Clinical Significance and Molecular Characterization of Extended Spectrum β-Lactamases (ESBLs)-Producing *Escherichia coli* Associated with Urinary Tract Infection in Bangladesh

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Degree of Philosophy

Submitted by

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Certificate

I hereby certify that the research work embodying the results reported in this thesis entitled: "Clinical Significance and Molecular Characterization of Extended Spectrum β-Lactamases (ESBLs)-Producing *Escherichia coli* Associated with Urinary Tract Infection in Bangladesh", submitted by Belal Ahmed Ibne Mahmood, bearing Registration No. 23, Session 2006-2007 for submission in the form of thesis, which has been carried out under our guidance for the degree of Doctor of Philosophy (PhD) in the Department of Microbiology, University of Dhaka.

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Declaration

I do hereby declare that the work submitted as a thesis entitled: "Clinical Significance and Molecular Characterization of Extended Spectrum β-Lactamases (ESBLs)-Producing *Escherichia coli* Associated with Urinary Tract Infection in Bangladesh" to the Department of Microbiology, University of Dhaka for the degree of Doctor of Philosophy (PhD) by the results of my own investigations and carried out under supervision of Dr. Donald James Gomes, Professor, Department of Microbiology, University of Dhaka. In addition, the experiments in the laboratory were carried out by me under the co-supervision of Dr. Kaisar Ali Talukdar, Senior Scientist, Laboratory Sciences Division (LSD), International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Mohakhali, Dhaka 1212, Bangladesh. The research work has not previously been submitted for any degree.

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Abbreviations

ATCC : American Type Culture Collection
bp : Base pair
CA : Clavulanic acid
CDC : Center for Disease Control and Prevention
cfu : Colony forming unit
DDD : Double disc-diffusion
DDST : Double disc synergy test
DNA : Deoxyribonucleic acid
dNTP : Deoxynucloside triphosphate
EDTA : Ethylenediaminetetraacetic acid
ESBL : Extended-spectrum β-lactamase
E-test : Epsilometer
ICDDR,B : International Center for Diarrhoeal Disease Research, Bangladesh
kb : Kilobase
kDa : Kilo Dalton
mA : Milliampere
MDa : Mega Dalton
PAGE : Polyacrylamide gel electrophoresis
PCR : Polymerase chain reaction
PFGE : Pulsed-filed gel electrophoresis
rpm : Rotation per minute
SDA : Sabouraud dextrose agar
SDS : Sodium dodecyl sulfate
YE : Yeast extract

Technical Terms

bla$_{CTX-M}$: Genes producing CTX-M type β-lactamase
bla$_{OXA}$: Genes producing OXA type β-lactamase
bla$_{SHV}$: Genes producing SHV type β-lactamase
bla$_{TEM}$: Genes producing TEM type β-lactamase
CTX-M : Cefotaxime M
eae : Gene for attaching and effacing phenotype
e$_{Agg}$: Gene for aggregative phenotype
gyrA : Gene for A subunit of DNA gyrase
gyrB : Gene for B subunit of DNA gyrase
ial : Gene for invasion associated locus
int1 : Gene for class 1 integron
int2 : gene for class 2 integron
ipaH : gene for invasive plasmid antigen
lt : gene for heat labile toxin
OXA : Oxacillinase
SHV : Sulphydryl variable
st : Gene for heat soluble toxin
stx1 : Gene for shiga toxin 1
stx2 : Gene for shiga toxin 1
TEM : Temoniera
*Xba*I : DNA restriction enzyme from *Xanthomonas badrii*

Abstract

Patients having urinary tract infections (UTIs) with multidrug resistance bacteria suffer from various complications. This is due to β-lactamase-production by certain group of bacteria. This study was undertaken to explore the molecular mechanisms of drug resistance caused by ESBL-producing *Escherichia coli* isolated from UTI patients in the Sylhet community of Bangladesh. A total of 100 urine samples from symptomatic UTI patients were used in this study. Twenty three isolates were found to be *Klebsiella*, which were discarded. Remaining 77 isolates were identified as *Escherichia coli* by standard methods in the laboratory. Of these isolates, 25 were further confirmed as ESBL-producing *Escherichia coli* by double disc-diffusion synergy test and these 25 were used in the present study. The patients' age ranged from 4 to 60 years. The highest age incidence of UTI patients was 21-30 years (28%), followed by 31-40 years (20%), 50 years and above (19%) and 41-50 years (15%).

The antibiotic susceptibility test for non-ESBL-producing organisms showed 100% sensitivity to imipenem (IMP) and meropenem (MPM). There were multidrug resistance (MDR) to amoxyclavulanic acid (AMC, 30%), ceftazidime (CAZ, 40%), ceftriaxone (CRO, 35%) and ciprofloxacin (CIP, 60%). Some isolates were sensitive to AMC (70%), CAZ (60%), CRO (65%) and CIP (40%). The ESBL-producing isolates were also 100% sensitive to carbepenems and some were resistant to AMC (76%), CAZ (55%), CRO (66%) and CIP (73%). Serogrouping showed that all ESBL isolates were heterogeneous and did not match with non-ESBL isolates. Nine ESBL isolates could not be typed and they were designated as untypeable.

Plasmid profile analysis was done both for ESBL and non-ESBL producing isolates. The plasmid profile showed both ESBL and non-ESBL exhibits high molecular weight plasmids (>140 MDa), which were 68 and 75% respectively. These findings suggest that both ESBL and non-ESBL-producing isolates harbor large size plasmid, indicating that the isolates carry transmissible plasmids and they are responsible for multidrug resistance (MDR) in Sylhet community. The investigations were further carried out about the detection of genes encoding ESBL types by polymerase chain reactions (PCRs). The ESBL positive strains showed $bla_{\text{CTX-M-1}}$ gene (60%), $bla_{\text{OXA-1}}$ gene (41%) and 45% isolates constituted $bla_{\text{TEM-1}}$ gene. PCRs were also done in all

these 20 ESBL isolates for virulent genes. Only 2 isolates showed pilus-associated pylonephritis (*pap*) genes and one isolate showed afrimbial adhesion (*afa*) gene. Further data analysis revealed that out of 20, 12 (60%) isolates were having single gene, 7 (35%) isolates had double genes and 1 (5%) isolate had triple gene. Interestingly, it was seen that isolates having single gene (60%) were harboring 140 MDa plasmids. These large-sized plasmids are transmissible in the community of Sylhet District. These results suggest that multidrug resistance (MDR) is probably due to TEM-1 gene harboring 140 MDa plasmids in the community. Pulsed-field gel electrophoresis results showed no clonal relatedness among the ESBL isolates. Same findings were found in non-ESBL-producing isolates.

In conclusion, the ESBL uropathogenic *E. coli* isolates were responsible for multidrug resistance (MDR) in the community of Sylhet Town. They also harbor large sized transmissible plasmids and these plasmids are encoded by genes such as TEM-1, OXA-1 and CTX-M-1. It is CTX-M-1 that constitutes the highest percentage (60%) in the district of Sylhet in Bangladesh.

Chapter 1 Introduction

1 Introduction

1.1 Historical Background and Review of Literature

The emergence and spread of antibiotics resistance is a foremost world wide problem especially in developing countries. Antibiotic resistance is one of the major causes of failure in the treatment of infectious diseases that results in increased morbidity, mortality and costs of health care. Understanding the molecular mechanism by which resistance genes are acquired or transmitted, may contribute to the creation of new antimicrobial strategies as well as some preventive measure to stop further spreading of resistance determinants among the pathogens.

Microbes undergo mutation of genes which can spread from cell to cell by mobile genetic elements such as plasmids, transposons and bacteriophages. Resistant bacteria flourish in areas of heavy antibiotic use such as hospitals and intensive care unit (ICU). Hospital outbreaks of multi-drug resistant Enterobacterioceae are now frequently caused by extended-spectrum beta-lactamase (ESBL) producers (Thomson, 2001). Incidence of ESBL producing strains among clinical isolates have been steadly increasing over past years resulting in limitation of therapeutic options. Recently, the use of second and third generation cephalosporins has led to the selection of Gram-negative organisms resistant β-lactamase stable cephalosporins. This resistance is attributed to the production of extended spectrum β-lactamases (Coque *et al*., 2002; Rahman *et al*., 2004a).

Ideally, surveillance of antimicrobial resistance should involve the collection of both clinical and microbiological data. By establishing surveillance systems that integrate clinical and laboratory data, not only can the necessary data be captured but also the strengths of both data sets can be combined. The continuing emergence of pathogenic microorganisms that are resistant to multi drug resistance (MDR) including extended expectrum β-lactamases (ESBLs) is a cause of increasing concern (Ho *et al*., 2000; Essack *et al*., 2001; Gniadkowski, 2001). Although the mechanisms by which organisms acquire resistance are often well understood, including the selective pressures arising from exposure to antimicrobials, the precise role of drug usage in selection of drug resistance has yet to be fully elucidated. Describing the distribution of infection due to resistant organisms within population together with the changes in

pattern of those infections over time, provides the basic information for action both to control disease caused by resistant microorganisms and to contain the emergence of resistance. Used in conjunction with disease prevention and infection control procedures and data on antibiotic usage, strategies can be developed to protect the public health now and in the future. Indiscriminate use of antimicrobial by healthcare provider or by way of self-prescribing and over-the counter availability are major risk factor for the development of high levels of antimicrobial resistance, which is common in rural Bangladesh and other developing country. These factors, coupled with the overcrowding, poor hygienic practices prevalent in rural people of low socio-economic status and an increasingly mobile population might have contributed to facilitate the dissemination of antibiotic resistance determinations among the pathogens.

