2. REVIEW OF LITERATURE

Antibiotic resistance is a problem of increasing incidence since the early 1960s and is currently viewed as a major threat to clinical practice in the treatment of infectious diseases. Antibiotic resistant organisms appear to be biologically fit and are capable of causing serious, life-threatening infections that are difficult to manage because treatment options are limited (Mulvey and Simor 2009). The spread of multiple antimicrobial-resistant pathogenic bacteria has been recognised by the World Organisation for Animal Health, the Food and Agriculture Organisation (FAO) and the World Health Organization (WHO) as a serious global human and animal health problem.

Resistance has been observed to majority of currently approved antimicrobial agents in human and veterinary clinical medicine. This makes the selection of an appropriate agent an increasingly more challenging task. This situation has made clinicians more dependent on data from in vitro antimicrobial susceptibility testing, and highlights the importance of the diagnostic laboratory in clinical practice (OIE, 2008). With the discovery and development of antibiotics and their medical applications, drug resistance took on new relevance. No sooner were new antibiotics announced than reports of drug resistance appeared: sulfonamide resistance in 1939, penicillin resistance in 1941, and streptomycin resistance in 1946 (Summers, 2008).

Antimicrobial Susceptibility Testing (AST) methods vary in different clinical laboratories and they should follow a common golden standard to make a necessary comparison between the susceptibility data (OIE, 2012). National Committee for Clinical Laboratory Standards (NCCLS) now called the Clinical and Laboratory Standards Institute (CLSI) provides standard representation of antimicrobial testing procedures. Each document is reviewed every three years and either discontinued or revised. In that way, CLSI standards are living documents that are updated (Schwalbe et al., 2007). Results of in vitro susceptibility tests can at best be used as an educated guess to predict the therapeutic outcome of standard antibiotic dosage regimens in normal patients. Many factors such as pharmacokinetics and pharmacodynamics of drug and drug effects on bacteria affect the potential clinical efficacy of a particular antibiotic (Wanger, 2007).
The present study analyses the problem of antimicrobial resistance in urinary isolates with the help of the reviews as discussed below:

2.1. History and development of Antimicrobials
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2.7. Conclusion
2.1. History and Development of antimicrobials

History of medicine has a profound effect upon human life and society with the development of power to control infectious diseases. The publications of Pasteur and Koch family established that microorganisms are the cause of infectious disease. Chemotherapy however really began with Paul Ehrlich during the period of ten years from 1902 which governed many concepts of antimicrobial agents. Ehrlich continued his research towards the search of compounds with more curative effect and low toxicity to be safe as chemotherapeutic agents. In 1910, the famous drug salvarsan, an organoarsenial compound was discovered for treating syphilis (Greenwood, 1995).

The term “chemotherapy” was invented by Ehrlich and he made a belief that infectious diseases could be treated by synthetic chemicals. According to Ehrlich, a chemotherapeutic substance has two functional features, the haptophore or binding group that helps to bind the drug to the specific receptor and the toxophore or toxic group which makes an adverse effect on the cell. After some 60 years of effort, the synthetic antibacterial compounds in current use include, sulphonamides, trimethoprim, imidazole, oxazolidinones etc., and semisynthetic β-lactam, aminoglycosides, macrolides etc., (Franklin and Snow, 2005d).

2.1.1. Antibiotic Revolution

In 1928 Alexander Flemming discovered penicillin from the mold *Penicillium notatum*, which was found to be active against gram positive bacteria. The success of penicillin quickly diverted a great deal of scientific effort towards the search of other antibiotics. Selman Waksman discovered streptomycin and soon followed by several thousand antibiotics. Out of all 50 had some sort of clinical use and only a very few are employed in the regular therapy of infectious diseases (Franklin and Snow, 2005d). The traditional first-line available options for treating serious infections caused by enterobacteria include penicillins, cephalosporins, monobactams, carbapenems, fluorquinolones, and in certain situations, aminoglycosides (Valverde et al., 2013).

2.1.2. Classes of antimicrobials

Antimicrobials are classified into many types based on their structure and mode of action. They are classified into five functional groups:

- Inhibitors of cell wall synthesis
- Inhibitors of protein synthesis
- Inhibitors of membrane function
- Antimetabolites
- Inhibitors of nucleic acid synthesis

Based on their structure they are mainly classified into two groups

1. β-lactams
2. Aminoglycosides

Among this β-lactam forms the major group. In 2002, the global market for antibiotics was estimated at 25 billion dollars, of which about 50% was β-lactam antibiotics (Coates et al., 2002). In addition β-lactam antibiotics are the most frequently applied in treatment of bacterial infections. β-lactam antibiotics all share the presence of the β-lactam ring, a four-membered ring in which a carbonyl and a nitrogen are joined in an amide linkage. Details of antibiotics available and sold during 2005-2009 in India are reported below (Ganguly et al., 2011):

![Graph showing details of antibiotics sold during 2005-2009 in India]

**Details of Antibiotics sold during 2005-2009 in India**

### 2.1.3. β-lactam antibiotics

The clinical success of the first β-lactam, penicillin G (benzylpenicillin), prompted the search for and development of additional derivatives and gave rise to the β-lactam antibiotics in clinical use today (penicillins, narrow and extended-spectrum cephalosporins,
monobactams, and carbapenems). The common structural feature of these classes of antibiotics is the highly reactive four-membered \( \beta \)-lactam ring (Babic et al., 2006).

The large number of natural, semisynthetic and synthetic \( \beta \)-lactam antibiotics can be subdivided into 6 different structural subtypes:

(i) penams (e.g. benzylPenicillin, Ampicillin);

(ii) cephems which include classical cephalosporins, 2nd generation cephalosporins (e.g. cefotiam, cefuroxime), and also representatives of 3rd generation cephalosporins (e.g. cefotaxime, ceftazidime);

(iii) cephamycins as 7-\( \beta \)-methoxy Cephalosporins (e.g. cefoxitin);

(iv) monobactams as monocyclic \( \beta \)-lactam molecules (e.g. aztreonam);

(v) penems with a 2,3-double bond in the fused thiazoline ring (e.g. faropenem); and

(vi) carbapenems (e.g. Imipenem) with an unsaturated fused 5-membered ring differing from penem structure by possession of a carbon atom at position 1.

