Antibiotics resistant have become a worldwide problem with serious consequences on the treatment of infectious diseases. The primarily contributing to the phenomenon is heightened use/misuse of antibiotics in human medicine, agriculture and veterinary. Multidrug resistances (MDR) in bacteria that cause either community infections or hospital acquired infections are continuously increasing. Of particular importance are the MDR pathogens, e.g. *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, penicillin-resistant *Streptococcus pneumoniae*, and extensively drug-resistant *Mycobacterium tuberculosis* (Alekshun and Levy, 2007).

The most common treatment for bacterial infections exhibited by beta-lactam antimicrobial agents and it also persist to be the major source of resistance to these antibiotics among Gram-negative pathogens worldwide. Continuous production and mutation of β-lactamases have emerging due to persistent exposure of bacterial strains to a multitude of β-lactams, leading to expanding their activity even against the newly developed β-lactam antibiotics. These enzymes are known as extended-spectrum β-lactamases (ESBLs) (Pitout and Laupland, 2008; Paterson and Bonomo, 2005). Treatment of these MDR organisms is a due scientific concern. Recently, a significant increase in the incidents of ESBL-related infections has been observed throughout the globe (Rupinder et al., 2013; Abhijit et al., 2013; Majda et al., 2013; Meeta et al., 2013; Kritu et al., 2013; Fatemeh et al., 2012; Gupta, 2007).

Accurate detection of ESBL types helps in selection of appropriate antibiotics, institution of antibiotic usage policies as well as in tracking trends and evolution of beta-lactamases. Estimation of prevalence and distribution of ESBL producers helps in determining the extent of ESBL mediated drug resistance. Detection and identification
of ESBL types by molecular techniques is important in understanding their epidemiology. Global incidence of ESBL production among *Enterobacteriaceae* members is on the rise ever since their discovery. In the absence of regional or national level surveillance programs, accurate prevalence rates of ESBL producing *E. coli* or *K. pneumoniae* is currently unavailable. Isolated reports that vary in research methodologies can only provide sketchy data.

There are several published reports on the detection of ESBLs among *Enterobacteriaceae* from India but not many among them have characterized ESBL genes by molecular biological techniques. Phenotypic detection of ESBLs is not complete as these methods suffer from false positive and false negative results. Therefore, it becomes essential to undertake molecular techniques in the detection of ESBLs. Although detection of beta-lactamase genes by PCR is a sensitive method of detection, it may not be entirely specific as some of them may not code for the ESBL phenotype at all. Hence, PCR amplification of the beta-lactamase gene must be followed by sequencing the entire gene to accurately identify the ESBL type.

1.1. **How do antibiotics work?**

Five major modes of antibiotic mechanisms of activity have been determined and here are some examples.

1.1.1. **Interference with cell wall synthesis**

Beta-lactams antibiotics like penicillin, cephalosporin impedes enzymes that are responsible for the formation of peptidoglycan layer (Benton et al., 2007).
1.1.2. Inhibition of protein synthesis

The oxazolidinones, is a newest classes of antibiotics, interact with the A site of the bacterial ribosome where they should interfere with the placement of the aminoacyl-tRNA. Tetracyclines interfere with synthesis of protein by binding to 30S subunit of ribosome, thereby deteriorating the ribosome-tRNA interaction. Macrolides bind to the 50S ribosomal subunit and inhibit the elongation of nascent polypeptide chains. By binding to the 50S ribosomal subunit chloramphenicol block the peptidyl transferase reaction. Aminoglycosides inhibit initiation of protein synthesis and bind to the 30S ribosomal subunit (Leach et al., 2007).

1.1.3. Interference with nucleic acid synthesis

Rifampicin interferes with a DNA-directed RNA polymerase. Quinolones interference with type II topoisomerase, DNA gyrase and type IV topoisomerase during replication cycle causing double strand break and inhibit DNA synthesis (Strohl, 1997).

1.1.4. Inhibition of a metabolic pathway

The inhibitory effect of sulfonamides (e.g. sulfamethoxazole) and trimethoprim is exerted by blocking the key steps in folate synthesis, which is a cofactor in the biosynthesis of nucleotides, the building blocks of DNA and RNA (Strohl, 1997).

1.1.5. Disorganizing of the cell membrane

The primary site of action is the cytoplasmic membrane of Gram-positive bacteria, or the inner membrane of Gram-negative bacteria. It is hypothesized that the inhibitory effect of polymyxins exert by increasing bacterial membrane permeability, causing leakage of bacterial content. By binding to the cytoplasmic membrane in a
calcium-dependent manner and oligomerizing in the membrane, the cyclic lipopeptide daptomycin displays rapid bactericidal activity leading to an efflux of potassium from the bacterial cell and cell death (Straus et al., 2006). Antibiotic resistant versus antimicrobial activity mechanism has been shown in figure 1.1.