ESBL producers are very common in urinary tract infections (UTIs). UTI is a common community-acquired bacterial disease, which frequently affects female outpatients (Dromingy *et al*., 2005). *Escherichia coli*, the most common member of the family Enterobacteriaceae, accounts for 75-90% of all urinary tract infections in both inpatients and outpatients (Gupta *et al*., 2002). Increasingly, rates of resistance among bacterial uropathogen have caused growing concern in both developed and developing countries (Gupta *et al*., 2002). It has been estimated the symptomatic UTIs results in as many as seven million visits from outpatient clinics, one million visits to emergency departments and 100,000 hospitalized annually (Schappert, 1999). It has been reported that annual cost to health care system of the United States attributable to community-acquired UTI alone is estimated to be approximately US$ 1.6 billion (Wilson *et al*., 2004). ESBL production was detected in 9.3% of *E. coli* and 23.0% of *Klebsiella pneumoniae* in Korea (Ryoo *et al.*, 2005). Among 259 isolates, 9 (124 urine isolates, 120 isolates from pus and 15 isolates from blood) belonging to the family Enterobacteriaceae, 31 (12%) were reported as ESBL producers (Gangoué-Piéboji *et al.*, 2005) in Cameroon. In other studies, *E. coli* and *K. pneumonae* isolates were reported as ESBL-producers in France (Eckert *et al.*, 2004; 2006). Although there is no systemic report on ESBL-producer in Bangladesh, however a study was done on prevalence of ESBL-producing *E. coli* and *K. pneumonae* that were isolated from an urban hospital at Dhaka, Bangladesh in which 43.2% *E. coli* and 39.5% *K. pneumonae* were ESBL-producers (Rahman *et al*., 2004a).

1.2 Urinary Tract Infection (UTI)

1.2.1 Definition

The urinary tract infection can be defined as the presence of actively multiplying organisms within the urinary tract and growth of at least $>10^5$ colony forming unit per ml from mid stream clean 'catch urine'. Urinary tract infections (UTI) are a serious health problem affecting millions of people each year. Urinary tract infection usually occurs when bacteria enter the opening of the urethra and multiply in the urinary tract (Figure 1.1). This is the second most common type of infection in the body. Women are especially prone to UTIs for reasons that are not yet well understood. One woman in five develops a UTI during her lifetime. UTIs in men are not as common as in women but can be very serious when they do occur. Children also develop UTI. Antibiotics are the typical treatment for urinary tract infections (Calbo *et al*., 2006).

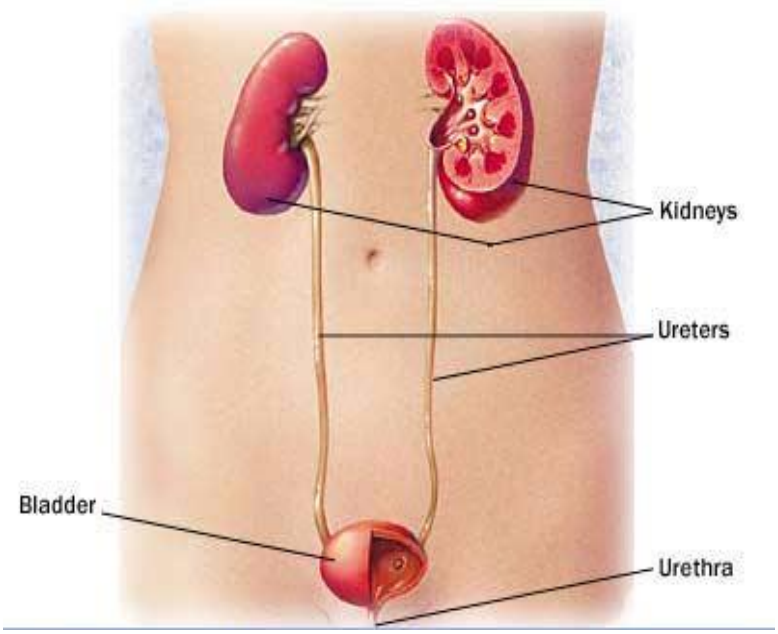

Figure 1.1: Anatomic features of urinary tract system.

1.1.2 Classification of UTI

UTI is classified in different ways both for clinical as well as scientific purpose. (a) Uncomplicated UTI: when UTI occurs in anatomically and functionally normal urinary tract. (b) Complicated UTI: Infections occurring with anatomic or urological abnormalities, neurogenic or obstructive lesions. Vesicoureteral reflux (VUR) recurrent catheterization, uretercele, diverticula, neurogenic bladder dysfunction etc.

UTI can also be classified according to region of the urinary tract involved and presence or absence of symptoms. (a) Symptomatic or asymptomatic: This group includes acute cystitis acute pyelonephritis unspecified UTI's and convert's or asymptomatic bacteriurias. (b) According to the regions involved. Upper UTI's, this includes pyelitis, pyelonephritis and ureteriitis. Lower UTI's includes cystitis and urethritis.

The clinical complications such cystitis, acute pyelonephritis, urethritis and catheter-associated urinary tract infections have the general clinical presentation as stated below:

1. Frequency of maturation,
2. Dysuria,
3. Suprapubic tenderness,
4. Fever with chill and rigor (≥39.4ºC), and
5. Loin pain, nausea, vomiting, diarrhoea and pyuria.

1.2.3 Etiology of UTI

Many different microorganisms are involved in the urinary tract infections (UTIs), but far most common agents are Gram-negative bacilli. *Escherichia coli* accounts for 80% of acute infections in patients of uncomplicated UTI. Other Gram-negative rods are especially *Proteus* and *Klebsiella* and occasionally *Enterobacter*, accounts for smaller proportion in uncomplicated infections.

Serratia and *Pseudomonas* assume increasing importance in recurrent infections and in infections associated with urologic manipulation, renal calculi or obstruction. They play a major role in nosocomial, catheter-associated infections (Nicolle, 2005). Gram-positive cocci have a lesser role in UTIs. However, *Staphylococcus saprophyticus* accounts for 10-15% of acute symptomatic urinary tract infections in young female. Enterococci and *Staphylococcus aureus* cause infections in patients with renal stone.

1.2.4 Pathogenesis of UTI

The following are the factors responsible for pathogenesis:

1. *Gender and sexual activity:* Female urethra appears to be particularly prone to colonization with colonic Gram-negative bacilli other reasons are close proximity

to the anus, its short urethra, and to its termination beneath the labia. Sexual intercourse can cause the introduction of bacteria into the bladder and may lead to cystitis.

2. *Pregnancy:* About 2-8% are seen in pregnant women.
3. *Obstruction:* Any obstruction such as tumor, stricture, stone or prostate hypertrophy, results in hydronephrosis.
4. *Neurogenic bladder dysfunction:* Spinal injury, tabes dorsalis, diabetes mellitus may interfere with nerve supply to bladder.
5. *Vesicoureteral reflux:* Defined as reflux of urine from bladder cavity up into the ureters and some time into the renal pelvis. It is common in children with anatomic abnormalities of urinary tract.
6. *Bacterial virulence factors:* Those strains that have uropathogenic properties will cause UTIs (Maheswari *at al*., 2013). Strains having serogroups like O, K and H and other factors such as pili-associated pylonephritis (*pap*), haemolysin (*hly*), aerobectin (siderophore scavenging iron, *aer*), cytotoxic necrotizing factor (*cnf*), etc.

The continuing emergence of pathogenic microorganisms that are resistant to multi drug resistance (MDR) including extended spectrum β-lactamases (ESBLs) is a cause of increasing concern. ESBL-producing organisms are frequently found in urinary tract infection (UTI). The most important ESBL is *Escherichia coli* causing global multi drug resistance.

1.3 *Escherichia coli*

1.3.1 The Species *Escherichia coli*

The strains *Escherichia coli* are Gram-negative bacilli and a member of the family Enterobacteriaceae (Choudhury, 1999). Most *E. coli* ferment lactose, reduce nitrates and are methyl-red positive. Approximately 10% of the species are late lactose fermenters; some of them are non-lactose fermenters (Thomas, 1988). *E. coli* produce indole, shows a negative Voges-Proskauer reaction and do not utilize citrate as the sole carbon source. Isolates of these genera usually do not produce extra cellular DNase, H_2S, phenylalanine deaminase, urease and do not grow in inositol containing media. Typical gas production from glucose, indole, lysine, arabinose, mannitol,

ortho-nitrophenyl galactoside (ONPG), trehalose and xylose is found in this genus (Edward and Ewing, 1972).

E. coli represent a wide cluster of biotypes. For many years, *E. coli* was considered to be the only species in the genus *Escherichia*. However, four additional species are now recognized (Farmer *et al.,* 1985). The other three species *E. fergusonii*, *E. vulneris*, and *E. hermannii* were isolated from human sources. However, these new *Escherichia* species are uncommon, and the species, *E. coli* accounts for more than 99% of the *Escherichia* isolates (Schaechter and Neidhardt, 1987).

E. coli is found habitually in the large intestine of vertebrates usually as a part of the normal flora. Strains of *E. coli* are widely distributed in nature, being present in soil and surface water and in animal and human faeces. As many as 10^9 cells of *E. coli* may be present per gram of faecal material.

