2. Review of literature

Antibiotics are a special kind of chemotherapeutic agents usually obtained from living organisms. The word 'antibiotic' has come to refer to a metabolic product of one microorganism that in very small amounts is detrimental or inhibitory to other microorganisms. It has been known for many years that antagonisms can exist between microorganisms which grow in a common environment. Antibiotics are a large group that also includes antiviral, antifungal, and antiparasitic drugs. They are relatively harmless to the host, and therefore can be used to treat infection. Antibiotics are among the medications most frequently prescribed today although their continued efficacy is threatened by microbial resistance caused by evolutionary pressures (Davies et al., 2010).

Antibiotics developed by chemical modifications have been very useful in treating infectious disease and has increase spectrum of activity, reduced toxicity, reduce side effects and leading to enhanced killing of pathogens. The development of resistance by many bacteria is the inevitable result of high clinical use of antimicrobial agents. The extent of resistance and the variety of resistance mechanisms that are now relatively common among many bacteria are truly astounding (Davies et al., 2010).

The first effective antibiotic discovered was penicillin. French physician Ernest Duchesne noted in his 1896 thesis that certain *Penicillium* molds killed bacteria. Duchesne died within a few years, and his research was forgotten for a generation, until an accident intervened, Alexander Fleming in 1928 had been culturing bacteria on agar plates, one of which was ruined by an accidental fungal contamination. Rather than discarding the contaminated plate, Fleming noticed a clear zone surrounding the colony of mold. Having previously studied the ability of the enzyme lysozyme to kill bacteria, Fleming realized that the mold was secreting something that stopped bacterial growth. He knew that substance might have enormous utility to medicine. Although he was unable to purify the compound (the β-lactam ring in the penicillin molecule was not stable under the purification methods he tried), he reported it in the scientific literature. Since the mold

was of the genus *Penicillium*, he named this compound penicillin (Kong et al., 2009; Flemming, 1929).

2.1 Classification of antibiotics

Antibiotics were classified by their mechanism of action (the mechanism by which they selectively poison bacterial cells **(**Mims et al., 2008; Willey et al., 2008; Walker 1998).

2.1.1 Antibiotics which interfere with cell wall synthesis: β-lactam antibiotics, including penicillins, cephalosporins, carbapenems, monobactam, glycopeptides and cycloserine.

2.1.2 Antibiotics which interfere with bacterial protein synthesis and antibiotics which bind to the 50S ribosomal subunit: Lincosamines/ lincosides including clindamycin and lincomycin, chloramphenicol, macrolides.

2.1.3 Antibiotics which interfere with 30S ribosomal subunit: Tetracyclines and aminoglycosides.

2.1.4 Drugs which inhibit folate synthesis: Sulfonamides and trimethoprim.

2.1.5 Drugs which interfere with RNA synthesis: Rifampin (rifampicin).

2.1.6 Drugs which interfere with cell membrane function: Polymixin B, gramicidin.

2.2 β-lactam antibiotics:

β-lactam antibiotics are bactericidal antibiotics containing β-lactam ring in their molecular structure (Figure 1). These are one of the most important groups of antimicrobial agents administered worldwide and also covering as much as 50% of all recommended drugs. This is because they are highly effective and the side-effects are minimal. The β-lactams belongs to the group of antimicrobial agents inhibiting the cell wall synthesis. They have a bactericidal effect on the microorganisms and are classified into several groups; penicillins, cephalosporins, carbapenems, monobactams, and the β-lactamase inhibitors. The different groups consist of both natural and synthetic

compounds varying in their chemical structure with the β-lactam ring as the common property. Each group has different ring structures and acyl side chains attached to the β-lactam ring (Mims et al., 2008; Essack et al., 2001).

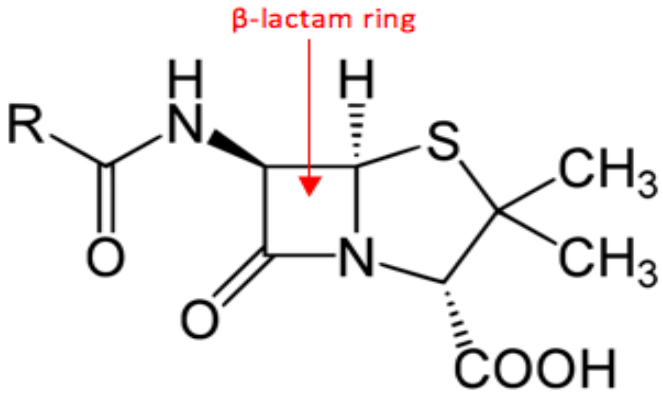

Figure 1: structure of β-lactam antibiotics (**Source:** http://lnls.cnpem.br/the-role-of-the-cystein-residue-in-the-inactivation-of-%CE%B2-lactam-antibiotics/)

2.3 Mechanism of action of β-lactam antibiotics:

They inhibit building of bacterial cell wall by interference with the synthesis of peptidoglycan. The β-lactam ring acts as an analogue of acyl-D-alanine-D-alanine and binds tightly to the active site of transpeptidase enzymes that catalyze the transpeptidation of MurNAc units within the cell wall. This transpeptidation is important because cross linking completes the murin sacculus and provides it with the tensile strength which needed to resist the osmotic lysis. The β-lactam antibiotics act on bacteria through two mechanisms targeting the inhibition of cell wall synthesis **(**Kong et al., 2009; SamahaKfoury and Araj 2003).

- **Firstly,** they are incorporated in the bacterial cell wall and inhibit the action of the transpeptidase enzyme responsible for completion of the cell wall.
- **Secondly,** they attach to the penicillin binding proteins that normally suppress cell wall hydrolases, thus freeing these hydrolases, which in turn act to lyse the bacterial cell wall. (Walker 1998; Pitout et al., 1997; Jacoby et al., 1991).

2.4 Groups of β-lactam antibiotics:

There are four groups of β-lactam antibiotics viz; Pencillins, Cephalosporins, Carbapenems, and Monobactam (Figure 2).

2.4.1 Penicillins: The group can be divided in two subgroups: Natural Penicillins and semisynthetic penicillins (Figure 2).

2.4.1.1 Natural Penicillins: They have narrow spectrum containing gram-positive and –negative cocci (streptococci, pneumococci, enterococci, meningococci), gram-positive bands (corynebacteria, *L. monocytogenes*), spirochetes (*Leptospira* sp., *Treponema* sp., *Borrelia* sp.), and most of anaerobes (peptostreptococci, clostridial species, Actinomyces). Penicillin G and Penicillin V are natural penicillins (Figure 2) (Walker 1998).

2.4.1.1.1 Penicillin G:

The nucleus of Penicillin G is a double ring structure consisting of a thiazolidine ring and a β-lactam ring as well as the phenylacetic acid is attached to the β-lactam ring via peptide bond. The active site of the penicillin is the β-lactam ring (Walker 1998). Penicillin G has primarily been used as treatment against gram positive bacteria such as pneumocoocci, staphylococci and streptococci. It has been used to treat infections caused by *Neisseria gonorrhoeae* and *Neisseria meningitides,* which are gram negative organisms. However Penicillin G unstable in gastric acid juice but suitable only for intravenous administration (Mims et al., 2008, Willey et al., 2008; Walker 1998).

2.4.1.1.2 Penicillin V:

Penicillin V represents the major advance because it was much more stable than Penicillin G in stomach acid. It can be given orally because it is more resistant to acid (Walker 1998).

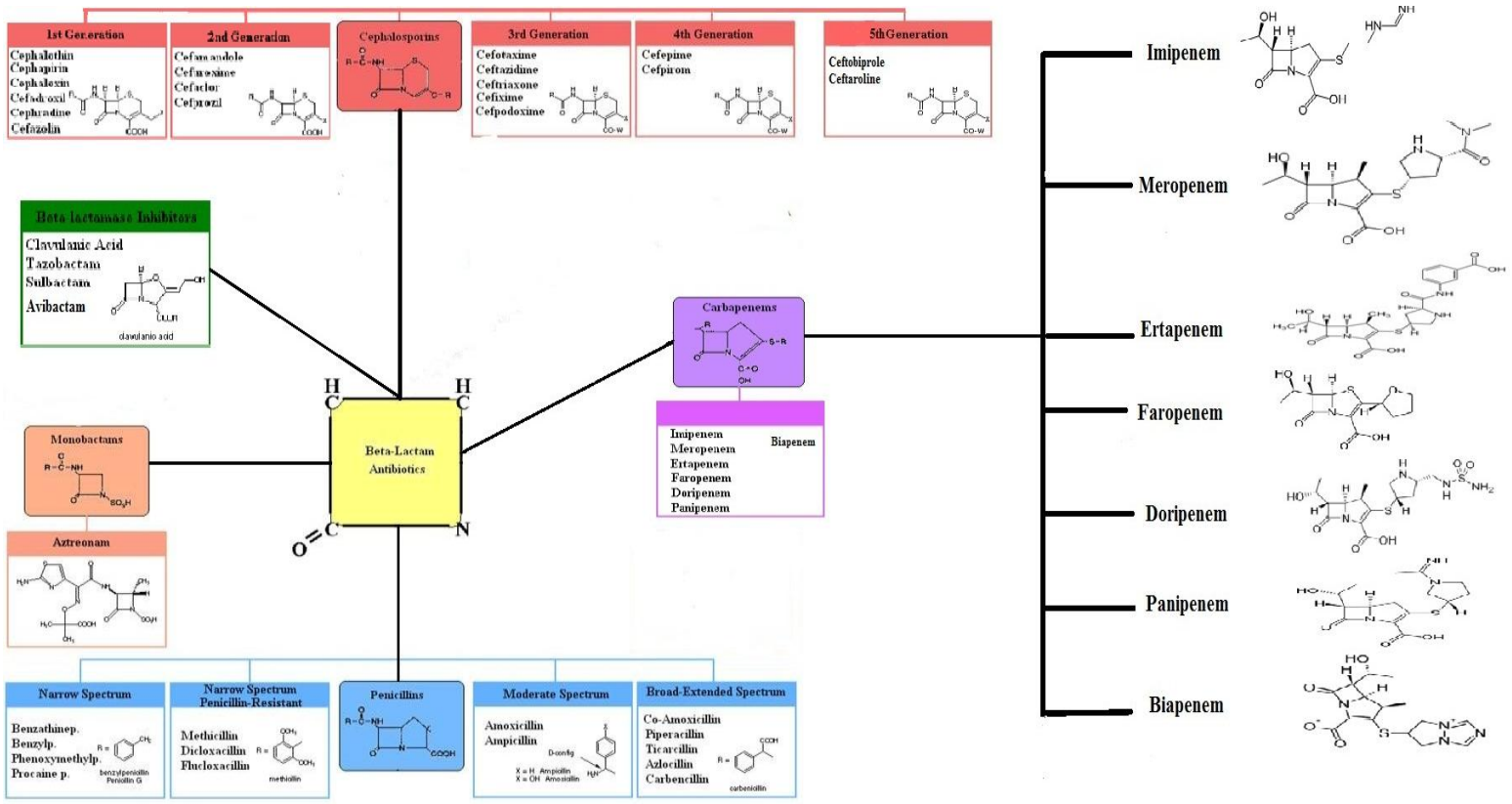

Figure 2: Different groups of β-lactam antibiotics (**Source:** http://www.scielo.br/img/revistas/jped/v82n5s0/en_v82n5s0a08f01.gif and http://www.shutterstock.com/pic-206261929/stock-vector-structural-chemical-formulas-of-beta-lactam-antibiotics-carbapenems-imipenem-meropenem.html)

2.4.1.2 Semisynthetic penicillins:

There are three major goals drove the development of semisynthetic penicillins:

- ✓ **First**, there was desire to produce more acid stable penicillins that could be administered orally, rather than parentally (Willey et al., 2008; Walker 1998).
- ✓ **Second,** some bacterial strains that originally had been susceptible to penicillin therapy later because resistant to it. This acquire resistance was due to the ability of bacteria to produce an enzyme (a penicillinase) that inactivates penicillin G by cleaving the β-lactam ring. So, investigators sought to attach R groups onto penicillins that would protect the penicillin from β-lactamase hydrolysis but allow the antibiotic to reach its target transpeptidase (Wiley et al., 2008; Walker 1998).
- ✓ **Third**, penicillin G and V had narrow spectrum of activity, while they were effective against many gram positive organisms, they were only effective against a handful of gram positive organisms. So, investigators searched for R group substitutions that would allow penicillins to kill members of enterobacteriacae and *Pseudomonas aeruginosa* (Willey et al., 2008; Walker 1998).

Three groups of semi synthetic penicillins were subsequently developed: **(i)** Penicillianse resistant penicillins, **(ii)** extended spectrum penicillins and **(iii)** antipseudomonal penicillins.

2.4.1.2.1 Penicillianse resistant penicillins: For treatment of the emerging strains of staphylococci that were resistant to penicillin G, a series of Penicillianse resistant penicillins were designed. It includes methicillin, nafcillin and the isoxazolyl penicillins like oxacillin, cloxacillin, dicloxacillin and flucloxacillin. These penicillins are more toxic and less active than penicillin G (Willey et al., 2008; Walker 1998).

2.4.1.2.2 Extended spectrum penicillins: This group was developed to extend the spectrum of penicillins to include gram negative bacteria. This includes aminopenicillins (amicillin, amoxicillin and bacampicillin) and amdinocilin (pivmecillinam) (Willey et al., 2008; Walker 1998; Bodey G P 1990). The aminopenicillins are effective against many

gram negative bacteria and are about one half as effective against gram positive bacteria as is penicillin G. Amdinocillin is unusual in that it is extremely active against *Escherichia coli, Enterobacter* spp.and *Klebsiella* spp. but is relatively inactive against gram positive bacteria (Willey et al., 2008).

2.4.1.2.3 Antipseudomonal Penicillins: Three types of penicillins have been developed to attack *Pseudomonas aeruginosa*: the carboxypenicillins, the peperazine penicillins and the uridopenicillins (Willey et al., 2008). However carbenicillin and ticarcilln, which are carboxypenicillins, were the first antipseudomonal penicillins. They are not effective against gram negative bacteria but have excellent activity against enterobacteriacae and *Pseudomoans.* These drugs are given in intensive care infections. The only route of administration is intravenous (Bodey G P 1990).

2.4.2 Cephalosporins: Cephalosporins are semisyntheitc derivatives of 7-aminocephalosporanic acid, which contains a β-lactam ring and a six membered dihydrothiazine ring (Figure 2) **(**Demain et al., 1999**).** Cephalosporins are a group of antibiotics produced by a species of marine fungus, *Cephalsporium acremonium,* which bears considerable resemblance to *Penicillin* spp. The mode of action of cephalosporins is that of inhibition of the cross linking transpeptidase. They are bactericidal to growing cells. Cephalosporin-fluoroquinolones esters act as cephalosporins and as prodrugs for fluoroquinolones (Georgopapadakou et al., 1993; Georgopapadakou 1989). Cephalosporin antibiotics are divided in four subgroups called generations. The individual drugs are arranged into generations according their spectrum of antibacterial activity including the susceptibility/resistance to β-lactamases.

2.4.2.1 First generation cephalosporins

The earliest cephalosporin was first generation cephalosporins. These antibiotics are effective primarily against a group of gram- positive bacteria that includes *Pneumococci, Streptococci, Clostridium perfringes, Corynebacterium diptheriae, Staphylococcus epidermis, and*methicillin resistant *Staphylococcus aureus* (MRSA) (Walker 1998). The drugs are predominantly used for treatment of skin and soft tissue infections and for

prophylaxis in surgical procedures (except colorectal surgery and situations when methicillin resistant staphylococci are spread in the surgery department) (Walker 1998) (Figure 2). **Examples:** Cefazolin, Cefacetrile, Cefadroxil, Cefaloridine, Cefalotin, Cefapirin, Cefatrizine, Cefazedone, Cefazaflur, Cefradine, Cefroxadine and Ceftezole.

2.4.2.2 Second generation Cephalosporins

The second-generation cephalosporins are more extensively modified than are the first generation cephalosporins. These are more active against gram- negative bacteria. Cefuroxime has proved to be highly effective against β-lactamase producing strains of *Haemophilus influenzae* and *Nissereia meningitidis*. Additionally, cefotetan and cefoxitin are effective against *N. gonorrhoeae,* including penicillinase- producing strains. The cephamycin, cefotetan has proved to be so effective against gram- negative enteric rods (Walker 1998) (Figure 2). **Examples:** Cefaclor, Cefamandole, Cefminox, Cefonicid, Ceforanide, Cefotiam, Cefprozil, Cefbuperazone, Cefuroxime, Cefuzonam, Cefphamycin (Cefoxintin, Cefotetan, Cefmetazole), and Carbacephem (Loracarbef).

2.4.2.3 Third generation Cephalosporins

Third generation cephalosporins are highly resistant to the actions of β-lactamases because of their large and unusual R groups. Although, third- generation cephalosporins have the widest spectrum of activity against gram negative bacteria and they have the poorest coverage against gram positive bacteria. In general, expanded spectrum cephalosporins are highly effective agents against the following gram-negative organisms. *N. gonorrhoeae* (including penicillinase producing strains) *N. meningitides*, *H. influenza*, *Moraxella catarrhalis* and most enteric bacteria (including many *Citrobacter* spp., *E. coli*, *Klebsiella* spp., *Morganella* spp., *Proteus* spp., *Providencia* spp., *Salmonella* spp., and *Shigella* spp.). However ceftazidime has the good activity against *P. areuginosa* along with cefoperazone having reasonable antipseudomonal activity (Walker 1998) (Figure 2). **Examples:** Cefixime, Ceftriaxone, *antipseudomonal* (Ceftazidime, Cefoperazone), Cefotaxime, Cefpimizole, Cefpiramide, Cefpodoxime, Cefsulodin, Cefteram, Ceftibuten, Ceftiolene, Ceftizoxime, and *oxacephem* (Flomoxef, Latamoxef).

2.4.2.4 Fourth generation Cephalosporins

This is recently developed group of cephalosporins and comprises cefepime and cefpirome and though that this class of cephalosporins is much related to the third generation cephalosporins in activity against gram negative bacteria. These agents also have particular therapeutic usefulness in treatment of infections due to aerobic gram negative bacilli resistant to third generation Cephalosporins (Walker 1998) (Figure 2). **Examples:** Cefepime, Cefozopran, Cefpirome, and Cefqinome.

2.4.2.5 Fifth generation Cephalosporins

Fifth generation cephalosporins are most recently launched for commercial use includes **Ceftaroline** and **Ceftobiprole** (Figure 2). The mechanism of action of **Ceftaroline's** is mediated by binding to the penicillin binding protein (PBP), the enzyme which mediating the cross-linking transpeptidation of the peptidoglycan which is the terminal step in completing formation of the bacterial cell wall. It also possesses an ethoxyimino side-chain mimicking a portion of a cell wall structure, which acts as a "Trojan horse", allosterically opening and facilitating access to the active site of the PBP2a (Joseph 2011; Christopher 2011). Alternatively **Ceftobiprole** rapidly binds and forms a stable inhibitory acyl-enzyme complex with PBP 2 (PBP 2a) and PBP 2x, which provide activity against β-lactam resistant staphylococci and streptococci, respectively. The stability of the acyl enzyme complex, in combination with the long side chain that sits deep in the PBP 2´-binding pocket, enhances the stability of the bond and inhibition of the enzyme (Jamie et al., 2008).

2.4.3 Penem:

A penem is a type of unsaturated β-lactam antibiotics. **For example**: Faropenem and Carbapenem (Figure 3). These are much related in organization to carbapenems. Still penems have sulfur and carbapenems have one another carbon. Moreover all the penems are synthetically made.

2.4.3.1 Structure of penems:

There are five main subgroups of penem: **thiopenems, oxypenems, aminopenems, alkylpenems,** and **arylpenems**. All these have been produced and are distinguished by the side chain (at position 2), of the unsaturated five membered ring. One structurally distinct penem is BRL 42715. This molecule has no substitution at the above position, but has a bulky group attached to the β-lactam ring, and it displays effective inhibition of class C β-lactamases but it has no antimicrobial activity. However one possible consequence of these structural differences of penems from other β-lactams may be reduced immunogenicity and immunogenic cross-reactivity.