Subdivisions of \( \beta \)-lactam antibiotics

2.1.4. Mechanism of action of \( \beta \)-lactam antibiotics

\( \beta \)-lactam antibiotics exhibit their bactericidal effects by inhibiting enzymes involved in cell wall synthesis. The integrity of the bacterial cell wall is essential to maintain the cell shape in a hypertonic and hostile environment. Osmotic stability is preserved by a rigid cell wall comprised of alternating N-acetyl- muramic acid (NAM) and N-acetylglucosamine (NAG) units. These glycosidic units are linked by transglycosidases.
A pentapeptide is attached to each NAM unit, and the cross-linking of two D-alanine–D-alanine NAM pentapeptides is catalyzed by PBPs, which act as transpeptidases. This cross-linking of adjacent glycan strands confers the rigidity of the cell wall. The β-lactam ring is sterically similar to the D-alanine–D-alanine of the NAM pentapeptide, and PBPs “mistakenly” use the β-lactam as a “building block” during cell wall synthesis. This results in acylation of the PBP, which renders the enzyme unable to catalyze further transpeptidation reactions. As cell wall synthesis slows to a halt, constitutive peptidoglycan autolysis continues. The breakdown of the murein leads to cell wall compromise and increased permeability. Thus, the β-lactam mediated inhibition of transpeptidation causes cell lysis, and the specific details of penicillin’s bactericidal effects are still being unravelled (Drawz1 and Bonomo 2010).

Different β-lactams exhibit different affinities for the various penicillin binding proteins and these in turn can be correlated with different morphological effects. Drugs that bind most strongly to penicillin binding proteins 1a and 1b cause cell lysis at low bacterial concentration. Compounds such as cephalosporin, cephalexin bind more strongly to penicillin binding protein and inhibit septation, leading to the formation of filaments, which are greatly elongated cells (Franklin and snow 2005e).

2.2. Antimicrobial resistance mechanisms

Antibiotics work by interacting with specific bacterial targets, inhibiting bacterial cell-wall synthesis, protein synthesis or nucleic acid replication. To accomplish this, the antibiotic must have access to and bind to its bacterial target site. Whether antibiotic resistance is intrinsic or acquired, the genetic determinants of resistance encode specific biochemical resistance mechanisms that may include
Enzymatic inactivation of the drug,
Alterations to the structure of the antibiotic target site, and
Changes that prevent access of an adequate concentration of the antimicrobial agent to the active site (Neu, 1992 and Koneman et al., 1997).

There are four primary mechanisms by which bacteria can overcome β-lactam antibiotics (Babic et al., 2006):

- Production of β-lactamase enzymes is the most common and important mechanism of resistance in gram-negative bacteria.
- Changes in the active site of PBPs can lower the affinity for β-lactam antibiotics.
- Decreased expression of outer membrane proteins (OMPs).
- Efflux pumps, as part of either an acquired or intrinsic resistance phenotype, are capable of exporting a wide range of substrates from the periplasm to the surrounding environment.

### 2.2.1. Enzymatic resistance mechanism

Bacteria may produce enzymes that modify or destroy the chemical structure of an antibiotic, which renders it inactive. This mechanism of resistance is probably best exemplified by the β-lactamase family of enzymes, which act by hydrolyzing the β-lactam ring of penicillins, cephalosporins and carbapenems. There are hundreds of β-lactamase enzymes that may be distinguished by their substrate profiles and activities. Some β-lactamase genes are chromosomal, whereas others are located on plasmids or transposons. Penicillin resistance in *S. aureus* and *Neisseria gonorrhoeae*, ampicillin-resistance in *Haemophilus influenzae*, and resistance to extended-spectrum Cephalosporins in *E. coli* and in *Enterobacter* species are all commonly mediated by the production of β-lactamases. Resistance to extended-spectrum Cephalosporins (e.g., Cefotaxime, Ceftriaxone, and Ceftazidime) has arisen primarily by 1 of 2 mechanisms, both of which involve the production of β-lactamases (Bradford, 2001).

#### 2.2.1.1. β-lactamases

β-lactamases (β-lactamhydrolyases, EC 3.5.2.6) are enzymes that open the β-lactam ring, inactivating the antibiotics. Reports on β-lactamases have been increasing in many countries. β-lactamases are the main cause of bacterial resistance to penicillins and
cephalosporins. Definitive identification of these enzymes is only possible by gene or protein sequencing aspects (Livermore and Brown, 2001).

β-lactamas (ESBL) are enzymes that confer resistance to most β-lactam antibiotics, including penicillins, cephalosporins, and the monobactam-aztreonam. Community-acquired ESBL producing Enterobacteriaceae are prevalent worldwide (Rodriguez and Jones 2002). The first plasmid-mediated β-lactamase in gram-negative bacteria was discovered in Greece in the 1960s. It was named TEM after the patient from whom it was isolated (Temoniera). The first β-lactamase enzyme was identified in Bacillus (Escherichia) coli before the clinical use of penicillin. In a sentinel paper published nearly 70 years ago, E. P. Abraham and E. Chain described the B.coli as “Penicillinase” (Abraham and Chain 1940).