E. coli has been widely used in research laboratories for nearly a century. The reasons for the popularity of this species in microbiological research are its easy accessibility, virulence in most cases and fast growing capacity. This organism grows readily at 37ºC on most of the laboratory media and produces large colonies upon overnight incubation. Much of our present knowledge on basic molecular mechanisms, DNA replication and regulation of gene expression is based on works done on *E. coli*. Moreover, many biological processes (*e.g*., genetic transformation) that frequently occur in bacterial species were not clear until these phenomena were artificially induced and studied in this bacterium.

1.3.2 The *Escherichia coli* Genome

The *E. coli* chromosome is very simple in structure consisting of a single covalently bonded circular molecule of DNA with a size of about 3,800 kilo base pairs. The *E. coli* chromosome was previously thought to be simply a naked molecule of DNA, but now it is known that there are associated proteins, which form complex with the DNA and maintain the chromosome in a highly condensed or compact state. Four specific nucleoid-associated proteins have been identified, and it is likely that these proteins maintain the nucleoid structure in the same way as specific histone and non-histone

proteins which are responsible for the structural organization of eukaryotic chromosome (Smith-Keary, 1988).

The length of *E. coli* chromosome is enough to harbour nearly 2,500 genes. So far fourteen hundred and three gene loci have been detected (Bachmann, 1990). All these genes code mainly for the products involved in metabolic pathways, structural components and various type of resistances. The unrevealed genes may control integrated cell physiology, *e.g*., responses to environmental changes, survival under stress and cell division etc. Studies in the past have shown that extraintestinal *E. coli* are responsible for 80-90% of all community-acquired urinary tract infections (UTIs). The environmental stress/changes can cause mutation of the genome. Recent studies in strain CFTO73 of uropathogenic *E. coli* contains 13 large genomic islands ranging in size from 32 kb to 123 kb (Figure 1.2). Three genomic islands mutants gene had been identified and they are specific for UPEC (Lloyd *et al*., 2007). Studies have also documented that UPEC strains contain a greater number of iron acquisition system than do faecal *E. coli* strains (Lloyd *et al.,* 2007). However, all the genes in *E. coli* may not be located in the chromosomal DNA; some of them may be located on plasmids and transposons or bacteriophages. The acquisition of plasmids and bacteriophages also plays an important role in generating genomic diversity (Lloyd *et al*., 2007).

Plasmids are extra-chromosomal, covalently closed, circular molecule and are generally dispensable to the cell. A transposons is a combination of a structural gene within a transposable element and is a discrete DNA segment (within a large DNA replicon), which can actually or effectively be translocated from one site to another site in the same replication or in a different replication in the cell. A restriction map of the entire *E. coli* chromosome is now available and hence chromosomal deletions can be performed in a way that indicates whether a region of the chromosome contains essential genes or not. It has been found that most of the chromosomally located genes are essentials and are stably maintained; whereas plasmid borne genes are usually not as stable as chromosomally located genes. They are prone to both vertical and horizontal propagation, whereas the chromosomal DNA propagate only vertically (*e.g*., parent cell to daughter cell). Plasmids are often responsible for the virulence of the bacteria that cause diseases in man, animals, fishes and plants (Hardy,

1986). Some coliphage DNA may also exit in the *E. coli* cell and may be maintained within the chromosomal DNA through lysogenic cycle. These DNA contain a number of genes, *e.g*., the phage 933 carry the Shiga like toxin-1 (SLT-1) gene and induce its production in enterohaemorrahgic *E. coli* strains by phage conversion (O'Brien *et al.,* 1984).

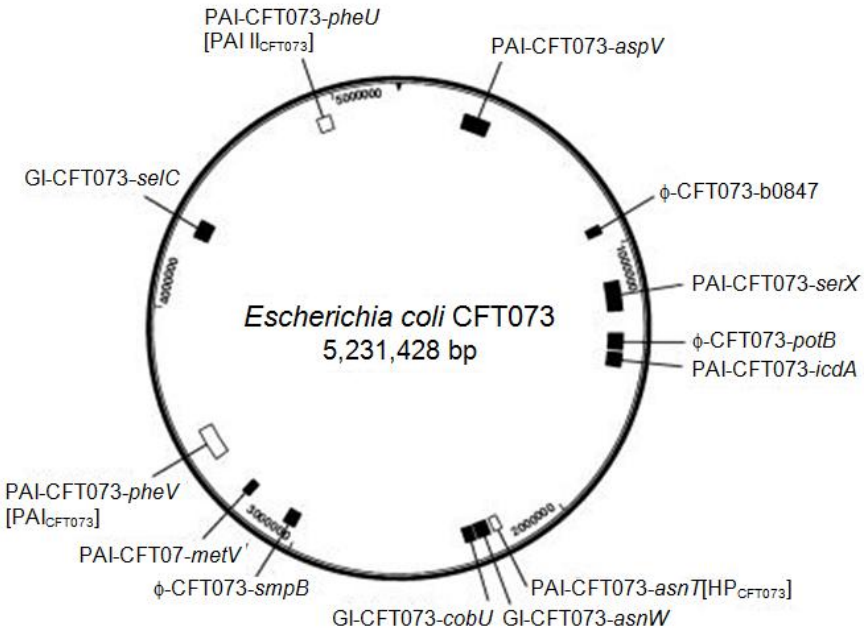

Figure 1.2: The genomic islands of uropathogenic *Escherichia coli*. Three of them are shown in white boxes. The 10 novel genomic islands were identified by comparative genomic hybridization analysis are shown in black boxes (Lloyd *et al*., 2007).

1.3.3 Classification and Natural History of *Escherichia*

E. coli and several little known related species, including Escherichia blattae, which was isolated from the hindgut of the oriental cockroach form 1 of 19 genera of the Gram-negative family enterobacteriaceae (Bergan, 1984). Isolates of *E. coli* and *Shigella* spp., including biochemically "atypical" strains formerly designated as the "Alkalescens-Dispar" group, show 70% or more DNA relatedness (percent relative binding) under conditions optimal for DNA reassociation (60ºC).

Genome size (including chromosomal and extra chromosomal DNA) in strains of *E. coli* varies from 2.3 x 10^9 to 3.0 x 10^9 Daltons, and guanine-plus-cytosine content ranges from 49% to 52%. The genera most closely allied with *Escherichia* are

Citrobacter and *Salmonella,* both of which show about 45% DNA relatedness (Brenner, 1984).

Most strains of *E. coli* are harmless commensal members of the intestinal flora of mammals and, to undetermined extent, birds, in which some strains adhere to the intestinal mucosa while other are only temporary transients in the lumen of the colon. *E. coli* is the major anaerobic of the large intestine, occurring in normal densities of about 10^6 cells per g of colon contents, but it is a major component of the total intestinal flora, which consists largely of obligate anaerobes and in the aggregate reaches 10^{11} cells per g of colon contents (Boyd *et al.,* 1994).

1.3.4 Common Features of *Escherichia coli* Virulence

The *Escherichia coli* are Gram-negative organisms and known as normal flora of the intestinal tract of human subjects. The continuing emergence of pathogenic microorganisms that are multidrug resistance (MDR) with manifestation of extended spectrum β-lactamases (ESBLs) is a cause of increasing concern. ESBL- producing *E. coli* are frequently found in urinary tract infection (UTI), wound infection and post surgical infections of haemorrhoids, infected anal fissures, fistulaes and abscesses.

Like most mucosal pathogen, *E. coli* also follows a requisite strategy of infections:

1. Colonization of mucosal site,
2. Evasion of host defences,
3. Multiplication of the organisms, and
4. Host damage.

The most highly conserved features of diarrhoeagenic *E. coli* strains is their ability to colonize the intestinal mucosal surface despite perstalsis and competition for nutrients by the indigenous flora of the gut (including other *E. coli* strains). The presence of surface adherence fimbriae is a property of virtually all *E. coli* strains including nonpathogenic varieties. However, diarrhoeagenic *E. coli* strains possess specific fimbrial antigens that enhance their intestinal colonizing ability and allow adherence to the small bowel mucosa, a site that is not normally colonized (Levine *et al.,* 1988). Once colonization is established, the pathogenic strategies of the diarrhoeagenic *E.*

coli strains exhibit remarkable varieties. Three general paradigms have been described by which *E. coli* may cause diarrhea:

1. Enteropathogenic *E. coli* (EPEC),
2. Enterotoxigenic *E. coli* (ETEC),
3. Enterohemorrhagic *E. coli* (EHEC),
4. Enteroinvasive *E. coli* (EIEC), and
5. Enteroaggregative *E. coli* (EAEC).

However, the interaction of the organisms with the intestinal mucosa is specific each category. The versatility of *E. coli* genome is conferred mainly by two genetic configurations virulence-related plasmid and chromosomal pathogenicity islands. All the six categories of diarrhoeagenic *E. coli* carry at least on virulence-related property upon a plasmid. The EIEC, EHEC, EAEC, and EPEC strains typically harbour highly conserved plasmid families, each encoding multiple virulence factors (Nataro *et al.,* 1987; Hales *et al.,* 1992). McDaniel and Kaper (1997) have showed recently that the chromosomal virulence genes of EPEC and EHEC are organised as a cluster, referred to as a pathogenicity island. Such island have been described for uropathogenic *E. coli* strains (Donnenberg and Welch, 1996) and systemic *E. coli* strains (Bloch and Rode, 1996) as well and may represent a common way in, which the genomes of pathogenic and nonpathogenic *E. coli* strains diverge genetically.

