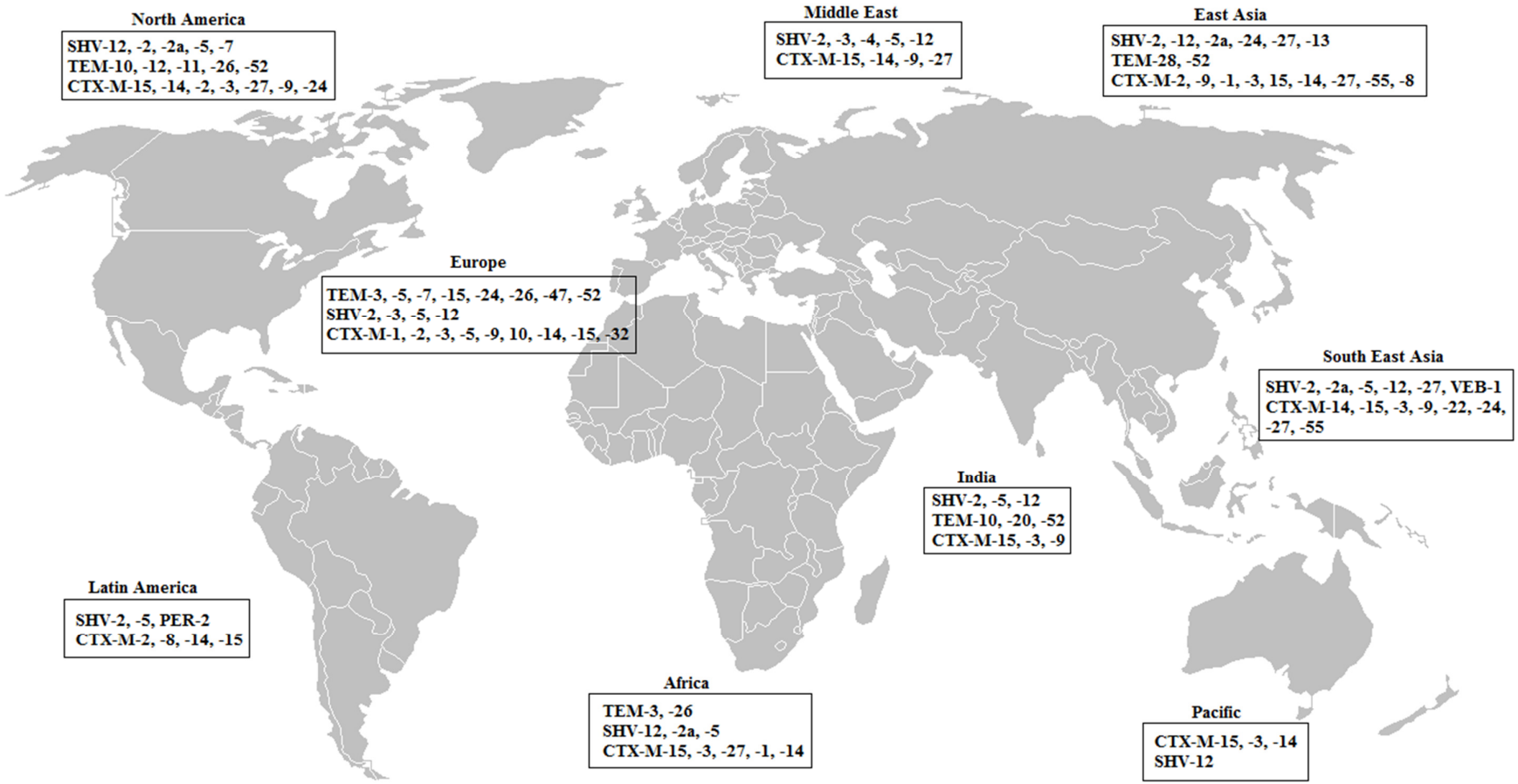
4.6 Prevalence of ESBL producers in India

There are no national surveillance program to monitor the prevalence of ESBL producers in India, hence data was collected from international surveillance studies, which included multiple centers from India. As a part of the MYSTIC surveillance programme, bacterial samples were collected from six cities (New Delhi, Lucknow, Indore, Mumbai, Bangalore and Vellore) across India prior to 2000. The prevalence of ESBL producers in that study was very high; 92% of *E. coli* and 96% of *K. pneumoniae* were found to be ESBL producers [121]. Interestingly, another publication of the same study reported ESBL production in >61% and >55% among *E. coli* and *Klebsiella spp*, respectively [122].

India was included in the Asia-Pacific SMART study in 2007 and samples collected from nine centers in that period showed prevalence of ESBLs in 79% of *E. coli* and 69.4% of *K. pneumoniae* isolates [123]. In one of the centers included in that study, a prevalence rate of 94.1% was observed among *E. coli* isolates. ESBL producers were found responsible for 78.9% of both nosocomial and community acquired infections in that study.

In the 2008 Asia-Pacific SMART study, samples collected from seven centers across India reported a prevalence of ESBLs in 61.2% of *E. coli* and 46.8% *K. pneumoniae* isolates [124]. The prevalence rates increased marginally in the subsequent study. The 2009 Asia-Pacific SMART study, reported a prevalence rate of 67.1% among *E. coli* and 56.8% among *K. pneumoniae* isolates [125].

Figure 14: World map showing distribution of various ESBLs



In the same period, multinational Asian Network for Surveillance of Resistant Pathogens (ANSORP) surveillance study reported similar prevalence (57.1%) of ESBL-KP from Indian centers [126]. The regional Resistance Surveillance (RRS) program in the Asia-Pacific (APAC) region reported a slightly increased prevalence of ESBLs; 78% of *E. coli* and 64% of *K. pneumoniae* isolates were found to be ESBL producers [127].

Although there are numerous studies from various parts of India, they differ widely in patient population, sample type and size, detection methodology and the study period. Hence, even with a vast amount of data, meaningful interpretation is difficult to achieve. In the period 2013-14, the rates of ESBL detection among *E. coli* ranged from 24-90% and that of *K. pneumoniae* ranged from 9-80% in the Northern parts of India [128,129]. In the Eastern parts, the rates ranged from 12-89% among *E. coli* and 26-93% among *K. pneumoniae* isolates [130-132]. In the central parts of India, the rates ranged from 41-50% among *E. coli* and 26-48% among *K. pneumoniae* isolates [133, 134]. In the western India, the rates ranged from 20-62% among *E. coli* and 15-67% among *K. pneumoniae* isolates [135-137]. In the southern India, the rates ranged from 18-73% among *E. coli* and 33-63% among *K. pneumoniae* isolates [138-140]. The ESBL detection rates in India during the 2013-14 period is displayed in Figure 15. Compilation of several studies across India from 1995 to 2014 along with references is presented in Annexure 2.