**Figure 1.1. Antibiotic resistance vs. antimicrobial activity mechanism**

1.2. Antibiotic resistance mechanism

Antibiotic resistance is the reduction in effectiveness of a drug such as an antineoplastic or an antimicrobial in therapeutic a condition or disease. When the antibiotic is not intended to kill or inhibit a pathogen, then the term is equivalent to dosage failure or drug tolerance. When an organism is resistant to more than one drug,
it is said to be multidrug-resistant (Fisher et al., 2010). Bacterial strains may possess different types of resistant mechanisms which are shown in figure 1.2 and are explained following:

![Antibiotic resistance mechanism diagram](image)

**Figure 1.2. Biochemical and genetic aspects of antibiotic resistance mechanisms**

### 1.2.1. Antibiotic inactivation

#### 1.2.2. By hydrolysis

Many antibiotics have chemical bonds like esters and amides that are hydrolytically susceptible. Various enzymes are known to damage antibiotic activity by targeting and cleaving these bonds. These enzymes can often be excreted. ESBLs mediate resistance to all penicillins, third generation cephalosporins (e.g. cefazidime, cefotaxime, ceftriaxone) and aztreonam, but not cephemycins (cefoxitin and cefotetan) and carbapenems (Bonnet et al., 2004).
1.2.3. By redox process

Infrequently exploited oxidation or reduction of antibiotics has been carried out pathogenic bacteria. Though, there are little examples of this strategy (Yang et al., 2004). One is the oxidation of tetracycline antibiotics by the TetX enzyme. *Streptomyces virginiae*, producer of the type A streptogramin antibiotic virginiamycin M1, defend itself from its own antibiotic by condensing a vital ketone group to an alcohol at position 16.

1.2.4. Antibiotic inactivation by group transfer

The utmost varied family of resistant enzymes is the group of transferases. They inactivate antibiotics (aminoglycosides, chloramphenicol, streptogramin, macrolides or rifampicin) by substitution of chemical (adenylyl, phosphoryl or acetyl groups are added to the periphery of the antibiotic molecule). The modified antibiotics are damaged in their binding to a target. Chemical strategies include O-acetylation and N-acetylation (Blanchard et al., 2004, Schwarz et al., 2004), O-phosphorylation (Matsuoka et al., 2004), O-nucleotidylation (Brisson-Noel et al., 1988), O-ribosylation, O-glycosylation, and thiol transfer. These covalent change strategies all require a co-substrate such as ATP, acetyl-CoA, NAD⁺, UDP-glucose, or glutathione for their activity and consequently these processes are restricted to the cytoplasm.

1.2.5. Antibiotic resistance via target modification

Modification of the antibiotic target site so that the antibiotic is impotent to bind properly is the second major resistance mechanism. Though, it is likely for mutational changes to take place in the target that decrease vulnerability to inhibition while retaining cellular function (Spratt et al., 1994).
1.3. Genetics of Antibiotic Resistance

1.3.1. Antibiotic resistance via mutations

The biochemical mechanisms of antibiotic resistance that based on mutational events, like the mutation in the genes encoding the target of certain antibiotics (e.g. resistance to rifampicin and fluoroquinolones are caused by mutations in the genes encoding the targets of these molecules, RpoB and DNA-topoisomerases, respectively) (Ruiz et al., 2003). Mutation (e.g. the reduced expression or absence of the OprD porin of *P. aeruginosa* reduces the cell wall permeability to carbapenems) also modified the variation in the expression of antibiotic uptake or of the efflux systems (Wolter et al., 2004).

1.3.2. Antibiotic resistance via horizontal gene transfer

The horizontal transfer of genetic material is a principal mechanism for the spread of antibiotic resistance. Different mechanisms like conjugation, transformation or transduction are responsible for transfer of antibiotic resistance genes. Beta-lactamase enzymes that have an extended spectrum of activity (ESBL) against the majority of β-lactams, including cephalosporins but not carbapenemases, have evolved over the last 15 years. One of these, CTX-M-15, originally found in *E. coli* but now found in other members of *Enterobacteriaceae* and commonly connected with a specific lineage, uropathogenic clone ST131 (Bush and fisher, 2011; Woodford, 2011), has spread worldwide. It is often located on highly mobile IncFII plasmids and associated with mobile genetic element IS26. Individuals in association with prolonged catheterization, hospitalization, nursing home residency, previous antibiotic treatment, underlying renal
or liver pathology, and travel to high-risk areas; the risk of infection is particularly high in such individuals (Nordmann et al., 2011).

1.4. ESBL definition and classification

There is no accord of the exact definition of ESBLs. They are a group of enzymes that break down antimicrobial drugs belonging to the cephalosporin and penicillin groups and leave them ineffective. ESBL has commonly been defined as transmissible β-lactamases that can be inhibited by clavulanic acid, tazobactam or sulbactam. CTX-M is the most common genetic variant of ESBL (Paterson and Bonomo 2005; Walsh, 2003).