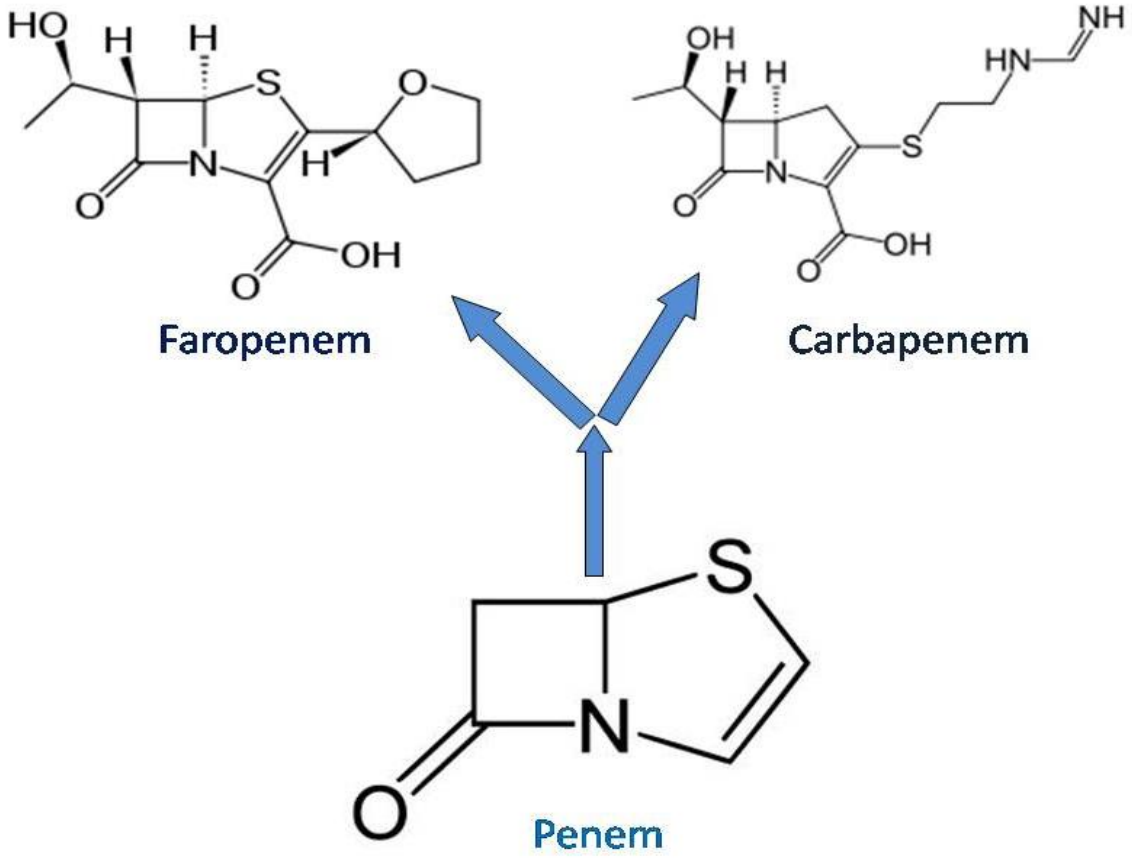

Figure 3: Structure of Penems (**Source:** http://en.wikipedia.org/wiki/Penem).

Faropenem is a unique antimicrobial penem being developed for oral administration as the pro-drug ester, faropenemdaloxate. Penems share structural similarities with both penicillins and cephalosporins (Figure 3), and are characterized by a broad antibacterial spectrum, a potent penicillin-binding protein affinity and good β-lactamase stability (Milazzo et al., 2003). Faropenem has good activity against *E. coli* and *Klebsiella* spp. with Extended spectrum β-lactamases (ESBLs), including the CTX-M types which is now a day's proliferating in Europe, but was less active against AmpC-derepressed and ESBL-producing *Enterobacter* spp (Mushtaq et al., 2007).

2.4.4 Carbapenems:

Carbapenems are derived synthetically from thienamycin, an antibiotic that is produced by bacterium *Streptomyces cattleya*. Carbapenems have a double ring structure that includes a β-lactam ring, but five membered rings retain a C instead of an S at position. Imipenem inhibits transpeptidation by binding tightly to PBP-1 and PBP-2. It is stable in the presence of most β-lactamases, including chromosomal class I β-lactamases that degrade third generation cephalosporins. Imipenem, Meropenem, Ertapenems, Doripenem, Panipenem and Biapenem are carbapenem antibiotics (Figure 2 and 3) **(**Krisztina et al., 2011; Bodey G P 1990**).** They are very potent antibiotics of extremely broad spectrum including majority of gram-positive and gram negative pathogenes. The group of non affected microbes embraces methicillin resistant staphylococci, *Clostridium difficile, Stenotrophomonas maltophilia, Pseudomonas cepacia* and some exceptionally resistant strains of enterococci, *Acinetobacter*, and *Pseudomonas* (Walker 1998).

Imipenem induces the expression of chromosomal class I β-lactamases. The spectrum of imipenem can be augmented by administering it in combination with an aminoglycoside. This antibiotic is metabolized by a naturally occurring dihydropeptidase in the renal brush order. Imipenem/cilastatin should not be administered to individuals who have central nervous system lesions (such as strokes or head injuries), a history of convulsions, or renal insufficiency, since reports indicate that 12- 32% of these patient develop convulsions as a consequence of receiving this form of treatment. Because of its propensity for eliciting seizures in these patients and because of its cost, imipenem is

generally reserved for use in patients who are gravely ill with nosocomial infections caused by multiple pathogens (Rodloff et al., 2006; Walker 1998; Bodey G P 1990) (Figure 2).

Meropenem is an ultra-broad spectrum injectable antibiotic used to treat a wide variety of infections. It can be administered without cilastatin and carries a lower risk of causing seizures. It penetrates well into many body fluids and tissues including the cerebrospinal fliud, peritoneal fluid, bile, heart valves and lung (Lowe et al., 2000). Meropenem is bactericidal except against *Listeria monocytogens* where it is bacteristatic. Spectrum of action of meropenem includes many gram negative and gram positive bacteria (including *Pseudomonas*) and anaerobic bacteria. Meropenem is frequently given in the treatment of febrile neutropenia. This condition frequently occurs in patients with hematological mailgnancies and cancer patients receiving anticancer drugs that cause bone marrow suppression. It is approved for complicated skin and skin structure infections, complicated intra abdominal infections and bacterial meningitis (Lowe et al., 2000) (Figure 2).

Ertapenem has been designed to be effective against gram negative and gram positive aerobes and anaerobes. But it is not active against MRSA, ampicillin resistant enterococci, *Pseudomonas aeruginosa* or *Acinetobacter* species. Ertapenem also has clinically useful activity against anaerobic bacteria **(**Zhanel et al., 2005) (Figure 2).

Doripenem, Food and Drug Administration (FDA) has been approved this antibiotics in 2007. It is an ultra-broad-spectrum injectable antibiotic. Firstly it was launched by Shionogi Company of Japan under the brand name Finibax in 2005 and it is being marketed outside Japan by Johnson and Johnson. It is particularly active against *Pseudomonas aeruginosa*. This antibiotic can be used for bacterial infections for instance: complex abdominal infections, pneumonia within the setting of a hospital, and complicated infections of the urinary tract including kidney infections with septicemia. Doripenem decreases the process of cell wall growth, which eventually leads to elimination of the infectious cell bacteria altogether (Figure 2).

Panipenem possesses potent activity against both gram-positive and gram negative bacteria and it is very significant for the successful treatment of patients troubled with serious infections. This antibiotic is marketed by Daiichi Sankyo Company of Japan and was launched in 1993 under the brand name Carbenin (Figure 2).

Biapenem has in vitro activity against many gram negative and gram positive aerobic and anaerobic bacteria, including those producing β-lactamases and was approved in Japan in 2001. Biapenem is stable to hydrolysis by human renal dihydropeptidase-I (DHP-I) and it does not require the co-administration of a DHP-I inhibitor (Thamlikitkul et al., 2010) (Figure 2).

2.4.5 Monobactam:

The first monobactam antibiotic is aztreonam (Figure 2). It is so named monobactams due to presence of only naked single β-lactam ring with central nucleus being the totally synthetic 3- aminobactamic acid.It is inactive against gram positive organisms and anaerobic bacilli due to it bind poorly to their PBPs. It is active against most aerobic gram negative organisms, including *P. aeruginosa*, *H. influenzae,* and *Neisseria* spp. as well as β-lactamase producing strains. It is also active against many gentamicin and carbenicillin resistant strains of *P. aeruginosa* (Walker 1998; Bodey G P 1990).

Aztreonam is commercially available only as a parenteral preparation. Intramuscular and intravenous administration result in similar serum concentrations. Aztreonam can be used to treat serious nosocomial infections caused by gram-negative bacilli. In potential mixed organism infections, involving gram positive organisms or anaerobic bacilli (as seen in the lower respiratory tract, abdomen, or pelvis), an antimicrobial agent with activity against these organisms must be included. Aztreonam combined with vancomycin has been used successfully for the initial therapy of fever in neutropenic patients (Walker 1998; Bodey G P 1990). Other major advantage of aztreonam is lack of its cross-allergenicity with other β-lactam antibiotics. Animal and human studies indicate a lack of cross-reactivity to aztreonam with antibodies produced against penicillin G. Therefore, aztreonam is a reasonable alternative for patients with penicillin or cephalosporin

allergies. Nevertheless, allergic reactions can be caused by aztreonam itself (Walker 1998) (Figure 2).

2.5 β-lactamase inhibitors

β-lactamase inhibitors are those which inhibit or destroy the efficacy of β-lactamase enzymes. They are also known as suicide inhibitors and form an irreversible acyl enzyme complex by a covalent bond throughout the catalysis reaction with the β-lactamase. It leads to activity loss of the enzyme**.** These β-lactamase inhibitors are divided into two groups: clavulanic acid and penicillanic acid sulfones (Figure 4 and 5). Clavulanic acid acts synergistically with multiple penicillins and cephalosporins against gram-negative bacteria that produce β-lactamases enzyme. The penicillanic acid sulfones, sulbactam and tazobactam are structurally related and sulbactam is combined with ampicillin, while tazobactam is combined with piperacillin (Moosdeen et al., 1988).

2.6 Commercially available β-lactamase inhibitors

2.6.1 Clavulanic acid or clavulanate: commonly used in combination with amoxicillin (Augmentin) or ticarcillin (Timentin)

2.6.2 Sulbactam: commonly used in combination with ampicillin (Unasyn) or Cefoperazone (Sulperazon)

2.6.3 Tazobactam: commonly used in combination with piperacillin (Zosyn or Tazocin)

2.6.4 Avibactam: It is non β-lactam β-lactamase inhibitor antibiotic and a class of inhibitors called the diazabicyclooctanes (DBOs), which is used in clinical development combined with β-lactam partners, for the treatment of bacterial infections including Gram-negative organisms. It is a structural class of inhibitor that does not contain a β-lactam core but upholds the capability to covalently acylate its β-lactamase targets (Ehmann et al., 2012) (Figure 4).

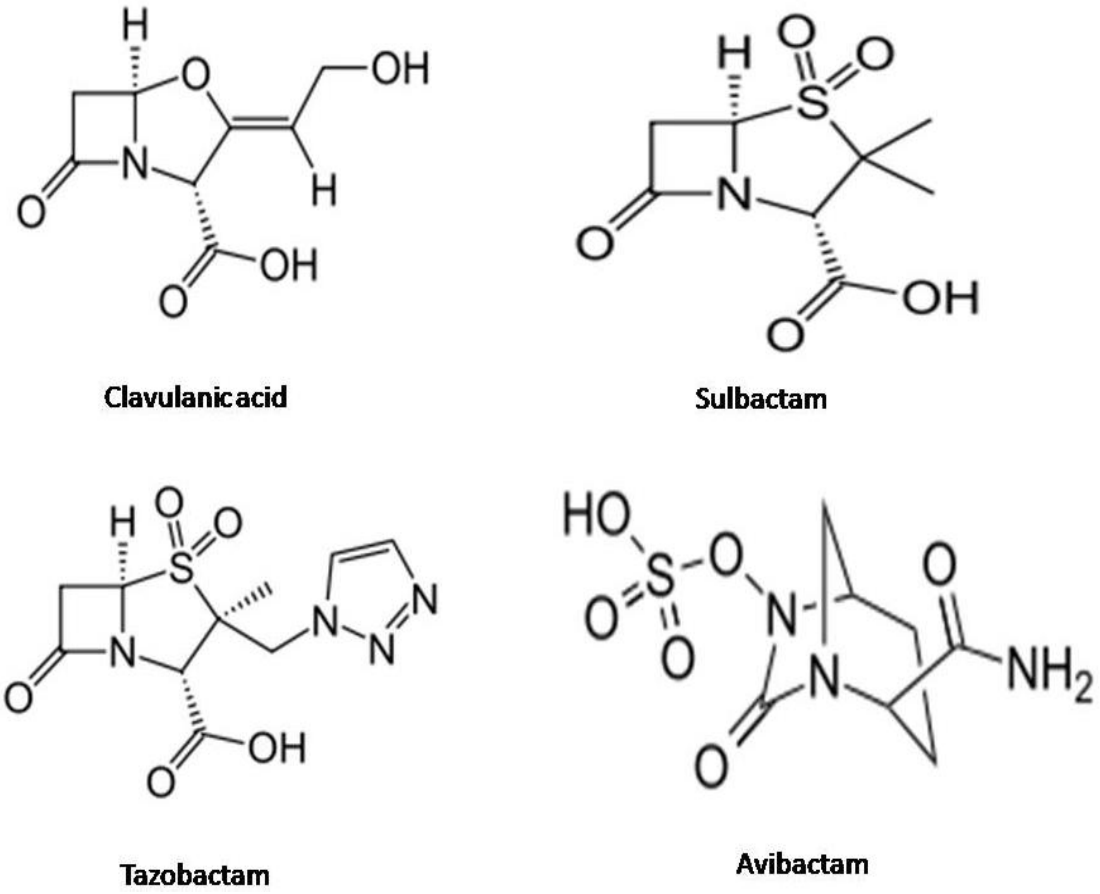

Figure 4: Commercially available β-lactamase inhibitors (**Source:** Ehmann et al., 2012).

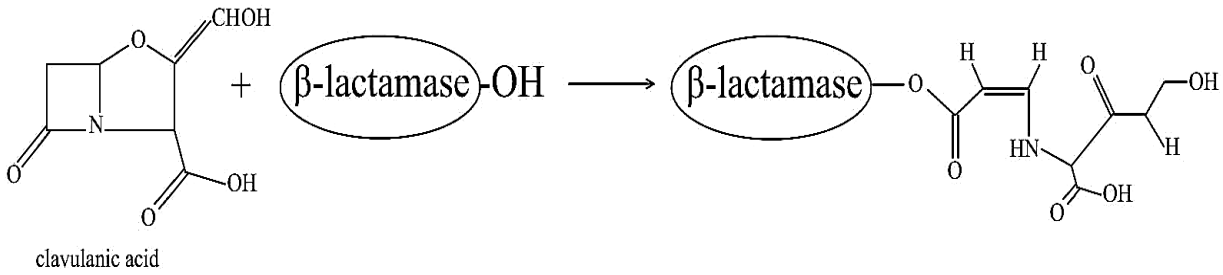

Figure 5: Action of β-lactamase inhibitors: the inhibitor binds and inactivates the β-lactamase. (Source: http://homepage.ntlworld.com/diamonddove/08BLInhibitors/BLInhibitors.htm).

2.7 Bacterial resistance to β-lactam antibiotics:

An organism to be resistant to an antimicrobial agent when it is inhibited in vitro by a greater concentration than highest achievable concentration in the human body (Poole 2004; Hawkey 1998).

2.7.1 Types of resistance:

There are two types of resistance: **Intrinsic and acquired resistance**

2.7.1.1 Intrinsic resistance:

This type of resistance is due to either the lack of the target for the action of drug or inability of drug to enter the bacterial cell (Davies and Davies 2010; Greenwood et al., 2006; Normark and Normark 2002).

2.7.1.2 Acquired resistance:

This type of resistance includes: **Mutational resistance and transferable resistance.**

Mutational resistance may occur either by point mutation, deletion, inversion or insertion in the bacterial genome resulting in a very few individuals, among the huge bacterial populations, exhibiting spontaneous resistance. These resistant mutants proliferate under the action of antibiotic selective pressure to constitute the majority or even the whole population (Greenwood et al., 2006; Normark and Normark 2002). Other is **transferable resistance** in which a resistance gene (or genes) transfers from resistant to susceptible bacterial cell. Among the different DNA elements those transfer antibiotic resistances are plasmids, phages, transposons and integrons (Greenwood et al., 2006; Normark and Normark 2002).

2.7.2 Mechanisms of resistance to β-lactam antibiotics:

The efficiency of β-lactam antibiotics is dependent on the target accessibility, the degree of resistance to enzymatic inactivation by β-lactamase enzymes and the capability of β-lactam to inhibit the target PBPs. Combinations or modifications of these parameters may

result in resistance. The clinical significant of mechanisms of resistance are alteration of the target site, enzymatic inactivation and inaccessibility to the target site (Poole 2004).

2.7.2.1 Resistance by alteration of the target site

Resistance caused by modifications in PBPs may occur by acquisition of a resistant PBP, with increased target PBP number and reduced affinity of the target PBP. However PBPs with reduced affinity are a significant mechanism of resistance to β-lactams when β-lactamases are absent (Poole 2004; Pitout 1997).

2.7.2.2 Resistance by alteration in access to the target site

Although β-lactam antibiotics diffuse through porin channels in the outer membrane to get entree to their target site: the PBPs. But sudden changes in the porin genes of gram-negative bacteria confer a decrease in permeability in the outer membrane. However synergistic effects can also take place with the expression of β-lactamases enzymes or an active efflux pump which are making the bacteria more resistant (Poole 2004; Pitout 1997).

2.7.2.3 Resistance by enzymatic inactivation

Antibiotic inactivation enzymes such as β-lactamses which are the most significant and single cause of resistance to β-lactam antibiotics. Although there are more than 700 unique enzymes which have been known and they are either chromosomally or plasmid mediated. These enzymes create biologically inactive products of the antibiotic by proficient hydrolysis of the amide bond in the β-lactam ring (Poole 2004; Pitout et al., 1997).

2.8 β-lactamases:

β-Lactamases are enzymes (EC 3.5.2.6) produced by gram positive and gram negative bacteria and are responsible for their resistance to β-lactam antibiotics (Bush et al., 1995). These were described in 1940s before the use of Penicillin became widespread (Drawz et al., 2010).These enzymes open the β-lactam ring by adding a water molecule to the common β-lactam bond, and this inactivates the β-lactam antibiotic from penicillin to

carbapenems (Figure 2). This hydrolyzation was first observed in 1940 by Abraham and Chain (penicillinase) in a strain of *E. coli* (Abraham and Chain 1940). However, the clinical effect of such hydrolyzation was not well-known until the beginning of the 1950s, when the first β-lactam-resistant *S. aureus* isolates appeared in hospitals (Jacoby 2009). There are two types of β-lactamase production in bacteria: **Constitutive β-lactamases** and **Inducible β-Lactamase**s.

Constitutive (Chromosomal) β-lactamase: This type of β-lactamase production occurs in *Pseudomonas aeruginosa*, or plasmid mediated (inducible) as in *Aeromonas hydrophila* and *Staphylococcus aureus* (SamahaKfoury and Araj 2003).

However **Constitutive β-lactamase** results from the regular expression of genetic material that encodes for β-Lactamase proteins.

And formation of **inducible β-lacatmases** enzyme occurs from exposure to a stimulus that causes expression of genetic material encoding for β-lactamase enzyme in both gram positive and gram negative bacteria (Jacoby 2005).