4.7 ESBL types in India

The first report of an ESBL producer from India came in 1998, when SHV-5 ESBL was detected among *Salmonella senftenberg* isolated from patients during an outbreak in a burns ward at Delhi [141]. In 2001, Karim *et al* reported detection of

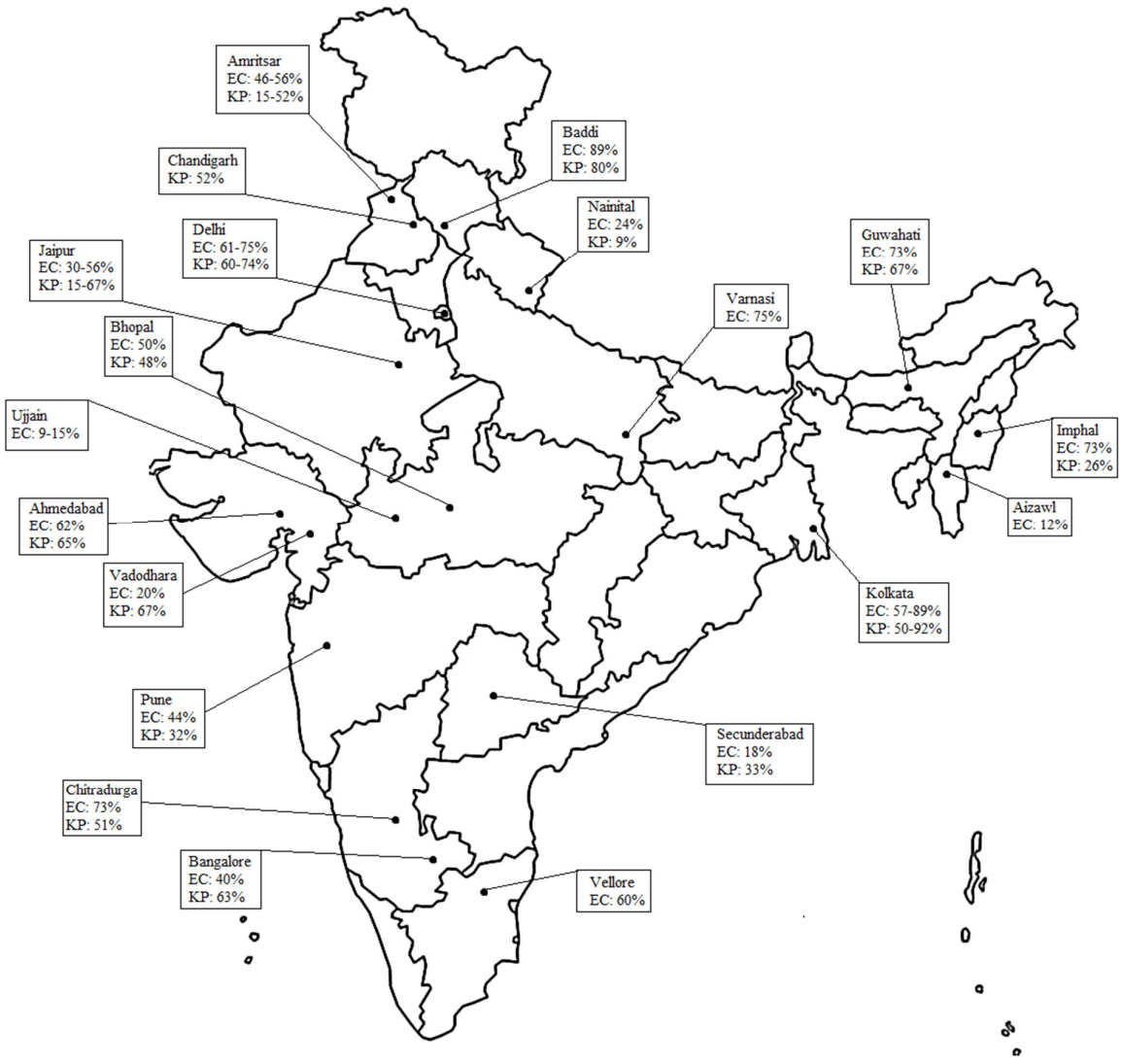
CTX-M-15 in clinical isolates of *E. coli*, *K. pneumoniae*, and *E. aerogenes* collected in 1999 at a hospital in Delhi [76]. This was the first account of a CTX-M enzyme that was also capable of hydrolyzing ceftazidime.

In 2006, Ensor *et al* reported detection of CTX-M-15 in 73% of *E. coli* and *K. pneumoniae* isolates collected from centers located in Aligarh, Varanasi and Hubli [142]. In response to this publication, Walsh *et al* reported that their study team, which had collected samples from New Delhi, Mumbai, Indore, Lucknow, Bangalore and Vellore prior to 2000, had detected CTX-M-15 in 83% of *E. coli* and 75% of *Klebsiella sps* [121]. CTX-M-15 still continues to be the dominant CTX-M type in India. Several studies from India have detected *bla*CTX-M genes among the clinical isolates by amplifying only a part of the *bla*CTX-M gene. Many of these studies had used multiplex PCR protocols, which allow the researchers to detect only the CTX-M groups but not their individual members.

In 2008, a study from Chennai reported detection of CTX-M-1 ESBL among Enterobacteriaceae members [143]. Authors of that study, used the same primer pairs for another study and reported detection of CTX-M-28 [144]. CTX-M-1, CTX-M-15 and CTX-M-28 belong to the CTX-M-1 cluster; CTX-M-15 differs from CTX-M-1 by four amino acids but CTX-M-28 differs from CTX-M-15 by one amino acid at position 289. The primers used by the authors of those two studies are more suitable for detecting CTX-M-15 ESBLs than CTX-M-1 or CTX-M-28. The possibility of misidentification of CTX-M-28 by using primers designed for CTX-M-15 has been pointed out by Menezes *et al* [145].

As a part of the Tigecycline Phase 3 Clinical Trials conducted during 2002-06, *E. coli* and *K. pneumoniae* strains collected from India were reported to possess CTX-M-2, CTX-M-5, CTX-M-15 and CTX-M-28 genes [146].

Figure 15: Prevalence of ESBL producers in parts of India (2013-14)



Until this report, CTX-M-15 was the only CTX-M type reported in India. First report of CTX-M-9 group ESBL in India came from Kolkata in 2011; the CTX-M-9 group genes were detected in isolates of *E. coli* and *K. pneumoniae* from the feces of infants [147]. Authors of that study also reported presence of CTX-M-14-like and CTX-

M-27-like sequences in those isolates. A pharmaceutical company based in Baddi, Himachal Pradesh, which undertook a study involving multiple clinical samples from several hospitals, reported CTX-M-9 group gene in an *E. coli* isolate [129]. In a study on diabetic foot ulcers conducted at Varanasi, 70% of *E. coli* were positive for *bla*CTX-M-15, and one isolate each possessed *bla*CTX-M-1, *bla*CTX-M-3 and *bla*CTX-M-9 [148]. Tripathi *et al* from Kolkata reported detection of CTX-M-72-like enzyme from *K. pneumoniae* isolate [149].

In 2009, a report from Chennai detected SHV-28 in a clinical isolate of *K. pneumoniae* and claimed it to be an ESBL since the isolate gave positive ESBL phenotypic test and isoelectric focusing revealed only a single band [150]. As of now, SHV-28 is not confirmed to be an ESBL. Among the few reports on SHV type ESBLs, SHV-12, followed by SHV-2, SHV-4 and SHV-5 and are the only ones reported from India [129-131, 148, 151]. There are two reports of TEM type ESBLs from India, both of which were found in *E. coli* isolates. While TEM-10, TEM-20 and TEM-52 were detected at Varanasi, TEM-3 was detected at Baddi (HP) [148, 129].

5. Laboratory detection of ESBLs

In 1999, the National Committee for Clinical Laboratory Standards (NCCLS), which is now known as CLSI, published methods for screening and confirming the presence of ESBLs in *K. pneumoniae*, *K. oxytoca*, and *E. coli* (M100-S9) [152]. The detection involved two steps; a screening test to detect resistance to oxyimino-cephalosporins and a confirmatory test based on inhibition of ESBL by clavulanic acid. Since then, the guidelines on the performance and interpretation of ESBL detection methods have been revised by CLSI. In 2010, CLSI recommended in its document M100-S20 that "routine ESBL testing is no longer necessary before reporting results"

and that it was no longer necessary to edit results for cephalosporins, aztreonam, or penicillins to resistant if the isolate was identified as an ESBL producer [153]. However, CLSI also maintains that "until laboratories implement the new interpretive criteria, ESBL testing should be performed as described". Over the years, several rapid methods including chromogenic media, automated systems and molecular methods have been devised to decrease the reporting period and to increase the sensitivity as well as the specificity of ESBL detection.