Plasmids and pathogenicity island carry cluster of virulence traits, yet individual traits may be transposons encoded such as Shiga toxin (So and McCarthy, 1980) or phage encoded (*e.g*., shiga toxin) (O'Brien *et al.,* 1984). The following are the virulence factors involved in the pathogenesis of urinary tract infections:

1. Haemolysin productions,
2. Mannose sensitive type-1 fimbriae,
3. Mannose resistant F-fimbriae,
4. Siderophore production,
5. Adhesions,
6. Iron uptake systems, and
7. Synthesis of cytotoxins production.

1.3.5 Serogrouping

According to the modified Kauffman scheme (Nataro *et al*., 1987), *E. coli* are serotyped on the basis of their O (somatic), H (flagellar) and K (capsular) surface antigen profiles (Edward and Ewing, 1972; Lior, 1996). A total of 170 different O antigens, each defining a serogroup are recognized currently. The presence of K antigens was determined originally by means of bacterial agglutination tests: An *E. coli* strain that was inagglutinable by O antiserum but became agglutinable when the culture heated, was considered to have a K antigen. A specific combination of O and H antigens defines the "serotype" of an isolate, *E. coli* of specific serogroups can be associated reproducibly with certain syndromes, but it is not in general the serologic antigens themselves that confer virulence. Rather, the serotypes and serogroups serve as readily identifiable chromosomal markers that correlate with specific virulence clones (Whittam *et al.*, 1993)

1.3.6 Plasmids

Many important bacterial genes are not part of main chromosome but are on separate circles of DNA called plasmids. Plasmids are exrachromosomal, autonomous DNA, *i.e.,* (*replicon*) found in many bacterial cells. It also has been found in lower eukarytes, *e.g*., the 2-μm plasmid of the yeast. The term 'plasmid' was proposed by Lederberg (1952) for all extrachromosomal genetic structures that can replicate autonomously. The adjective 'autonomous' here means self-governing and separate from the chromosome of the host rather than an entity capable of separate existence, in that plasmids are obligate 'endosymbionts' of bacteria using the replication enzymes of their host to ensure their own propagation, but retaining control over replication. Most plasmids whether from Gram-negative or Gram-positive bacteria are circular elements but linear plasmids have been reported in *Streptomyces* spp., and in *Rhodococcus* spp. (Hinnebush and Tilly**,** 1993).

Plasmids are characterized either by size, as measured in kilo bases (kb), or molecular weight, as measured in millions of Daltons or mega Daltons (MDa). Most strains of enterobacteriaceae have a molecular weight of between 2 x 10^9 and 3 x 10^9 Daltons, or between 6 x 10^3 and 9 x 10^3 kilo base (kb) pairs. Plasmids vary in size between 1.5 and 300 kb pairs (for conversion 1 MDa is equivalent to 1.51 kb). They are not indispensable except under certain circumstances. For example growth of bacteria in

presence of ampicillin, due to resistance plasmid encoding β-lactamase, it follows that the plasmid is indispensable in presence of the drug.The first plasmid to be identified was the *E. coli* fertility factor F, the causative factor of mediating transfer of chromosomal marker from one strain of *E. coli* to another (Bennett and Grinsted, 1984). But the importance of this discovery was realized in Japan in 1959 when Elwell *et al*. (1977) first demonstrate that multiple infectious drug resistance (MDR) in epidemic strains of *Shigella* in Japan was carried by these extrachomosomal elements.

1.3.6.1 Chemical and Physical Characteristics

Bacterial plasmids are molecules of double stranded DNA. No RNA bacterial plasmid has been found. All bacterial plasmids exist predominantly as covalently closed circular molecules in their host cell. Under the electron microscopes they appear as tightly coiled and branched structures (Sambrook *et al.*, 1989).

1.3.6.2 Classification of Plasmids

One way of grouping plasmids is by their ability to transfer to other bacteria. Conjugative plasmids contain *tra* genes, which perform the complex process of conjugation, the transfer of plasmids to another bacterium (Brown, 2010). Non-conjugative plasmids are incapable of initiating conjugation; hence they can be transferred only with the assistance of conjugative plasmids. Intermediate classes of plasmids are mobilizable, and carry only a subset of the genes required for transfer. They can parasitize a conjugative plasmid, transferring at high frequency only in its presence. Plasmids are now being used to manipulate DNA and may possibly be a tool for curing many diseases.

It is possible for plasmids of different types to coexist in a single cell. Several different plasmids have been found in *E. coli*. However, related plasmids are often incompatible, in the sense that only one of them survives in the cell line, due to the regulation of vital plasmid functions. Thus, plasmids can be assigned into incompatibility groups.

Another way to classify plasmids is by function (Brown, 2010). There are five main classes:

1. Fertility F-plasmids, which contain *tra* genes. They are capable of conjugation and result in the expression of sex pilli.
2. Resistance (R) plasmids, which contain genes that provide resistance against antibiotics or poisons. Historically known as R-factors, before the nature of plasmids was understood.
3. Col plasmids, which contain genes that code for bacteriocins, proteins that can kill other bacteria.
4. Degradative plasmids, which enable the digestion of unusual substances, *e.g*., toluene and salicylic acid.
5. Virulence plasmids, which turn the bacterium into a pathogen.

Plasmids can belong to more than one of these functional groups. Plasmids that exist only as one or a few copies in each bacterium are, upon cell division, in danger of being lost in one of the segregating bacteria. Such single-copy plasmids have systems that attempt to actively distribute a copy to both daughter cells. These systems, which include the parABS system and parMRC system, are often referred to as the partition system or partition function of a plasmid (Ebersbach and Gerdes, 2005).

1.3.6.3 Functions of Plasmids

The horizontal spread of plasmids among natural bacterial populations serves as an evolutionary function for adaptation to the ups and downs in nature (Tschäpe, 1994). Recent evolutionary challenges are the introduction of antibiotics and the creation of new environmental conditions in agriculture and medical care. As a consequence, surviving bacterial populations have acquired new genetic determinants which enable the colonisation and maintenance in distinct ecological niches. The functions of plasmid included:

1. Resistance properties namely to aminoglycosides, chloramphenicols, penicillins, sulphonamides and tetracyclines.
2. Resistance to heavy metals cations such as mercury and organo mercurials, nickels, cobalt, lead, antimony, silver etc.
3. Resistance to radiation and bacteriophages.
4. Metabolic properties namely antibiotic production, metabolism of carbohydrate, proteins etc., nitrogen fixations, citrate utilization, H_2S production, leucine biosynthesis etc.

5. They conjugal properties contributing to sex pili formation and fertility inhibition.
6. They have properties concerning replication and maintenance. Such as sensitivity to curing agents and range of hosts that can be harbored by the bacteria.
7. They have the properties for the production of ESBLs. It was estimated by agarose gel electrophoresis, plasmids determining extended spectrum ranged in size from 80 to 300 kb (Jacoby and Sutton, 1991).

1.4 The Pattern of Antimicrobial Resistance

Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. Antibiotic resistant bacteria can live and multiply in the presence of therapeutic levels of antibiotics. They can be resistant in their natural state or they can acquire resistant genes by mutation or from other bacteria. There is a large reservoir of resistant genes in bacterial genomes and in extra-chromosomal pieces of DNA that encode different mechanisms of drug resistance (Soulsby, 2005).

1.4.1 Causes of Microbial Resistance to Antibiotics

In the last 50 years bacteria have demonstrated a remarkable ability to develop and share resistance to every antibiotic often by quite unexpected mechanisms (Spratt, 1994). Antibiotic resistance is a consequence of evolution via natural selection or programmed evolution. The antibiotic action is an environmental pressure; those bacteria which have a mutation allowing them to survive, will live on to reproduce. They will then pass this trait to their offsprings, which will be a fully resistant generation (Soulsby, 2005).

Patterns of antibiotic usage greatly affect the number of resistant organisms that develop. Overuse of broad-spectrum antibiotics greatly hastens the development of resistance, even in organisms that have never been exposed to the selective pressure of these antibiotics. Other factors contributing towards resistance include incorrect diagnosis, unnecessary prescriptions improper use of antibiotics by patients and the use of antibiotics as livestock feed additives for growth promotion (Bouza and Cercenado, 2002).

1.4.2 Antibiotic Resistance Mechanism

The four main mechanisms by which microorganisms exhibit resistance to antimicrobials are (Soulsby, 2005).

1. *Drug inactivation or modification:* For example, enzymatic deactivation of penicillins G in some penicillin-resistant bacteria through the production of β-lactamases.
2. *Alteration of target site:* For example, (a) alterations in topoisomerase genes (*gyrA*, *gyrB* and *parC*), which are associated with quinolone and/or fluroquinolones resistance; (b) alteration of penicillin-binding protein (PBP)-the binding target site of penicillins-resistant bacteria − in MRSA (methicillin-resistant *Staphylococcus aureus*) and other penicillin-resistant bacteria.
3. *Alteration of metabolic pathways:* For example, some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA) − an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides. Instead, like mammalian cells, they turn to utilizing preformed folic acid.
4. *Reduced drug accumulation:* For example, by decreasing drug permeability and/or increasing active efflux.

1.4.3 Transfer of Drug Resistance Genes among Organisms

During the time of stress, commensals and pathogenic bacteria demonstrate the ability to become hypermutable and they duplicate and share survival information, such as resistance genes that can be encoded on plasmids, transposons and integrons (Shapiro**,** 1999). Spontaneous mutations that produce new resistance or strenghthen existing resistance occur readily in bacteria. Bacteria may acquire resistance genes through a number of routes. They may inherit the genes from their resistant forerunners. Alternatively they may acquire resistance genes through transfer via a virus or plasmid vector, or by free DNA uptake.