Hydrolysis β-lactam ring in Penicillin by β-lactamase enzymes

Extended spectrum β-lactamases are the enzymes with broad substrate specificity to β-lactam antibiotics and were first identified in the year 1983 (Knothe et al., 1983). They are associated with increased morbidity and mortality, especially amongst patients on intensive care and high-dependency units (John et al., 2002). Thus β-lactamases predate the antibiotic era. The widely accepted molecular classification places β-lactamases into four classes: three serine-dependent enzyme classes (classes A, C, and D) and one metal-dependent (class B). Class A β-lactamases

This is the largest and best mechanistically characterized serine β-lactamase class. Historically, these β-lactamases were described as “penicillinases” as their ability to catalyze penicillin hydrolysis was greater than that for cephalosporins. The class A β-lactamases are closely related in sequence to low molecular weight class C PBPs such as PBP4 of E. coli,
**H. influenza**, and *Mycobacterium tuberculosis* (Massova and Mobashery, 1998). New class A β-lactamases that are active against the more recent cephalosporins (ceftazidime and cefotaxime and the monobactam aztreonam) and others that are active against the carbapenems are known collectively (also with other class C and D enzymes) as “expanded-spectrum β-lactamases” (ESBL) (Bradford, 2001).

### Class B β-lactamases

These metal-dependent (almost always divalent zinc) β-lactamases have a broad β-lactam substrate tolerance that encompasses many of the newer generation cephalosporins, carbapenems, and other β-lactamase inhibitory (clavulanate and penam sulfones) β-lactams important to the treatment of gram-negative infection (Livermore and Woodford, 2006, Nordmann and Poirel 2002). This enzyme was first observed in 1967 by Kawabata and Abraham as chromosomal enzymes of the innocuous gram positive *Bacillus cereus* – a spontaneous mutant strain producing class B β-lactamase constitutively (Walsh and Wright 2005). The structure and dynamics of metallo β-lactamases have been studied (Concha et al., 1996 and Scrofani et al., 1999).

### Class C β-lactamases

Class C β-lactamases share with the class A β-lactamases a similar mechanisms active site acylation and hydrolytic deacylations for β-lactam hydrolysis. The class C β-lactamases originally termed as cephalosporinases due to a substrate preference for cephalosporins. They are found, with few exceptions, in most Gram-negative bacteria and are chromosomally encoded in several organisms (including *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae*) (Rice and Bonomo 2000, Hanson, 2003). An increased incidence of plasmid-encoded class C β-lactamases was observed 15 years after their first discovery (Hall and Barlow, 2004). Plasmid-encoded class C enzymes have been found in *E.coli*, *Klebsiella pneumoniae*, *Salmonella* spp., *C.freundii*, *Enterobacter aerogenes*, and *Proteus mirabilis* (Bauernfeind et al. 1998, Livermore, 1995, Philippon et al., 1994). Most worrisome is that the rate of incidences of these enzymes is highest in *Klebsiella pneumoniae* and *E.coli*, organisms common to the hospital and community settings (Rice and Bonomo, 2000).

### Class D β-lactamases

The class D β-lactamases are increasingly encountered among the defensive β-lactamase ensemble of certain gram-negative pathogens (Hall and Barlow, 2004; and Thomson and Moland, 2000; Nordmann and Poirel, 2002). These β-lactamases were first termed as oxacillinases for their ability to hydrolyze the 5-methyl-3-phenylisoxazole-4-carboxy side chain penicillin class, exemplified by oxacillin and cloxacillin. Over 50 class D
OXA variants are now known (Heritier et al., 2004). The first studies on class D β-lactamases showed that the domain folding was similar to serine β-lactamases (Paetzel et al., 2000). But later it was demonstrated that lysine lies in the active site of this class of enzyme (Maveyraud et al., 2002). Class D gene in gram positive bacteria possesses structural and functional relationship with Penicillin binding protein (Colombo et al., 2004).

2.2.1.2. β-lactamase inhibitors

β-lactamase inhibitors generally inhibit ESBL producing strains. β-lactamase inhibitors, such as clavulanic acid, sulbactam, or tazobactam, are largely prescribed in association with amino and ureido penicillins for treating gram-negative infections. Clavulanic acid, the first β-lactamase inhibitor introduced into clinical medicine, was isolated from Streptomyces clavuligerus in the 1970s, (Reading and Cole, 1977). Clavulanate (the salt form of the acid in solution) showed little antimicrobial activity alone, but when combined with amoxicillin, clavulanate significantly lowered the amoxicillin MICs against S. aureus, K. pneumoniae, Proteus mirabilis and E. coli (Brown, 1986). However with the increased use of amoxycilav resulted with resistance to them (Guibout et al., 2000).

There are suicide inactivators of Ambler class A β-lactamases (CTX-M and the ESBL derivatives of TEM-1, TEM-2, and SHV-1). Several mechanisms, however, allow enterobacteriaceae to overcome the efficacy of these molecules, such as overproduction of a cephalosporinase or of narrow-spectrum class A enzymes, limited uptake of the antibiotics, production of OXA-type enzymes, and production of β-lactamase inhibitor-resistant TEM or SHV derivatives (Therrien and Levesque, 2000).

Clavulinic acid                                Sulbactam                                        Tazobactam

Structure of β-lactamase inhibitors
Boronic acid is used as an effective inhibitor for class C \( \beta \)-lactamases whereas clavulanic acid acts as an effective inhibitor for ESBL (Liebana \textit{et al.}, 2004). \( \beta \)-lactamase enzyme activity was also inhibited by inhibitors such as EDTA, iodine, \( \text{Cu}^{2+} \), and \( \text{Hg}^{2+} \), sulbctam. Mercaptoacetic acid thiol esters (Yang and Crowder, 1999) and thiomandelic acid (Mollard \textit{et al.}, 2001) had been identified as metallo-\( \beta \)-lactamase inhibitors.