The first plasmid mediated β-lactamase was detected in Gram negative bacteria in Greece, in the 1960s and which was designated as TEM, named after the name of the patient (Temoneira) who carried the pathogen (Datta and Kontomichalou 1965). However this TEM-1 is the most common β-lactamase genes in gram negative bacteria, that can be hydrolyze penicillins (ampicillin). The β-lactamases also rapidly spread to other bacteria, and shortly after changes in only one or a few amino acids, these enzymes were able to hydrolyze narrow spectrum cephalosporins. These were found in *Enterobacteriaceae, Neisseria gonorrhoeae*, and *Haemophilus influenzae* (Brunton et al., 1986). Moreover these β-lactamases are secreted to the outside membrane environment as exoenzymes in gram positive bacteria. But in the gram negative bacteria they secreted in the periplasmic space, by that they attack the antibiotic before reaching its receptor site (Kong et al., 2009; SamahaKfoury and Araj 2003).

2.9 Mechanism of action of β-lactamases:

β-lactamases enzyme destroys the β-lactam ring by two major mechanisms of action. **Firstly,** most common β-lactamases have a serine based mechanism of action. In which the β-lactamases enzyme first associates non-covalently with the antibiotic to form the non-covalent Michaelis complex. Afterwards, β-lactam ring is attacked by the free hydroxyl on the side chain of a serine residue at the active site of the enzyme, yielding a covalent acyl ester. Hydrolysis of the ester finally liberates active enzyme and the hydrolyzed, inactive drug (Figure 6) (Livermore 1995).

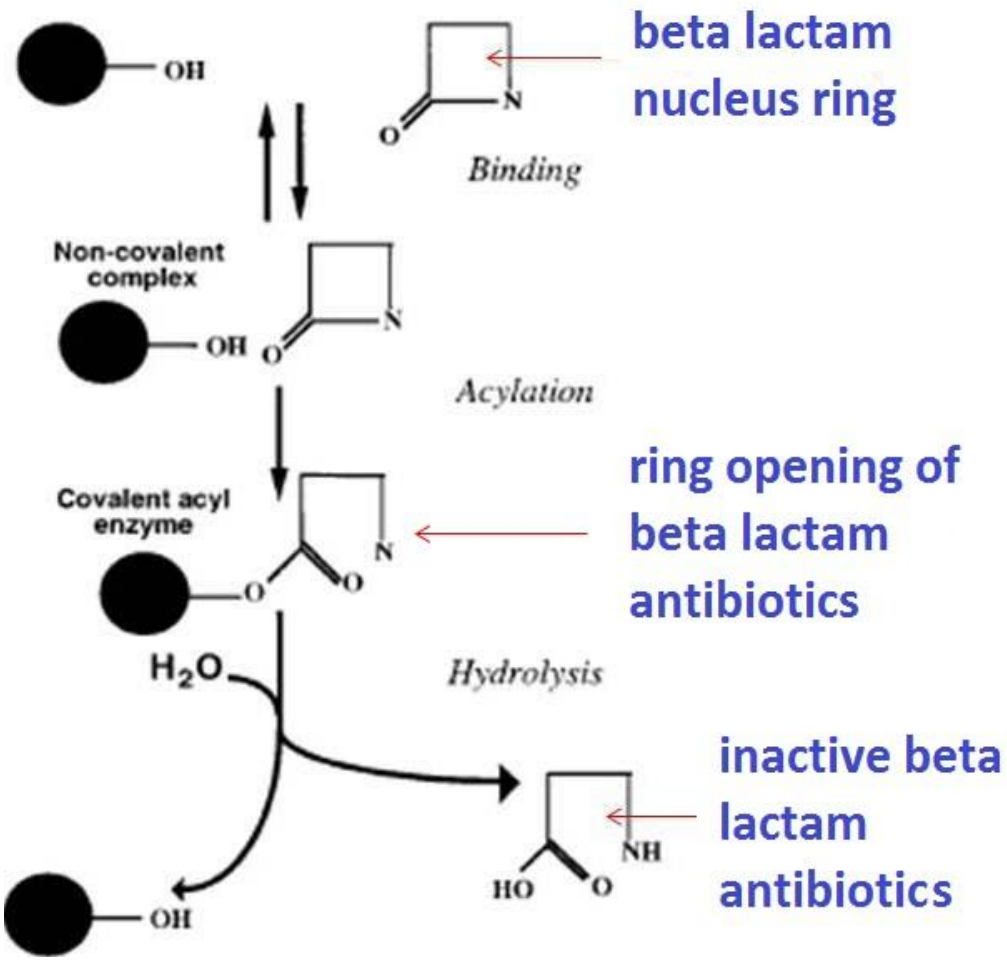

Figure 6: Action of a serine β-lactamase (Adopted and modified with permission: Livermore 1995).

Secondly, a less commonly encountered group of β-lactamases is the metallo β-lactamases, or class B β-lactamases. These use a divalent transition metal ion, most often zinc, linked to a histidine or cysteine residue or both, to react with the carbonyl group of the amide bond of most penicillins, cephalosporins, and carbapenems, but not monobactam (SamahaKfoury and Araj 2003).

2.10 Classification of β-lactamases:

There is various classification schemes have been proposed by many researchers:

- ✓ The classification of Sawai et al., (Sawai et al., 1968) in 1968, describing penicillinases and cephalosporinases by using the response to antisera as an additional discriminator.

- ✓ The Richmond and Sykes (Richmond and Sykes1973) scheme in 1973 that included all type of the β-lactamases from gram-negative bacteria described at that time and classifying the enzymes into five major groups on the basis of substrate profile.

- ✓ The extension of the Richmond and Sykes scheme by Sykes and Matthew (Sykes and Matthew1976) in 1976, they emphasizing the plasmid-mediated β-lactamases which could be differentiated by isoelectric focusing.

- ✓ However the Molecular structure classifications were first proposed by Ambler (Ambler, 1980) in 1980 when only four amino acid sequences of β-lactamases were known.

- ✓ The scheme proposed by Mitsuhashi and Inoue (Mitsuhashi and Inoue 1981) in 1981 in which the group ‘‘cefuroxime-hydrolyzing β-lactamase’’ was added to the ‘‘penicillinase and cephalosporinase’’classification;

- ✓ The groupings proposed by Bush (Bush1989a; Bush1989b; Bush 1989c) in 1989 included enzymes from all bacterial sources and it was the first scheme to try to correlate substrate and inhibitory properties with molecular structure.

- ✓ Further Bush et al., in 1995 proposed "A Functional Classification Scheme for β-Lactamases and Its Correlation with Molecular Structure" based on their substrate and inhibitor profiles.

- ✓ In the year 2005, Hall and Barlow gave a classification based on their amino acid sequences "A revised ambler classification of β-lactamases".

- ✓ In the year 2009, Bush and Jacoby gave a classification of β-lactamases "An Updated Functional Classification of β-Lactamases". In this functional classification, enzymes were aligned based on their ability to hydrolyze specific β-lactam classes and on the inactivation properties of the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam.

- ✓ In the year 2010, Bush and Jacoby proposed "Updated Functional Classification of β-Lactamases". This classification was based on hydrolytic and inhibition properties (Table 1).

2.11 Updated functional classification of β-lactamases:

Group 1 cephalosporinases: These cephalosporinases enzymes belonging to molecular class C which are encoded on the chromosomes of many *Enterobacteriaceae* and a few other organisms (Jacoby 2009).

There are new **subgroup 1e** enzymes in which group 1 variants with greater activity towards ceftazidime and some other oxyimino β-lactams as a result of amino acid substitutions, insertions, or deletions (Nordmann and Mammeri 2007).

Group 2 serine β-lactamases. This functional group 2 β-lactamases, including molecular classes A and D, which represent the largest group of β-lactamases, due primarily to the increasing identification of ESBLs during the past 20 years (Bush and Jacoby 2010).

Subgroup 2a in this subgroup penicillinases correspond to a small group of β-lactamases with a comparatively restricted spectrum of hydrolytic activity. These are the principal β-lactamases in Gram positive cocci, together with the staphylococci and rarely enterococci (Bush and Jacoby 2010).

This **Subgroup 2a** β-lactamases are **inhibited** by clavulanic acid and tazobactam with 50% inhibitory concentrations The majority of these enzymes are chromosomal even though some staphylococcal penicillinases are plasmid encoded (Bush and Jacoby 2010).

Subgroup 2b this group of β-lactamases readily hydrolyzes penicillins and early cephalosporins, like cephaloridine and cephalothin, and is strongly inhibited by clavulanic acid and tazobactam (Roy et al., 1983).

Subgroup 2be this subgroup comprises ESBLs. These are broad-spectrum enzymes which retain the activity against penicillins and cephalosporins of subgroup 2b β-lactamases and additionally they are able to hydrolyze one or more oxyimino-β-lactams, such as cefotaxime, ceftazidime, and aztreonam (Queenan et al., 2004).

Subgroup 2br These are broad-spectrum β-lactamases enzymes have acquired resistance to clavulanic acid and associated inhibitors while retaining a subgroup 2b spectrum of activity (Bush and Jacoby 2010).

Subgroup 2ber this subgroup includes TEM enzymes which combine with an extended spectrum through relative resistance to clavulanic acid inhibition (Robin et al., 2005).

Subgroup 2c this group comprises penicillinases which are characterized functionally by their ability to hydrolyze carbenicillin or ticarcillin with cloxacillin or oxacillin hydrolyzed at rates less than half those for benzylpenicillin (Bush 1995).

Subgroup 2ce this subgroup contains the newly described extended spectrum carbenicillinase RTG-4 (CARB-10) with expanded activity against cefepime and cefpirome (Potron et al., 2009).

Subgroup 2d this subgroup includes β-lactamases which is well-known by their ability to hydrolyze cloxacillin or oxacillin and therefore they are known as OXA types enzymes. Many β-lactamases of this subgroup are inhibited by NaCl and some of them are inhibited by clavulanic acid **(**Bush and Jacoby 2010).

Subgroup 2de these are cloxacillin- or oxacillin-hydrolyzing enzymes with an extended spectrum which includes oxyimino β-lactams but not carbapenems (Aubert et al., 2001).

Subgroup 2df these β-lactamases are OXA enzymes which have carbapenem-hydrolyzing abilities (Bush and Jacoby 2010).

However the subgroup of 2df enzymes has been divided into nine clusters based on amino acid homologies (Walther and Hoiby 2006).

Subgroup 2e the cahacteristics of this subgroup cephalosporinases include the ability to hydrolyze extended-spectrum cephalosporins and inhibited by clavulanic acid or tazobactam (Bush 1989).

Subgroup 2f these are serine carbapenemases and are the distinctive substrates for these enzymes, which may be inhibited better by tazobactam than by clavulanic acid (Queenan and Bush 2007).

Group 3 MBLs This functional group comprises theMetallo β-lactamases (MBLs), which is a unique group of β-lactamases according to their structure and function. However these β-lactamases were differ structurally from the other β-lactamases by their requisite for a zinc ion (Zn^{2+}) at their active site. Meanwhile as a substitute, they are inhibited by metal ion chelators such as EDTA (Marchiaro et al., 2008). However these metallo β-lactamse enzymes have been subdivided, based on either structure (subclasses B1, B2, and B3) (Garau et al., 2004) or function (subgroups 3a, 3b, and 3c).

Conversely this **subgroup 3a** includes the mostly plasmid-encoded MBL families, like IMP and VIM enzymes which have appeared globally and most frequently in non fermentative bacteria but also in *Enterobacteriaceae* (Queenan and Bush 2007).

Subgroup 3b contains a minor group of MBLs which preferentially hydrolyze carbapenems in comparision to penicillins and cephalosporins (Segatore et al., 1993).

Group 4β-lactamases: This group has been included in functional classification in 1995 but it has been omitted in the present classification scheme (Bush and Jacoby 2010).

Table 1: Classification schemes of β-lactamases

Bush Jacoby group	Molecular class	Bush Jacoby Medeiros group	Active site	Type of enzyme	Substrate	Inhibited by		Characteristics	Examples
						Clavulanic Acid or Tazobactam	EDTA		
1	C	1	Serine	Cephalosporinase	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycin	AmpC, ACT-1, CMY-2, FOX-1, MIR-1
1e	C	NI	Serine	Cephalosporinase	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often oxyimino-β-lactamases	GC1, CMY-37
2a	A	2a	Serine	Penicillinases	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	A	2b	Serine	Broad spectrum	Penicillins and early cephalosporins	Yes	No	Similar hydrolysis of benzyl penicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	A	2be	Serine	ESBL	Extended spectrum cephalosporins and monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (Cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	A	2br	Serine	Penicillinases	Penicillins	No	No	Resistance to clavulanic acid, sulbactam and tazobctam	TEM-30, SHV-10
2ber	A	NI	Serine	ESBL	Extended spectrum cephalosporins and monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams with resistance to clavulanic acid, sulbactam, tazobactam	TEM-50
2c	A	2c	Serine	Carbenicillinases	Carbenicillin	Yes	No	Increased hydrolysis of carbinicillin	PSE-1, CARB-3
2ce	A	NI	Serine	Carbenicillinases and ESBL	Carbenicillin and cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, cefepirome	RTG-4
2d	D	2d	Serine	Cloxacillinases	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	D	NI	Serine	Extended spectrum	Extended spectrum cephalosporins	Variable	No	Hydrolyzes colxacillin, oxacillin, oxyimino-β-lactams	OXA-11, OXA-15
2df	D	NI	Serine	Carbapenemases	Carbapenems	Variable	No	Hydrolyzes cloxacillin, oxacillin, carbapenems	OXA-23, OXA-48
2e	A	2e	Serine	Extended spectrum	Extended spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins inhibited by clavulanic acid but not aztreonam	CepA
2f	A	2f	Serine	Carbapenemases	Carbapenems	Variable	No	Increased hydrolysis of carbapenms, oxyimino-β-lactams, cephamycins	KPC-2, IMP-1, SME-1
3a	B (B1)	3	Zn^{2+} metal ion	Carbapenemases	Carbapenems	No	Yes	Broad- spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, IND-1
3b	B(B2)	3	Zn^{2+} metal ion	Carbapenemases	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	Unknown	4	--	--	--	--	--	--	--

2.12 Ambler's Molecular classification of β-lactmases:

Molecular classification of β-lactaamses was firstly proposed by RP Ambler in 1980 based on the amino acid sequence of the β-lacatamases. Presently β-lactamases are classified in to four classes viz; A, B, C and D which are correlating with the functional classification. Class A, B and class C are serine β-lactamases where as class B β-lactamases has Zinc divalent ion for their action and they are known as metallo β-lactamases (Ambler 1980).

2.12.1 Class A serine β-lactamases:

This class of β-lactamses is of chromosomal and plasmid-mediated penicillinases, cephalosporinases and carbapenemases and has serine in their active site. However the enzymes of this class are susceptible to the commercially available β-lactamase inhibitors like clavulanate, tazobactam, and sulbactam (Drawz and Bonomo 2010). Although first plasmid-mediated β-lactamase was identified in *E. coli* and it was named as "TEM" after the patient from whom it was isolated. SHV, this is another common β-lactamase which found primarily in *K. pneumoniae*, and was named from the term "*sulfhydryl variable*" (Paterson and Bonomo 2005). However these both TEM and SHV are common β-lactamases detected in clinical isolates of *E. coli* and *K. pneumoniae*, pathogens which are responsible for urinary tract, hospital-acquired respiratory tract, and bloodstream infections (Drawz and Bonomo 2010).

2.12.2 Class B metallo-β-lactamases:

These are Zn^{2+} dependent β-lactamases which demonstrate a hydrolytic mechanism different from that of the serine β-lactamases of class A, C, and D (Bush et al., 1995). Although they have capability to hydrolyze penicillins, cephalosporins, carbapenems, and they are resistant to inhibition by the common β-lactamase inhibitors. Meatllo β-lactamases genes are located on the chromosome, plasmid, and integrons (Walash et al., 2005).

2.12.3 Class C serine cephalosporinases:

This class of β-lacatamases has cephalosporinases with serine in their active sites. They are mostly encoded by the genes which are located on the bacterial chromosome, although plasmid encoded enzymes are becoming common (Philippon et al., 2002). Mostly the organisms of this group expressing the AmpC β-lactamases which are typically resistant to penicillins, β-lactam β-lactamase inhibitor combinations, and cephalosporins, including cefoxitin, cefotetan, ceftriaxone, and cefotaxime. However AmpC β-lactamases enzymes poorly hydrolyze cefepime and are inhibited by cloxacillin, oxacillin, and aztreonam (Bush et al., 1995).

2.12.4 Class D serine oxacillinases:

Class D β-lactamases are well known as "oxacillinases" or OXA type β-lactamases. These β-lactamases are active serine like Ambler class A and C β-lactamases which are differing in the amino structure from class A and C (Ambler 1980). They have ability to hydrolyze the isoxazolylpenicillin oxacillin faster than classical penicillin like benzylpenicillin. However these classes of β-lactamases have most diverse enzymes and have an expanded spectrum of activity. These β-lactamses genes have a source of acquired resistance in most of gram negative bacteria and also naturally produced in clinically significant pathogens and in environmental species (Nass and Nordmann 1999). Furthermore they are not inhibited by clavulanic acid, tazobactam, and sulbactam but somehow they are inhibited in vitro by sodium chloride (NaCl) (Poirel and Heritier et al., 2004; Aubert et al., 2001).

2.13 Extended Spectrum β-Lactamases (ESBLs)

Extended spectrum β-lactamases are heterogeneous group of plasmid encoded enzymes which are responsible for resistance to expended spectrum cephalosporins like cefotaxime, ceftazidime, ceftriaxone and monobactam (Bush 1996). The exposure of bacterial strains towards the β-lactam antibiotics has induced the dynamics, over production and mutation of β-lactamases in organisms which results expanding their activity against third generation cephalosporins and monobactam group of antiobiotics

(Bush 2001). However they are blocked by inhibitors like clavulanic acid, sulbactam, and tazobactam.

However the name ESBLs was usually originally to refer to TEM and SHV β-lactamases enzymes which have the capability to hydrolyze oxyimino-cephalosporins. Afterward, this word has been expanded to consist of the Enzymes derived from other source and have resistance spectra related to that of TEM and SHV mutants *viz*. CTX-M and VEB types. Mostly these enzymes exhibit wider resistance than their parents but do not belong to 2be group for example: OXA mutants which have increased activity towards cefepime (Livermore 2008). These enzymes are plasmid as well as chromosomal mediated and capable of hydrolyzing a wide variety of β-lactam antibiotics including penicillins, third generation cephalosporins group and monobactam. These enzymes are results originated due to mutation of TEM-1, TEM-2 and SHV-1. These are commonly found in members of *Enterobacteriaceae* family. Mostly TEM-1, TEM-2 and SHV-1 enzymes confer high label of resistance to early penicillis and low level resistance to first generation cephalosporins. However there are high use of third generation cephalosporins and aztreonam is understood to be the major cause of the mutations in these enzymes which has led to the emergence of the ESBLs (Nathisuwan et al., 2001).

However their emergence and rapid dissemination have been responsible for numerous outbreaks of infection throughout the world.The survival of ESBL producing variants is strictly dependent on their being given selective advantage. Both TEM-1 and SHV-1 have most favorable structures as penicillinases. So the change in specificity to a cephalosporin resistant ESBL makes the organism somewhat less resistant to penicillins. Thus, it is only in the context of cephalosporins use through which the resistant variants get emerge. Infection with ESBL producing bacteria can result in preventable failure of treatment and increased cost in patients who have received inappropriate antibiotic treatment. However nosocomial outbreaks of this form of resistant are most often linked with intensive care units and oncology, burns and, neonantal wards. They can result in prolongation of hospital stay, as well as devastating or even fatal consequences (Bisson et al., 2002).

2.14 Diversity of ESBLs

2.14.1 TEM:

These types of ESBLs are derivatives of TEM-1 and TEM-2. TEM-1 was detected for the first time in 1965 in Greece in an *E. coli* isolate (Datta and Kontomichalou 1965). However this TEM-1 enzyme hydrolyzes ampicillin at a rate higher than that of carbenicillin, oxacillin, and cephalothin although it fails to hydrolyze the extended-spectrum cephalosporins. But TEM-2 has the same hydrolytic activity of TEM-1 and has more active native promotor and a different isoelectric point (5.6 instead 5.4). The plasmid mediated β-lactamase TEM-3 was detected in 1987 in *Klebsiella pneumoniae* isolate in France. It was originally named CTX-1 due to its higher activity against cefotaxime (Bradford 2001).