These four methods of gene transfer are described as transformation, transductions, conjugation and transposition. Transformation is the transfer of genes from donar cell to recipient cell by means of naked DNA. In this process DNA enters the recipient cell across the cell membrane.

In the process of conjugation, bacteria transfers DNA or plasmids by cell to cell contact. This contact is made by hair like projection called sex pili. Transductions refer to transfer of genes from one cell to another cell via a phage vector (virus) without cell to cell contact.

In recent years transpositions have been recognized (jumping genes). Certains DNA segments called transpositions have the ability to move from place to place on the chromosome and into and out of plasmids. The replicative transposon leaves a copy of itself at the original location. Figure 1.3 shows the mechanism of action of transposons. Many plasmids contain mobile DNA sequence that can move between plasmids and between plasmids and the chromosome. Genes encoding ESBL type are typically plasmid-borne. Therefore the relationship between extended spectrum β-lactamases producing *E. coli* with plasmids and transposon needs further investigations.

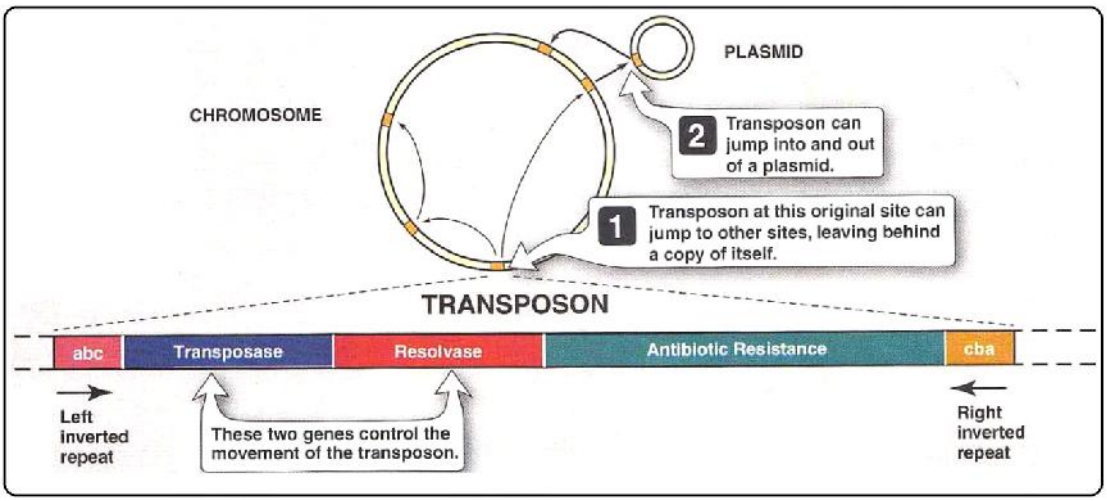

Figure 1.3: The mechanisms of action transposons (jumping genes) and its interaction with plasmid.

1.5 Extended Spectrum β-Lactamases (ESBLs)

1.5.1 Definition and General Properties of ESBL

ESBLs are defined as β-lactamases capable of hydrolyzing oximino-cephalosporins that are inhibited by clavulanic acid and are placed into functional group 2be (Ambler, 1980). Extended spectrum β-lactamases is a major source of antimicrobial resistance in Gram-negative pathogens. Generally encoded by plasmid-borne genes, these enzymes confer resistance to penicillin, cephalosporins and aztreonam and were

inhibited by clavulanic acids (Bush *et al*., 1995; Bradford, 2001). Most of the ESBL are derived from beta-lactamases TEM-1, TEM-2 and SHV-1 (Levimore, 1995; Bradford, 2001). CTX-M enzymes are also inhibited by clavulanic acid and are in the category of ESBLs. In addition, some of the enzymes of the OXA family, although belonging to ESBLs, infections caused by multidrug resistant (MDR) bacteria expressing ESBLs pose serious challenges to clinicians. Because ESBL-producing bacteria are resistant to a broad range of β-lactam, including third generation cephalosporins, nosocomial infection caused by these organism complicate therapy and limited treatment options (Rampal and Ambrose, 2006).

1.5.2 Organisms Producing ESBLs

Majority of ESBL-producing strains are *Klebsiella pneumonae, K. oxytoca* and *E. coli* (Rawat and Nair, 2010). Other organisms reported to harbour ESBL include *Enterobacter* spp., *Salmonella* spp., *Morganella morganii*, *Proteus mirabilis*, *Serratia marcesens*, *Shigella dysenteriae*, *Pseudomonas aeroginosa*, *Burkholderia cepacia* and *Capnocytophagia ochracea* (Rawat and Nair, 2010). Organisms previously known to produce β-lactamase occasionally such as *E. coli*, *Moraxella catarrhalis* now are frequent. Additionaly, those originally β-lactamase negative, such as *Haemophilus influenzae* and *Neisseria gonorrhoea* are now β-lactamase producers (Sensakovic and Smith, 1995).

1.5.3 Types of ESBL

1.5.3.1 TEM (Temoniera)

TEM-1 is the most commonly encountered β-lactamases in Gram-negative bacteria. So far 90% of ampicilin resistance in *E. coli* is due to the production of TEM-1 (Levimore**,** 1995)**.** TEM-1 is able to hydrolyze penicillins and cephaloridine. TEM-2, the first derivative of TEM-1, had a single amino acid substitution from the original β-lactamases) (Branger *et al.,* 1998). This caused a shift in the substrate profile. TEM-3 originally reported in 1989, was the first TEM derivatives that displayed the ESBL phenotype (Sougakoff *et al.,* 1988). In the years since that first report, over 90 additional TEM derivatives have been described. The amino acid substitutions that occur within the TEM enzyme occur at a limited number of positions. The combination of these amino acid changes result in various subtle alternations in ESBL

phenotypes, such as the ability to hydrolyse specific oxyimino-cephalosporin such as ceftazidime and cefotaxime or a change in their isoeletric points, which can range from a pI of 5.2 to 6.5 (Vahabogula *et al*., 1998). A number of amino acid residues are especially important for producing the ESBL phenotype when substitutions occur at that position (Figure 1.4). They include glutamate to lysine at position 104 arginine to either serine or histidine at position 164 glycine to serine at position 238 and glutamate to lysine at position 240 (Bradford, 2001).

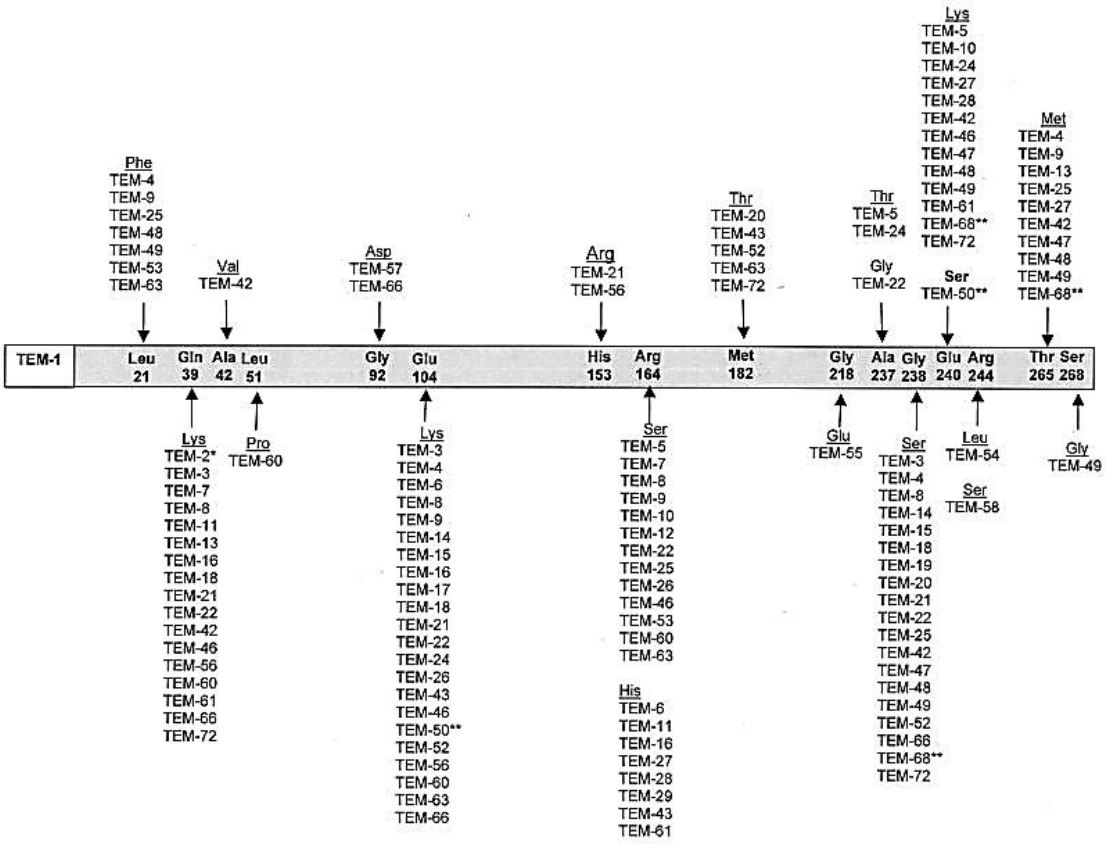

Figure 1.4: The amino acid substitutions in the different TEM variants in comparison with TEM-1 adapted from Bradford (2001).