Li \textit{et al.} (1998) reported that \( \beta \)-lactamase inhibitors like cloxacillin acted as substrates for Multidrug efflux pumps of \textit{Pseudomonas aeruginosa} The MexAB-OprM Multidrug efflux system exported a number of antimicrobial compounds, including \( \beta \)-lactams and their inhibitors. MIC determinations demonstrated that, clavulanate, cloxacillin, and BRL42715, were accommodated by the pump. With \( \beta \)-lactams which were poorly hydrolyzed, however, the inhibitors failed to enhance the susceptibility. Thus \( \beta \)-lactam and \( \beta \)-lactamase inhibitor combinations are routinely used for treating bacterial infections.

\textbf{2.2.2. Alternation of the antibiotic target site}

Antibiotics must bind to a specific bacterial target site, which varies depending on the class of antibiotic. A change in the structure of the target may result in the inability of the antibiotic to bind to its target. For example, \( \beta \)-lactam antibiotics act by binding to structures in the bacterial cell wall called penicillin-binding proteins. Methicillin-resistant strains of \textit{S.aureus} (MRSA) possess a genetic element called staphylococcal cassette chromosome mec (SCC mec), which contains the mecA gene that codes for the production of an altered penicillin-binding protein (PBP2a) that does not effectively bind \( \beta \)-lactam antibiotics. As a result, MRSA is resistant to all of the currently available penicillins, cephalosporins and carbapenems (Katayama \textit{et al.}, 2000).

\textbf{2.3. Genetics of antimicrobial resistance}

Genes can encode proteins or ribosomal RNA that enables bacteria to evade the actions of antibiotics. Antibiotic-resistance genes, however, are not confined to bacterial genomes. They are also frequently found on mobile genetic elements (plasmids, transposons, and integrons) that readily pass horizontally from organism to organism, even across species boundaries, thereby circumventing the standard parent-to-progeny route of genetic flow (Levy and Marshall, 2004). Such antibiotic resistance may either be intrinsic or acquired.
2.3.1. Intrinsic resistance

Intrinsic resistance is associated with the inherent genetic makeup and the organism can be resistant to a specific class of antibiotics. This form of resistance is predictable, which makes antibiotic selection straightforward. For example, all *Streptococci* are intrinsically resistant to Aminoglycosides (e.g., gentamicin and tobramycin), and all gram-negative bacilli are intrinsically resistant to vancomycin.

2.3.2. Acquired resistance

Antibiotic resistance may also be acquired. This involves a change in the organism’s genetic composition. This may occur by 1 of 2 mechanisms:

- Mutation in the bacterial chromosomal DNA, or
- Mobilization of the genetic material such as plasmids and transposons

Mutations are generally uncommon events, perhaps occurring at a frequency of event per $10^7–10^{10}$ bacteria, but may result in the development of resistance during therapy in organisms that are initially susceptible. An important example of this type of resistance is isoniazid resistance that can occur in *Mycobacterium tuberculosis*. This form of resistance is not transferable to other organisms. The probability of multiple resistance mutations occurring in a single organism is equal to the product of their individual probabilities.

Multiple antibiotic resistance genes may be transferred at the same time. There are numerous examples of this type of resistance, including plasmid-mediated production of β-lactamase enzymes, which are capable of inactivating penicillins or cephalosporins in *Staphylococcus aureus*, *Escherichia coli* or *Enterobacter* species (Mulvey and Simor, 2009). Acquisition of antibiotic resistance gene may be either through community acquired and hospital acquired infections. Both of them have their own limitations (Perrin *et al.*, 1998).

Examples include:

- Streptomycin-resistance genes, *strA*- and *strB*, carried on plasmid can transfer resistance
- Sulfa drug resistance caused by plasmids carry the drug insensitive enzyme.
- The plasmid *qnr* (quinolone resistance) gene encodes a pentapeptide, binds to the DNA gyrase, thereby causing low-level resistance.

### 2.3.3. β-lactamase genes

β-lactamase genes are generally located on large transferable plasmids that often carry other resistance determinants such as those for aminoglycosides, tetracycline, sulphonamides and chloramphenicol (Jacoby and Mederios 1991). All enterobacteriaceae can harbor plasmid-mediated ESBL genes (Bouchillon et al., 2002 and Lautenbach et al., 2001). β-lactamase TEM is found to be predominant and very lesser amount of *blaSHV* and *blaOXA-1* were reported in Enterobacteriaceae. *blaTEM* and *blaSHV* genes were also found to be in combination form (Colom et al., 2003). β-lactamase genes families of *blaTEM*, *blaSHV*, *blaVEB* and *blaCTX-M*, were reported to be highly prevalent in many countries (Tribudharat and Fennewald, 2002; Bradford, 2001; Chanawong et al., 2007; Empel et al., 2007). Plasmid analysis of β-lactamase genes in *Klebsiella pneumoniae* showed that Kp4940 and Kp1 resistance gene was carried by one of two small plasmids with estimated sizes of 6 and 14 kb, respectively. The small size of the Kp1 plasmids suggested that they were not self-transferable, but probably mobilized by the 60 kb plasmid (Laksania et al., 2000). Mobilisation of ESBL genes in environment led to the rise of *blaCTX* family enzyme in Enterobacteriaceae (Bonnet, 2004).

Molecular methods, particularly PCR, are widely used for confirmation and determination of β-lactamase genes, although there are some limitations as there are many more β-lactamase genes than *blaTEM*, *blaSHV*, *blaVEB* and *blaCTX-M* families. *blaTEM* hyperproduction is a frequently described mechanism by which resistance to the β-lactam and β-lactamase inhibitor combinations is mediated in *E. coli*. Resistance to aminoglycosides is most often conferred by plasmid-encoded *blaTEM*-type β-lactamase production (Nicolaschanoine, 1997). Detection of antibiotic resistant genes in gram positive bacteria through microarray based hybridization has large potential applications in screening of antibiotic resistant property in different strains (Perreten, et al., 2005).
2.3.4. β-lactamase TEM gene

TEM and SHV-derived extended-spectrum β-lactamase genes producing enterobacteriaceae had been reported from throughout the world, but there has been limited data for the molecular characterization of these enzymes (Tash and Bahar, 2005). Zeba et al. (2004) had reported that β-lactamases bla SHV gene may be common among Klebsiella spp and E.coli species. Klebsiella pneumoniae strain BDK0419 contained a transferable plasmid with a molecular size 21 kbp that carries both blaSHV-2a and blaCTX-M-54 β-lactamase genes, along with two other plasmids. The blaCTX-M-54 gene was flanked upstream by an ISEcp1 insertion sequence and downstream by an IS903-like element (Bae et al., 2006).