So far, there is 218 TEM variants have been identified to date, many of them displaying an ESBL phenotype (http://www.lahey.org/Studies/; accessed on 21/5/2014), all are TEM-1 or TEM-2 derivatives. TEM enzymes can be divided into four groups according to their biochemical characteristics **(i)** broad-spectrum TEM variants such as TEM-1 include only penicillins and early-generation cephalosporins in their hydrolysis spectra, **(ii)** inhibitor resistant TEM variants (IRT) possess similar hydrolysis spectra but are not inhibited by clavulanate (Salverda et al., 2010) **(iii)** TEM-type ESBLs possess an hydrolysis profile similar to that of other clavulanate inhibited Ambler class A ESBLs, including activity against penicillins and broad-spectrum cephalosporins with highest activity against ceftazidime than cefotaxime, and sparing cephamycins and carbapenems (Robin et al., 2007) and **(iv)** complex mutant TEM variants (CMT) include TEM variants that possess an extended spectrum of hydrolysis toward cephalosporins together with an inhibitor resistant hydrolysis profile (Partridge 2011). Additionally there are two types of variants can be well known, some exhibiting single amino acid changes that is sufficient for assignment of a new number (for example TEM-3, TEM-4, etc.), and some others being variants that possess only silent substitutions (no amino acid change) like $bla_{\text{TEM-1a}}$ or $bla_{\text{TEM-1b}}$ genes (Partridge 2011).

2.14.2 SHV:

Sulfhydryl reagent Variable (SHV) ESBLs are point mutants of narrow spectrum SHV-1 or SHV-11 β-lactamases which is mainly originated from the chromosome of *Klebsiella pneumoniae*. Although these types of ESBL showing a hydrolysis profile together with activity against penicillins and broad-spectrum cephalosporins with maximum activity against cefotaxime than ceftazidime, and sparing cephamycins and carbapenems (Poirel et al., 2012). It refers to *sulfhydryl* variable because it was thought that the inhibition of the enzyme activity by p-chloro mercuri benzoate which was substrate dependent and variable according to the substrate used in the assay (Sykes and Bush 1982).

To date 184 variants of SHV β-lactamase has been reported to date (http://www.lahey.org/studies; accessed on: 21/5/2014). In 1983, a new SHV-β-lactamase was reported and designated as SHV-2 which efficiently hydrolyzes cefotaxime and to lesser extent ceftazidime has been detected in *Klebsiella ozaenae* in Germany (Knothe et al., 1983). However there are some of the SHV-type ESBLs which are globally distributed throughout the world and particularly the SHV-2, SHV-2a, SHV-5, and SHV-12. But in Europe, SHV-12 and SHV-5 have been identified in clinical isolates in Poland, Hungary, France, Italy and Spain (Coque et al., 2008). Also SHV-5/-12 ESBLs have been also recovered in North America (Mulvey et al., 2004). However SHV-2 spread globally due to the selection pressure exerted by third generation cephalosporins which have been detected in a wide range of *Enterobacteriaceae* but mainly *Klebsiella* spp. (Paterson and Bonomo 2005). Even though bla_{SHV} genes mainly found in enterobacterial isolates, however there have been some reports in *Acinetobacter baumannii* and *P. aeruginosa.* It has been reported that $bla_{\text{SHV-5}}$ ESBL gene has been identified in *A. baumannii* in New York City, USA, and the $bla_{\text{SHV-2/-12}}$ genes was identified in *A. baumannii* isolates from the Netherlands (Naas et al., 2007). Furthermore, it has been seen that SHV-5 producing *P. aeruginosa* has been described through an outbreak incident in Greece, and SHV-2/-12-producing *P. aeruginosa* in the Netherlands (Zhao and Hu 2010). Recently SHV-147, 148, 149, 150, 151, 153, 154, 155, 156, 158, 159, 161, 163, and 165 was reported (Castanheira et al., 2013).

2.14.3 CTX-M:

This type of ESBL was first reported in 1989 in a clinical isolate of *E. coli* in Munich, Germany (Bauernfeind et al., 1990). These are CTX-M ESBL types and confer resistance to penicillins and expanded-spectrum cephalosporins with frequently higher hydrolysis of cefotaxime than ceftazidime for most variants (Bonnet 2004). However dissemination of genes encoding CTX-M like ESBLs is currently a troublesome problem, with a global and increasing trend of dissemination. So far, there are 154 variants of CTX-M ESBL have been reported to date (http://www.lahey.org/studies; accessed on: 21/5/2014).

Though this enzyme can be classified in to five clusters based on their amino acid sequences (Bonnet 2004). The CTX-M-1 cluster, CTX-M-2 cluster, CTX-M-8 cluster, CTX-M-9 cluster and CTX-M-25 cluster (Bonnet 2004). However CTX-M-1 cluster includes β-lactamases of major clinical impact, particularly CTX-M-15, CTX-M-3, and CTX-M-1, but also the CTX-M-10-12/; 22/; 23/; 28-30/; 32-34/; 36/; 37/; 42/; 52-54/; 57/; 58/; 60/; 61 variants (Figure 7) (Rossolini et al., 2008). This cluster is now widely distributed all around the world including Asia, Europe and North America (Bush 2008).The CTX-M- 2 cluster includes the CTX-M-2/; 4-7/; 20/; 31/; 35/; 43/; 44 variants, the CTX-M-8 cluster includes the CTX-M-8/; 40/; 63 variants, the CTX-M-9 cluster includes the CTX-M-9/; 13/; 14/; 16/; 17-19/; 21/; 24/; 27/; 38/; 46-51/; 55/; 65 variants, and the CTX-M-25 cluster includes the CTX-M-25/; 26/; 39/l 41 variants (Rossolini et al., 2008). Conversely $bla_{\text{CTX-M}}$ genes originated from the chromosome of different *Kluyvera* species, *Kluyvera georgiana* and they have been the progenitor of $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-9}}$ like genes, however *Kluyvera ascorbata* and *Kluyvera cryocrescens* they have $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-2}}$ like genes (Bonnet 2004) while CTX-M enzymes have been rarely found in non fermenters, a single *A. baumannii* isolate producing CTX-M-2 which was identified in Japan (Nagano et al., 2004).

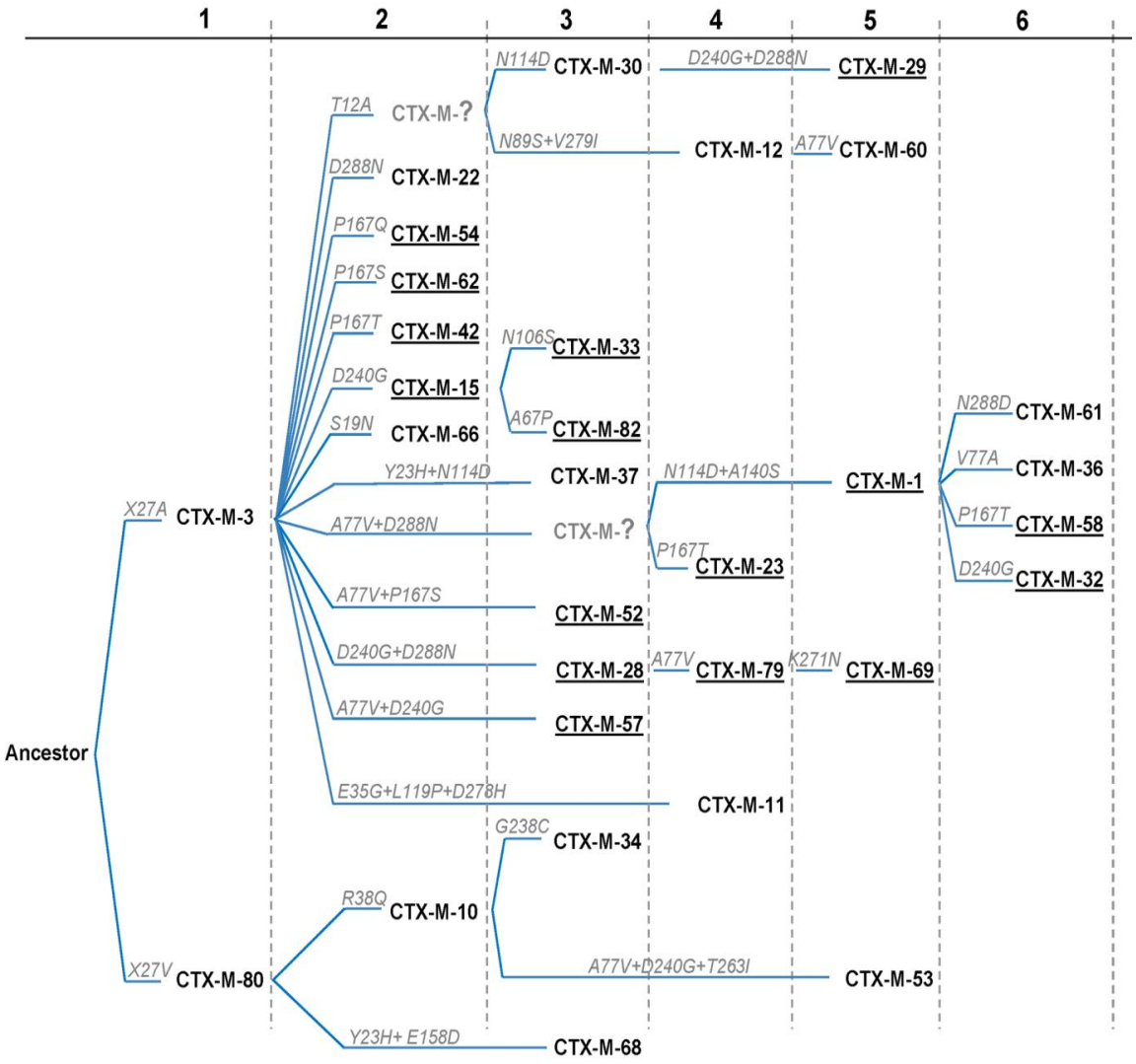

Figure 7: Cluster of CTX-M-1 ESBL

(Source:http://www.plospathogens.org/article/info%3Adoi%2F10.1371%2Fjournal.ppat.1000735)

Although an interspecies transfer of the *bla*$_{\text{CTX-M-2}}$ gene from *Proteus mirabilis* to *A. baumannii* was also found (Nagano et al., 2004). Moreover spread of the *bla*$_{\text{CTX-M}}$ genes is predominantly worrying since they are identified in nosocomial but also in community-acquired pathogens. However in some of the geographical areas, like India; they are identified mostly in *E. coli* isolates. Additionally their worldwide spread within the last 10–15 years is one of the most rapid and important incident which has been observed in term of antibiotic resistance (Poirel et al., 2012). Recently CTX-M-39 (Vervoort et al.,

2012); CTX-M-87 (Cheng et al., 2009); CTX-M-93 (Djamdjian et al., 2011); CTX-M-94 (Vervoort et al., 2012); CTX-M-100 (Vervoort et al., 2012); CTX-M-116 (Fursova et al., 2013); CTX-M-117 (Hachler et al., 2013); CTX-M-123 (Dandan et al., 2013); and CTX-M-124 (Poirel et al., 2012) were reported.

2.14.4 OXA β-lactamase:

These types of β-lactamses refer to the oxacillin-hydrolyzing capability. They have ability to hydrolyze oxacillin and cloxacillin at a rate greater than 50% that of benzyl penicillin (Bush et al., 1995). And this OXA β-lactamases are mostly reported in *P. aeruginosa* (Weldhagen et al., 2003). These groups of ESBLs are commonly harbored in *P. aeruginosa*, hence they are also acknowledged as PSE-2 β-lactamases. However few reports are also on record regarding their occurrence in members of enterobacteriacae family (Huovinen et al., 1988). OXA-type β-lactamases hydrolyse penicillins, third generation cefalosporins and carbapenems. Among them OXA-10, OXA-2 groups are regarded as ESBLs (Figure 8). These are known to be located within chromosome as well as plasmid and also reported to have association with gene cassette (Walther and Hoiby 2006; Poirel et al., 2010b). Moreover there are the majority of OXA-type β-lactamases but most common is OXA-1. It has been detected in 10% of *E. coli* isolates (Livermore 1995). However most OXA-type β-lactamases do not hydrolyze the extended spectrum cephalosporin and therefore are not regarded as ESBLs. There are some point mutation derivatives of OXA-10, such as OXA-11, OXA-13, OXA-16, OXA-28, OXA-35, and OXA-74, possessing increased activities toward expanded-spectrum cephalosporins. OXA-type ESBLs includes, OXA-10, -11, -14, -16, -17. -19, -15, -18, -28, -31, -32, -35 and -45 (Poirel et al., 2010). Recently OXA-161 (Juan et al., 2009); and OXA-147 (Fournier et al., 2010) were reported in *P. aeruginosa*.

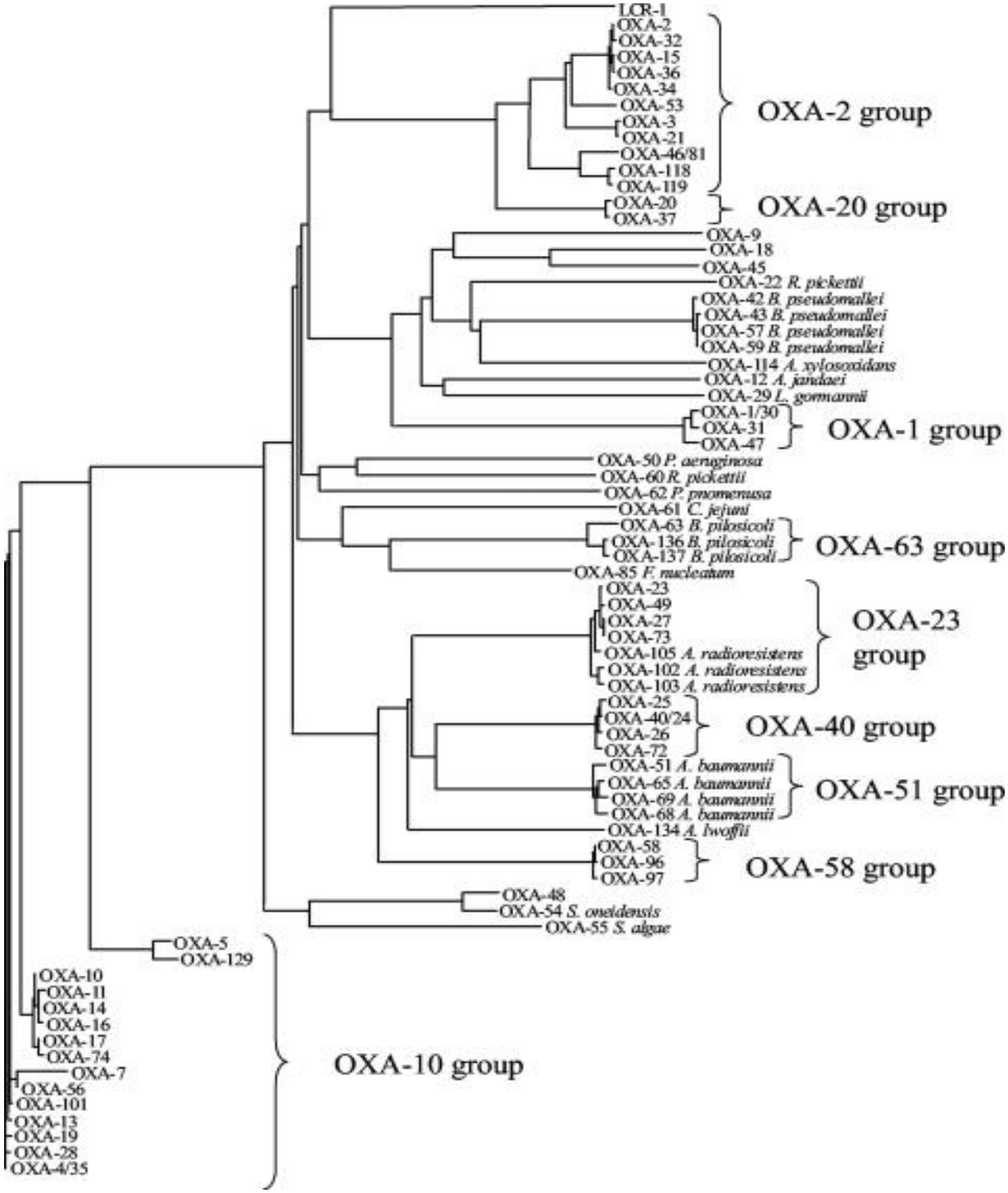

Figure 8: Diversity of OXA-type β-lactamases

[Source: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2798486/figure/f2/].

2.14.5 PER:

Pseudomonas extended resistance (PER) ESBL was firstly found in clinical isolate of *P. aeruginosa* from a Turkish patient who was hospitalized in France (Nordmann et al., 1993). The PER type ESBLs share only around 25 to 27% homology with known TEM and SHV type ESBLs (Paterson et al., 2005) however this β-lactamases efficiently hydrolyzes penicillins and cephalosporins and is susceptible to clavulanic acid inhibition (Paterson et al., 2005). To date 7 variants of PER β-lactamse are known (http://www.lahey.org/studies; accessed on: 21/5/2014).

However there are a number of PER-like variants which have been known subsequent to two subgroups made of PER-1 like point mutant derivatives (PER-3, PER-4, PER-5 and PER-7), and of PER-2 and PER-6, this differing by 22 amino acids from each other and also showing 85% of amino acid identity with PER-1 β-lactamase (Bonnin et al., 2011a). However $bla_{\text{PER-1}}$ gene is globally spread in *Acinetobacter* spp. and *P. aeruginosa* in Turkey, although these β-lactamases were also be isolated from *Salmonella enterica* serovar *Typhimurium* and *Providencia rettgeri* (Ranellou et al., 2012). PER-1 has also been found in *Proteus mirabilis* and *Alcaligenes faecalis* in Italy. Furthermore there were high occurrence of PER-1 producers which was found among *Acinetobacter* spp. in Korea (Paterson et al., 2005), and *P. aeruginosa* in Greece (Bonnin et al., 2011b). Alternatively PER-2 has only been found in South America (Paterson et al., 2005). Additionally the coexistence of these enzymes provides an organism resistant to virtually all β-lactam antibiotics (Paterson et al., 2005). However it has been reported in Argentina, Uruguay, and Bolivia. While $bla_{\text{PER-1}}$ is commonly described in Turkey and Korea (Naas et al., 2008). Recently PER-1 in *P. aeruginosa* (Ranellou et al., 2012); PER-6 in *Aeromonas allosaccharophila* (Girlich et al., 2010); PER-7 in *Acinetobacter baumannii* (Bonnin et al., 2011b); and PER-3 in *A. baumannii* (Al-Hassan et al., 2013) has been described.

2.14.6 VEB:

Vietnam Extended Spectrum β-lactamase (VEB), *bla*$_{VEB-1}$ was firstly identified in an *E. coli* isolate which was recovered in France from a Vietnamese child (Poirel et al., 1999). Although this Novel VEB-1 has greatest homology with PER-1 and PER-2 (38%), it confers high-level resistance to ceftazidime, cefotaxime, and aztreonam, which is reversed by clavulanic acid. The gene encoding VEB-1 was found to be plasmid mediated; such plasmids also confer resistance to non-β-lactam antibiotics (Poirel et al., 1999). To date 9 variants of VEB β-lactamase has been reported to date (http://www.lahey.org/studies; accessed on: 21/5/2014).

A variety of other β-lactamases which are plasmid mediated or integron-associated class A enzymes have been recently discovered. They are not simple point mutant derivatives of any known β-lactamases and are remarkable for their geographic diversity. An indistinguishable β-lactamase has also been found in *E. coli*, *K. pneumoniae*, *Enterobacter cloacae*, *Enterobacter sakazakii*, and *P. aeruginosa* isolates in Thailand (Bradford 2001). However this *bla*$_{VEB}$ gene was described among enterobacterial isolates from Europe but also in *P. aeruginosa* from Thailand and Kuwait (Girlich et al., 2002) and *A. baumannii* from France and Argentina (Pasteran et al., 2006; Poirel et al., 1999). There are several outbreaks which involving the isolates producing VEB-1-like enzymes which have been known and together with a nation wide outbreak of VEB-1 *A. baumannii* in France and Belgium, of *P. mirabilis* in Korea, and VEB-3-producing *E. cloacae* in China (Naas et al., 2008; Jiang et al., 2005) and VEB-4 has been reported in *Proteus mirabilis* (Aragon et al., 2008).