5.1 Screening methods

Screening methods involve detection of resistance to any of the third generation cephalosporin antibiotics such as cefotaxime, ceftazidime, ceftriaxone, cefpodoxime or aztreonam by disk diffusion method or by MIC estimation. The current breakpoints for resistance to antibiotics are shown below [154]:

Antibiotic		Disk diffusion		MIC determination	
	µg	*E. coli, K. pneumoniae & K. oxytoca*	*P. mirabilis*	*E. coli, K. pneumoniae & K. oxytoca*	*P. mirabilis*
aztreonam	30	≤27 mm	-	≥ 2 µg/ml	-
ceftriaxone	30	≤25 mm	-	≥ 2 µg/ml	-
ceftazidime	30	≤22 mm	≤22 mm	≥ 2 µg/ml	≥ 2 µg/ml
cefotaxime	30	≤27 mm	≤27 mm	≥ 2 µg/ml	≥ 2 µg/ml
cefpodoxime	10	≤17 mm	≤22 mm	≥8 µg/ml	≥ 2 µg/ml

CLSI recommends use of multiple antibiotics to increase the sensitivity of screening. Breakpoint MIC levels of the screening agents can also be obtained by automated antibiotic susceptibility systems such as MicroScan Walk-Away (Dade Behring Inc, Phoenix) and VITEK-2 (bioMerieux, France). These methods provide accurate results in a short period of time, but are expensive.

Commercial screening media incorporate third-generation cephalosporins and resistance exhibited by the test isolates are detected by either growth on those media or a change in colour of colonies. While BLSE agar (AES Laboratory, France) is a non-chromogenic medium, ChromID ESBL (BioMèrieux, Lyon, France), Brilliance ESBL (Oxoid, Basingstoke, United Kingdom), ESBL-Bx (bioMerieux, France) and CHROMagar ESBL (CHROMagar, Paris, France) are chromogenic media [155, 156]. The chromogenic substrates may differ among the various chromogenic agars, but ESBL producing bacteria are identified by the colour of their colonies as recommended by the manufacturer. The advantage of chromogenic ESBL screening media is that by directly culturing the clinical samples on these media, ESBL-producing Enterobacteriaceae members can be detected in a short period of time. In a comparative study, non-chromogenic media were found to possess sensitivity ranging from 74.6 to 86.5% and specificity ranging from 82 to 96.8%. The sensitivity of ChromID ranged from 88.2 to 97.3% and specificity ranged from 90.4 to 95.5%. The sensitivity of Brilliance ESBL ranged from 94.9 to 100% and specificity ranged from 93.3-95.7% [157]. Quicolor agar (QC ES) (Salubris Inc., Massachusetts, USA) is a chromogenic medium, which changes colour within 4-6 hours due to the metabolic activity of growing bacteria. This medium can be used instead of Mueller Hinton agar. Upon growth, the red colour of this medium turns to yellow whereas the inhibition zones remain red [158]. While these media have high sensitivities, they often have lower specificities.

5.2 Detection of ESBL production by phenotypic methods

The methods to detect ESBLs rely on a) inactivation of a chromogenic substrate by a rapid test, b) inactivation of indicator antibiotic by the extracts of the cells by three-

dimensional tests, or more specifically by c) inhibition of ESBLs by clavulanic acid (clavulanate).

5.2.1 Rapid tests:

Commercially available rapid tests such as β-Lacta test (Bio-Rad, France) and Cica-β-Test (Kanto Chemical, Tokyo, Japan) employ HMRZ-86, a novel chromogenic cephalosporin to detect beta-lactamase activity [159-161]. The former test gives positive results with ESBLs, MBLs, and de-repressed AmpC beta-lactamases, but not by broad-spectrum beta-lactamases such as TEM-1 or SHV-1. The latter test is more specific as it uses specific beta-lactamase inhibitors for each class of beta-lactamases. While the appearance of red colour in the former test is considered positive for the presence of beta-lactamases, failure to develop red colour is considered positive in the latter test. With the former test, it is not possible to differentiate between the three beta-lactamase types. The sensitivity and specificity of the β-Lacta test were reported to be 96% and 100%, respectively when tested for *E. coli* and *K. pneumoniae* [159]. Another study, which investigated its use in the detection of ESBL producers directly in urine samples, reported sensitivity of 94% and specificity of 100% [160]. The sensitivity and specificity of Cica- β-Test was found to range from 74 to 96% and from 57 to 98%, respectively [157, 161]. Further tests are required to establish their utility in detection and differentiation of the prevalent beta-lactamases.

5.2.2 Three dimensional tests:

Three-dimensional (3D) tests are employed to detect hydrolytic activity of ESBLs against indicator antibiotics. In case of direct 3D tests, the extracts of test isolates are tested against itself, whereas in indirect 3D test, the extracts of test isolates are tested against a standard susceptible strain [162, 163]. In direct 3D test, the extracts

of the test isolate is prepared first and lawn culture of the same isolate is made on a Mueller Hinton agar plate. A disk containing indicator antibiotic (e.g., cefotaxime) is placed at the center. A small volume of the extract is dispensed close to the antibiotic disk in a variety of ways, such as a narrow trough, a slit or a well. If the extract contains ESBL, it would inactivate the antibiotic that has diffused in the medium and following incubation, the zone of inhibition would get distorted. This method works only if the isolate produces some zone of inhibition around the indicator disc. This limitation can be overcome in the indirect 3D test by using a fully susceptible standard strain (*E. coli* ATCC 25922) to make lawn culture. Both these methods are labour intensive and may yield non-specific or false positive results.

5.2.3 Tests based on inhibition by clavulanic acid

Since inhibition by clavulanic acid is a cardinal property of ESBLs, tests that use this inhibitor to reverse ESBL mediated resistance are naturally preferred. Clavulanate based tests can be performed as disk diffusion test, agar screen test, agar or broth dilution test, or E-tests. Even the commercially available automated systems work on this principle.

5.2.3.1 Double Disk Synergy Test (DDST)

The test, which is also known as Double Disk Diffusion test (DDDT) was introduced by Jarlier *et al* in 1988 [43]. It relies on the action of clavulanic acid to augment the zone of inhibition. In this test, following the lawn culture of the test isolate, a clavulanate containing disk (such as amoxycillin+clavulanic acid 30/10 µg) is placed at the center of the plate and disks of cefotaxime (30 µg), ceftazidime (30 µg), aztreonam (30 µg) or cefpodoxime (10 µg) are placed around it at a variable distance of 20-30 mm and incubated. Augmentation of zone of inhibition towards the centrally

place disk is considered as positive for ESBL production. The test suffers from poor sensitivity due to variations in the distance of disk placement, indeterminate results and subjective reading by the researcher. The sensitivity of the test is also affected by the choice of antibiotic, the type of ESBL produced by the organism and the distance of placement of disks. The sensitivity has been shown to vary from 55-100% but the specificity vary from 72-100% [164-166].

5.2.3.2 Inhibitor-potentiated disk diffusion (IPD)

Limitation of DDST test can be overcome by this method as it gives a quantitative result. This method involves use of two plates of Mueller Hinton agar; one with 4 µg/ml clavulanic acid and the other without. Standardized inoculum of the test isolate is swabbed on the surface of both agar plates and disks of cefotaxime, ceftazidime, ceftriaxone and aztreonam are placed on both of them and incubated overnight at 37°C. A difference in the zone diameter of ≥ 10 mm around disc(s) in plates with clavulanic acid versus disks on plain Mueller Hinton agar is considered positive for ESBL production [167]. The sensitivity of this method is also affected by the choice of antibiotic and type of ESBL produced by the organism. The sensitivity has been shown to vary from 55 - 100% whereas the specificity is generally high (~100%). When the interpretative zone size difference is changed from 10 to 5 mm, the sensitivity has been shown to increase but with a fall in specificity [164, 167]. This method gives good results but the limitation of this method is the requirement of a stock solution of clavulanic acid, which is highly temperature sensitive and has short shelf-life.