The Ambler molecular classification and the Bush-Jacoby-Medeiros functional classification are two general schemes for classification beta-lactamases (Bush and Jacoby, 1995; Ambler, 1980). According to the protein homology of enzymes the Ambler scheme classifies β-lactamases into four classes. Beta-lactamases of class A, C, and D are serine β-lactamase while class B enzymes are metallo-β-lactamases. On the other hand, the Bush-Jacoby-Medeiros functional scheme is based on functional properties of enzymes, i.e the inhibitor and substrate profiles.

1.4.1. SHV type

This family of β-lactamases appears to be consequent from *Klebsiella spp.* SHV-1, progenitor of the SHV class of enzymes, is universally found in *K. pneumoniae*. In many *K. pneumoniae* strains, the gene encoding SHV-1, or its apparent precursor, LEN-1, resides within the bacterial chromosome too; it may be that the gene for SHV-1 β-lactamase evolved as a chromosomal gene in *Klebsiella* and was later incorporated into a plasmid which has spread to other *enterobacteria* species.
SHV-1 show resistance to broad-spectrum penicillins such as tigecycline, ampicillin and piperacillin but not to the oxyimino substituted cephalosporins (Livermore, 1995). Up to, 20% of the plasmid-mediated ampicillin resistance in *K. pneumoniae* species has been associated with SHV-1 β-lactamase (Tzouvelekis, 1999).

### 1.4.2. TEM type

TEM-1 has substrate and inhibition profiles similar to those of SHV-1 and first reported from an *E. coli* isolate in 1965 (Datta, 1965). This enzyme is able of hydrolyzing first generation cephalosporins and penicillins but is incapable to bother the oxyimino cephalosporin. TEM-3 was the first TEM variant with better activity against extended spectrum cephalosporins (Soughakoff et al., 1988; Sirot et al., 1987). TEM-2 had a single amino acid substitution from the original β-lactamase, was the first derivative of TEM-1 (Barthelemy et al., 1985). Due to this, a shift in the isoelectric point from a pI of 5.4 to 5.6, but it did not alter the profile of substrate. In 1989, TEM-3 has originally reported. It was the first TEM-type β-lactamase that exhibited the ESBL phenotype. In retrospect, TEM-3 may not have been the first TEM-type ESBL. *Klebsiella oxytoca* was first isolated in Liverpool, England, in 1982 harboring a plasmid carrying a gene encoding ceftazidime resistance (Du Bois et al., 1995). Interestingly, the strain came from a neonatal unit which had been stricken by an outbreak of *K. oxytoca* producing TEM-1 (Du Bois et al., 1995).

### 1.4.3. CTX type

CTX type is new family of β-lactamases that preferentially hydrolyzes cefotaxime. *Salmonella enterica* serovar, *Typhimurium, E. coli* mainly and some other species of *Enterobacteriaceae* harbor CTX type beta-lactamases (Gazouli et al., 1998;
These enzymes are not very strongly related to SHV or TEM beta-lactamases (Tzouvelekis et al., 2000). The distinctive characteristic of these enzymes is that they are better inhibited by the β-lactamase inhibitor tazobactam than by sulbactam and clavulanate (Bradford et al., 1998; Ma et al., 1998).

Thought to originate from chromosomal ESBL genes found in *Kluyvera* spp., an opportunistic pathogen of the *Enterobacteriaceae* found in the environment, CTX-M β-lactamases are found entirely in functional group 2 (Bush and Jacoby, 2010). In the late 1980’s, the first CTX-M proteins were discovered and today more than 100 variants have been sequenced (Bonnet, 2004). They can be divided in five groups based on their amino acid sequences (CTX-M group 1, 2, 8, 9, and 25) (Bonnet, 2004).

The origin of the CTX-M enzymes is unlike in comparison to SHV and TEM ESBLs. The amino acid substitutions of parent enzymes generated the SHV-ESBLs and TEM-ESBLs, while CTX-M ESBLs were acquired by the horizontal gene transfer from other bacteria using genetic apparatuses like transposon or conjugative plasmid. Beta-lactamases of *Kluyvera* species show a high similarity to gene sequences encoding CTX-M enzymes. Additionally, the gene sequences neighboring to the CTX-M genes of *Enterobacteriaceae* are also comparable to those adjacent the β-lactamase genes on the chromosomes of *Kluyvera* species (Olson et al., 2005; Humeniuk et al., 2002; Poirel et al., 2002). It has been shown by kinetic studies that the CTX-M-type β-lactamases hydrolyze cephaloridine or cephalothin better than benzyl penicillin and they preferred hydrolyze cefotaxime over ceftazidime (Tzouvelekis et al., 2000; Bradford et al., 1998).