2.14.7 GES:

This type of ESBL is known as Guiana Extended Spectrum β-lactamase and is very rare and uncommon. Till date 24 variants of GES type of β-lactamases has been reported (http://www.lahey.org/studies; accessed on: 21/5/2014). *bla*$_{GES-1}$ gene was at first identified as plasmid borne in a *K. pneumoniae* clinical isolate recovered in France from a patient originating from French Guyana (Poirel et al., 2000). This β-lactamase possesses an hydrolysis profile which is very similar to that of other clavulanate inhibited Ambler class A ESBLs (Poirel et al., 2000), as well as activity against penicillins and

broad spectrum cephalosporins with maximum activity against ceftazidime than cefotaxime, not cephamycins and carbapenems but unlike most ESBLs does not hydrolyze monobactams, and are inhibited by clavulanate, tazobactam and imipenem (Poirel et al., 2000). GES-1 is not closely related to any other plasmid-mediated β-lactamases but it showes 36% homology to a carbenicillinase from *Proteus mirabilis* (Bradford 2001). The bla_{GES-1} was characterized in a *K. pneumoniae* isolate from France in the past, however recently this β-lactamase also well known in Brazil, Portugal, Netherlands and Argentina from *K. pneumoniae*, *P. aeruginosa*, and *S. marcescens* (Naas et al., 2008).

Furthermore GES-2 was reported from South Africa in *P. aeruginosa* clinical isolate (Poirel et al., 2001), however GES-5, GES-6, GES-7 and GES-8 were reported from Greece, and GES-3 and GES-4 were from Japan. Additionally the carbapenemase bla_{GES-5} has been known in Korea, China and Brazil (Naas et al., 2008).

So, these GES-type enzymes are still rare and they are more and more reported and particularly in environment while GES-7 was found in environmental isolate of *Aeromonas* sp. which was isolated from Seine River in France together with PER-like and VEB like enzymes (Girlich et al., 2011). However these GES types of enzyme are also increasingly known in *A. baumannii* ever since GES-11, GES-12 and GES-14 was also described in Belgium (Bogaerts et al., 2010). RecentlyGES-13, a β-Lactamase Variant Possessed Lys-104 and Asn-170 in *Pseudomonas aeruginosa* (Kotsakis et al., 2010) and GES-14 possessed a Gly170Ser change extending the hydrolysis spectrum toward carbapenems and cephamycins and a Gly243Ala change which extended the hydrolysis spectrum toward monobactam, has been reported (Bonnin et al., 2011c). However this type of constitutes therefore the first recognition of a GES-type enzyme which includes all β-lactam antibiotics in its substrate profile as a result a combination of these two substitutions (Bonnin et al., 2011c).

2.15 Prevalence of ESBLs: Global and local scenario

2.15.1 Global scenario

2.15.1.1 Europe

ESBL producing organisms were first detected in Europe in 1983. Even though the initial reports were from Germany (Knothe et al., 1983) and England (Du Bois et al., 1995), the vast majority of reports in the first decade after the discovery of ESBLs were from France (Sirot et al., 1987). The first large outbreak in France to be reported occurred in 1986; 54 patients in three intensive care units were infected and spread of the infection to four other wards then occurred (Brun et al., 1987). However the proliferation of ESBLs in France was quite dramatic. By the early 1990s, 25 to 35% nosocomially acquired *K. pneumoniae* isolates in France were ESBL producing (Marty et al., 1998). However the frequency of ESBL producing *Enterobacteriaceae* is very much variable from geographical region to another and from country to country. Moreover the published studies reflect that lower ESBL prevalence in Northern European countries compared to Southern and Eastern European countries (Bradford 2001 and Coque et al., 2008). New TEM and the SHV enzymes are still evolving in Europe, and specific epidemic clones have been found, for example *Salmonella* isolates with TEM-52 in Spain (Fernandez et al., 2006) and *E. coli* and *K. pneumoniae* isolates with SHV-12 in Italy (Perilli et al., 2011).

Nowadays, *E. coli* and the CTX-M enzymes are not uncommon in outpatients. Furthermore, the resistance shown by *K. pneumoniae* has reached a higher level with emergence of carbapenemases such as OXA-48, which was first found in Turkey (Coque et al., 2008). A novel derivative of OXA – 10 (numbered OXA – 28) was found in *P. aeruginosa* isolate (Poirel et al., 2001). In France TEM derivatives were found to be predominant among *Enterobacteriaceae* in mid 2000s (Bert et al., 2005; Lavigne et al., 2004). In another study, consisting 10 public hospitals and 3 private clinics showed 0.6% of ESBL incidence in *Enterobacteriaceae* and the predominant enzymes were TEM – 24, CTX – M – 15 and three new enzymes CTX – M – 61 was identified (Brasme et al., 2007). There was a recent report of TEM – 158 (CMT – 9), a new member of CMT type

ESBL from France (Robin et al., 2007). But, there have also been larger nosocomial outbreaks of clonal ESBL strains: one at a neonatal care unit with ESBL-related mortalities, a large outbreak in Uppsala involving *K. pneumoniae* with CTX-M-15, and in Kristianstad caused by a multiresistant CTX-M-15-producing *E. coli* strain (Heimer et al., 2009; Lytsy et al., 2008). As in most parts of Europe, the following enzyme types were found: CTX-M group 1 predominated (67%) followed by CTX-M group 9 (27%), although other types have also been found, for example CTX-M group 2 and TEM- and SHV enzymes (Fang et al., 2008).

In Spain, only 1.5% among1962 invasive *E. coli* isolates in 2001 were found to produce ESBL (Oteo et al., 2002). However CTX-M-10, TEM-24, -27 producing *Enterobacter* spp. were identified from a hospital in a study conducted for 12 years duration (Canton et al., 2002). CTX- M-9, TEM-64, -18 ESBLs were also reported from community acquired infections in 2004 (Rodriguez B 2004) and in the same year there was dramatic increase of ESBLs in the faecal carriage during a non outbreak situation (Valverde et al., 2004). However, there was a report of dissemination of CTX-M-1 like enzymes in *Enterobacteriaceae* that were associated with IncFII and plasmids (Novais et al., 2007). In a separate study it was shown that the same plasmid was carrying CTX-M-1 group and CMY-2 enzymes in isolates obtained from animal farms (Cortes et al., 2008). In an epidemiological study, a diverse range of β-lactamase genes (TEM-110, SHV-11, - 12, CTX-M-14, -15) were found in *K. pneumoniae* from a single institution in Madrid (Valverde et al., 2008). In France, a Novel integron associated GES-11 in *Acinetobacter baumannii* was detected in *A. baumannii* BM4674 (Moubareck et al., 2009) and CTX-M-93, a CTX-M variant lacking penicillin hydrolytic activity was reported (Djamdjian et al., 2011). Recently contribution of IncFII and broad host IncA/C and IncN plasmids to the local expansion and diversification of phylogroup B2 *E. coli* ST131 clone carrying $bla_{\text{CTX-M-15}}$ and *qnrS1* genes was reported from Spain (Novais et al., 2012).

In Italy, β-lactamase production was 52% in *Proteus mirabilis*, the enzymes were characterized as TEM- 52 and PER-1 (Perilli et al., 2007; Perilli and Santis et al., 2007). From the same hospital in northern part of Italy multiple CTX-M (CTX-M-1, -2, -15) type ESBLs were detected in nosocomial isolates of *Enterobacteriaceae*. There was a

report of spread of PER-1 ESBL gene among *Enterobacteriaceae* family (Perilli and Santis et al., 2007). A new type of TEM variant (TEM-134) was isolated from *Citrobacter koseri* from an Italian hospital (Perilli et al., 2007).

In Greece there was report of SHV ESBL producing *Enterobacter* (Tzelepi et al., 2000) and SHV-5, TEM-52 and CTX-M-32 harbouring *Salmonella* spp from human as well as animal product (Politi et al., 2005). However recently, GES-13 variant possessing Lys-104 and Asn-170 was found in *P. aeruginosa* (Kotsakis et al., 2010) and CTX-M-117, a Pro174 Gln variant of CTX-M-15 ESBL, from a bovine *E. coli* isolate was found (Hachler et al., 2013).

In Germany during 2001, a study was completed by Paul Ehrlich Gesellschaft (PEG) covering many medical centers in Germany revealed that the prevalence of ESBL-producing *K. pneumoniae* 8.2%, *E. coli* 0.8% and *K. oxytoca* 1.3% (Sturenburg and Mack, 2003). Another study carried out by Grobner *et al.,* at the University Hospital of Tübingen during the period from 2003 to 2007 reported that the overall ESBL prevalence still low (1.6%), however, the study recorded slight but continuous increase in the percentage of ESBL-producing *Enterobacter* spp. from 0.8% in 2003 to 6.4% in 2007 and in *E. coli* from 0.5% in 2003 to 3.8% in 2007. Percentage of ESBL-producing *Klebsiella* spp. ranged from 1.3% in 2003 to 2.9% in 2007 (Grobner et al., 2009). A previous study carried out in Giessen, Germany by Mshana et al., on 63 *E. coli* isolates, collected in the period from August 2006 to April 2007 recorded high prevalence (77.7%) of $bla_{\text{CTX-M}}$ encoding isolates (Mshana et al., 2009).

Beside these countries, there were report of SHV gene harbouring *K. pneumoniae* from nosocomial outbreak in Netherlands (Gruteke et al., 2003) and CTX – M type ESBLs from *E. coli* in a similar outbreak in Sweden (Fang et al., 2004). However in Belgium, GES-14 and GES-12 ESBL detected in *Acinetobacter baumannii* Isolates (Bogaerts et al., 2010). On the other hand, recently in Czech Republic, dissemination of IncFIIK-type plasmids in multiresistant CTX-M-15 producing *Enterobacteriaceae* isolates from children in hospital paediatric oncology wards waqs reported (Dolejska et al., 2012).

2.15.1.2 North America:

First report of ESBL producing organisms in the United States occurred in 1988 (Jacoby et al., 1988). In 1989, significant infections with TEM-10 producing *K. pneumoniae* were noted in Chicago (Quinn et al., 1989). It has been shown that ESBL producing isolates were found were found in 75% of 24 medical centers in the United States (Moland et al., 2002). CTX-M type ESBLs have recently been described in the United States and Canada (Pitout et al., 2007; Pitout and Gregson et al., 2005). Sanchez et al., investigated data obtained from The Surveillance Network (TSN) concerning *in vitro* antimicrobial resistance in US outpatients between 2000 and 2010, and their results showed that resistance to ceftriaxone rose from 0.2% to 2.3% and resistance to cefuroxime increased from 1.5% to 5%, but the bacterial isolates in focus were not tested for ESBLs (Sanchez et al., 2012).

In the United States the prevalence of ESBL encoding *Enterobacteriaceae* was around 3% (Bradford, 2001). There are ESBL genes which are commonly encountered worldwide like SHV-5 and CTX-M-15 (Bradford, 2001). Global dissemination of an antibiotic resistance determinant encoded by a bacterial host can be achieved either by human travelers, migrating birds, imported animals or imported agricultural and meat products (Okeke and Edelman, 2001). Travel plays an important role in the dissemination of antibiotic resistance (Naseer and Sundsfjord, 2011). Recently SHV-147, 148, 149, 150, 151, 153, 154, 155, 156, 158, 159, 161, 163, and 165 were reported (Castanheira et al., 2013).

2.15.1.3 Latin America:

In 1988 and 1989 isolates of *K. pneumoniae* from Chile and Argentina were reported as harbouring SHV-2 and SHV–5 (Casellas et al., 1989) and recently SHV–5 producing *K. pneumoniae* was reported in Mexican hospital (Miranda et al., 2004). Organisms with CTX–M–2 have spread throughout many parts of South America (Radice et al., 2002). Other CTX-M enzymes (CTX-M-8, -9 and -16) have been discovered in Brazil (Bonnet et al., 2001). TEM–116 and PER-2 gene encoding *E. coli* is recently detected in Uruguay (Vignoli et al., 2005). Two novel ESBLs have been reported from South America: GES-

1, isolated from an infant previously hospitalized in French Guiana (Poirel et al., 2000). ESBLs have been found in 30 to 60% of klebsiellae from intensive care units in Brazil, Columbia and Venezuela (Sader et al., 2000; Otman et al., 2002; Pfaller et al., 1999). A survey carried out among members of a healthy population of children living in Bolivia and Peru revealed that faecal carriage of *E. coli* strain resistant to cephalosporins was remarkably increased and the isolates were harbouring CTX-M-2, -56, 14, -15, -24, and SHV-2 (Pellecchi et al., 2007). As in other parts of the world, the SHV-5 and SHV-12 ESBLs have been found in South America, and the TEM-10 enzyme has been observed in Argentina (Villegas et al., 2008). Data from 33 centres in Latin America collected over the period 2004–2007 within the Tigecycline Evaluation and Surveillance Trial (TEST) showed ESBLs in 36.7% of *K. pneumoniae* isolates and in 20.8% of 932 *E. coli* isolates (Rossi et al., 2008). However recently, a CTX-M-15 was reported from wild coastline birds as reservoirs of broad spectrum β-Lactamase producing *Enterobacteriaceae* in Miami Beach, Florida, USA (Poirel et al., 2012b).

2.15.1.4 Africa

Several outbreaks of infections with ESBL producing *Klebsiella* have been reported from South Africa (Cotton et al., 2000), but no national surveillance figures have been published. However, it has been reported that 36.1% *Klebsiella pneumoniae* isolates collected in a single South African hospital in 1998 and 1999 were ESBL producers (Bell et al., 2002). In some other studies from the same country, characterization of ESBLs has revealed TEM and SHV types (SHV-2 and SHV–5) (Hanson et al., 2001; Pitout et al., 1998). *Salmonella enterica* serovar Isangi and Typhimurium harbouring TEM-63, TEM-131 and CTX-M-37 and a nosocomial outbreak of *P. aeruginosa* expressing GES-2 is reported from South Africa (Govinden et al., 2006; Kruger et al., 2004; Poirel et al., 2002). Outbreaks of *Klebsiella* infections with strains resistant to third generation cephalosporins have been reported in Nigeria and Kenya without documentation of ESBL production (Musoke et al., 2000; Akindele et al., 1997). A novel CTX-M enzyme (CTX-M-12) has been found in Kenya (Kariuki et al., 2001). SHV-12, SHV-28 and CTX-M-15 producing *Enterobacteriaceae* has been isolated from septicemia patients and from other nosocomial origin from a intensive care unit of a tertiary care hospital in Tanzania

(Tellevik et al., 2007; Ndugulile et al., 2005). There are also reports of nosocomial outbreak of *Salmonella enterica* serotype Livingstone producing CTX–27 and *P. aeruginosa* harbouring OXA–18 from Tunisia (Blagui et al., 2007; Godet et al., 2005), CTX–M and SHV genes are also reported from *E. coli* (Jouini et al., 2007). An investigation conducted at a tertiary hospital in Mwanza, Tanzania, the overall prevalence of ESBLs in all Gram-negative bacteria (377 clinical isolates) was 29%. The ESBL prevalence was 64% in *K. pneumoniae* but 24% in *E. coli* (Mshana et al., 2009). Dramatic figures were also obtained in a small study at an orphanage in Mali, where 63% of the adults and 100% of the children were found to carry ESBL producing *Enterobacteriaceae* that showed extensive co-resistance to other antibiotics (Tande et al., 2009). Furthermore, in Madagascar, Herindrainy et al., observed that 10% of non-hospitalized patients carried ESBLs, in the majority of the cases CTX-M-15, and these investigators also found that poverty was a significant risk factor for carriage (Herindrainy et al., 2011).

In Egypt, the first study that referred to the potential high rate of ESBL production that was reflected by the reduced rate of susceptibility of *E. coli*, *Klebsiella* and *Enterobacter* to ceftazidime recording 62%, 40% and 46%, respectively (El-Kholy et al., 2003). The first study that aimed at the determination of the molecular basis of ESBL resistance in clinical *E. coli* isolates derived from Egyptian University Hospitals was carried out by Mohamed Al-Agamy *et al.,* who reported a very high ESBL rate (60.9%). All isolates were positive for bla_{TEM} and $bla_{\text{CTX-M}}$ genes. $bla_{\text{CTX-M}}$ was further differentiated into $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-27}}$ (Al-Agamy et al., 2006). A study investigating 520 enterobacterial isolates collected during the period from May 2007 till August 2008 at Theodor Bilharz Research Institute, Cairo, Egypt reported that a total of 16% of all isolates, 19% of *E. coli* and 14% of *K. pneumonae* were ESBL producers (Fam et al., 2011).

There are, thirty-five different sequence types have been detected among the avian *E. coli*. The majority of them (ST10, ST90, ST648 and ST69) have been detected among human clinical isolates suggesting interspecies transmission, while the avian ST746 has not been detected among the clinical isolates (Guenther et al., 2011). CTX-M-producing

E. coli are likely to be globally present in chickens. CTX-M variants that have previously been isolated from chickens include CTX-M-1 (the most prevalent variant), CTX-M-2, CTX-M-14 and CTX-M-15. But the pandemic CTX-M-15-producing/ST131 *E. coli* clone has not been isolated from poultry (Randall et al., 2010). The existence of ESBL producing organisms in the intestinal tract increases the risk of transmission to other individuals through human to human transmission or through the environment **(**Andriatahina et al., 2010). Emergence of ESBL producers in the community could be attributed to acquisition of an ESBL producer by a patient during hospitalization or due to antibiotic overuse by community patients. However recently, PER-3 was detected in *A. baumannii* (Al-Hassan et al., 2013).

2.15.1.5 Australia

The first ESBLs to be detected in Australia were isolated from a collection of gentamicin resistant *Klebsiella* spp. collected between 1986 and 1988 from Perth (Mulgrave 1990). These were characterized as being of SHV derivation (Mulgrave et al., 1993). In the last decade, ESBL producing organisms have been detected in every state of Australia and in the Northern Territory (Howard et al., 2002; Bell et al., 2002; Royle et al., 1999). It appears that proportion of *K. pneumoniae* isolates that produces ESBL are about 5% (Bell et al., 2002). Recently four cases of CTX-M-15 producing *Shigella flexneri* was reported from New Zealand (Upton et al., 2007).

2.15.1.6 Asia

In 1988, isolates of *K. pneumoniae* from China which contained SHV-2 were reported (Jacoby and O'Brien et al., 1988). In reports comprising limited numbers of isolates collected in 1998 and 1999, 30.7% of *K. pneumoniae* and 24.5% of *E. coli* isolates were ESBL producers (Bell et al., 2002). In a major teaching hospital in Beijing, 27% of *E. coli* and *K. pneumoniae* blood cultures isolates collected from 1997 through 1999 were ESBL producers (Du et al., 2002). In Palestine 3.3% of *E. coli* isolates from community acquired urinary tract infections were ESBL producers (Astal et al., 2004). In south Asian countries, prevalence rate was up to 50% in Pakistan (Jabeen et al., 2005), 40% in Bangladesh (Rahman et al., 2004) and 29.6% in Nepal (Hammer et al., 2007) and there

was a report from Nepal of ESBL production in *Salmonella enterica* serovar Paratyphi A (Pokharel et al., 2006). There was a report of dissemination of CTX–M–3 genes among *K. pneumoniae* in central China (Li et al., 2003). A nosocomial outbreak of *E. coli* occurred in that country which was producing CTX–M–15 and OXA–30 (Pai et al., 2006). From Japan, CTX-M-2 producing *A. baumannii* was isolated from a neurosurgery ward (Nagano et al., 2004a), there was also a report of ESBL producing Shiga toxin gene positive *E. coli* O26:H11 in that country (Ishii et al., 2005). In Israel, 42.5 % enterobacterial isolates were ESBL producers (Venezia et al., 2003), in another study CTX-M-2, CTX-M-39, TEM, SHV were detected in *E. coli*, most of the isolates were multiple β-lactamase harbouring and OXA–2 was first time reported in *E. coli* (Chmelnitsky et al., 2005). However recently, Two New CTX-M-25-Group like CTX-M-39, CTX-M-94, and CTX-M-100 ESBL variants identified in *E. coli* isolates from Israel (Vervoort et al., 2012). Reports from other parts of Asia have indicated a variety of ESBLs, such as VEB enzymes, and have also shown a significant increase in ESBL carrying *Enterobacteriaceae* in both out and inpatients, as well as in stool samples from healthy volunteers (Hawkey 2008). Luvsansharav et al., analysed stool samples from healthy volunteers in Thailand in 2009, and the results showed that 30–50% of these subjects in three different regions were ESBL carriers (CTX-M types) (Luvsansharav et al., 2012).