5.2.3.3 CLSI Phenotypic Confirmatory Test (PCT)

Also known as Combined Disk Method, this test is recommended by the CLSI for confirmation of ESBL production in isolates of *E. coli, K. pneumoniae, K. oxytoca*

and *P. mirabilis* [154]. The test can be performed either as disk diffusion or by MIC estimation and both these methods are mostly in agreement with each other. Since CLSI PCT method is quantitative, it eliminates the subjectivity in interpretations.

In the disk diffusion method, a standardized inoculum of the test isolate is swabbed on the surface of a Mueller Hinton agar plate. 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 overnight at 37^{o}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.

In microbroth dilution tests, 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. A reduction in MIC by ≥ 3 two-fold dilutions for cephalosporin with clavulanic acid versus the MIC of cephalosporins alone confirms ESBL production.

Even though the two methods yield similar results, disk diffusion method is preferred as it is easier to perform and is cheaper than the microbroth dilution method. Both the sensitivity and specificity of this method shown to be >90% [158, 160]. False positive results may occur with isolates producing KPC-beta-lactamases or chromosomal K1 beta-lactamases. False negative results may occur in isolates that also produce high levels of AmpC beta-lactamases.

5.2.3.4 E-test

ESBL E-test (bioMérieux, France) strips are thin, inert and non-porous plastic carriers measuring 5 x 60 mm. 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 E-test strips for ESBL detection; one strip (CT/CTL) contains cefotaxime gradient at one end and cefotaxime+clavulanic acid gradient at the other end. The other strip (TZ/TZL) contains ceftazidime gradient at one end and ceftazidime+clavulanic acid at the other end. Testing for ESBL must be performed with both the strips. The MIC is read as the point where the inhibition ellipse intersects the edge of the E-test strip. Following concentration gradients are used with these strips: Cefotaxime (0.25-16 µg/ml) at one end and cefotaxime+clavulanic acid (0.016 - 1 µg/ml) plus 4 µg/ml of clavulanic acid at the other end. Ceftazidime (0.5 - 32 µg/ml) at one end and ceftazidime+clavulanic acid (0.064 - 4 µg/ml) plus 4 µg/ml clavulanic acid at the other end. The presence of an ESBL is confirmed if MIC of cefotaxime or ceftazidime is reduced by ≥3 two-fold dilutions in the presence of clavulanic acid. Production of ESBL is also inferred by the appearance of a phantom zone or deformation in the ellipse. E-test confirmatory strips are convenient but are expensive. The sensitivity of E-test has been shown to vary from 94-98% and the susceptibility vary from 59-84% [166, 168, 169]. Since the concentration gradient in E-test strip is not as extensive as recommended by the CLSI, it yields more indeterminate results than CLSI phenotypic confirmatory test. Indeterminate result can also occur due to co-production of AmpC beta-lactamases.

5.2.3.5 Automated systems:

Automated ESBL detection tests such as Vitek Legacy and Vitek 2 (BioMérieux), MicroScan (Siemens Medical Solutions Diagnostics), Sensititre (TREK Diagnostic Systems), and Phoenix (BD Diagnostic Systems) detect ESBLs on the principle of inhibition by clavulanic acid. Studies have shown that these systems have sensitivities ranging from 84-100% and specificities ranging from 52-98% [166, 169-171]. Automated systems offer the advantage of rapid detection of ESBLs in clinical isolates but are also prohibitively expensive.

5.3 Detection of ESBL production in isolates also producing AmpC beta-lactamases

Various strategies have been developed to overcome the false negative results in clavulanate based tests due to co-expression of AmpC beta-lactamases in the test isolates. AmpC beta-lactamases are able to interfere in these tests, as they are not inhibited by clavulanic acid. Hence, the activity of clavulanic acid can be unmasked by selectively inhibiting AmpC beta-lactamases. Amino-phenylboronic acid (APB) and cloxacillin are good inhibitors of AmpC beta-lactamases. Disk diffusion tests involving clavulanate can be modified by incorporating 200 µg/ml of cloxacillin in the Mueller Hinton agar [172]. CLSI PCT can be modified by supplementing the antibiotic disks with 400 µg/ml of APB [173]. Isolates co-producing ESBLs and AmpC beta-lactamase would produce a zone of $\geq$ 5 mm around disk having cefotaxime+ clavulanic acid+APB over disk with cefotaxime+ clavulanic acid only. Another approach to detect ESBLs in isolates co-producing both these enzymes is to use cefepime, as this fourth generation cephalosporin is unaffected by AmpC beta-lactamases. A 30 µg disk of cefepime has been used in addition to the other cephalosporin disks in DDST test [174]. CLSI-PCT

has been modified by including cefepime disk and cefepime+ clavulanic acid disk [175]. Similarly, additional E-test strips with cefepime at one end and cefepime+ clavulanic acid at the other end has also been introduced [176]. In all these tests, clavulanic acid based inhibition of ESBL becomes apparent since cefepime suppresses AmpC beta-lactamase activity.

5.4. CLSI vs EUCAST guidelines

Currently, there are two widely popular guidelines on antimicrobial susceptibility testing, the CLSI and EUCAST (European Committee on Antimicrobial Susceptibility Testing) [154]. CLSI guidelines originating from USA are more popular and are followed in many countries across the globe. The EUCAST guidelines are used in the European countries (available at http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/EUCAST_guidelines_detection_of_resistance_mechanisms_121222.pdf). The two guidelines are not in complete agreement with each other and there are few differences between the two regarding screening and detection of ESBL. For screening purposes, CLSI recommends disk diffusion or MIC microbroth dilution method whereas EUCAST additionally recommends MIC agar dilution method. CLSI recommends use of cefpodoxime, cefotaxime, ceftriaxone, ceftazidime or aztreonam for screening by disk diffusion or MIC estimation. EUCAST recommends ceftazidime, ceftriaxone and cefotaxime for both MIC estimation and disk diffusion. EUCAST guidelines mentions that although cefpodoxime is a sensitive indicator for detection of ESBL production, it is less specific than the combination of cefotaxime (or ceftriaxone) and ceftazidime. While CLSI guidelines are restricted to be applied only on *E. coli*, *K. pneumoniae*, *K. oxytoca* and *P. mirabilis*, the EUCAST has developed algorithm applicable for several Enterobacteriaceae members. As per EUCAST guidelines MIC value of ≥1 µg/ml of

cefotaxime, ceftriaxone or ceftazidime is considered as a positive screening test. According to CLSI guidelines, MIC ≥8 µg/ml for cefpodoxime or MIC ≥2 µg/ml for ceftazidime, aztreonam, cefotaxime, or ceftriaxone; and for *P. mirabilis*, MIC ≥2 µg/ml for cefpodoxime, ceftazidime, or cefotaxime is considered as a positive screening test. There are differences between EUCAST and CLSI guidelines in the prescribed zone diameters for disk diffusion testing. While the diameter of zone of inhibition of ceftazidime is common to both CLSI and EUCAST at 22 mm, they are different for cefotaxime and ceftriaxone. It is 21 mm and 27 mm for cefotaxime whereas 23 mm and 25 mm for ceftriaxone according to EUCAST and CLSI, respectively. Unlike CLSI guidelines, which recommend 30 µg disk for cefotaxime, ceftriaxone and ceftazidime antibiotics, EUCAST recommends 5 µg cefotaxime disk, 10 µg ceftazidime disk and 30 µg ceftriaxone disk. There are no guidelines from CLSI on detection of ESBLs in other Enterobacteriaceae members or in cases where isolates may also co-produce AmpC beta-lactamases.