Serine residue at position 237, plays an important role in the extended-spectrum activity of the CTX-M-type β-lactamases and it is present in all of the CTX-M enzymes (Tzouvelekis et al., 2000). The crystallographic data for the Toho-1 enzyme suggested
that there was increased elasticity of the interacting β 3 strands and ω loop of this enzyme in association to other class A β-lactamases. Additionally, the lack of hydrogen bonds in the vicinity of the ω loop could account for the extended-spectrum phenotype (Ibuka et al., 1999).

1.4.4. OXA type

Because of their oxacillin-hydrolyzing abilities the OXA-type β-lactamases are so named. These β-lactamases are characterized by hydrolysis rates for cloxacillin and oxacillin greater than 50% that for benzyl penicillin (Bush et al., 1995). They mostly found in *P. aeruginosa* (Weldhagen et al., 2003) and been reported in many other Gram-negative bacteria. OXA-1 has been found in 1 to 10% of *E. coli* isolates is the most common OXA-type β-lactamase (Livermore, 1995). They were originally revealed in *P. aeruginosa* isolates from a single hospital in Ankara, Turkey. A new derivative of OXA-10 (numbered OXA-28) was reported in a *P. aeruginosa* isolate in France (Poirel et al., 2001). Further, a novel ESBL (OXA-18) and an extended-spectrum derivative of the narrow spectrum OXA-13 β-lactamase (numbered OXA-19) have also been found in *P. aeruginosa* isolates in France (Philippon et al., 1997). There are very little epidemiologic data on the geographical increase of OXA-type ESBLs (Philippon et al., 1997).

1.4.5. PER type

The PER-type ESBLs share only about 25 to 27% homology with known TEM and SHV-type ESBLs. This beta-lactamase proficiently hydrolyzes cephalosporins and penicillins and is inhibited by clavulanic acid. PER-1 ESBL was first reported in *P. aeruginosa* (Neuhauser et al., 2003), and later in *Salmonella enterica* serovar
Typhimurium and Acinetobacter isolates as well (Vahaboglu et al., 2001). 46% of nosocomial isolates of Acinetobacter spp. and 11% of P. aeruginosa were found to produce PER-1 in Turkey (Vahaboglu et al., 1997). PER-2 has been reported in S. enterica serovar Typhimurium, K. pneumoniae, E. coli, Proteus mirabilis and Vibrio cholerae O1 El Tor and it shares 86% homology to PER-1 (Petroni et al., 2002).

1.4.6. GES type

GES-1 was originally reported in a K. pneumoniae isolate from a neonatal patient just transferred to France from French Guiana (Poirel et al., 2000). GES-1 has hydrolytic activity against penicillins and extended-spectrum cephalosporins, but not against carbapenems or cephemycins. GES-1 ESBL is inhibited by β-lactamase inhibitors. The enzymatic properties similar to those of other class A ESBLs; therefore, GES-1 was known as a member of ESBLs.

1.4.7. VEB-1, BES-1, and Other ESBLs type

Other remarkable enzymes having ESBL have also been reported (e.g. BES-1, CME-1, VE-B-1, PER, SFO-1, GES-1) (Bradford, 2001). These novel enzymes are found uncommonly, details of these enzymes are reviewed elsewhere (Naas et al., 2008).

1.5. Detection

Examination of pathogens having ESBLs provides clinicians with supportive information. Cure of infections or disease caused by ESBL-producing pathogens with extended-spectrum aztreonam or cephalosporins may effect in treatment failure even when the causative pathogen show to be susceptible to these antimicrobial drugs by usual susceptibility testing (Paterson and Bonomo, 2005; Paterson et al., 2001).
Further, in order to avoid hospital transmission, patients colonized or infected with ESBL-producing pathogen should be placed under contact precautions (Siegel et al., 2006). These benefits summons the detection of ESBL-producing organisms in clinical laboratories. Additionally, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has revised cephalosporin breakpoints and is under way by the Clinical and Laboratory Standards Institute (CLSI) for better prediction of clinical outcome by MIC values (Kahlmeter, 2008). This revision might allow clinical laboratories to dispense with ESBL detection is still controversial (Paterson and Bonomo, 2005; Kahlmeter, 2008).

1.5.1. Phenotypic detection

The guidelines for ESBL screening in Enterobacteriaceae family particularly for \textit{E. coli}, \textit{Klebsiella spp} and \textit{Proteus spp} has been published by the US Clinical and Laboratory Standards Institute (CLSI) and the UK Health Protection Agency (HPA) (CLSI, 2002; HPA, 2008). Other species, such as \textit{Salmonella spp} has also been included in the HPA guidelines. These guidelines worked on the principle that the majority of ESBLs hydrolyze third-generation cephalosporins while they are inhibited by clavulanate, and propose preliminary screening with either 8 mg/L (CLSI) or 1 mg/L (HPA) of cefpodoxime, 1 mg/L each of cefotaxime, ceftazidime, ceftriaxone, or aztreonam, followed by confirmatory tests (including the E-test ESBL strips) with both cefotaxime and ceftazidime in combination with clavulanate at a concentration of 4 \(\mu g/mL\). In North America and certain European countries automated systems that use similar detection principles have proved to be popular in clinical laboratories (Spanu et al., 2006). The CLSI and HPA published methods confirm high sensitivity of up to 94% and specificity of 98% for detecting ESBLs in \textit{E. coli}, \textit{Klebsiella spp} and \textit{Proteus}
spp, if clinical laboratories adhere to the published guidelines for detecting ESBLs (Wiegand et al., 2007).