In addition to β-lactamases TEM-1 through TEM-92, there has been a report of naturally occuring TEM-like enzymes, TEM-AQ, which contained a number of amino acid substitutions and one aminoacid deletion that have not been noted in other TEM enzymes (Perilli *et al*., 1997). It is interesting that laboratory mutants of TEM-1 that contain mutation at positions other than the ones described in nature have been constructed (Petrosino and Palzkill, 1996; Blazquez *et al*., 2000). It has been suggested that the naturally occurring TEM-type ESBLs are the result of fluctuating

selective pressure from several β-lactams within a given institution rather than selection within a given institution rather selection with a single agent.

Although, TEM-type β-lactamases are most often found in *E. coli* and *K. pneumonae,* they are also found in other species of Gram-negative bacteria with increasing frequency. TEM-type ESBLs have been reported in genera of Enterobacteriaceae such as *Enterobacter aerogenes*, *Morganella morganii*, *Proteus mirabilis*, *Proteus rettgeri* and *Salmonella* spp. (Morosoni *et al*., 1995; Paltzkill *et al*., 1995; Bonnet *et al.,* 1999; Marchandin et al, 1999; Perilli *et al.,* 2000). Furthermore, TEM-type ESBLs have also been found in non-Enterobacteriaceae Gram-negative bacteria.

1.5.3.2 SHV (Sulphydryl Variable)

The SHV-1 β-lactamase is most commonly found in *K. pneumonae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species (Tzouvelekis and Bonomo, 1999). In many strains *K. pneumoniae*, $bla_{\text{SHV-1}}$ or a related gene is integrated into the bacterial chromosome (Livermore, 1995). Although it has been hypothesized that the gene encoding SHV-1 may exist as part of a transferable element, it has been proven (Bush *et al*., 1995). The majority of SHV variants possessing an ESBL phenotype are characterized by the substitution of a serine for glycine at 238 (Figure 1.5) (Nüesch-Inderbinen *et al*., 1996; Bradford, 2001).

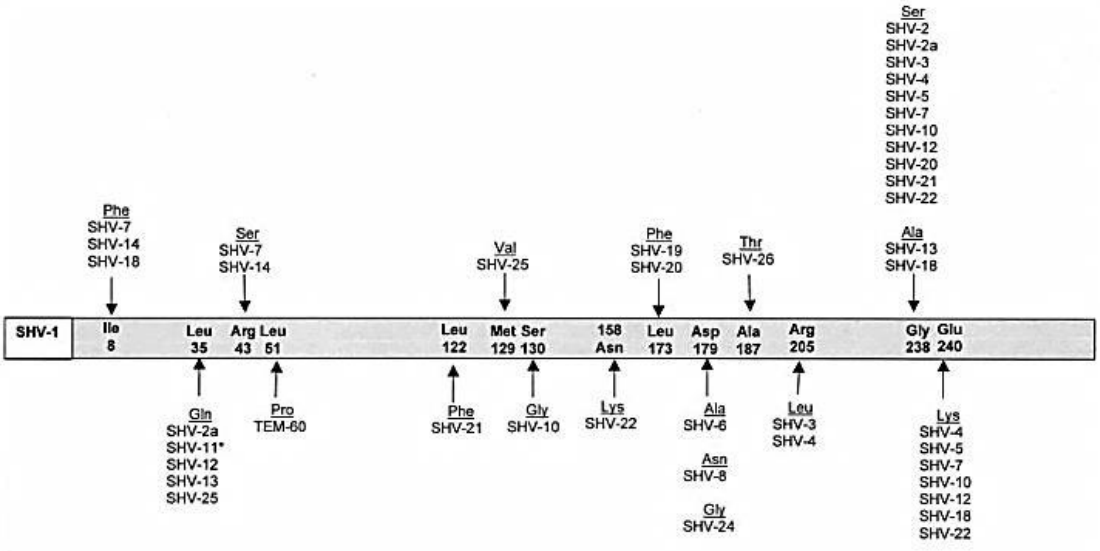

Figure 1.5: The amino acid substitutions in the different SHV variants in comparison with SHV-1 adapted from Bradford (2001).

To date, the majority of SHV-type derivatives possess the ESBL phenotypes. However, one variant, SHV-10, is reported to have an inhibitor-resistant phenotypes. It is interesting to note that the inhibitor-resistant phenotype conferred by the ser140Gly mutation seems to override the strong ESBL phenotype usually seen in enzymes containing the Gly238Ser and the Glu240Lys mutations seen in other SHV-5 type enzymes.

1.5.3.3 CTX-M (Cefotaxime-M)

In recent years, a new family of plasmid-mediated ESBLs called CTX-M, which preferentally hydrolyze cefotaxime has arisen (Medeiros, *et al*., 1982; Medeiros, *et al*., 1985). They have mainly been found in strains of *Salmonella enterica* serovar Typhimurium and *E. coli*, but have also been described in other species of Enterobacteriaceae. They include the CTX-M type enzymes CTX-M-1, CTX-M-2 through CTX-M-10 (Bauernfriend *et al*., 1990; Barthélémy *et al*., 1992; Bauernfeind *et al*., 1992; Bauernfriend *et al*., 1996) as well as Toho enzymes 1 and 2. These enzymes are not very closely related to TEM or SHV β-lactamases in that they show only approximately 40% identity with these two commonly isolated β-lactamases (Tzouvelekis and Bonomo, 1999; Tzouvelekis *et al*., 2000). However, it was recently reported by Huang and Stafford (2002) that there is a high degree of homology between the chromosomal AmpC enzymes of *Kluyvera ascorbaa* and the CTX-M type enzymes, suggesting that the latter probably originated from this species.

1.5.3.4 OXA (Oxacillinase)

The OXA-type enzymes are another growing family of ESBLs. These β-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d (Bush *et al.,* 1995). The OXA-type β-lactamases confers resistance to ampicillin and cephalothin and is characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid (Bush *et al.*, 1995). The OXA-type β-lactamases family was originally created as a phenotypic rather than a genotypic group for a few β-lactamases that had a specific hydrolysis profile.

Inhibitor-resistant β-lactamases: Although the inhibitor-resistant β-lactamases are not ESBLs, they are often discussed with ESBLs because they are also derivatives of the classical TEM- or SHV-type enzymes (Miró *et al*., 2002). In the early 1990s β-lactamases that were resistant to inhibition by clavulanic acid were discovered nucleotide sequencing revealed that these enzymes were variants of the TEM-1 or TEM-2 β-lactamase. These enzymes were at first given the designation IRT for inhibitor-resistant TEM β-lactamases; however all have subsequently been renamed with numerical TEM designation. There are at least 19 distinct inhibitor-resisitant TEM β-lactamases. In recent survey of amoxicillin-clavulanic resistant *E. coli* in a hospital in France, Leflon-Gouibout *et al*. (2000) found that up to 41% of these isolates produced inhibitor-resistant TEM variants. Point mutation that leads to the inhibitor-resistant phenotypes occurs at a few specific amino acid residues within the structural gene for the TEM enzymes, Met-69, Arg-244, Arg-275 and Asn-276 (Belaaouaj *et al*., 1994; Henquell *et al*. 1995, Zhou *et al*., 1994). The sites of these amino acid substitutions are distinct from that lead to these ESBLs phenotype.

1.5.4 Association between Other Antimicrobial Resistance and ESBLs

Multidrug resistance (MDR) profiles involving non-β-lactam antibiotics in ESBL-producing isolates may also contribute to the increase in colonization pressure. Fluoroquinolone resistance is becoming a common feature rather than an exception in ESBL-producing isolates (Velasco *et al*., 2001; Vila *et al*., 2002; Valverde *et al.,* 2004). Previous fluoroquinolone use has been demonstrated to be a risk factor for the acquisition of ESBL-producing isolates, particularly isolates producing the CTX-M-type enzymes in the community setting (Valverde *et al.,* 2004). The epidemiology of ciprofloxacin resistance and its relationship to ESBL production among *K. pneumoniae* strains has been described (Paterson *et al*., 2000; Yu *et al*., 2002; Edelstein *et al*., 2003).

Genes encoding ESBL are usually located on conjugative plasmids such as bla_{TEM} or bla_{SHV}, although many of the most recently described ESBL genes are frequently found within integrons-like structures such as $bla_{\text{CTX-M}}$ (Jacoby and Medeiros, 1991; Bradford, 2001; Canton *et al*., 2003; Bonnet *et al*., 2004). Integrons are naturally efficient recombination and expression systems able to capture genes as part of

genetic elements known as gene cassettes (Rowe-Magnus *et al*., 2002). Class 1 integrons are most commonly found in nosocomial and community environments, followed by class 2 integrons, other integrons classes being scarcely reported to date (Rowe-Magnus *et al*., 2002). ESBLs located on integrons-like structures are being increasingly reported worldwide (Canton *et al*., 2003; Bonnet *et al*., 2004).

In Gram-negative organisms, the primary target of fluoroquinolone antibiotics is the DNA gyrase, a type II DNA topoisomerase, which is essential for DNA synthesis (Everett *et al*., 1996). DNA gyrase is composed of two subunits A and B. Most changes have been found in the A subunit (*gyrA*) in a specific region termed the quinolone resistance-determining region (QRDR) (Thomson, 1999). This region is highly conserved within bacterial species and alterations within it have been documented in a variety of species, including *Shigella* spp., *E. coli*, *Salmonella* spp. and *Campylobacter* spp. (Thomson, 1999).