2.3.5. β-lactamase SHV gene

SHV type β-lactamases can be easily detected using PCR and melting curve analysis in which enzymes of nearly 32 clinical isolates can be detected within one hour (Randegger and Hachiler, 2001). LCR typing permitted for the definition the SHV families with simplicity and reliability and can be applied to the detailed characterization and molecular epidemiology of SHV type β-lactamases (Kim and Lee, 2000).

2.3.6. Mutations in β-lactamase genes

Mutations in genes that encode resistance to β-lactam antibiotics were described in different studies (Jacoby and Sutton, 1991), which commonly include mutations in blaTEM at position 21,164 and 265. These mutations may be associated with either increase or decrease in β-lactam resistance (Stobberingh, et al., 1999).

A substantial number of blaTEM was associated with mutations with absence of a fragment of 136 base pairs located upstream the promoter region including the -35 region and not the -10 region of the promoter. This finding was associated with increased resistance to cefaclor when compared to the normal isolates. In addition 3.9% of isolates carried the bla Rob gene (Molina et al., 2003). Single nucleotide specific PCR was used to discriminate the polymorphic nucleotides at positions 32 and 317 of the blaTEM genes from a collection of TEM-positive strains(Tristram et al., 2005).According to the amino acid sequence, SHV β-lactamases in Taiwan were basically derived through stepwise mutation from SHV-1 or SHV-11 and further subdivided by four routes. The stepwise mutations initiated from SHV-1 or SHV-11 to SHV-2, SHV-5, and SHV-12 comprise the evolutionary change responsible for extended-spectrum β-lactamase (ESBL) production in Taiwan (Chang et al., 2001).
2.3.7. Amino acid substitutions in β-lactamase genes

Zeba et al. (2004) had highlighted a typical blaSHV11 (clone 5) gene of 861 bp isolated from *K. pneumoniae* N°39 which was not identical with blaSHV-11 genes available in gene Bank (NCBI-DB). The nucleotide alignment of blaSHV-11 gene and that of clone 5 revealed substitutions at position 324 (cytosine replace thymine), 357 (thymine replace cytosine), 762 (cytosine replace thymine) and 795 (cytosine replace thymine). This report shows that the aminoacid change always occurs between cytosine and thymine. Therefore, on the basis of nucleotide sequences, the two genes (classical blaSHV-11 and clone 5) are slightly different, but the translation of the two genes yields the same variant SHV-11 β-lactamase.

Common blaSHV variants such as blaSHV-1 to blaSHV-2, blaSHV-2a, blaSHV-11, and blaSHV-12 were differentiated by detecting four mutations affecting the amino acids at positions 35, 205, 238 (Gly-Ser), and 240. Other blaSHV variants that are uncommon may be underestimated due to the lack of methods suitable for screening these genes in instances when many isolates are to be characterized (Chanawong et al., 2001). Kwon, (2006) identified novel CTX-M enzymes from clinical isolates with an expanded activity towards Ceftazidime through a single amino acid substitution. Ceftazidime-hydrolysing CTX-Mutant designated CTX-M-54. CTX-M-54 differed from CTX-M-3 only by the substitution Pro-167→Gln and is the third CTX-M enzyme harbouring an amino acid substitution at position 167 after CTX-M-19 and CTX-M-23.

2.4. Antimicrobial resistance issues

Antimicrobial resistance is now recognized as an increasingly global problem, both in gram positive and gram-negative bacteria (Slama, 2008). For example, Methicillin resistant *Staphylococcus aureus* (MRSA) killed 19,000 people in US every year, which exceeded the death caused by any other infectious diseases. Penicillin resistant pneumonias and vancomycin resistant Enterococci (VRE) are more frequently incriminated from many industrialised countries forcing frequent changes and recommendations of management of diseases caused by these bugs (Vashishtha, 2010).

Appropriate use of antimicrobials might lead to an eventual, but acceptable, decrease in effectiveness, whereas overuse or misuse would lead to an inappropriate or unacceptable loss of effectiveness and the society is adversely affected. There are many challenging tasks
in susceptibility testing. Susceptibility breakpoints vary from one region of the world to another. In North America, most fluoroquinolones are typically dosed 250 to 750mg one to two times daily, while in Japan these drugs are typically dosed 100 to 200mg two to three times daily. This difference in dosing would suggest a higher North American susceptibility break point compared to that in Japan. Such regional differences in susceptibility breakpoints can affect comparisons between countries.

2.4.1. Issues of antimicrobial resistance in urinary tract infections

Urinary tract infection (UTI) is a very common infection both in the community and hospital patients and ranks high amongst the most common reasons that compel a patient to seek medical attention (Gastmeier et al., 1998; Mobley, 2000; Hassan et al., 2007).