1.5.2. Genotypic detection

Point mutations around the active sites of the SHV and TEM enzyme sequences have lead to amino acid changes that enhance the spectrum of activity of the parent enzymes, such as in TEM1, TEM2, and SHV1, therefore, determination of whether a specific ESBL present in a clinical isolate related to TEM and SHV enzymes is a complicated process (Bradford, 2001). PCR is the commonly used molecular method for amplification of the bla<sub>TEM</sub> and bla<sub>SHV</sub> genes with oligonucleotide primers, followed by sequencing. Sequencing is crucial to distinguish between the non-ESBL parent enzymes (e.g. TEM1, TEM2, or SHV1) and diverse variants of TEM or SHV enzymes (e.g. TEM3, SHV2, etc) (Bradford, 2001).

The PCR amplification of CTX-M-specific products without sequencing, in an isolate that produces an ESBL, generally provides adequate indication that a bla<sub>CTX-M</sub> gene is accountable for this phenotype. This is distinct TEM and SHV types of ESBLs. Various molecular approaches for the fast screening of ESBL-positive pathogens for the presence of diverse bla<sub>CTX-M</sub> genes have been described by many studies. This included amplification of a universal DNA fragment specific for most of the different groups of CTX-M β-lactamases (Batchelor et al., 2005), a PCR assay that used four sets of primers to amplify group specific CTX-M β-lactamase genes (Pitout et al., 2004), real-time PCR (Birkett et al., 2007), duplex PCR (Pitout et al., 2007), multiplex PCR (Woodford et al., 2006), pyrosequencing (Naas et al., 2007) and reverse-line hybridization (Ensor et al., 2007). Molecular techniques play a crucial part in the laboratory setting for the screening, tracking, and monitoring of the spread of large
numbers of pathogens harboring CTX-M enzymes from the hospital settings and community in real time.

1.6. Epidemiology

The epidemiology of ESBLs is relatively complex. Originally, there are certain unusual levels to consider that is the wider geographical area, the country, the hospital, the community, and the host (in most cases a single patient or a healthy carrier). Furthermore, the mobile genetic elements of bacteria, usually plasmids and the bacteria itself (E. coli is more endemic, and K. pneumoniae is more epidemic). The environment (e.g. soil and water), wild animals, farm animals and pets are various reservoirs responsible for dissemination of antibiotic resistant. The final factor entails spread from water and food and also through direct or indirect contact (person to person) (Carattoli, 2008). In Germany in 1983, the first ESBL was found, but initial nosocomial outbreaks occurred in France in 1985 and in the United States at the end of the 1980s and the beginning of the 1990s (Rice and Willey, 1990). In France in the early 1990s, many of the K. pneumoniae isolates that are responsible for nosocomial infections were ESBL producers (Sirot et al., 1987).

In Sweden from an international facet, the use of antibiotics, particularly broad-spectrum agents, was narrow (Cars et al., 2001). Clinical laboratories are required to account all cases linking ESBL-producing Enterobacteriaceae strains to the Swedish Institute for Communicable Disease Control and the number of such cases increased by 100% from 2008 to 2011, since February 2007 (SIIDC, 2012). There have also been larger nosocomial outbreaks of clonally 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 Kristiansand caused by a multi resistant CTX-M-15-producing
E. coli strain (Alsterlund et al., 2009). 2.6% of E. coli and 1.7% of K. pneumoniae strains in Sweden were resistant to third-generation cephalosporins in 2010 according to data from the European Antimicrobial Resistance Surveillance System (EARSS) (EARSS, 2011).

In Europe, new TEM and SHV ESBLs are still rising, and distinct epidemic clones have been observed, for instance in Spain Salmonella isolates with TEM-52 (Fernandez et al., 2006) and E. coli and K. pneumoniae isolates with SHV-12 in Italy (Perilli et al., 2011). In Spain, pathogens with the CTX-M-9 group are common and strains with the CTX-M-3 enzymes have been reported predominantly in Eastern Europe, although clones producing CTX-M group 1 (including the CTX-M-15 type) are the most prevalent throughout Europe (Coque et al., 2008; Canton et al., 2008; Coque et al., 2008). Today, E. coli and the CTX-M enzymes are not infrequent in outpatients.

In addition, K. pneumoniae resistance has reached a higher level with emergence of carbapenemases such as OXA-48, which was first found in Turkey (Aktas et al., 2008).