2.15.1.7 The Middle East:

The overall data on ESBL producing *Enterobacteriaceae* in the countries of the Middle East are extremely worrisome, and this region might indeed be one of the major epicentres of the global ESBL pandemic. Most of the available data concern isolates from hospital inpatients, and only a limited amount originates from the community. In a study of *E. coli* isolates collected at five hospitals in Egypt in 1999-2000, it was found that 38% were resistant to expanded spectrum cephalosporins (El-Kholy et al., 2003). In addition, another investigation conducted in that country in 2001 showed that 61% of *E. coli* produced ESBLs of the CTX-M-14, CTX-M 15, and CTX-M 27 types, and all of strains harboured the TEM enzyme (Al-Agamy et al., 2006). No data have been published regarding the community prevalence of ESBLs in Egypt. Other researchers in Lebanon

(Khanfar et al., 2009) observed that the proportion of ESBL producing isolates was significantly larger among inpatients (15.4%) than in outpatients (4.5%). Moreover, data collected over three years in Kuwait showed that the levels of ESBLs were lower in community isolates of *K. pneumoniae* (17%) and *E. coli* (12%) than in the corresponding hospital isolates (28% and 26%, respectively). In addition, there have been large outbreaks of *K. pneumoniae* caused by strains producing OXA-48 in Morocco (Benouda et al., 2010) and KPC in Israel (Goren et al., 2011) which perhaps gives us a glimpse of what can be expected in the future.

In the **United Arab Emirates**, five (11.3%) multidrug-resistant enteroaggregative *E. coli* strains demonstrated ESBL production (Sonnevend et al., 2006). The first detection of CTX-M ESBL type in Saudi Arabia was documented in 2009 by Al-Agamy *et al.*, in a study targeting the estimation of the prevalence of ESBL-producers among 400 *K. pneumoniae* isolates. In which, high ESBL rate (55%) has been detected. Among those 97.3%, 84.1% and 34.1% were positive for bla_{SHV}, bla_{TEM} and $bla_{\text{CTX-M}}$ β-lactamase genes, respectively. 60% of the $bla_{\text{CTX-M}}$ belonged to $bla_{\text{CTX-M-1}}$ group and the remainders belonged to the $bla_{\text{CTX-M-9}}$ group (Al-Agamy et al., 2009).

2.15.2 Indian scenario

In India, the first report of ESBL was possibly in the year 1995 from south India (Abigail et al., 1995). conversely in 1997, from Nagpur, there were 17 out of 66 isolates of *Klebsiella* isolates showed ESBL production (Hansotia et al., 1997) and additionally from JIPMER, Pondicherry, 20.5% isolates were found to be ESBL producers from surgical wound infections and burn patients (Ananthakrishnan et al., 2000). Although in Delhi 61% of burn wound patients isolates were ESBL producers (Singh et al., 2003). Another Study conducted in north India, about 30.18 % of *Klebsiella pneumoniae* were ESBL producer in Aligarh (Shukla et al., 2004). From Mumbai which is the western part of India, around 53% of Gram negative isolates were reported to be ESBL producers (Rodrigues et al., 2004) although 63.4% and 27% of *Enterobacteriaceae* were found to be ESBL producer in Nagpur (Raut et al., 2005). In other parts of the country, studies from Karnataka showed the frequency of ESBL in neonatal septicemic cases raised from

13.54% to 22.7% in a span of two years (Kumar et al., 2006; Kumar et al., 2004), conversely a related study from Lucknow showed high levels of ESBL production (63-86.6%) (Jain et al., 2003). In Hyderabad and in Bangalore which is in south India, nearly 19.8% of *Enterobacteriaceae* were ESBL producers (Kumar et al., 2006), and 28% of *Acinetobacter* isolates showed ESBL production (Sinha et al., 2007).

However there are not much molecular studies from India. CTX-M-15 was for the first time reported from New Delhi (Karim et al., 2001). An isolate of *P. aeruginosa* carrying VEB gene was isolated from a tertiary referral hospital Chennai (Aubert et al., 2004). Additionally bla_{SHV} and bla_{TEM} producing *K. pneumoniae* was reported from two separate studies from Delhi (Lal et al., 2007; Grover et al., 2006). CTX-M-15 was isolated from *Enterobacteriaceae* family from three different medical centers from India (Ensor et al., 2006). OXA-2 carrying by *E. coli* was reported from India which is second report from the world (Bhattacharjee 2007). However the prevalence of ESBL producing isolates of *E. coli* and *K. pneumoniae* in Pune was found to be 22% (80 out of 357). This was significantly lower than the data available from other hospitals (Agrawal et al., 2008). Also 36.5 % of ESBL producers were reported and they showed a high degree of resistance to piperacillin (93.1 and 90.9%), amoxycillin-clavulanic acid (93.4 and 90.9%), aztreonam (79.4 and 78%), cefepime (76.7 and 78%), and ampicillin-sulbactam (76.7 and 70.4%) (Taneja et al., 2008). Though in Tamil Nadu production of ESBL was observed in 71.5% of gram negative bacilli. However of these ESBL producing isolates 6.18% were inhibitor resistant and 70.17% of *Enterococcus* isolates were found high level of resistance to aminoglycosides and ciprofloxacin (Ramesh and Sumathi 2008). In a study the activity of different inhibitors of ESBLs were evaluated (Bhattacharjee 2008). However the presence of $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV}, and bla_{ampC} in clinical isolates was noticed in 52.2%, 60.9%, 21.7%, and 43.5%, respectively in Aligarh (Shahid 2009). However there were 20 $bla_{CTX\text{-}M}$ were identified as CTXM-3 first time in India which shows High co-resistance to non-β-lactam antibiotics (Goyal et al., 2009). Observation of integron carriage among SHV and CTX-M within nosocomial isolates of *Klebsiella pneumoniae* were also observed in a university hospital of Varanasi (Bhattacharjee 2010). ESBL production was higher in *E. coli* when compared to *K. pneumoniae* and *Enterobacter* spp. AmpC β-lactamases were also seen more among *Citrobacter* spp.

followed by *Proteus* spp. and *E. coli* in Karnataka (Rudresh and Nagarathnamma 2011). Further in 2011, from a multicentric Indian surveillance study it was reported that there were excellent susceptibility among the strains to imipenem (100%), meropenem (100%) and ertapenem (98.7%); good susceptibility to amikacin (89.7%) and piperacillin/tazobactam (85.3%) was observed. TEM and CTX-M were predominantly found in *E. coli* (39.2%) while, among the *Klebsiella* spp., TEM, SHV and CTX-M occurred together in 42.6% of the isolates (Manoharan et al., 2011). There is another report in 2011 among 39 resistant isolates of *K. pneumoniae*, 17 were ESBL producers detected by double disk synergy test (DDST) and phenotypic confirmatory disc diffusion test (PCDDT) ESBL producers were more in the hospital isolates (28%) compared to community isolates (6%). Maximum percentage of ESBL producers were noticed from blood sample with 57.14% in Anantapur, India (Sarojamma et al., 2011). Additionally in 2011 about 87% and 88% of the ESBL producing *E. coli* and *K. pneumoniae* respectively showed multi-drug resistance to amoxicillin- clavulanic acid, gentamicin and ciprofloxacin in Pondicherry (Uma Devi et al., 2011). Moreover the occurrence of ESBL-producing *K. pneumoniae* and *E. coli* among pediatric population in South India was also reported (Harini and Ananthan 2012). Gaurav Dalela in 2012 reported the high prevalence of ESBL production in Tertiary Care Hospital at Jhalawar, Rajasthan, India (Dalela 2012). However in 2012 in Tamilnadu Harini et al., phenotypically reported the presence of ESBLs in different organism like two *K. pneumoniae* and one isolate of *E. coli* in paediatrics population (Laxminarayan et al., 2012). Additionally in 2012; in tamilnadu it was found that Multidrug resistance was significantly higher (63.2%) in ESBL positive isolates than non ESBL isolates (26.3%) (Swaminathan 2012). In 2013 the multiple drug resistance patterns of *E. coli* and the correlation between ESBL and biofilm production in *E. coli* was also determined (Ponnusamykonar et al., 2013). However the frequency of ESBL producing organisms was found to be 5.3% and ESBL infection incidence densities were found to be 3.4 per 1000 Patient days in New Delhi (Vijayakanthi et al., 2013). Most recently there were one study revealed the daunting state of occurrence of multidrug resistant *E. coli* and its infection dynamics in both community and hospital settings (Rath et al., 2014).

2.16 Detection of ESBLs

ESBL producing organisms are major challenge for clinical microbiology laboratory due to the variable affinity of these enzymes for different substrates and inoculum effect, some ESBL isolates may appear susceptible to third generation cephalosporins *in vitro* condition. However, treatment of infections due to an ESBL producing organism with third generation cephalosporins may results in clinical failure if infection is outside the urinary tract (Nathisuwan et al., 2001). There are epidemiologic implications for the detection of ESBL producing organisms and this resistance issue may not be as apparent if organisms are simply reported as intermediate or resistant to individual cephalosporins. Detection of ESBL production in organisms from samples such as urine may be important because this represents an epidemiological marker of colonization and therefore the potential for transfer of such organisms to other patients (Paterson et al., 2005). So, there are two reasons for detection of ESBLs in laboratory.

First, there is value of the knowledge of presence of ESBLs in terms of aiding infection control. **Second**, in the presence of high inoculum infections (for example, intra abdominal absences, some cases of pnemonia) or infections at sites in which drug penetration may be poor (for example, meningitis, endocarditis, or osteomylitis), physicians should avoid cephalosporins if an ESBL producing organism is present (Paterson et al., 2005).

2.16.1 CLSI recommended Methods for ESBL detection

2.16.1.1 Screening of ESBL producers

2.16.1.1.1 Screening by agar dilution method: The clinical laboratory standard institute (CLSI) has proposed the dilution methods for screening of ESBL production by *E. coli*, klebsiellae and *Proteus mirabilis*. Third generation cephalosporins like cefotaxime, ceftazidime or ceftriaxone may be used at 1 µg/ml of screening concentration (CLSI 2011). However the growth of bacteria at this screening concentration is suspicious of ESBL production.

2.16.1.2 Phenotypic Confirmatory Tests for ESBL Production

2.16.1.2.1 Combined Disk diffusion method: The CLSI has given an idea about the use of cefotaxime (30μg) or ceftazidime disks (30 μg) with or without clavulanate (10 μg) for phenotypic confirmation of the presence of ESBLs in *E. coli*, klebsiellae and *P. mirabilis*. Moreover, CLSI recommended that the disk tests be performed with confluent growth of bacteria on Mueller Hinton agar plate. A difference of ≥5 mm zone diameters of either of the cephalosporin disks and their individual cephalosporin/clavulanate disk is taken to be phenotypic confirmation of ESBL production (CLSI 2011).

2.16.1.2.2 Combined Disk diffusion method Broth microdilution: Phenotypic confirmatory testing may also be performed by broth microdilution assay methods using ceftazidime (0.25 to 128 μg/ml), ceftazidime plus clavulanic acid (0.25/4 to 128/4 μg/ml), cefotaxime (0.25 to 64 μg/ml), and cefotaxime plus clavulanic acid at a fixed concentration of 4 μg/ml concentration. However all over again it should be emphasized that both ceftazidime and cefotaxime should be used (Queenan et al., 2004). Broth microdilution is performed using standard methods. Phenotypic confirmation is considered as a ≥ three-twofold/ or eight fold serial dilution decrease in minimum inhibitory concentration (MIC) of either cephalosporin in the presence of clavulanic acid compared to its MIC when tested alone.

2.16.2 Quality control when performing screening and phenotypic confirmatory tests for ESBL detection:

Quality control suggestions are that simultaneous testing with a non-ESBL producing organism (*E. coli* ATCC 25922) and an ESBL-producing organism (*K. pneumoniae* ATCC 700603) also be performed (CLSI 2011). The ESBL control organism, *K. pneumoniae* K6 (ATCC 700603), has been newly characterized. This organism produces SHV-18 and has lost the OmpK35 and OmpK37 porins (Rasheed et al., 2000).

2.16.3 Commercially available methods for Detection of ESBL

2.16.3.1 E-test ESBL strips:

AB Biodisk (Solna, Sweden) manufactures drug-impregnated strips, in which one end of strip contains a gradient of ceftazidime (MIC test range 0.5 to 32 µg/ml) and the other with a gradient of ceftazidime plus a constant concentration of clavulanate (4 µg/ml). However other similar strips containing cefotaxime and cefotaxime/clavulanate which are also available. These strips are useful for both screening and phenotypic confirmation of ESBL production. The reported sensitivity of the method as a phenotypic confirmatory test for ESBLs is 87 to 100% (Brown et al., 2000; Cormican et al., 1996) and their specificity rate is 95 to 100%. The sensitivity and specificity of this method depend on the percentage of MICs of the cephalosporin versus cephalosporin/clavulanate combination used the manufacturer as recommends. Moreover the MIC of the cephalosporin alone is difficult to read because the inhibition zone is distorted by the clavulanic acid diffusing from the opposite ends of the strip.

2.16.3.2 Vitek ESBL cards:

These are a specific card which includes tests for ESBL production and it has been approved by FDA. The Vitek ESBL test (bioMerieux Vitek, Hazelton, Missouri) uses cefotaxime and ceftazidime, alone (at 0.5 µg/ml), and in combination with clavulanic acid (4 µg/ml). Although inoculation of the cards are the same to that performed for regular Vitek cards. Moreover analysis of all the wells is carried out mechanically once the 4 to 15 h of incubation has been completed. However programmed decline in growth of the cefotaxime or ceftazidime wells having clavulanic acid, in comparision with the intensity of growth in the well with the cefotaxime and ceftazidime alone, this indicates a positive result (Sanders et al., 1996).

2.16.3.3 BD Phoenix Automated Microbiology System:

This system has been established by Becton Dickinson Biosciences (Sparks, Md). BD phoenix is a short-incubation system for bacterial identification and susceptibility testing. In the BD Phoenix system, ESBL test uses growth in response to cefpodoxime, ceftazidime, ceftriaxone and cefotaxime, with or without clavulanic acid, for detection of production of ESBLs. By this system results are generally available within 6 hours. Algorithm of this test has been described by Sanguinetti et al., (Sanguinetti et al., 2003).

2.16.3.4 Micro Scan panels:

This micro scan panels are Dade Behring MicroScan (Sacramento, Calif.) for microdilution antibiotic susceptibility testing. In Micro Scan panels study, gram negative urine MIC 7 and gram negative MIC Plus 2 panels has been examined by the Walk away system, in which the panels constantly indicated the presence of ESBL production (Moland et al., 1998). However in the field of Micro Scan conventional panel, users consistently report ESBL producers as ceftazidime resistant but are probable fewer to report cefotaxime or ceftriaxone as resistant (Steward et al., 2000). Additionally there are a wider system which is a recently developed computer-supported image-processing device that analyzes commercial bacterial detection and susceptibility testing panels. Although use of MicroScan panels in combination with the Wider system for the purpose of antibiotic susceptibilities in a sequence of 100 clinical *Enterobacteriaceae* isolates with characterized resistance mechanisms has also been reported (Canton et al., 2000).

2.17 Molecular detection methods for ESBLs:

Molecular detection of ESBL in clinical isolate is more complicated. But in earlier time isoelectric point was used and sufficient for determination of ESBL in clinical isolates. However this method was no longer possible because somehow related situation was found in the SHV, CTX-M, and OXA families of ESBLs.

2.17.1 Polymerase chain reaction (PCR):

This is the easiest and common method which is used to detect the presence of ESBLs gene however it cannot distinguish between ESBLs and non ESBLs. Oligonucleotide primers can be designed from sequence available in public databases like Genebank (GenBank, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/Genbank/index.html). Although primers are usually selected to anneal to regions wherever different point mutations are not well known to occur. Moreover PCR will not differentiate between different variants of TEM or SHV or others ESBLs (Bradford 2001).

2.17.2 DNA probes:

Genotypic detection of ESBLs was used primarily. Mostly using this techniques DNA probes for detection of ESBLs is very labor intensive. Furthermore this may not differentiate between ESBLs and non ESBLs (Bradford 2001).

2.17.3 Oligotyping:

This is the first molecular method for characterization of the ESBLs which was developed by Quellette et al.,, mostly this method is used for discrimination between TEM-1 and TEM-2 or other ESBL types (Ouellette et al., 1988). In this method used oligonucleotide probes which are designed to distinguish point mutations below stringent hybridization conditions. However by using this method, there were several new TEM variants were identified within a set of clinical isolates. The probes used in oligotyping tests for TEM β-lactamases have been labeled either with a radioisotope or with biotin (Mabilat and Courvalin 1990).

2.17.4 PCR Restriction fragment length polymorphism (PCR- RFLP):

In this method, amplified PCR products were subjected to digestion with some of the selected restriction endonucleases and consequently digested fragments were separated by gel electrophoresis. The sizes of the fragments produced by every

restriction enzyme may specify point mutations within the structural gene. This simplest method was recommended by Nuesch-Inderbinen et al., (Nuesch et al., 1996).

2.17.5 PCR Single stranded conformational ploymorphism (PCR-SSCP):

This method has been used to detect a single base mutation at specific locations within the *bla*$_{SHV}$ gene (M'Zali et al., 1998). In PCR-SSCP, *bla*$_{SHV}$ gene were amplified by using oligonucleotide primers and then digested with restriction enzyme *Pst*I. Denatured freagments were separated on a 20% polyacrylamide gel. So, by this method gene for SHV-1, -2, -3, -4, -5, and -7 β-lactamases can be identified (M'Zali et al., 1998).

2.17.6 Ligase Chain Reaction (LCR):

By the use of a thermostable ligase with four oligonucleotide primers, ligase chain reaction permits the discrimination of DNA sequences which vary a single base pair. However the oligonucleotide primers are complimentary to the target sequence and hybridize adjacent to each other. So, by the reaction a single base difference in the oligonucleotide junction will not be ligated and subsequently it may be amplified. The LCR product is detected by an enzymatic reaction using NADPH alkaline phosphatase. So, by this LCR method, it is possible to detect seven of the SHV variants (Bradford 2001).

2.17.7 Nucleotide sequencing:

Nucleotide sequencing is the most of the standard determination of the specific β-lactamase gene present in a strain. However nucleotide sequence can give variable results depending on the method used (Bradford 2001).

2.17.8 Real-time PCR:

This is one of the genotypic detection of ESBLs which can be used in practical medical diagnostics. This is mainly due to the compact cycle times, deduction of separate post PCR detection procedures and the use of sensitive fluorescence

detection equipment, which allowing the earlier amplicon detection. Unlike conventional PCR method, real-time PCR allows constant monitoring of accumulating amplicon in real time by labeling primers, oligoprobes or amplicons with molecules which capable of fluorescing. Although there are several types of detection using intercalating fluorescent dyes such as SybrGreen or LCGreen, dual-labeled probes, FRET probe systems in real-time amplification. A practical application in detection of extended-spectrum β-lactamases has been found by consequent melting curve analysis in Real-time PCR. Additionally this method could be useful locally for investigating epidemic outbreaks and would be suitable for use in regional or national reference facilities (Chroma et al., 2010).