Although mutations in B subunit (*gyrB*) associated with quinolone resistance have been described (Vila *et al*., 1996), their prevalence in clinical isolates is very rare. Topoisomerase IV, a secondary target of fluoroquinolones in Gram-negative bacteria (Thomson, 1999), consists of two subunits, *parC* and *parE*, which share significant sequence similarity to the *gyrA* and *gyrB* subunits. Unlike *parC*, the alterations in the regions of the *parE* gene are not significantly related to the resistance level (Villa *et al*., 2000).

Recent reports indicate that quinolone resistance can be mediated by plasmids (Wang *et al*., 2004; Corkill *et al*., 2005). The gene responsible for plasmid-mediated resistance, *qnr*, was found on plasmids varying in size from 54 to ≥180 kb in clinical isolates of *Klebsiella pneumoniae*, *Escherichia coli* and *Shigella flexneri* 2b from which low-level quinolone resistance could be transferred to a sensitive recipient by conjugation (Wang *et al*., 2004; Hata *et al*., 2005; Jones *et al*., 2005). However, the exact mechanism of DNA gyrase protection conferred by *qnr* gene has yet to be established.

1.5.5 ESBL Epidemiology

ESBLs are now a problem in hospitalized patients worldwide. The ESBL phenomenon began in Western Europe, most likely because extended-spectrum β-lactam antibiotics were first used there clinically. However, it did not take long before ESBLs had been detected in the United States, South America and Asia (Radice *et al*., 2002; Komatsu *et al*., 2003; Raksha *et al*., 2003; Jeong *et al*., 2004; Wang *et al*., 2004). The prevalence of ESBLs among clinical isolates varies from country to country and from institution to institution.

In Europe, the prevalence of ESBL production among isolates of Enterobacteriaceae varies greatly from country to country. In Spain, a nationwide epidemiological study was conducted in 2000, revealing that the prevalence of ESBL-producing *Klebsiella pneumoniae* and *Escherichia coli* strains were 2.7% and 0.5% respectively (Hernández *et al*., 2005). A recent finding in January 2011 showed the emergence of CTX-M-15 ESBL isolates in the community in Spain (Perez *et al.*, 2007). In Turkey, *E. coli* strains isolated from community showed ESBL-CTX-M-15 and they are showing resistance to non-β-lactam antimicrobial. Thus the treatment methods got complicated at outpatients (Azap *et al*., 2010).

TEM-10 has been responsible for several unrelated outbreaks of ESBL-producing organisms in the United States for a number of years (Bradford, 2001). However, TEM-10 has only recently been reported in Europe with the same frequency (Bradford, 2001). Similarly, TEM-3 is common in France, but has not been detected in the USA. In recent years, there have been reports of outbreaks of TEM-47-producing organisms in Polland (Bradford, 2001).

In Bangladesh, extended-spectrum β-lactamase (ESBL)-mediated third generation cephalosporin resistance in *Shigella* isolates has been reported by ICDDR,B, Dhaka (Rahman *et al*., 2004b). Two *S. sonnei* and one *S. boydii* were positive by double disc synergy test (DDST) and were resistant to β-lactams, but susceptible to β-lactam/β-lactamase inhibitor combination cefoxitin and imipenem indicating the presence of a class A ESBL. In Bangladesh, another study was done on prevalence of ESBL-producing *E. coli* and *K. pneumoniae* in an urban hospital in Dhaka, where the DDST method was performed. *E. coli* (43.2%) and *K. pneumonia* (39.5%) were found to

have ESBL phenotypes (Rahman *et al*., 2004a). Another study (Alim *et al*., 2009) showed that about 23% Gram-negative bacteria are ESBL-producing organisms including *Klebsiella* spp. (41%), *Proteus* spp. (27%), *E. coli* (27%) and *Pseudomonas* spp. (5%). Further study was also conducted by Islam *et al*. (2013) regarding multidrug-resistant New Delhi metallo-β-lactamase-1 (NDM-1)-producing bacteria that highlights the occurrence of NDM-1-producing organisms in Bangladesh in 2008 (Islam *et al*., 2013). The clonal diversity of the isolates and the transferability of bla(NDM-1) plasmids suggest a wider distribution of NDM-1-producing bacteria in Bangladesh (Islam *et al*., 2013).

1.6 Phenotypic Detection Methods for ESBL

The detection methods are divided into (1) Phenotypic methods, and (2) Molecular methods. Phenotypic methods are based on the resistance of ESBL-producers to oxyimino-β-lactams such as cefotaxime, ceftriaxone, ceftazidime and aztreonam and the ability of β-lactamase inhibitors to inhibit this resistance. Several tests have been developed to detect the presence of ESBLs. Serogrouping for ESBL following the procedure developed by National Committee for Clinical Laboratory Standard (NCCLS, 1999) and double disc diffusion synergy test (Jarlier *et al.,* 1988) were commonly used for this purpose. The NCCLS (1999) has developed broth microdilution and disc diffusion screening tests using selected antimicrobial agents. Each *K. pneumoniae*, *K. oxytoca*, or *E. coli* isolate should be considered a potential ESBL-producer.

The sensitivity of screening for ESBLs in enteric organisms can vary depending on which antimicrobial agents are tested. The use of more than one of the five antimicrobial agents suggested for screening will improve the sensitivity of detection. Cefpodoxime and ceftazidime show the highest sensitivity for ESBL detection.

1.6.1 Double Disc-Diffusion Synergy Test

The test was earlier performed by swabbing the organism onto a Muller-Hinton agar plate then a disc containing amoxicillin-clavulanates (20 μg/10 μg) was placed in the center of the plate and discs containing 30 μg of ceftazidime, ceftriaxone, cefotaxime and aztreonam were placed at a distance of 30 mm (center to center) (Jarlier *et al*.,

1988). Enhancement of the zone of inhibition of the oxyimino-β-lactam caused by the synergy of the clavulanate in the amoxicillin-clavulanate disc was interpreted as positive test indicating the production of ESBL. The use of cefpodoxime as the oxyimino-cephalosporin of choice is recommended. It gives test sensitivity up to 97% and test specificity up to 100%. If the test is negative with an isolate that is highly suspected to be an ESBL-producer the test should be repeated with closer distance (20 mm) but generally the test is reliable, convenient and non-expensive method for screening of ESBL production (Drieux *et al*., 2008).

1.6.2 Three-Dimensional Test

This test provides the advantage of simultaneous determination of antibiotic susceptibility and β-lactamase substrate profile (Thomson and Sanders, 1992). It is sensitive, easy to interpret. It is not specific for ESBL and labour is extensive.

1.6.3 Disc Approximation Test

Cefoxitin (inducer) disc is placed at a distance of 2.5 cm from cephalosporin disc (Revathi and Singh, 1997). Production of inducible β-lactamase is indicated by flattening of the zone of inhibition of the cephalosporin disc towards inducer disc by >1 mm.

1.6.4 MIC Reduction Test

By this method the MIC of cefotaxime or ceftazidime with and without clavulanic acid (4 μg/ml) is determined (Nathisuwan *et al*., 2001). An 8-fold reduction in the MIC of cephalosporin in the presence of clavulanic acid compared with cephalosporin alone indicates production of ESBL.

1.6.5 Automated Vitek 2 ESBL Test (BioMerieux, France)

This is an automated microbial susceptibility test system that utilizes ceftazidime or cefotazime alone and in combination with clavulanic acid. Cards containing four wells are inoculated (Sanders *et al.,* 1996). Presence of ESBL was indicated if there is a reduction in growth of cephalosporin well containing clavulanic acid compared with the level of growth in well with cephalosporin alone.

1.6.6 Automated Phoenix ESBL Test (Becton Dickinson, USA)

This method depends also on the growth response to certain expanded-spectrum cephalosporins and the result also interpreted via computerized system (Drieux *et al*., 2008).

1.6.7 E-Test

The E-test ESBL strip carries two gradients, on the one end, ceftazidime and on the opposite end ceftazidime plus clavulanic acid (Vercauteren *et al*., 1997). MIC is interpreted as the point of intersection of the inhibition ellipse with the E test strip edge. Ratio of ceftazidime MIC and ceftazidime clavulanic acid MIC equal to or greater than 8 indicates the presence of ESBL.

1.6.8 Isoelectric Point

This was used to identify ESBL in early days. However, with >90 TEM-type β-lactamases, many of which possess identical isoelectric points, determination of the ESBL by isoelectric point is no longer possible (Bradford, 2001). A similar situation is found in the SHV, CTX-M, and OXA families of ESBLs.

1.7 Genotypic Detection Methods for ESBL

Double disc test, three dimensional test, MIC reduction test, E-test etc. presumptively identify the presence of an ESBL, whereas the following molecular methods can identify specific (TEM, SHV, OXA, CTX-M) ESBL present in a clinical isolate (Bradford, 2001).

1.7.1 DNA Probe

Early detection of β-lactamase genes was performed using DNA probes that was specific for TEM and SHV enzymes. However, using DNA probes can sometimes be rather laborious and intensive (Huovinen *et al*., 1988).

1.7.2 Polymerase Chain Reaction (PCR)

The easiest and most common molecular method used to detect the presence of a β-lactamase belonging to a family of enzymes is PCR with oligonucleotide primers that

are specific for a β-lactamase gene. These primers are usually chosen to anneal to regions where various point mutations are not known to occur. However, PCR will not discriminate among different variants of TEM or SHV (Bradford, 2001).