Another study conducted at a tertiary hospital, among the overall ESBL producing isolates, 35% of being community origin and 65% from hospitals in Nigeria. The ESBL isolates exhibited high resistance to array of antibiotics tested such as tetracycline, gentamicin, pefloxacin, ceftriaxone, cefuroxime, ciprofloxacin and Augmentin (Amoxicillin and clavulanic acid combination). Conjugation studies exhibited non-transference of resistance determinants between the ESBL transconjugants and recipient isolates. Likewise, the plasmid curing studies exposed that the acridine orange could not distress a cure on the pathogens as they still retained high resistance to the antibiotics after the treatment (Ruth et al., 2011).
A study conducted at National Public health laboratory (NPHL), Kathmandu Nepal reported 31.57% *E. coli* were confirmed as Extended Spectrum β-lactamase producer, these isolates further exhibited co-resistance to several antibiotics (Thakur et al., 2013).

Another research conducted at a tertiary hospital in Mwanza, Tanzania, the overall prevalence of ESBLs in all Gram-negative bacteria (377 clinical isolates) was 29%. The occurrence of ESBL was 64% in *K. pneumoniae* while 24% in *E. coli* (Mshana et al., 2009). In a small study at an orphanage in Mali, dramatic figures were also obtained where 63% of the adults and 100% of the children were found to harbor ESBL-producing *Enterobacteriaceae* that exhibited extensive co-resistance to other antibiotics tested (Tande et al., 2009). Moreover, in Madagascar, Herindrainy et al. (2011) reported that 10% of non-hospitalized patients harbor ESBLs, CTX-M-15 in majority of the cases, and these studies also determined that significant risk factor for carriage was poverty. Fatemeh et al. found 26.5% *E. coli* and 43% *K. pneumoniae* were ESBL positive in their investigation conducted at Imam Reza hospital of Mashhad, IR Iran. They indicated the increase occurrence of ESBL producing *Enterobacteriaceae* particularly in inpatients (Fatemeh et al., 2012).

In the countries of the Middle East the overall data on ESBL-producing *Enterobacteriaceae* are extremely worrying. Study conducted in that country exhibited that 61% of *E. coli* produced ESBLs of the CTX-M-14, CTX-M 15, and CTX-M 27 types, and all of strains carried the TEM enzyme (Al-Agamy et al., 2006). Tawfik and colleagues found that 26% of *K. pneumoniae* isolates produced ESBLs, the majority of which were SHV-12 and TEM-1 enzymes, and 36% were CTX-M-15 in Saudi Arabia (Tawfik et al., 2011). Another research conducted in the same country in 2004–2005
reported that 10% of clinical urinary *E. coli* isolates from inpatients and 4% of such isolates from outpatients were ESBL producers (Khanfar et al., 2009). In Lebanon in 2003, Moubareck and colleagues analyzed faecal samples and noted that ESBL carriage differed somewhat between patients (16%), healthcare workers (3%), and healthy subjects (2%) and also that there was a prevalence of the CTX-M-15 enzyme (83%) (Moubareck et al., 2005). Further, Khanfar and co-workers reported that the proportion of ESBL-producing pathogens was remarkably high among inpatients (15.4%) than in outpatients (4.5%) in Lebanon (Khanfar et al., 2009). In contrast to this, the levels of ESBLs were lower in community isolates of *E. coli* (12%) and *K. pneumoniae* (17%) than in the corresponding hospital pathogens (26% and 28%, respectively) as revealed by data collected over three years in Kuwait (Al Benwan et al., 2010).

Extent of the ecological disaster related to ESBL-producing *Enterobacteriaceae* in parts of Asia and the Indian subcontinent, have also determined and the number of reports of very high frequency of such bacteria in those regions continues to emerge. It has been determined that some of the successful ESBL-producing clones originate from Asia. Probable major factors that have promoted the development of resistance are deficient sewage routines and drinking water of poor quality, in combination with a need of control over prescription and sales of antibiotics. The population of Asia to be 4.2 billion in 2012, has estimated by the United Nations and hence it is a very challenging task to try to stop the emerging resistance to antibiotics stemming from this part of the world as exemplified by the rapid spread of the carbapenemase NDM-1 (Kumarasamy et al., 2010). In China and Japan, a few articles published as early as the end of the 1980s and the beginning of the 1990s have reported occurrence of the SHV-2 and Toho-1 (CTX-M-44) enzymes (Hawkey, 2008). There is rapid occurrence of ESBL-producing *K. pneumoniae* (up to 60%) and *E. coli* (13–35%) in different parts
of China, with a prevalence of the CTX-M-3 and CTX-M-14 enzymes (Hawkey, 2008; Hirakata et al., 2005) according to the SENTRY surveillance program. It has been determined that 66% of third generation cephalosporin resistant to *E. coli* and *K. pneumoniae* from three medical centers in India carried the CTX-M-15 type of ESBL, which was also the only CTX-M enzyme reported (Ensor et al., 2006), and study of 10 other centers in that country exhibited that rates of ESBL-producing *Enterobacteriaceae* reached 70% (Mathai et al., 2002). Recently, in Patiala, Punjab ESBL production was observed in 44% of *K. pneumoniae*, 48% of *E. coli* and 50% of *P. aeruginosa* pathogens in a tertiary hospital (Rupinder et al., 2013). In other recent studies, authors observed ESBL rates of 46% and 50% in out- and inpatients, respectively (Sankar et al., 2012), and Nasa and co-workers detected ESBL production in almost 80% of clinical isolates (Nasa et al., 2012). Studies from India and Pakistan exhibited an alarming and rapid increase in the occurrence of *Enterobacteriaceae* with NDM-1 with incidence rate from 6.9% in a hospital in Varanasi, India, to 18.5% in Rawalpindi, Pakistan (Perry et al., 2011) and possibly the spread of these enzyme could be even more rapid than the spread of the CTX-M enzymes.