![Percentage of occurrence of different hospital acquired infections in humans](NAO, 2004)

UTI is caused by the bacteria originating from the patient’s own faecal flora. *E.coli*, the dominant species in the faecal flora cause 80-90 % of urinary tract infections (Sabath and Charles 1980). Urinary tract infections are the second most common bacterial infection in children, and are important in view of their acute morbidity and the long term risk of renal scarring often present with few signs or symptoms other than fever (Bagga, 2002).Urinary tract infection is the most common and frequent cause of illness in humans bacterial infections, which includes cystitis, pyelonephritis, asymptomatic bacteriuria, and acute urethra syndrome (Badaruddin and Memon 2007). Gram negative UTI strains were more prevalent with ESBL (Behroozi et al., 2010).
Based on the microbial sensitivity test results, drugs that are usually administered against uropathogens include cotrimoxazole, amoxicillin, ampicillin, aminoglycosides, cephalosporins, nalidixic acid and nitrofurantoin. However, many reports have indicated the presence of multi-drug resistance in organisms causing UTI (Yuksel et al., 2006 and Yildiz et al., 2007). Javad (2009) stated that the *E. coli* showed high resistance to Ampicillin (61.9 %), Nalidixic acid (43.6 %) respectively. Maragakis and Perl, 2008, reported the emergence of carbapenem-resistant *A. baumannii* in many parts of the world.

Reports of antimicrobial resistance recorded in urinary tract isolates were as follows:

**SENTRY** Antimicrobial Surveillance Program analysed 887 urinary tract infection (UTI) isolates from 20 European hospitals and reported that ninety percent of the referred species were *Escherichia coli* (52%), *Enterococcus* spp. (12%), *Klebsiella* spp. (7%), *Proteus* spp. (7%), *Pseudomonas aeruginosa* (7%), and *Enterobacter* spp. (5%). The susceptibility of *E. coli* isolates to penicillins was less than 60%, while almost all of the isolates were susceptible to piperacillin/tazobactam (98% susceptibility), cephalosporins (98%), and carbapenems (100%). Amikacin was the best aminoglycoside (99.8% susceptibility). The susceptibility to quinolones was only 88–89%. The susceptibility of *Klebsiella* spp. to the newer generations of cephalosporins was 82–95% and to the carbapenems was 100%. Amikacin was again the best aminoglycoside (94% susceptibility). The susceptibility of *Enterobacter* spp. to any β-lactam antibiotic was poor, except for the carbapenems (100% susceptibility) and cefepime (90% susceptibility), while the susceptibility to aminoglycosides was 80–89%. *Proteus* spp. showed complete susceptibility to cefepime, ceftaroloxone, the carbapenems, and piperacillin/tazobactam, while the susceptibility of *P. aeruginosa* isolates was poor, with best results for the carbapenems (susceptibility 89%), piperacillin/tazobactam (susceptibility 84%), and amikacin and ticarcillin (susceptibility to both 80%) *Enterococcus* spp. showed the highest susceptibility to vancomycin (98%), teicoplanin (98%), and ampicillin (94%) (Fluit et al., 2000).

UTI was considered as a common risk factor for resistance to the different antibiotics tested, and steps should be formulated for a better strategy that can be used to overcome antibiotic resistance. Among the antibiotics tested, the highest rates of resistance were found for AMZ (48.1%), Ticarcillin (46.9%), Piperacillin (40.6%), SXT (26.9%), AMC (20.3%), Pipemidic acid (12.9%), and FQs (5.3%) (Sotto et al., 2001). *Proteus mirabilis* NEL-1
isolated from a urine sample of a 93-year-old woman resident at the long-term care facility of the Hospital at France produced a β-lactamase with a pI of 5.2 conferring resistance to amoxicillin, amoxicillin-clavulanic acid, tetracycline, nitrofurantoin, and colistin and susceptible to most antibiotics tested (gentamicin, tobramycin, netilmicin, amikacin, Chloramphenicol, nalidixic acid, pefloxacin, ciprofloxacin, sulfamides, and trimethoprim) (Naas et al., 2003).

The ESBLs producing MDR uropathogen E. coli and Klebsiella species reported in different countries (Khanfar et al., 2009) were as follows: 40.9% in Egypt (Bouchillon et al., 2002); 20.8% in Nigeria (Ibukun et al., 2003); 71.5% (Mohanty et al., 2003) and 48.3% (Supriya et al., 2004) in India; 40.3% in Saudi Arabia (Kadar and Angamuthu, 2005); 42% (Mohammed et al., 2007); 53% in Sudan (Mekki et al., 2010).

Falagas et al., (2005) suggested the usage of colistin for Multidrug resistant gram negative bacilli. Among gram negative bacteria, Pseudomonas aeruginosa was most resistant isolates against tested antibiotics. Among gram positive cocci, Enterococcus fecalis isolates showed highest resistance followed by Staphylococcus isolates. Among β-lactam antibiotics, imipenem had the widest coverage against E. coli isolates. E. coli resistance to ampicillin peaked in preteens (76.4%) but was high in teens (65.7%), toddlers (53.4%), and infants (47.6%). Resistance to cotrimoxazole peaked in teens (68.3%) but was high in preteens (59.1%), infants (49.4%) and toddlers (47.6%). Klebsiella isolates also showed high susceptibility against imipenem (Mashouf et al., 2009). Nasehi et al. (2010) reported that all clinical isolates in his study were susceptible to imipenem in disk diffusion test. The MICs of ceftazidime in ESBLs producing isolates ranged from 4 to > 512µg/ml, 53% of which showed MICs ≥ 128 µg/ml. The ESBL-producing isolates were recovered mostly from urine (n= 46), sputum (n= 15) and wound (n= 8) specimens. Ciprofloxacin, Cefazolin and Cefuroxime may be considered as alternative therapies for MDR urinary isolates (Eryilmazi, et al., 2010). Osazuwa, et al., 2011 reported the nature of inconsistence in the susceptibility patterns of ESBL isolated from different samples (urine, stools etc.).

Treatment of bacterial urinary tract infections are often encountered with the problem of antimicrobial resistance. To combat these problems, epidemiological studies should be undertaken in hospital settings to monitor the source of infection. Proper antibiotic policy and measures to restrict the indiscriminative use of cephalosporins and carbapenems should be
taken to minimize the emergence of this multiple β-lactamase producing pathogens (Upadhyay et al., 2010).