For phenotypic detection of ESBLs, both CLSI and EUCAST recommend testing with ceftazidime, cefotaxime alone and in combination with clavulanic acid. While CLSI recommends the combination disk method and microbroth dilution method for detection of ESBLs, EUCAST also permits two other detection methods including Double Disk Synergy Test (DDST) and E-test method. CLSI guidelines on ESBL detection applies only to *E. coli, K. pneumoniae, K. oxytoca* and *P. mirabilis*, the EUCAST guidelines extend this recommendation to *Shigella spp* and *Salmonella spp* as well. Additionally, EUCAST guidelines recommend usage of cefepime/cefepime+clavulanic acid when *E.coli, Klebsiella spp., P. mirabilis, Salmonella spp*., and *Shigella spp* test negative by ceftazidime and cefotaxime based test. Cefepime/cefepime+clavulanic acid combination has been recommended by

EUCAST for detection of ESBL among *Enterobacter spp., Citrobacter freundii, Morganella morganii, Providencia stuartii, Serratia spp*., and *Hafnia alvei* whereas CLSI offers no such guidelines for these organisms.

Because of the disagreements in the guidelines and protocol, there is bound to be variation in the screening and detection of ESBLs depending on which of the two protocols is followed by the researcher.

5.5 Molecular techniques in the detection and identification of ESBLs

Detection and identification of beta-lactamase (*bla*) gene became accurate when nucleotide sequencing techniques were introduced. Earlier, the detection of *bla* genes was undertaken by DNA hybridization assay using labeled probes, but with the advent of Polymerase Chain Reaction (PCR) technology, detection and identification of *bla* genes have become more convenient.

5.5.1 Nucleic acid based molecular techniques

By designing suitable primers, entire *bla* gene can be amplified by PCR technique. By detecting specific parts of the *bla* gene, beta-lactamases can be identified to a certain extent but for complete accuracy, the entire *bla* gene must be amplified and sequenced. Conventional PCR can be run either as uniplex or multiplex reactions; the latter offers the convenience of detecting multiple *bla* genes at the same time and at a lesser cost [177, 178]. In order to skip the laborious post-PCR detection and validation methods, real-time PCR has been developed. Using well designed primers and probes, real-time multiplex PCR can detect multiple gene types in a much shorter time [179]. However, reproducibility of the results seems to be a limitation of this technique [180].

Conventional DNA sequencing methods such Sanger's dideoxy chain termination involves purification of PCR product, which adds to the delay in obtaining

results. Sequencing reactions are often outsourced to specialist service providers, which also contributes to the delay. New generation sequencing technologies such as pyrosequencing and ion semiconductor sequencing promises to provide better results at much shorter time [181, 182]. Some of the new generation sequencing technologies have been made as bench top instruments that can be used in diagnostic laboratories [183]. Despite these advancements, DNA sequencing is beyond the means of many researchers due to it prohibitive costs.

Alternatives to DNA sequencing for the identification of *bla* gene have been developed; these include PCR-RFLP (Restriction fragment length polymorphism) and Restriction Site Insertion-PCR (RSI-PCR). In PCR-RFLP, the PCR amplicons are digested with specific restriction endonuclease and then subjected to electrophoresis, which are then compared with the profiles of known *bla* genes [184]. For this technique, a thorough knowledge of nucleotide sequences, expected position of mutations and appropriate selection of endonuclease is necessary. Using this technique, only some of the already known *bla* types can be identified. A major limitation of this method is the absence of natural restriction site at the point of interest. This limitation has been overcome by introducing an artificial restriction site of choice by a technique called the Restriction Site Insertion-PCR (RSI-PCR). In this technique, the primers are designed to bind to areas where mutations are expected. These primers are modified at their 3' end so that they contain additional one or three nucleotides that are not complementary to the target, but their presence creates an artificial restriction site when the target gets amplified [185]. The restriction site would be created in the amplicon only when the PCR amplification is successful. Following PCR reaction, the amplicons are subjected to restriction digestion using specific endonuclease. By using a series of different primers and endonucleases, it is possible to accurately identify the point mutations. By

suitably modifying the restriction sites, new variants can also be detected. This method has been used to detect to several variants of *bla*SHV genes [185]. A major limitation of this technique is that detection of mutations is possible only where it is feasible to introduce a restriction site.

Other nucleic acid based technologies such as Ligase chain reaction (LCR), PCR-Single-Strand Conformational Polymorphism (PCR-SSCP) and Real-Time PCR - Melting Curve Analysis (MCMD) have been explored by a few researchers to identify the *bla* types. Although many of these new technologies have good discriminatory power to differentiate single nucleotide changes, they are restricted to the detection of existing types only. PCR-SSCP has been used to detect a limited SHV mutant forms and discrimination of inhibitor resistant TEM types [186, 187]. RT-PCR MCMD has been used to detect SHV genes and to differentiate non-ESBL from ESBL genes but this technique cannot identify individual SHV types [188, 189].

Microarray technology relies on the binding of specific sequences in the organisms with pre-designed probes that are immobilized on the array matrix. This way, a very large number of genes can be detected simultaneously. Check KPC/ESBL (Check-Points HealthBV, Netherlands) is a commercially available, microarray-based diagnostic test system that can rapidly detect and identify *bla* genes belonging to the TEM, SHV, CTX-M, and KPC types [190]. This technology has also been shown to detect genes coding for antibiotic resistance in bacteria from processed blood samples, thereby reducing the time involved in detection of specific genes [191]. Most of these systems take 6-8 hours for results to be available but their high costs make them beyond reach for many diagnostic establishments.

5.5.2 Non-nucleic acid based molecular techniques

Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) is new technique to analyze biological molecules. The substance to be evaluated is subjected to a laser beam, which ionizes the components. These ions are accelerated into a tube, which drift towards the detector at a rate proportional to their molecular weight. The time taken for the particles to reach the detector is measured, from which the mass-to-charge ratio of the ions is calculated. The generated mass spectra are analyzed by dedicated software and compared with stored profiles. This technology has been used in the identification of β-lactams and β-lactam degradation products. Bacterial cultures are incubated with β-lactam antibiotic, centrifuged, and then MALDI-TOF MS is used to analyze the supernatants for the presence of β-lactam degradation products [192]. This technology has been shown to discriminate ESBL-producing from non-ESBL producing clinical isolates of *E. coli, K. pneumoniae* and *P. aeruginosa* [193]. This technology is still in the nascent stage and requires through evaluation of its specificity.

6. Clinical significance of ESBLs

When the ESBLs were detected during late 1980s and early1990s, they were often seen in *K. pneumoniae* isolates causing hospital infections. ESBL-KP have been incriminated in several outbreaks of nosocomial infections [194]. Thereafter, ESBLs began to be detected in *E. coli* isolates especially those causing community acquired infections [195]. In many studies CTX-M ESBLs have been detected predominately among *E.coli* causing community acquired infections, especially the urinary tract infection [86].

In early studies, the intestinal colonization by ESBL producers were noticed in hospitalized patients or those living in long-term health care facilities. Now, intestinal colonization is known to occur in healthy individuals living in the community [86]. Prior colonization by ESBL producers at the time of hospital admission has been shown to be a significant risk factor for developing serious infections [195]. Thus, colonized individuals serve as a source for their own infection. At the same time, they can be a potential source of colonization or infection in other patients in the hospital. For this reason, some hospitals mandatorily screen the patients on admission for ESBL producers and quarantine them, if found positive [196]. Those patients who become carriers of ESBL producers in the hospital, remain so for a long time and then disseminate them in the community [197]. Therefore, it becomes important to undertake de-colonization measures [198].