Majda et al. reported 72% *E. coli* and 65.8% *K. pneumoniae* were ESBL producer at Microbiology laboratory of Shalamar Medical College, Lahore. Antibiotics susceptibility testing exhibited multidrug resistance in ESBL producing *K. pneumoniae* and *E. coli*. Highest resistance reported in *E. coli* (ESBL) was as cefotaxime (98.9%), Ceftazidime (96.7%) and Cefuroxime (93.4%) while minimum resistance was observed with Imipenem (0.8%), fosfomycin (1.2%) and Nitrofurantoin as well piperacillin/tazobactam (2.2%) each. The ESBL producing *Klebsiella* exhibited highest resistance to ceftazidime (100%), cefotaxime (89%), Cefuroxime (84%) while least
resistance reported with imipenem (4%) Nitrofurantoin and Piperacillin / Tazobactam (8%) (Majda et al., 2013).

Recently, Shakti and co-workers find 12.11% ESBL positive among ICU and NICU isolates and 22.47% ESBL positive from nosocomial pathogens. They further statically established that ESBL pathogens were equally disseminated in hospital units or community. Drug susceptibility testing of 23 antibiotics exposed progressive increased of drug resistant against each antibiotic with the highest resistant values were found against gentamycin: 92% and 79%, oxacillin: 94% and 69%, ceftriazone:85% and 58%, and Norfloxacin 97% and 69% resistance, in nosocomial and community pathogens, respectively (Shakti et al., 2014).

Though there are a little differences between countries, the maximum occurrence of ESBL-producing *K. pneumoniae* in the world is seen primarily in Latin America, data from 33 centers in Latin America collected over the period 2004–2007 within the Tigecycline Evaluation and Surveillance Trial (TEST) exhibited ESBLs in 36.7% of *K. pneumoniae* isolates and in 20.8% of 932 *E. coli* isolates (Rossi et al., 2008).

A huge diversity of unusual types of SHV has also been reported. In Calgary, Canada one wide epidemic of *Enterobacteriaceae* producing the CTX-M-14 enzyme reported (Pitout et al., 2005). In other study performed in 2001, it was established that about 5.3% of the *E. coli* in the United States carried ESBLs (Winokur et al., 2001), and an examination conducted in 2009 exhibited that 9% of *E. coli* isolates at a cancer centre in Texas were ESBL producers (Bhusal et al., 2011). Sanchez and co-workers examined data obtained from The Surveillance Network (TSN) relating to *in vitro* antimicrobial resistance in US outpatients between 2000 and 2010, and their results exhibited that resistance to ceftriaxone rose from 0.2% to 2.3% and resistance to
cephuroxime increased from 1.5% to 5%, but the bacterial pathogens in focus were not examine for ESBLs (Sanchez et al., 2012). The epidemiology of ESBLs producing organism has been shown in table1.1.
### Table 1.1. Epidemiology of ESBL producing organisms