2.17.9 DNA microarray:

DNA microarrays techniques are mainly used in three major clinical areas: **(A)** for gene expression profiling in which measuring the expression level of thousands of genes in any bacterial sample, **(B)** for genotyping determination of disease relevant genes or agents which causing diseases, and **(C)** DNA sequencing –by this, screening thousands of DNA base pairs for mutations in specific genes whose normal sequence is previously known (screening of single nucleotide polymorphisms, SNPs).

In addition to this DNA microarray looks to be a promising genotyping method with a high multiplexing capability. Microarrays allow thousands or tens of thousands of definite DNA sequences to be detected parallel on a small glass or silica slide with only 12 cm square, instead of detecting and studying one gene at a time. However the most important disadvantage of the above methods is the limited number of targets which can be detected and differentiated in each reaction (Chroma et al., 2010).

2.18 Clonal dissemination/diversity of ESBL genes:

Clonal dissemination involving genes which encoding ESBL enzymes can occur either by emerging bacterial clones or by horizontal gene transfer between bacterial species. But

sometimes in the latter cases, plasmids containing the resistance genes are spread between bacteria of the same and/or different species. Clonal dissemination also passes on to a particular bacterial cell line multiplying and disseminating in a community or causing an outbreak in hospital settings. The bacteria are then spread vertically and multiplying through cell division. In order to detect clonal expansions molecular epidemiological strain typing tools are important. They allow microbiologists to investigate when and how a particular strain is spreading. This is most important in order to be able to stop or diminish the source of clonal dissemination (Dolejskaa et al., 2012).

A number of clonal dissemination epidemics have been described. The great majority of the outbreaks occurred in tertiary hospitals. Epidemic plasmids have been implicated in the dissemination and the high prevalence of bla_{ESBL} in the European region and have been detected among local or international epidemic clones (Canton et al., 2008).

- In a Korean Hospital, clonally disseminated Extended-Spectrum Lactamase (ESBL) producing *K. pneumoniae* was reported. Of the 43 ESBL-producing *K. Pneumonia* isolates recovered from the Kang buk Samsung Hospital, all but one produced SHV enzymes (29 isolates of SHV-1, 5 of SHV-5, 8 of SHV-11, and one each of SHV-12 and SHV-14). Among them four isolates produced CTX-M-14 and 10 isolates were positive to $bla_{\text{TEM-1}}$. Most of the outbreak isolates belonged to ST2 and they produced SHV-1 and CTXM- 14, and were dissimilar from the features of the *K. pneumonia* isolates from other Korean hospitals (Kwan et al., 2008).
- In Tehran hospitals, Clonal dissemination of ESBLs was observed in the neonatal intensive care unit and intensive care unit of one hospital.The spread of multi-resistant clones of ESBL producers harboured bla_{SHV}, bla_{TEM} and $bla_{\text{CTX-M}}$ genes. PFGE differentiated the 71 ESBL-producing isolates into 62 different genotypes (Feizabadi et al., 2010).
- Conversely in South Korea, high prevalence of CTX-M-15 amongst 218 *K. pneumoniae* isolates belonged to diverse Clones as determined by multilocus sequence typing (MLST) from nine Asian countries. It was found that acquisition of IncFIIA-type plasmids carrying $bla_{\text{CTX-M-15}}$ genes and spread of certain clones

may have contributed to the high prevalence of CTX-M-15 producing *K. pneumoniae* isolates in Asian countries. However diversity of Sequence types (STs) amongst CTX-M-15-producing *K. pneumoniae* isolates suggests an independent and frequent transfer of $bla_{\text{CTX-M-15}}$ (Lee et al., 2011).

- However in one earlier study it was reported that dissemination of IncFIIK plasmids among various *Enterobacteriaceae* isolates was considered an important aspect of nosocomial colonisation in hospital paediatric oncology wards by *Enterobacteriaceae* species producing ESBLs and this dissemination of IncFIIK type plasmids in multiresistant CTX-M-15 producing plasmids into new variants containing novel antibiotic resistance elements and their important role in spreading ESBL-producing bacteria among hospitalised patients (Dolejskaa et al., 2012).

2.19 Fingerprinting of ESBLs:

The emergence of ESBL producers along with multiple resistant isolates poses a serious problem in the hospital settings. Molecular typing of ESBL producing organisms is useful for surveillance purposes, to monitor outbreaks and track nosocomial spread of the pathogens. Although pulsed-field gel electrophoresis (PFGE) is the current "gold standard" for bacterial molecular typing, MLST may offer advantages (Nemoy et al., 2005).There is Rapid and discriminative subtyping methods which are essential for determining the epidemiology of isolates in order to design rational control methods. However the accessible subtyping methods for *E. coli* include plasmid profiling, ribotyping and PCR-based typing methods such as arbitrary primed PCR, repetitive extragenic palindromes (REPs), and enterobacterial repetitive intergenic consensus (ERIC) (Lim et al., 2009; Versalovic et al., 1994).

There were several outbreaks have been reported globally. Where resistant organisms were considered epidemiologically as demonstrated by the following examples:

- ✓ In a French Hospital, SHV-5 expressing *K. pneumoniae* were isolated from six peripartum women and two neonates. PFGE profiles of these strains indicated that

all of the strains have PFGE-patterns identical to that of a strain isolated from contaminated ultrasonography coupling gel (Gaillot et al., 1998).

- ✓ In South Africa, an ESBL producing *K. pneumoniae* was carried by cockroaches infesting the neonatal intensive care unit (ICU) has the same PFGE-type of the strain that were implicated in an outbreak caused high mortality rate among neonates in that hospital (Cotton et al., 2000).

- ✓ In Spain, two clones was found responsible for nosocomial outbreak due to Extended-Spectrum-β-Lactamase producing *Enterobacter cloacae* in a Cardiothoracic Intensive Care Unit in Hospital Universitari de Bellvitge, Spain due to increased use of cefepime and quinolones during June and July 2005. Seven patients in the ICU with ESBL producing *E. cloacae* were identified (four males; median age, 73 years; range, 45 to 76 years); six patients had cardiac surgery. Four patients developed infections; three had primary bacteremia, one had ventilator associated pneumonia, and one had tracheobronchitis (Manzur et al., 2007).

- ✓ In India, Extended spectrum β-lactamase producing *Klebsiella pneumonia* was found for a nosocomial outbreak of septicaemia in neonatal intensive care unit, due to showing multiple mechanisms of drug resistance. The humidifier solution, water tap, hands of hands of health care workers (HCW) at neonatal intensive care unit (NICU) and the disinfectant solution from labour room (LR) as the probable sources of the outbreak strain. The most probable source of the outbreak seems to be the disinfectant solution (Rastogi et al., 2010).
- ✓ In one another outbreak in Spain, *A. baumannii* was found one of the major pathogens involved in nosocomial outbreaks at 19 public hospitals in 17 Spanish provinces. The clonal diversity of 729 epidemic strains isolated from 19 Spanish hospitals (mainly from intensive care units) and was analysed over an 11-year period. PFGE identified 58 types that were subjected to susceptibility testing, *rpoB* gene sequencing, and MLST (Villalon et al., 2011).

- ✓ In Sweden, ESBL producing *E. coli* were isolated from 28 infants, ages 1-72 months (median age 4 months), hospitalized patients from a neonatal post-surgery ward at Sahlgrenska University Hospital, Gothenburg, between September 2008 to June 2009; Ten distinct GECM-10 types were detected among 50 isolates. Two clusters of isolates with distinct GECM-10 types (G06-04 and G07-02), corresponding to two major PFGE types and the MLST based sequence types (STs) 131 and 1444, respectively, were confirmed to be responsible for the outbreak (Karami et al., 2013).
- ✓ In Italy, Extended-spectrum β-lactamase-producing *E. coli* sequence type 131 was found causing an outbreak of colonizations in the NICU of the University Hospital "AziendaOspedaliero-UniversitariaPoliclinico P. Giaccone", Palermo, during the first semester of 2012. There were 15 infants found to be colonized by ESBL *E. coli*. The strain belonged to the sequence-type 131 community-associated clone and this epidemic strain demonstrated continuous transmission throughout the outbreak period (Giuffre et al., 2013).
- ✓ In Turkey, two epidemic episodes of ESBL producing *K. pneumoniae* were evaluated in the NICU of Selcuk University Faculty of Medicine. The strains were isolated in six different samples (one wound, one blood, and four cerebrospinal fluid cultures) of the three neonates in the first episode and in 11 different samples (seven blood and four cerebrospinal fluid cultures) of the four neonates in the second episode. It was found two clones were responsible for these two outbreaks by PFGE (Sumer et al., 2014).

2.20 Genetic environment of ESBLs:

The appearance and spread of antibiotic resistance in bacterial pathogens is significant example of evolution of bacterial resistance. But these are like assembly platforms which integrate exogenous open reading frames (ORFs) by site-specific recombination and change them to functional bacterial genes by ensuring their correct gene expression (Figure 9). However these integrons are composed of three key elements which are compulsory for the capture of exogenous genes: intI; this is responsible for encoding an integrase which belongs

to the tyrosine recombinase family; *attI* site (a primary recombination site); and Pc promoter, an outward- orientated promoter that directs transcription of the captured bacterial resistance genes (Figure 9) (Hall et al., 1995). However in these integron encoded integrases enzyme can recombine distinct units of circularized DNA (Collis et al., 1993) which is well-known as gene cassettes. Integration occurs downstream of the resident Pc promoter at the *attI* site, allowing expression of the genes in the cassette in integrons (Mazel 2006). There is *attC* site in each integrons which is containing a single gene and an imperfect inverted repeat at the 3' End. These are also known as 59 be and are a diverse family of nucleotide sequences that function as recognition sites for the site-specific integrase. There length varies from 57 bp to 141 bp (Figure 9) (Mazel et al., 2006).

However Class 1 integrons are the most widespread and have been frequently found in ESBL producing clinical isolates of *Enterobacteriaceae* including *K. pneumonia* (Machado et al., 2007). Class 2 integrons occur less frequently in ESBL producing *E. coli* and *K. pneumoniae* and finally, class 3 integrons are rarely found in ESBL producing *K. pneumoniae* (Bhattacharjee et al., 2010). Antibiotic resistance genes have been identified as a form of gene cassettes and as part of class 1 integrons in *P. aeruginosa*. These genetic structures are vectors of co-localization and of co-expression of antibiotic resistance genes. They are associated with typical sequences (59 bp elements) recognized as target sites by the integrase, and that are essential for their mobility (Stokes et al., 1997). The 5' conserved segment of integrons contains the integrase gene (*intI*) and the recombination site *attI1* whereas the 3' conserved sequence carries the antiseptic resistance determinant *qacE*D*1*, and the *sul1* gene that confers resistance to sulfonamides (Levesque et al., 1995). Whereas genes encoding β-lactamases of class D (oxacillinases) are usually located in class 1 integrons, genes encoding VEB and GES type enzymes are the only genes encoding class A ESBLs that are associated with these genetic structures (Nordmann et al., 1998). Several integrons have been reported as transposon located; these structures may provide an additional means of mobility for these antibiotic resistance genes and may

explain plasmid and chromosomal locations of the same ESBL gene in *P. aeruginosa* (Dubois et al., 2002).

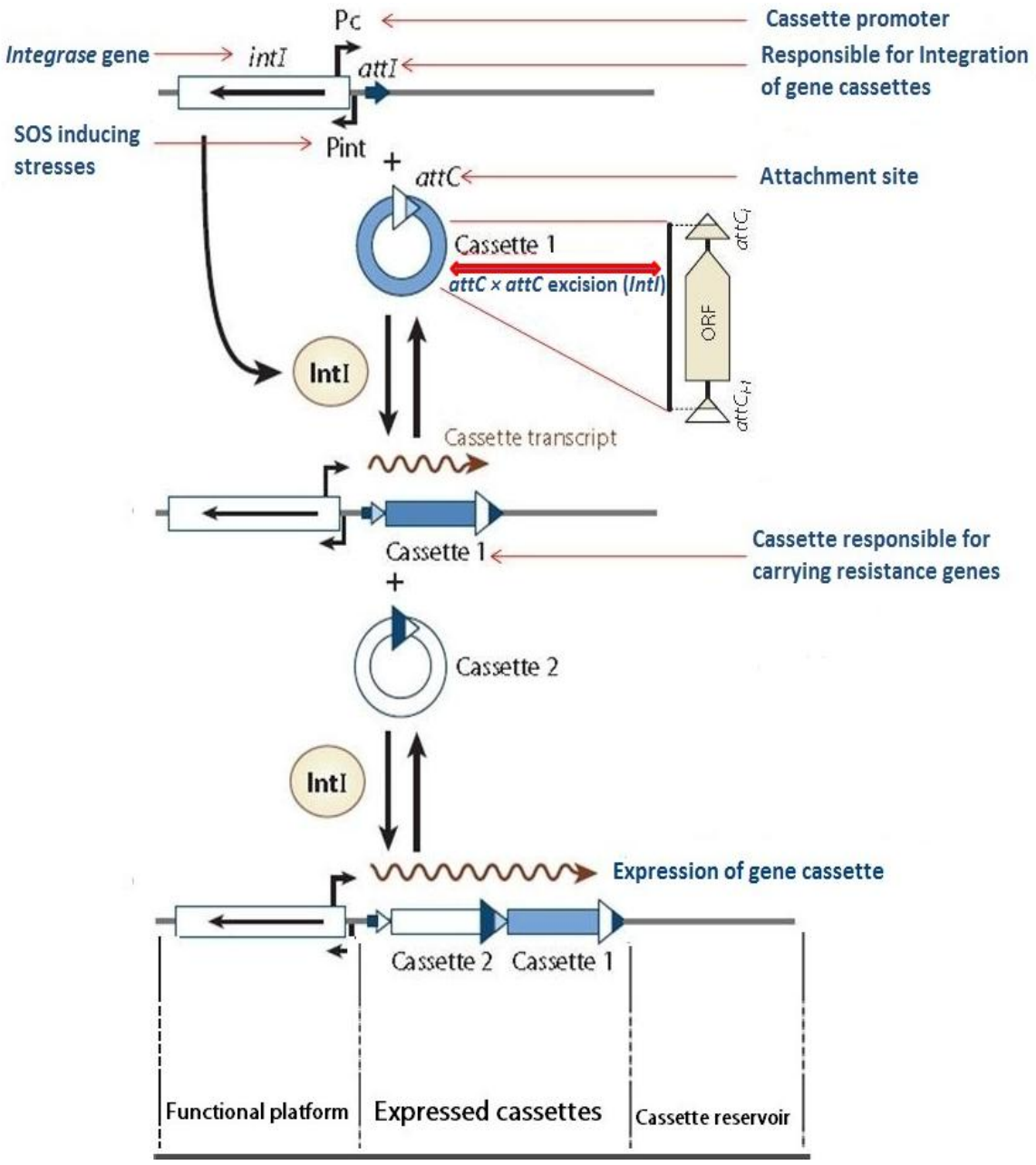

Figure 9: Schematic organization of integrons: Integron mediated genes capture and model for cassette exchange

Since *bla*$_{VEB}$ like and *bla*$_{GES}$ like genes are integron located, it is possible that their presence in *P. aeruginosa* may result from horizontal transfer from gram negative aerobes that are known as a source of integrons (Poirel et al., 2012) and that may be present in the same ecological niche. Dubois et al., reported that *bla*$_{TEM-21}$ gene was identified as part of a chromosome located Tn*801* transposon disrupted by insertion of an IS*6100* element (Dubois et al., 2002).

However the low diversity of class 1 integrons strength indicates a wide dissemination of specific genetic elements in which they are located. Furthermore in the environment, the spread of genetic elements encoding ESBL does not have any major impact on the dispersion of integrons, nor have a major impact on the spread of ESBL, except when *bla*$_{ESBL}$ genes are within an integron platform such as *bla*$_{CTX-M-9}$ (Machado et al., 2005). According to Lim et al., integron and plasmid encoded transmissible ESBL producing *E. coli* were detected in 55.3% of isolates, with class 1 integron encoded *intI1* integrase being the majority. In the 5'CS region of the integrons antibiotic resistance encoding gene cassettes *aadA5-dfrA17, dfrA7, aadA1-aadBcmlA6, dfrA12-aadA2-orfF* and *aadA1, dfrA1-aadA1-sat2* were present and may have led to the high prevalence of ESBL producers and multidrug resistance among *E. coli* hospital isolates in Malaysia (Lim et al., 2009). Chen et al., described that, clinical strains of bacteria with multiple ESBL genotypes have a greater opportunity to carry class 1 integrons and therefore, bacteria carrying both integrons and ESBL genes have much stronger multi resistance activity. However analysis of the relationship between class 1integrons and the β-lactamase genes in the ESBL producers revealed that detection rates for class 1 integrons in *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ gene carriers were 72.9%, 84.4% and 68.9% respectively and the occurrence of class 1 integrons in strains carrying the *bla*$_{SHV}$ gene was significantly higher than that in strains carrying the other two β-lactamase genes (Chen et al., 2013).

2.21 Transferability of ESBLs:

Plasmids are a major cause of spreading of bacterial resistance, as they can be transferred between Gram negative bacteria by transformation, conjugation and between Gram positive bacteria by bacterial viruses called transducing phages. However, presence of it

does not necessarily lead to bacterial resistance. This transferability results many outbreaks of resistance, and these outbreaks occurs when appropriate infection control measures are not followed or break in hospital settings (SamahaKfoury and Araj 2003). Bacterial resistance may possibly develop vertically in two ways: one is selection under antibiotic pressure and another way to develop resistance is chromosomal mutation in bacteria. However bacterial resistance may also develop horizontally when a susceptible bacterial cell acquires resistance genes from other resistant strain through transformation or conjugation or any modes of genetic exchange (Tenover 2006). There are processes through which bacterial pathogens transfer their DNA.

Mostly plasmid encoded genetic factors can arise from various sources being consequently disseminated by horizontal gene transfer (HGT). HGT is mainly responsible for the spreading of many characters linked with bacteria, including antibiotic resistance. Furthermore, broad host range plasmids play an important role in bacterial adaptation to new settings (Zhou et al., 2012). HGT conferring resistance too many classes of antimicrobials has resulted in a world wide epidemic of nosocomial and community infections caused by multidrug resistant microorganisms, leading to suggestions that we are in effect returning to the preantibiotic era (Warnes et al., 2012). A number of findings showing that $bla_{\text{CTX-M}}$ genes are commonly found on large plasmids that often carry other genes conferring resistance to other antimicrobial agents including aminoglycosides, fluoroquinolones, chloramphenicols, tetracyclins and others like $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$, *tetA*, *aac(6')-lbcr* (Leflon et al., 2004). In Lebanon, 96% of ESBL producing *E. coli* and *K. pneumoniae* clinical isolates from various sources harboured $bla_{\text{CTX-M-15}}$ and a number of these isolatescarried also *aac(6')-lβ-cr* gene. The encoding genes of the two enzymes were found on pC15-1a, and other related plasmids. The $bla_{\text{CTX-M-15}}$ gene located on these plasmids was found downstream of the *ISEcp1* insertion sequence implicated in its expression (Kanj et al., 2008).

According to Harajly et al., 2010; the frequency of transfer of antimicrobial resistance in non-clonal *Enterobacteriaceae* at the tertiary care center by conjugation was 49%. Conjugation occurred in isolates expressing the *tra* encoding transferase genes. Multiple conjugative strains harbouring the plasmid encoded antimicrobial resistant genes were

circulating in the medical centre. Molecular epidemiology analysis showed that conjugative isolates are neither clonal nor linked to a particular site and transfer of antimicrobial resistance is by horizontal transfer of plasmids (Harajly et al., 2010). The recent discovery of the gene on the IncFII plasmid which has been primarily responsible for the rapid dissemination of $bla_{\text{CTX-M-15}}$ is adding to concerns of a similar pandemic spread (Bonnin et al., 2012). Wang et al., identified that bla_{ESBL} harbouring conjugative plasmids present in multidrug resistant *E. coli* isolated from food producing animals and healthy humans. Nine isolates demonstrated the ability to transfer their cefotaxime resistance marker at high transfer rates. Plasmid profile reanalysis of these transconjugants identified 16plasmids. IncFIB and IncI1 were found the most prevalent replicon types (Wang et al., 2013).