2.5. Antimicrobial surveillance studies

The surveillance of antimicrobial resistance has as its goal in the gathering of information for several purposes at every level where health care is provided. No country has a reliable, longitudinal, full-service antimicrobial resistance surveillance program with comprehensive focus, nor is there a comprehensive database for monitoring trends in antimicrobial usage. Thousands of clinical and basic research laboratories throughout the world generate resistance data. But very few labs submit these data to appropriate databases that could allow local analysis or linking with a surveillance network (Obrien et al., 2001).

The effectiveness of surveillance data can be enhanced by integrating with other types of information. For instance, molecular studies of resistance can help to observe resistance phenotypes. Comparison with data on antibiotic usage allows estimation of and potential for the management of antibiotic selection. Surveillance of resistance can and should build on existing resources. Clinical laboratories in more than eighty countries have begun to build databases and link them into international networks using free software (WHONET) downloadable from a World Health Organization Web site (Obrien et al., 2001).

Surveillance data come essentially from three sources:

1. Active surveillance,
2. Passive surveillance involving reference laboratories, and
3. Outbreak investigations.

2.5.1. Types of AMR surveillance

Three types of surveillance can be done for AMR

- Comprehensive surveillance - gives actual estimate of AMR burden, includes the study of the whole population at risk / under study and needs the involvement of a large number of laboratories which is not practical, especially in our country.
- Point prevalence studies - useful for validation of the representativeness of the surveillance data.
- Sentinel surveillance studies -suitable mode of surveillance when prolonged and detailed data are needed. This seems to be the best approach for our country.
2.5.2. Local surveillance

Local level surveillance forms the foundation for the national and international comprehension of antimicrobial resistance. These data highlight the importance of understanding ‘microtrends’ within larger patterns. Susceptibility testing should be performed in all local laboratories and hospitals that help to draw a conclusion for a particular antibiotic (Osterholm, 1998).

2.5.3. National systems

Surveillance should not be limited to the health-care sector. Mechanisms of resistance to any new antibiotic may already exist in nature, so any resistance encountered in nonpathogenic organisms in the environment or antibiotic producers should also be entered into the database. Clinicians and developers of diagnostics would then be aware of resistance mechanisms that may be encountered in the clinic. Though there are definite policies / standard treatment guidelines for appropriate use of antimicrobials in specific national health programmes e. g. RNTCP (Revised National Tuberculosis Control Programme), NACP (National AIDS Control Programme), NVBDCP (National Vector Borne Disease Control Programme), the same are not available for other diseases of public health importance like enteric fever, diarrhoea / dysentery, pneumonia, etc.

In this regard a task force has been constituted with the following terms of reference (Srivastava, 2011):

- To review the current situation regarding manufacture, use and misuse of antibiotics in the country.
- To recommend the design for creation of a national Surveillance System for antibiotic resistance.
- To initiate studies documenting prescriptions patterns and establish a monitoring system for the same.
- To enforce and enhance regulatory provisions for use of antibiotics in human & veterinary and industrial use.
- To recommend specific intervention measures such as rational use of antibiotics and antibiotic policies in hospitals.
Diagnostic methods pertaining to monitor antimicrobial resistance:

The Central Drugs Standard Control Organization (CDSCO), headed by the Drugs Controller General (India) in the Directorate General of Health Services, is concerned with the regulatory control over the quality of drugs, cosmetics and certain notified medical devices under the Drugs and Cosmetics Act, 1940 and rules made thereunder.

2.5.4. International systems

The surveillance network should not only be nationwide, but linked to international efforts to integrate worldwide data seamlessly. Multiple surveillance activities around the globe are attempting in different ways and at different speeds to move toward the ideal depicted in this report, but these systems, as a group, are uncoordinated and unstandardized. Thus, the magnitude of the resistance problem and its impact are really unknown and may be considerably understated.

SENTRY is the first collaborative, worldwide, longitudinal antimicrobial surveillance program to provide timely data on both community and hospital acquired infections with standard methodology. This project was launched in February 1997 in four regions; currently, there are 38 program sites in North America, 27 sites in 13 European countries, 10 sites in 7 South American countries, and 3 sites in Turkey. Japan, Australia, and countries in Asia and Africa are slated to join in 1998. CEM/NET (Centre for Epidemiologic molecules / Network for epidemiologic tracking of antibiotic resistance pathogens) is an independent international alliance between clinical microbiologists and molecular biologists (Kristonsson, 1998).

2.6. Approach to new antimicrobials

There are several indications that new approaches are required to combat emerging infections and the global spread of drug-resistant bacterial pathogens.

One is the pattern in rates of death from infectious disease in the 20th century: from 1900 to 1980, the rate dropped from 797 per 100,000 people to 36 per 100,000 people, a reduction by a factor of more than 20 and a testament in part to the efficacy of antibiotics (Armstrong et al., 1999).

A second indication of the need for novel antibacterial therapeutics is the almost 40-year innovation gap between introductions of new molecular classes of antibiotics:
fluoroquinolones in 1962 and the oxazolidinone linezolid in 2000 (Walsh, 2003a,b). A third indication is the recent trend by several large pharmaceutical companies to leave the antibacterial and anti-fungal therapeutic arenas, suggesting a future decrease in scientific expertise in antibacterial-drug discovery and development skills (Projan, 2003, Shlaes 2003). It is very hard to displace the establishing resistance and newer resistance continues to emerge and proliferate at new sites. Consequently there remains strong need for new antibiotics particularly towards Multidrug resistance in gram negative isolates (Vashishtha, 2010).