<table>
<thead>
<tr>
<th>S.No.</th>
<th>ESBL producing organisms</th>
<th>Country/City</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>E. coli, K. pneumoniae</em></td>
<td>Sweden</td>
<td>Alsterlund et al., 2009</td>
</tr>
<tr>
<td>2.</td>
<td><em>Salmonella spp.</em></td>
<td>Spain</td>
<td>Fernandez et al., 2006</td>
</tr>
<tr>
<td>3.</td>
<td><em>E. coli, K. pneumoniae</em></td>
<td>Italy</td>
<td>Perilli et al., 2011</td>
</tr>
<tr>
<td>4.</td>
<td><em>K. pneumoniae</em></td>
<td>Turkey</td>
<td>Aktas et al., 2008</td>
</tr>
<tr>
<td>5.</td>
<td><em>Enterobacteriaceae, P. aeruginosa</em></td>
<td>Nigeria</td>
<td>Ruth et al., 2011</td>
</tr>
<tr>
<td>6.</td>
<td><em>E. coli</em></td>
<td>Nepal</td>
<td>Thakur et al., 2013</td>
</tr>
<tr>
<td>7.</td>
<td><em>E. coli, K. pneumoniae</em></td>
<td>Tanzania</td>
<td>Mshana et al., 2009</td>
</tr>
<tr>
<td>8.</td>
<td><em>E. coli, K. pneumoniae</em></td>
<td>Iran</td>
<td>Fatemeh et al., 2012</td>
</tr>
<tr>
<td>9.</td>
<td><em>E. coli</em></td>
<td>Middle East</td>
<td>Al-Agamy et al., 2006</td>
</tr>
<tr>
<td>10.</td>
<td><em>E. coli</em></td>
<td>Saudi Arabia</td>
<td>Tawfik et al., 2011</td>
</tr>
<tr>
<td>11.</td>
<td><em>K. pneumoniae</em></td>
<td>Saudi Arabia</td>
<td>Khanfar et al., 2009</td>
</tr>
<tr>
<td>12.</td>
<td><em>E. coli, K. pneumoniae</em></td>
<td>Lebanon</td>
<td>Moubareck et al., 2005</td>
</tr>
<tr>
<td>13.</td>
<td><em>E. coli, K. pneumoniae</em></td>
<td>Kuwait</td>
<td>Al Benwan et al., 2010</td>
</tr>
<tr>
<td>14.</td>
<td><em>E. coli, K. pneumoniae</em></td>
<td>China</td>
<td>Hawkey, 2008; Hirakata et al., 2005</td>
</tr>
<tr>
<td>15.</td>
<td><em>E. coli, K. pneumoniae</em></td>
<td>India</td>
<td>Ensor et al., 2006; Nasa et al., 2012</td>
</tr>
<tr>
<td>16.</td>
<td><em>E. coli, K. pneumoniae</em></td>
<td>Punjab</td>
<td>Rupinder et al., 2013</td>
</tr>
<tr>
<td>17.</td>
<td><em>E. coli</em></td>
<td>Odisha</td>
<td>Shakti et al., 2014</td>
</tr>
<tr>
<td>18.</td>
<td><em>Enterobacteriaceae</em></td>
<td>Pakistan</td>
<td>Perry et al., 2011</td>
</tr>
<tr>
<td>19.</td>
<td><em>E. coli, K. pneumoniae</em></td>
<td>Lahore</td>
<td>Majda et al., 2013</td>
</tr>
<tr>
<td>20.</td>
<td><em>E. coli, K. pneumoniae</em></td>
<td>Latin America</td>
<td>Rossi et al., 2008</td>
</tr>
<tr>
<td>21.</td>
<td><em>Enterobacteriaceae</em></td>
<td>Canada</td>
<td>Pitout et al., 2005</td>
</tr>
<tr>
<td>22.</td>
<td><em>E. coli</em></td>
<td>United state</td>
<td>Winokur et al., 2001; Sanchez et al., 2012</td>
</tr>
</tbody>
</table>
1.7. Aims and objective

This study has attempted to address the problem of “Multidrug Resistance” in bacterial pathogens and identified the best plausible combination of β-lactam antibiotic/β-lactamase inhibitor) for the patients infected by CTX-M-producing bacteria. In addition to clinical microbiology studies, a detailed investigation concerning the molecular interactions occurring between each of the ligands (selected for this study) with CTX-M beta lactamase enzymes as potent inhibitors were also explored by *in silico* docking simulations.

The specific goals of the present study were:

- To PCR amplify ESBL gene \( \text{bla}_{\text{CTX-M}} \) from bacteria isolated from clinical samples obtained from North Indian hospitals (Lucknow region).
- Homology modeling of enzyme-structures.
- Docking of drugs and inhibitors to the enzyme models.
- To suggest potent drug/inhibitor combinations against ESBL gene \( \text{bla}_{\text{CTX-M}} \) producing bacteria on the basis of relevant bioinformatics parameters and a set of microbiological test.

1.8. Significance

Multidrug resistance and production of ESBLs by enteric gram negative rods in hospitals and the community continue to be a problem of due scientific concern. The immediate benefit of the present study is that they are needed to update the empirical antibiotic regimen in a given region and would be useful for the development of drug-resistant antibiotics in the future. Additionally, the present study has found the most suitable antimicrobial therapy for CTX-M-producing pathogens which are an emerging threat to the global healthcare system with special reference to pathogens prevalent in North India. The study identified the best possible “beta lactam
antibiotic/beta lactamase-inhibitor” combination which would be able to treat infections by most of the CTX-M-producing bacterial pathogens prevalent in this setting. It is worth mentioning that “using molecular modeling and docking techniques to address clinical microbiology issues” such as multidrug resistance, was a relatively new approach in the present setting.