2.22 Genetic Fittness of ESBLs:

The occurrence and rates of ascent and dissemination of antibiotic resistance in bacterial populations are anticipated to be directly related to the volume of antibiotic use and inversely related to the cost that resistance imposes on the fitness of bacteria. All reports suggest that, resistance determining mutations and accessory elements cause some fitness cost, although those costs are likely to be reorganized by subsequent evolution (Andersson and Levin1999). The fitness of anantibiotic resistant pathogen is determined by the relative rates at which resistant and sensitive bacteria **(i)** grow and die in hosts and environment, **(ii)** are transmitted between hosts, and **(iii)** are cleared from infected hosts (Andersson and Levin 1999). The fitness cost associated with plasmid carriage can also be reduced or eliminated by allowing the host-plasmid to coevolve. According to Modi and Adams, there are five evolutionary changes are possible in response to the carriage of a costly plasmid. **First**, adaptive genetic changes can occur in the bacterial chromosomes that are independent of the presence of the plasmid. **Second,** there can be adaptive genetic changes inthe bacterial chromosome that better the harmful effect of the plasmid.**Third**, adaptive genetic changes in the plasmid can render the plasmid competitively superior inrelation to other plasmids within the cell. **Fourth,** adaptive genetic changes in the plasmid can result in overall improvement of the lethal effect of

the plasmid. **Fifth**, there may be adaptive genetic changes in both the bacterial chromosome and the plasmid which are precise to each other (Modi and Adams 1991).

The combination of plasmid loss, conjugative transfer, plasmid cost and the presence of selection will therefore determine whether or not a plasmid can persist in a population over evolutionary time (De Gelder et al., 2007). A nitrofurantoin resistance confers a reduction in fitness in *E. coli* in the absence of antibiotic as described by Sandegren et al.,. They named that in the presence of therapeutic levels of nitrofurantoin the resistant mutants are so disturbed in growth and that they are probably unable to become enriched and establish an infection (Sandegren et al., 2008). Conversely in *E. coli* fitness cost of antimicrobial resistance caused by chromosomal mutations and plasmid acquisition is influenced by stressful growth conditions as described by Petersen et al., 2009. They also noted that the biological relevance of fitness cost depends not only on the growth rate but also on the transmission/colonization ability and the survival ability ofthe resistant strain (Petersen et al., 2009). Humphrey et al., described that fitness impact of a particular antibiotic resistance plasmid confers on a given bacterial species which is dependent on the genotype of the specific host strain that it is in (Humphrey et al., 2012). In *E. coli* it was also noticed that tigecycline selects for low level resistance mutations with relatively high mutation rates and the majority of them come with a substantial fitness cost (Linkevicius et al., 2013). In one another reports the effect of a particular $bla_{\text{CTX-M-15}}$ carrying plasmid on the fitness of fluoroquinolone resistant *K. Pneumonia* may be sequence type specific and failed to substantially compromise the fitness of the ST15 major clone strain (Toth et al., 2014).

2.23 Genetic mapping of ESBLs:

Genetic mapping of bla_{TEM} ESBL genes revealed that they were carried by three of the most basic bacterial transposons recognized (Partridge and Hall 2005). However there were closely related transposons also known as the carriers of bla_{TEM} genes which were initially selected as *TnA*, although later on it was well-known as *Tn1*, *Tn2*, *Tn3*, and *Tn801*. However all these were completely depends upon the bla_{TEM} variant they harbored (Bailey et al., 2011) but transposons like *Tn 1*, *Tn 2*, and *Tn 3*; carried $bla_{\text{TEM-1b}}$, $bla_{\text{TEM-2}}$, and $bla_{\text{TEM-1a}}$ genes. Also these transposons enclose transposase and resolvase

gene *viz. tnp A and tnp R* (Figure 10) and also a *res* resolution site. *Tn3* is class II transposons which possesses 38-bp inverted repeats and are capable to transpose efficiently the bla_{TEM} ampicillin resistance gene marker, and in the same way resistance to expanded spectrum β-lactams when the TEM determinant encodes an ESBL variant. After the detailed analysis of these transposons it was confirmed that the most of the differences were confined to short regions flanking the *res* site, which signifying that they had been generated by a combination of site specific and homologous recombination between ancestral transposons (Poirel et al., 2012a; Partridge 2011) (Figure 10).

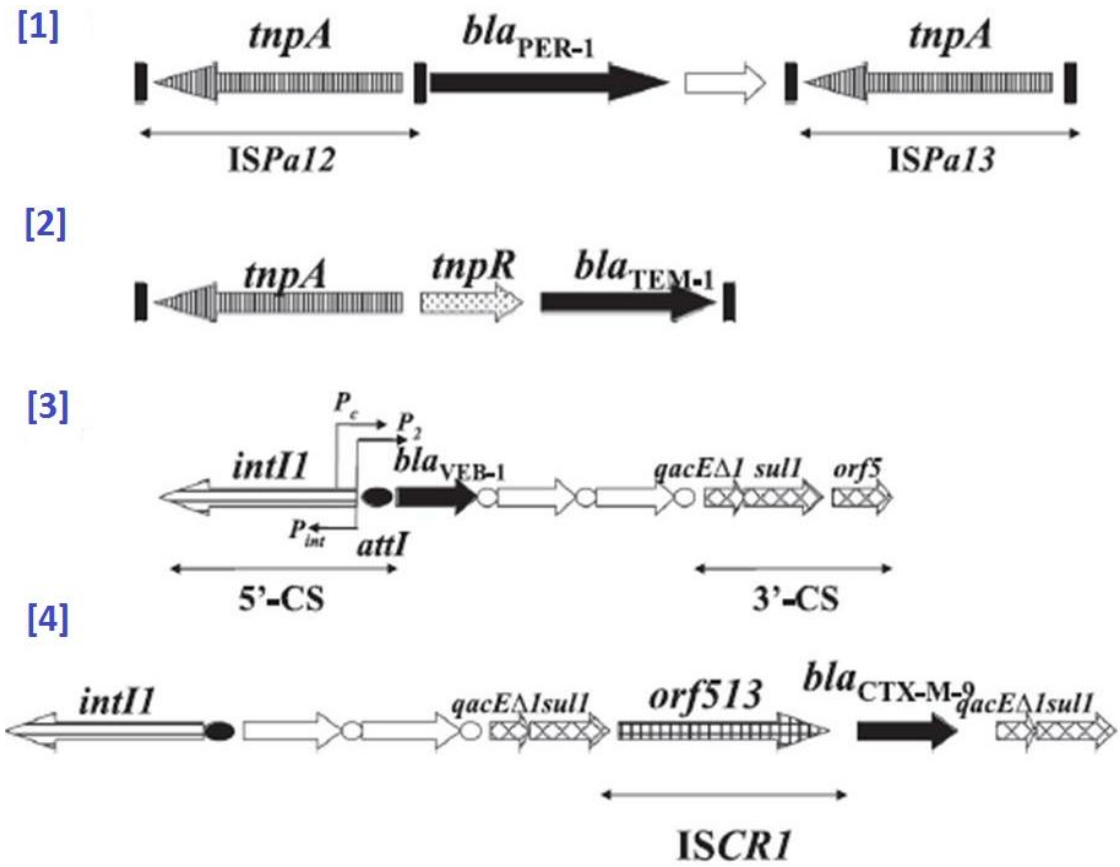

Figure 10: Schematic representation of three main genetic structures of ESBL genes acquisition **[1].** A composite transposon harbouring the ESBL PER-1, inverted repeats of insertion sequence are indicated by black rectangle [**2].** A *Tn3* transposon harbouring the bla_{TEM-1} gene (its inverted repeats are represented by black rectangles) **[3].** Class 1 integron harbouring the bla_{VEB-1} gene cassette **[4].** *sul1* type integron carrying the

bla$_{CTX-M-9}$ gene, comprising the class 1 integron and its gene cassettes associated with the *ISCR1* element and a duplication of the *qacED1/sul1* tandem. (**Source**: http://www.sciencedirect.com/science/article/pii/S1567134812000354#gr2).

On the other hand there are multiple types of genetic vehicles which are responsible for the acquisition of CTX-M ESBL gene. These are including insertion sequences *ISEcp1* and *ISCR1* and also there are some of the phage related elements in rare cases (Figure 10 and 11) (Oliver et al., 2005). However this *ISEcp1* characterized in association with genes encoding CTX-M-1, CTXM- 2 and CTX-M-9 clusters and belongs to *IS1380* family which demonstrated that it may mobilize *bla*$_{CTX-M}$ genes by a peculiar transposition process (Figure 11) (Poirel et al., 2003). Additionally *ISEcp1* enhances the expression of CTX-M gene by providing the promoter sequences which is very low in natural species although they are highly acquired in members of *Enterobacteriaceae* (Poirel et al., 2003). However there are some genetic elements like *IS91* or *Tn21* which are able to mobilize the neighboring sequences by one ended transposition process. (Poirel et al., 2012a).

Moreover there are only a very few studies on the genetics and acquisition of SHV β-lactamase genes. There were one insertion sequence element *viz IS26* was identified and this *IS26* provably present in the downstream region of *bla*$_{SHV-5}$ for its acquisition through a homologous recombination, rather than a transposition event, as suggested by the absence of their target site duplication (Figure 10 and 11). Direct repeated *IS26* elements were both truncated, that are forming a defective compound transposon (Preston et al., 2004) (Figure 11).

In some enterobacterial and *P. aeruginosa* isolates, there is only a single *IS26* element which was identified on upstream of the *bla*$_{SHV}$ like genes. Therefore possibly *IS26* is a characteristic feature in acquisition of *bla*$_{SHV}$ genes, through mobilization which have been occurred through at least two separate events. As among *bla*$_{TEM}$ genes, the *bla*$_{SHV}$ genes have never been identified as gene cassettes inside integron structures (Ford et al., 2004). However a much related organization was identified on plasmid pACM1 but the two PER-1 β-lactamase was found in several gram negative bacilli and analysis of the

sequences showed that this β-lactamase gene is a part of composite transposon which is named as *Tn1213* (Poirel et al., 2005).

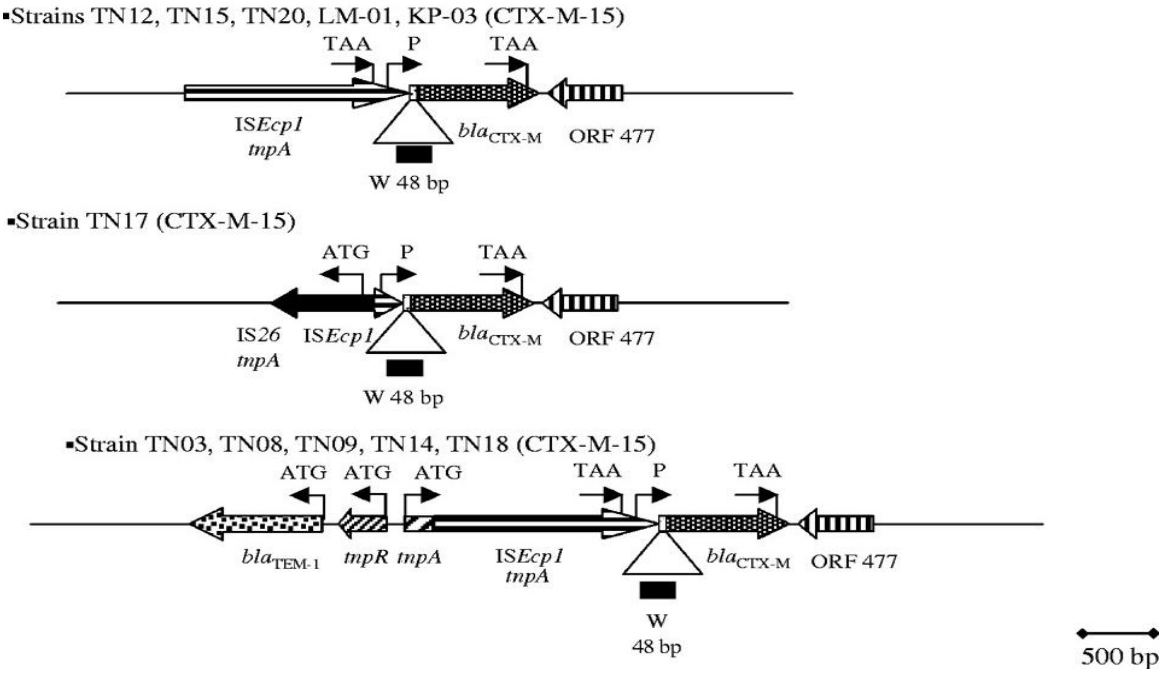

Figure 11: Schematic representation of the genetic environment of $bla_{\text{CTX-M-15}}$ genes in *Enterobacteriaceae* isolates producing CTX-M-15. (S**ource:** http://jac.oxfordjournals.org/content/57/1/14/F1/graphic-1.large.jpg).

Furthermore this composite transposon has been formed by two structurally related but considerably different insertion sequences *ISPa12* and *ISPa13* (Figure 10). However there are an 8-bp long duplication of the insertion site has been found at the left-hand extremity of *ISPa12* and at the right-hand extremity of *ISPa13*, this is also undoubtedly evidencing that this structure had transposed (Figure 10). But the investigation of *Tn1213* surrounding sequences in *P. aeruginosa*, *A. baumannii* and *Providencia stuartii* isolates disclosed that the transposition had occurred inside an IS element by interrupting its transposase gene and which is named as *ISPa14*. However *Tn4176* transposons are very analogous to *Tn1213* but inserted into a *Tn5393* derivative, which was additionally recognized in an Italian *Alcaligenes faecalis* isolate (Mantengoli and Rossolini 2005). However in recent times it was well noticed that $bla_{\text{PER-7}}$ gene encoding PER-7 β-lactamase which was identified in an *A. baumannii* clinical isolate from France was

recognized in association with the *ISCR1* element inside a *sul1*-type integron structure and in PER-1 also (Figure 12) (Bonnin et al., 2011b).

Figure 12: Genetic context of*bla*$_{PER-1.}$ IS*CR1* associated with*dfr* genes, *qnrA* genes, and *aacA4* genes **(Source:** http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1489542/figure/f1/).

Genetic mapping of VEB-1 gene cassette possessed perfect core and inverse core sites simultaneously with a 133 bp long 59 be recombination site. This *bla*$_{VEB-1}$ gene has been the foremost ESBL gene which is identified as a form of a gene cassette in a class 1 integron structure. And it was located inside the *In53* class 1 integron that carries an unusual array of eight functional gene cassettes further encoding antibiotic resistance determinants (Naas et al., 2001; Poirel et al., 2012a) (Figure 10 and 13).

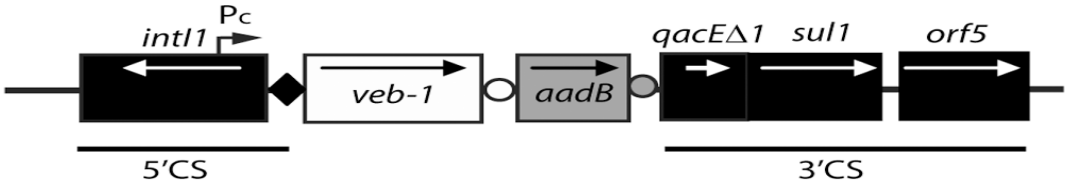

Figure 13: Schematic representations of the integron mediated *VEB-1* from *P. aeruginosa.* The 5' and 3'-CSs are underlined. ORFs are shown as boxes with an arrow indicating the orientation of the coding sequence. The promoter Pc is indicated by a broken arrow.

(**Source:**
http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0051602).

However this *In53* was identified as plasmid borne and which is a part of a composite transposon structure named as *Tn2000*. This was flanked on both of its extremities by an *IS26* element. On both extremities of *Tn2000*, an 8 bp duplication of the target site was the signature of the transposition process. Therefore, In53, as a substitute of being located

onto a defective *Tn402* based transposon, was mobilized by an *IS26* made composite transposon (Fournier et al., 2006). So, the genetic environment of VEB-1 gene in enterobacterial and *P. aeruginosa* isolates and in an *A. baumannii* epidemic clones from France. It has been seen that ESBL encoding gene was present in variety of class 1 integron structures (Figure 10 and 13) (Fournier et al., 2006).

The gene cassette of *bla*$_{GES-1}$ was identified at the first position in a class 1 integron structure which was named as *In52* (Figure 14). Consequently it was also well known in a *P. aeruginosa* isolate from France, at the second position of a class 1 integron. Interestingly, this *bla*$_{GES}$ gene was found to be an element of gene cassette but with a truncated recombination site (Figure 14) (Dubois et al., 2002).

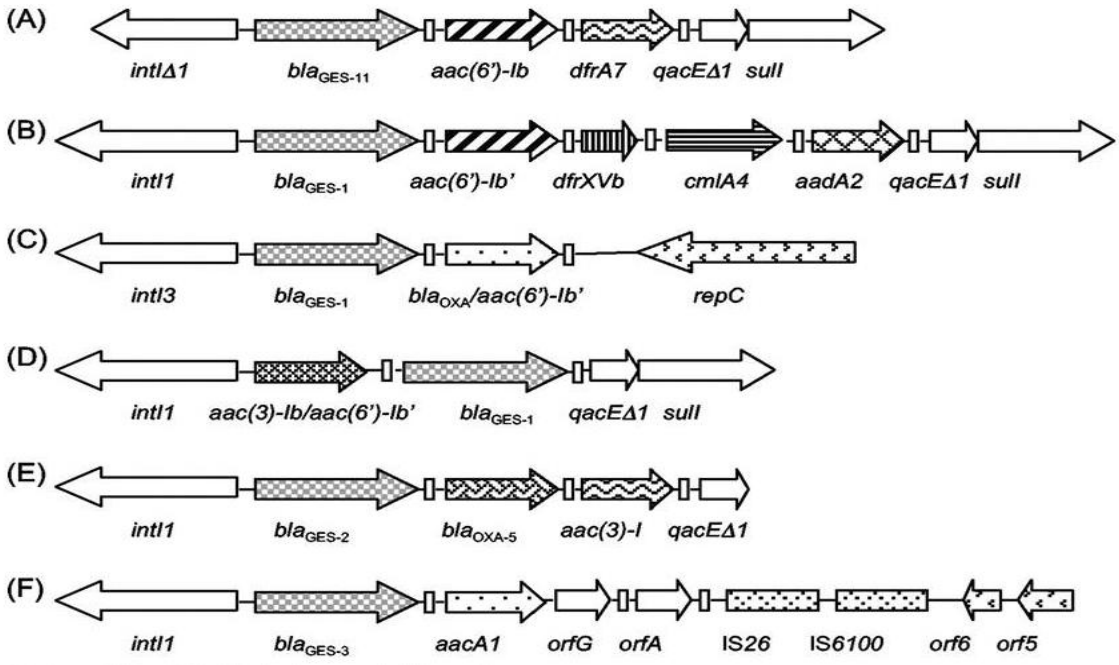

Figure 14: Schematic representation of integrons containing *bla*$_{GES}$ genes. **(A)** *bla*$_{GES-11}$ containing a class 1 integron **(B)** *bla*$_{GES-1}$ containing a class 1 integron **(C)** *bla*$_{GES-1}$ containing a class 3 integron **(D)** *bla*$_{GES-1}$ containing a class 1 integron with different resistant genes **(E)** *bla*$_{GES-2}$ containing a class 1 integron **(F)** *bla*$_{GES-3}$ containing a class 1 integron **(Source:** http://aac.asm.org/content/53/8/3579/F1.expansion.html)

However there are some other GES encoding genes which have been consequently identified in *Enterobacteriaceae* and *P. aeruginosa*, and it was noticed that this gene was always located inside class 1 integrons, with the exception of bla_{GES-1} from a *K. pneumoniae* and *E. coli*, which were surrounded into class 3 integron structure (Correia et al., 2003) but in *E. coli*, the bla_{GES-1} containing class 3 integron which was located onto a small IncQ type plasmid (Poirel et al., 2010a). Significantly bla_{GES-5} was found identified as a gene cassette with the *intI1* and *qacE* genes which being truncated by the Integron Mobilization Unit (IMU) elements (Poirel et al., 2010b).