Third generation cephalosporins are the antibiotics that are used commonly to treat a variety of infections produced by *E. coli* and *K. pneumoniae*. These are also used in the empirical treatment of serious infections such as intra-abdominal infection or sepsis. By inactivating the cephalosporin antibiotics, ESBL producing bacteria can result in treatment failure. Several controlled studies have been carried to determine relationship with infection by ESBL producers and the outcome of such infections. The results of these studies have been conflicting; while some studies have shown positive association, some others have not found significant association between ESBL production and the outcome of treatment [199-206]. However, in most of these studies infection by ESBL producing bacteria have been shown to be significantly associated with prolonged hospital stay and increased morbidity.

Treatment of infections by ESBL producers has been complicated by the fact that many of these bacteria produce multiple beta-lactamases, which confer resistance

to many class of antibiotics [207]. In some bacteria, resistance to carbapenems occur due to combination of ESBL production, low level production of AmpC beta-lactamases and loss of porin proteins [208]. It must also be borne in mind that resistant strains can emerge during the course of treatment, therefore repeated susceptibility testing during the course of treatment may be warranted in severe infections [209, 210]. In many hospitals, carbapenems have replaced cephalosporins as the preferred antibiotic. Just as excessive use of cephalosporins are thought to have led to widespread resistance, resistance to carbapenems is also on the rise.

7. Treatment of infections produced by ESBL producers

Until 2010, CLSI had suggested to report resistance to all cephalosporins if the organism was found to be an ESBL producer. Subsequently, this recommendation was withdrawn [152, 153]. Yet, not much enthusiasm is seen in using the cephalosporins as there are reported cases of treatment failure with cephalosporins even when the MIC values were in the susceptible range [199, 211]. Prior exposure to third generation cephalosporin antibiotics for treatment has been shown to be a risk factor for acquiring subsequent bacteremia by ESBL producing bacteria, hence these antibiotics must be used with caution [212]. Treatment failure can occur despite low MIC values because of the so-called inoculum effect, where a high microbial load leads to rise in MIC levels [213, 214]. In intra-abdominal infections, the microbial loads are often high and the level of antibiotic attained at site is often not sufficient, which may lead to treatment failure.

Cefepime, a fourth generation oxyimino-cephalosporin, is variably hydrolyzed by ESBLs. It is resistant to hydrolysis by stably de-represessed AmpC beta-lactamase but is hydrolyzed by CTX-M ESBLs including CTX-M-15. Susceptibility to cefepime from Asia-Pacific area has been reported to range from 33 to 93% in *E. coli* and from

25 to 100% in *K. pneumoniae* [215]. Only a few studies on the clinical utility of cefepime in the treatment of infections by ESBL producers have been reported. Failure rates of up to 30% has been reported in one study dealing with treatment of nosocomial pneumonia by ESBL producers [216]. In another study, use of cefepime as an empirical therapeutic agent for blood stream infections was found to be associated with increased mortality in patients with infections by ESBL producers [217]. In 2014, CLSI introduced a new susceptibility category for reporting cefepime results, called the "susceptible-dose dependent" (SDD) in place of intermediate category [154]. Inhibition zone sizes of 19-24 mm and MIC range of 4-8 µg/ml are now considered under SDD category. In CLSI document M100 S24, the following is mentioned: "SDD is recommended instead of "intermediate" when reporting cefepime results for Enterobacteriaceae isolates because there are multiple approved dosing options for cefepime, and SDD highlights the option of using higher doses to treat infections caused by isolates when the cefepime MIC is 4 or 8 µg/ml or the zone is 19 to 24 mm." In a recent meta-analysis, Nguyen HM *et al* have concluded that cefepime can be used for treatment of invasive infections caused by ESBL producing *E. coli* and *Klebsiella spp* if the MIC of the cefepime is $\leq$ 2 µg/ml [218].

Cephamycins such as cefoxitin, cefotetan, cefmetazole or flomoxef are not hydrolyzed by ESBLs. An in-vitro study showed that cefoxitin and cefotetan exhibited cross susceptibility but not cross-resistance [219]. All the isolates in that study, which were susceptible to cefoxitin were also susceptible to cefotetan indicating cross-susceptibility; however among the isolates that were resistant to cefoxitin, only 50% of them were also resistant to cefotetan, indicating lack of cross-resistance. Using a murine model of pyelonephritis, Lepeule *et al*, suggested that cefoxitin may be used in the treatment of UTIs caused by CTX-M-ESBL producing *E. coli* [220]. A major drawback

with the use of cefoxitin in treatment of infections by *K. pneumoniae* is the emergence of resistance due to the selection of porin mutants [221]. A study conducted in Taiwan on treatment of bacteremic patients with ESBL producing *K. pneumoniae* suggested that flomoxef was similar to carbapenem in clinically efficacy [222]. Doi A *et al* successfully used cefmetazole for the treatment of UTIs caused by ESBL-producing organisms and found no difference in clinical cure rate in the group treated with cefmetazole versus the group treated with carbapenem [223]. The authors opined that cefmetazole can be used for the treatment of UTIs as long as the organisms do not produce AmpC beta-lactamase.

Several combinations of beta-lactam and beta-lactamase inhibitors are available for clinical use. These include amoxicillin + clavulanic acid, ampicillin + sulbactam, cefoperazone + sulbactam, ticarcillin + clavulanic acid, piperacillin + tazobactam and cefepime + tazobactam. The combinations have not been very effective in the treatment of ESBL-producing Enterobacteriaceae because of poor drug concentrations achieved at site of infection, co-production of other beta-lactamases, hyperproduction of classical beta-lactamases such as TEM-1 or SHV-1 and induction of AmpC beta-lactamases by clavulanic acid [13, 224].

Despite the encouraging in-vitro results, there not many clinical studies on these combinations. Rodriguez-Bano J *et al* and Park SH *et al* have found amoxicillin + clavulanic acid and piperacillin + tazobactam (PCT) to be suitable alternatives to carbapenems for treating bloodstream infection and community acquired acute pyelonephritis caused by ESBL-producing *E. coli*, respectively, provided the in-vitro results displayed susceptibility [225, 226]. A Spanish study, which investigated 30-day mortality in patients with bacteremia by ESBL-EC suggested that PCT may be used in the treatment of bacteremia with origins from urinary tract despite elevated MIC levels,

but if the source is other than urinary tract, PCT may be used only if MIC is ≤2 µg/µl [227]. As a part of hospital policy, PCT was reportedly used for six years in a Korean hospital in lieu of oxyimino-cephalosporins. Their study revealed that not only the incidence of infection by ESBL producers came down but also no increase in resistance to PCT was observed in that period [228]. There are very few studies on the clinical efficacy of cefepime + tazobactam. A study by Ghafur *et al*, showed that this combination can be safely used in the treatment of bacteremia by Gram negative bacteria [229]. Cefoperazone, a second generation cephalosporin has been combined with tazobactam or sulbactam. A study from Mumbai reported susceptibility to cefoperazone + sulbactam and cefoperazone + tazobactam in 89% of ESBL-producing *E. coli* [230]. In that study cefoperazone + tazobactam was found to be more active than cefoperazone + sulbactam (83% vs 67%) against ESBL-KP. In an in-vitro study from Mumbai, cefoperazone + sulbactam was shown to be significantly more effective than PCT against ESBL producing Enterobacteriaceae [231].