Among fifth generation of cephalosporin antibiotics, two compounds were introduced: ceftobiprole medocaril (EMA - 2008) and ceftaroline fosamil (FDA - 2010, EMA 2012). Two new compounds CXA-101 and S-649266 are at the stage of clinical trials. In April 2010, Calixa Therapeutics, Inc. completed phase II of clinical trials on safety and efficacy of the CXA-101 (ceftolozane, FR264205) in comparison to ceftazidime, among patients with complicated urinary tract infections (cUTI). The advantage of this compound is low tendency to induce resistance and increased stability to β-lactamase type AmpC (Karpiuk and Tyski, 2013). The combination of CXA-101 with β-lactamase inhibitor – tazobactam in a concentration of 8μg/mL was found to be active against Enterobacteriaceae producing ESBL. This combination called CXA-201 shows efficacy against more than 90% of the Enterobacteriaceae producing extended spectrum β-lactamase type CTX-M (Bush, 2012).

2.6.1. Need for new molecules

The development of new antimicrobial agents is urgent. However, only a few new agents have entered full clinical development; these include newer aminoglycosides, β-lactams, β-lactam inhibitors, and tetracycline derivatives with activity against enterobacteria (Bush, 2012). New antibiotics can help stave off the catastrophe. But since 1987, no major antibiotic has been discovered. The science of antibiotics is complex and research on antibiotics is expensive and time consuming. It’s not profitable for global big pharma as antibiotics are for short-term use. As the root of the crisis is a “patent cliff”, a term coined for the sheer number of major drugs coming off patent between 2010-2014. Patents protect the rights of original makers of a branded drug for 20 years to sell it exclusively. Once it expires, others can make and sell cheaper versions. Loss of avenues is the biggest challenge to R&D innovation (Datta, 2013).
2.6.2. Status for new antibiotics

Oxazolidinones was the only new antibiotic class joined in 1990s. All other introductions have been variants of existing classes. Only a few new antibacterial agents have received approval by the US Food and Drug Administration in the last 10 years, including linezolid in 2001, cefditoren pivoxil and ertapenem in 2002, gemifloxacin and daptomycin in 2003, telithromycin in 2004, and tigecycline in 2005 (Raghunath, 2008). Only a single new antibacterial doripenem has been approved in the USA since 2006. Many of these agents are improved derivatives from established classes of antibiotics, and several are directed primarily at resistant gram-positive bacteria (e.g., linezolid and daptomycin). These modified agents suffer from the disadvantages of the parent molecules. In view of the crossover of resistance across related compounds, the future may see sharply depleting antibiotic resources (Vashishtha, 2010).

New antibacterial agents introduced during the period of 1983-2012

![Chart showing the number of new antibacterial agents introduced each year from 1983 to 2012.]

2.6.3. New Therapeutic Approaches in Combating Antimicrobial Resistance

Understanding the substrate evolution, properties and modes of spread of these clinically important β-lactamases can help in formulating effective antibiotic policies and developing new antimicrobial agents. One strategy employed to overcome these resistance mechanisms is the use of combination of drugs, such as β-lactams together with β-lactamase inhibitors. Several plant extracts have exhibited synergistic activity against microorganisms. Synergy and mechanism of action between natural products including flavonoids and essential oils with synthetic drugs can be used in effectively combating bacterial infections.
The secondary metabolites from plant are good sources for combination therapy and there are a wide range of phytochemicals which act as Multidrug resistance modifiers. The mode of action of combination differs significantly than that of the same drugs acting individually; hence isolating a single component may lose its importance thereby simplifying the task of pharma industries (Hemaishwarya et al., 2008).

Plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being. Using plants as the inspiration for new drugs provides an infusion of novel compounds or substances for healing disease (Iwu et al., 1999). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties Braga et al. (2005) have reported pomegranate extract can enhance the in vitro activity of certain antibiotics against strains of MDR Staphylococcus aureus and other pathogens. With an increasing demand to new drugs, plants are being utilized (Nasim et al., 2010). Allium sativum (Garlic) is a perennial, erect, bulbous plant and belongs to family Liliaceae. The juice of bulb is used to treat skin diseases, ear ache and cough (Pathmanathan et al., 2010). Allium sativum can be used as an adjunct with antibiotics to combat the effect of antibiotic resistance. (Abouelfetouch and Moussa 2012). Oxygenated sulfur compound in Allium sativum is reported as an important antimicrobial compound (Shobana et al., 2009).

Allicin is a volatile molecule, which gives garlic its characteristic odour. It has been shown that it is synthesised by the oxygenation of alliin, its stable precursor, in the presence of an enzyme termed allinase, which is also present in garlic cloves. The transformation of alliin to allicin is extremely rapid, being completed in seconds. Garlic is odour free until it is crushed and studies have shown that alliin and allinase are stored in different compartments. The antimicrobial activity is, however, diminished upon boiling, which is attributed to its key component allicin, which is denatured at high temperature (Jabar and Mossawi 2007).

The ability of some chemical compounds (called MDR inhibitors or resistance modifying agents) to modify the resistance phenotype in bacteria by working synergistically with antibiotics in vitro has since been observed. The search for such compounds which can be combined with antibiotics in the treatment of drug resistant infections may be an alternative to overcoming the problem of resistance in bacteria. Crude extracts of medicinal
plants stand out as veritable sources of potential resistance modifying agents (Sibanda and Okoh 2007). Garlic can be used as an adjunct with antibiotics for the treatment of infections caused by multidrug resistant isolates (Abouelfetouch and Moussa 2012).

2.7. Conclusion

Antibiotic resistance if unnoticed may result in excess mortality, morbidity (e.g., length of hospital stay and complications) at attribute costs (e.g., costs to the hospital, patient and society). The risk of such adverse outcomes has been found to be higher in patients with infections caused by an antibiotic-resistant organism compared with infections caused by susceptible strains of the same pathogen, even after adjustment for underlying comorbidities (Cosgrove et al., 2005 and Engemann et al., 2003). Complete knowledge about the specific mechanisms of resistance may lead to the identification of novel targets for new antimicrobial drug development. A better understanding of the relative importance of selective pressure related to antibiotic use compared to cross-infection as mechanisms for emergence and spread of antimicrobial resistance would also be important to design and evaluate effective infection prevention and control strategies (Mulvey and Simor, 2009).