Mecillinam, is an extended-spectrum oral penicillin with good in-vitro activity against Enterobacteriaceae members. Since the concentration of this drug is achieved at good levels in the urine, it is used in the treatment of UTIs by *E. coli* isolates. Mecillinam is not stable to hydrolysis by beta-lactamases and also suffers from the inoculum effect. Both the resistance and the inoculum effect have been shown to be reversed when it was combined with clavulanic acid. Thomas K *et al* and Lampri N *et al* have suggested that it may be useful in the treatment of uncomplicated lower UTIs caused by ESBL-EC with low MIC values [232, 233].

Temocillin, a derivative of ticarcillin is active only against Enterobacteriaceae members, with no significant activity against non-fermenters, anaerobes or Gram positive organisms [234]. It is more stable than the parent drug to hydrolysis by ESBLs

and AmpC beta-lactamases. The susceptibility breakpoint for temocillin is not available in CLSI document S100-S24. In a study by Livermore *et al* in UK, >88% of the isolates producing ESBL or AmpC beta-lactamases were susceptible to temocillin at ≤16 µg/ml and 99% of them were susceptible at ≤32 µg/ml [235]. In a clinical study from UK, temocillin use was associated with clinical cure in 93% of cases with UTI and 83% of cases with bloodstream infection by ESBL or AmpC producing Enterobacteriaceae members [236]. Because good levels of this antibiotic is achieved in the urine, this antibiotic has been suggested as an alternative to carbapenem in the treatment of UTI by ESBL/AmpC beta-lactamase producers.

Carbapenems such as imipenem, meropenem, doripenem and ertapenem are widely used as antibiotics of first choice for the treatment of serious infections due to ESBL producing Enterobacteriaceae members. These antibiotics are preferred since they are resistant to hydrolysis by different types of beta-lactamases, achieve good tissue concentrations, do not suffer from inoculum effect and are not potent inducers of AmpC beta-lactamases. A retrospective cohort study on bloodstream infections by ESBL producing *E. coli* and *K. pneumoniae* found similar outcomes when treated with ertapenem, imipenem or meropenem [237]. Use of ertapenem as a first-line antibiotic in treating different infections by ESBL producers has been associated with good clinical response [238, 239]. Vardakas KZ *et al* reported that use of carbapenems as empirical antibiotic in the treatment of bloodstream infection by ESBL producers was associated with lower mortality than those treated with combinations of beta-lactam + beta-lactamase inhibitors but the difference was not found to be significant [240]. Clinical failures have been reported to occur during the course of treatment with carbapenems, which have been attributed to selection of porin mutants and co-

production of AmpC beta-lactamase or KPC enzymes [241, 242]. Other important causes of carbapenem resistance is the production of metallo-beta-lactamases.

Ceftaroline fosamil, a fifth generation cephalosporin, is active against Enterobacteriaceae members but is ineffective against isolates producing ESBLs, cephalosporinases, and carbapenemases. Avibactam (previously known as NXL104) is a new non-beta-lactam beta-lactamase inhibitor that inhibits Ambler classes A (e.g., ESBL, KPC), C (AmpC), and D (OXA-like) beta-lactamases [20]. The combination of ceftaroline and avibactam is known to have expanded spectrum of activity. At a fixed concentration of 4µg/ml, ceftaroline + avibactam combination was shown to be active against Enterobacteriaceae strains producing ESBLs, plasmid-mediated AmpC, carbapenemases, and a combination of multiple enzymes [243]. In that study, the ceftaroline + avibactam MICs for ESBL producers ranged from 0.015/4 – 1/4 µg/ml. In another study, 98.2% of ESBL producing isolates were reportedly inhibited by ceftaroline + avibactam at MIC of < 0.5/4 µg/µl [244]. This combination was shown to be effective against producers of CTX-M, SHV as well as KPC beta-lactamases with activity comparable to that of imipenem. In another in-vitro study, the combination of ceftaroline + avibactam was shown to be active against 100% of ESBL-EC and 96% of ESBL-KP [245].

Ceftolozane + tazobactam is a novel cephalosporin and beta-lactamase inhibitor combination that has improved activity against *P. aeruginosa* and several Enterobacteriaceae members. Although interpretive criteria for ceftolozane + tazobactam susceptibility has not been established, an American study has shown that it was active against most ESBL-EC isolates at MIC of 0.5/4 µg/ml [246]. However, its potency was much lower against ESBL-KP, which was probably due to co-production

of carbapenemases in those isolates. Further clinical trials are awaited for its approval as a therapeutic agent.

Tigecycline, is a novel glycylcycline antibiotic, which inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit of the bacteria. Report from Tigecycline Evaluation and Surveillance Trial (TEST) conducted in USA reported a susceptibility rate of 100% to ESBL-EC and 92% to ESBL-KP [247]. A study from Argentina reported susceptibility of 99.8% against ESBL-EC and 93.7% against ESBL-KP [248]. Roy *et al* reported that all the ESBL-EC in their study were susceptible to tigecycline but 3.5% of ESBL-KP were resistant to it [131]. The authors also observed that over a period of four years (2007-10), the MIC levels of tigecycline increased by two-folds, which was comparatively higher among *K. pneumoniae* than *E. coli*.

Fosfomycin, which inhibits an enzyme that catalyzes the first step in bacterial cell-wall synthesis, has a broad spectrum of antimicrobial activity. The MIC breakpoints for susceptibility against urinary isolates is 64 µg/ml. A study on ESBL producing urinary isolates in USA showed that 91.3% of CTX-M and 100% of SHV or TEM type ESBL-EC were susceptible to fosfomycin [249]. In a study from India, all the ESBL producing urinary isolates of *E. coli* tested by them were susceptible to fosfomycin [250]. Clinical cure with fosfomycin in patients with UTI produced by ESBL-EC has been shown to vary from 78.8% to 93.8% [251, 252].

Apart from the aforementioned antibiotics, others such as aminoglycosides, fluoroquinolones and trimethoprim + sulfamethoxazole may be used if the ESBL producers are in the susceptible range. Park SH *et al*, reported that the efficacy of antibiotics such as amikacin, ciprofloxacin and trimethoprim + sulfamethoxazole were similar to that of carbapenems in treating community acquired acute pyelonephritis

caused by ESBL-EC [226]. Hsueh PR reported that aminoglycosides and polymyxins (colistin or polymyxin B) can be used for the treatment of multidrug-resistant organisms or serious UTIs when treatment with first line antibiotics fail [253]. However, many CTX-M ESBL producing *E. coli* possess plasmids that also code for resistance to fluoroquinolones and aminoglycoside [254, 255].

Thus, there are multiple approaches to treat infections by ESBL producing Enterobacteriaceae members such as beta-lactam + beta-lactamase inhibitor combinations, mecillinam, temocillin, fosfomycin and tigecycline. These antibiotics may be encouraged to prevent overuse of carbapenems.

Current interest in ESBL

A timeline in the history of significant discoveries is displayed in Figure 17. Ever since the discovery of the ESBL in 1983, these enzymes have been increasingly reported from all parts of the world. Due to the increased awareness, many researchers are taking interest in its detection and identification.

Both EUCAST and CLSI do not recommend testing for ESBLs in clinical isolates on a routine basis anymore. Despite that studies on ESBL continue to be published. By the first week of January 2015, 28 articles have been indexed in PubMed with keyword “ESBL”. A search on PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) with query of “ESBL” OR "extended spectrum beta-lactamase" OR "extended spectrum ß-lactamase" showed up over 5400 results. The actual number would be even more as several journals are not indexed in PubMed. Year wise data on number of literature published with the aforementioned search terms has shown sharp increase since 1989.

Figure 16: Number of publications indexed in PubMed citing ESBL

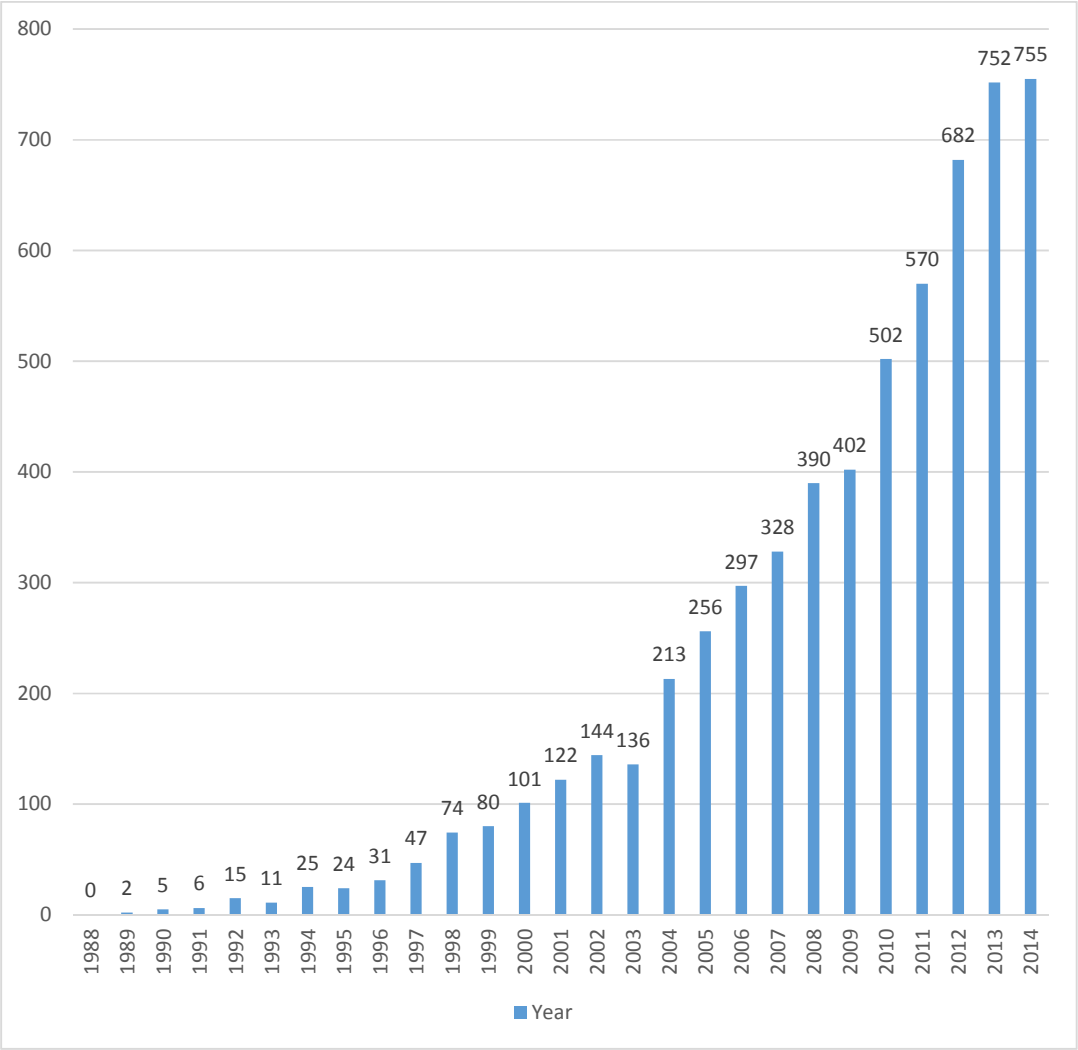
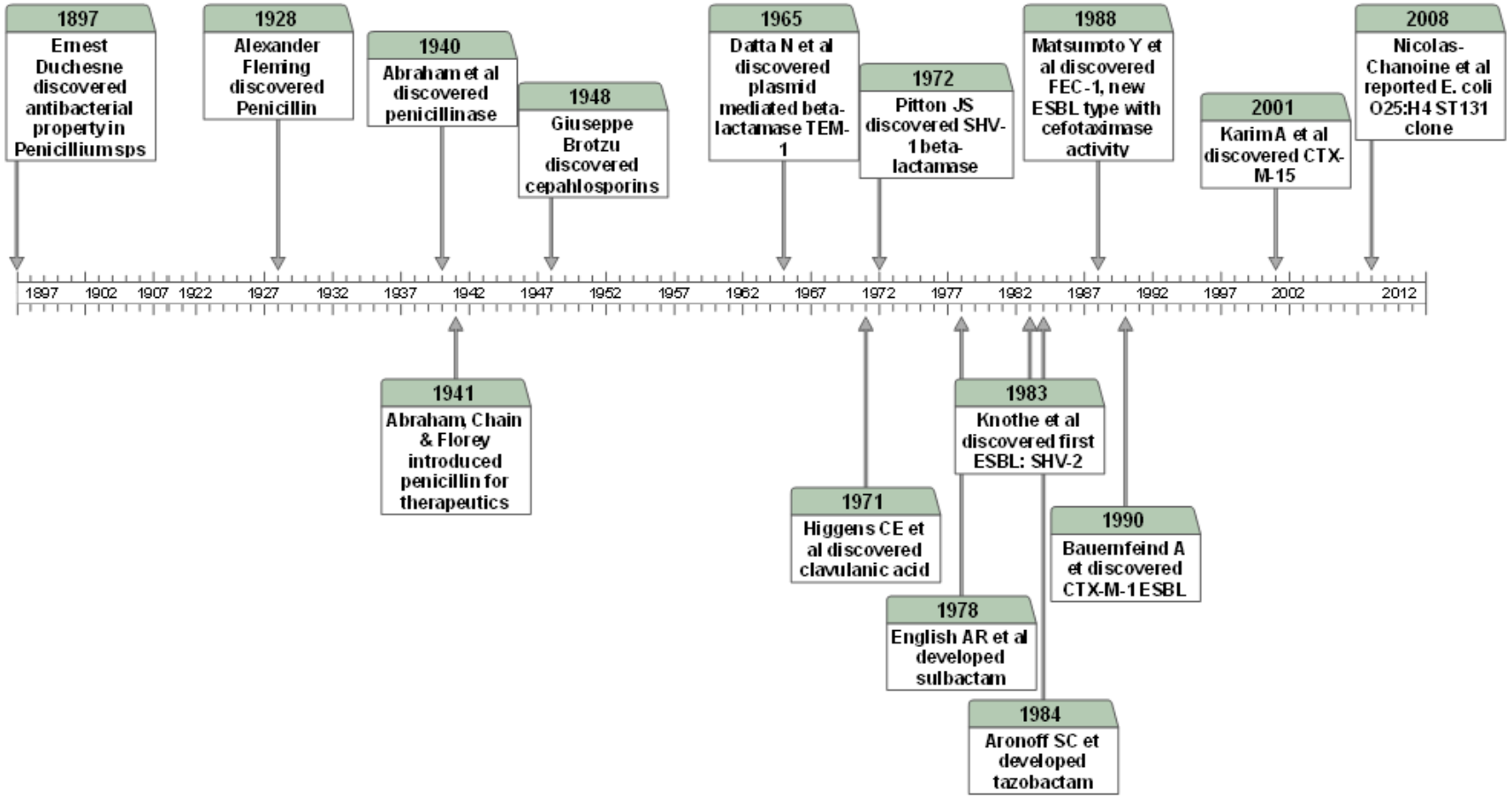


Figure 17: Timeline of significant milestones in the history of beta-lactamase mediated resistance and its inhibition



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