Polymerase chain reaction (PCR) is an ingenious new tool for molecular biology that has had an effect on research similar to that of the discovery of restriction enzymes and the Southern blot. PCR is so sensitive that a single DNA molecule can be amplified and single copy genes are routinely extracted out of complex mixtures of genomic sequence and visualized as distinct bands on agarose gels. PCR can also be utilized for rapid screening and or sequencing of insert directly from aliquots of individual phage plaques or bacterial colonies. Enhancement, such as the use of thermostable DNA polymerase and automation by standard method have fostered the development of numerous and diverse PCR applications throughout the research community. Unquestionably, no single protocol will be appropriate to all situations.

The theoretical basis of the PCR was probably first described in a paper by Klepee *et al.,* 1971. However this technique did not excite general interest until the mid 1980. Kary Mullis and co-workers (Lauerman, 2004) developed PCR into a technique that could be used to generate large amount of single copy genes from genetic DNA (Saiki *et al.,* 1985).

PCR is an *in vitro* method for system of nucleic acid in which a particular segment of DNA can be specifically amplified. The PCR uses multiple cycles of template denaturation, primer annealing and primer elongation to amplify DNA sequence and are oriented so that DNA synthesis by DNA polymerase proceeds across the region between the primers. Since the extension products themselves are also complementary to and capable of binding primers the successive cycles of amplification essentially double the amount of the target DNA synthesized in the previous cycles. The result is a exponential accumulation of specific target DNA fragment approximately up to 2n, when n is the numbers the cycles of amplification performed (Saiki *et al.,* 1985).

Typically, enough amplified product is generated after 20 to 30 cycles of PCR so that it can be visualized on an ethidium bromide stained gel. The reaction is composed of several components, *e.g.*, the template, primers, reaction buffer magnesium chloride dNTP mixture and thermostable polymerase. The template can be genomic or plasmid

DNA, RNA converted to cellular DNA, unpurified crude biological sample such as bacterial colonies or phage plaques.

The template must be sufficient to be able to visualize PCR products using ethidium bromide. Usually 100 ng of genomic DNA is sufficient to defect a PCR product from a single copy mammalian gene. A number of contaminants found in DNA preparations can decrease the efficiency of PCR. These include urea, the detergents SDS, sodium acetate and sometimes components carried over in purifying DNA from agarose gels.

An optimal primer set should hybridize efficiently to the sequence of interests with negligible hybridization to other sequences present in the sample; the distance between the primers is rather flexible ranging upto 10 kb. There is however a considerable drop of synthesis efficiently with distance >3 kb. A primer should be 20 to 30 bases in length. It is unlikely that longer primer will help increase specificity significantly.

The most frequently used thermo stable polymerase is *Taq* DNA polymerase. *Taq* DNA polymerase permitted the use of higher temperatures for annealing and extension which improved the stringency of primer template hybridization and this is the specificity of the products. A very important property of *Taq* DNA polymerase is its error rate, which was initially estimated at 2 x 10^{-4} nucleotides/cycle (Saiki *et al.,* 1985). The purified enzymes lack a proofreading 3→5 exonuclease activity, which also lowers error rates than other enzymes.

The magnesium ion concentration affects enzymes activity, primer annealing, melting temperature of the template and the PCR product, fidelity and primer-dimer formation. Reaction buffer supports the reaction condition and dNTPs mixture. Genes encoding ESBL producing *E. coli* bla_{TEM}, bla_{SHV}, bla_{OXA}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}8}$, $bla_{CTX\text{-}M\text{-}9}$, and *qnr* gene (virulent) detection can be carried out by using PCR-based method (Saiki *et al.,* 1985).

1.7.3 Oligonucleotide Method

The first molecular method for the identification of β-lactamase was the oligotyping method, which was used to discriminate between TEM-1 and TEM-2 (Mabilat and

Courvalin, 1990; Ouellette *et al*., 1988). The disadvantages are that it requires specific oligonucleotide probes. There is extensive labor and cannot detect new variants.

1.7.4 PCR-RFLP

Another approach for molecular characterization of the TEM β-lactamase gene was to add restriction fragment length polymorphism analysis to PCR (PCR-RFLP) (Arlet *et al*., 1995). Easy to perform, can detect specific nucleotide changes. The disadvantages are the nucleotide changes must result in altered restriction site for detection.

1.7.5 Ligase Chain Reactions (LCR)

The LCR (ligase chain reaction) method was proposed for the identification of SHV genes is the use of ligase chain reaction (Kim and Lee, 2000). It can distinguish among a number of SHV variants. The disadvantage is that it requires a large number of oligonucleotide primers.

1.7.6 PFGE Analysis for Molecular Typing

In the normal electrophoresis, all linear double-stranded DNA molecules that are larger than a certain size migrate through agarose gels at the same time giving no separation of distinct bands. To solve this problem Schwartz and Cantor (1984) first reported the development of pulsed-field gel electrophoresis (PFGE) capable of resolving DNAs up to 2 megabase pairs in length. PFGE typing is a very discriminatory and reproducible typing method, which is becoming more common and common.

In PFGE, a form of macrorestriction analysis, one uses restriction enzymes that are referred to as infrequent cutters, *i.e*., instead of 4 bases, these enzymes will recognize specific 8 bases or 6 base sequences (Figure 1.6). Therefore, one ends up with a small number (5 to 20) of very large well-resolved fragments. It seems that separation by pulsed-field gel electrophoresis of chromosomal DNA fragments generated after digestion with rarely cutting endonucleases provides fingerprint patterns that are particularly useful for epidemiologic work. In fact, macrorestriction patterns obtained by PFGE often became referred to as the "gold standard" of molecular fingerprinting techniques (Chung *et. al*., 2000). Many different factors are known to affect the

resolution of large sized DNA fragments during pulsed-field separations. Some of these include electric field strength, field angle and shape, agarose type and concentration, pulse time, and temperature.

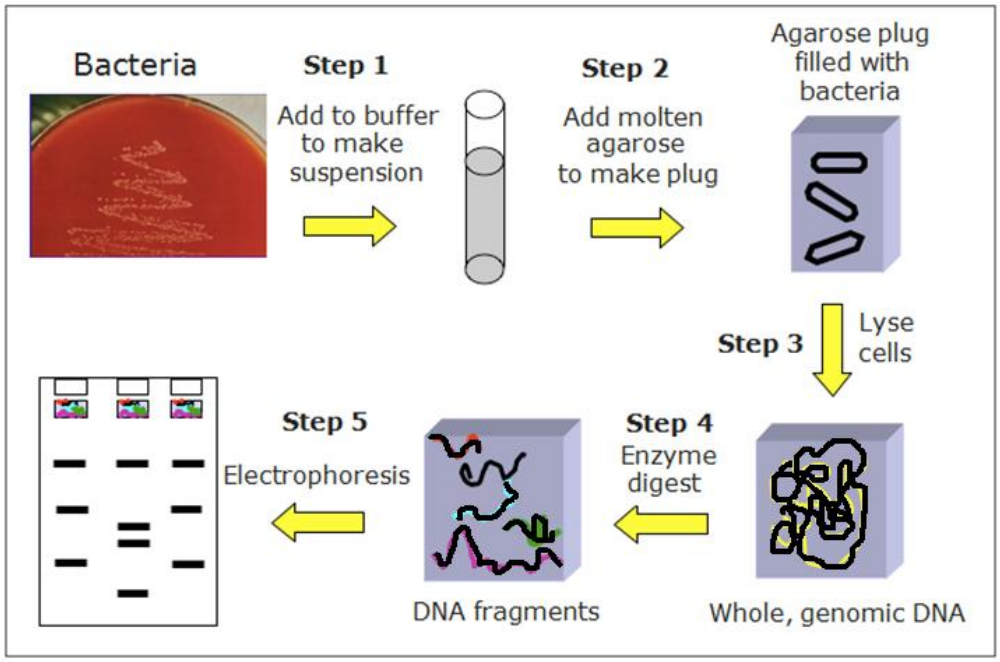

Figure 1.6: Macrorestriction analysis of chromosomal DNA by pulsed-field gel electrophoresis (PFGE). The first step of PFGE is to make a suspension of bacteria and buffer (Step 1). Molten agarose is added to the bacterial suspension and the agarose/suspension mixture is added to a mold (Step 2). The agarose is allowed to solidify then added to a lysis solution which breaks open the bacteria releasing the DNA. Subsequent washing steps remove extracellular debris leaving intact, circular genomic DNA in the agarose plug (Step 3). A small piece of the plug is cut off and added to a restriction endonuclease mixture which cleaves DNA at a specific sequence (Step 4) resulting in 10-20 DNA fragments of varying sizes. The large DNA fragments are then separated by size by PFGE (Step 5). PFGE facilitates the migration of the large DNA fragments through the agarose gel by regularly changing the direction of the electrical field during electrophoresis, allowing the fragments to maneuver through the agarose. The smaller DNA fragments will move faster through the agarose than the larger fragments and the result is a pattern of DNA fragments. The pattern from one isolate can be compared to other patterns to determine whether the samples may have originated from a common source.

1.8 Risk Factors

Extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria are emerging pathogens. Clinicians, microbiologists, infection control practitioners, and hospital epidemiologists are concerned about ESBL-producing bacteria because of the increasing incidence of such infections, the limitations of effective antimicrobial drug

therapy, and adverse patient outcomes (Lautenbach *et al*., 2001; Paterson *et al*., 2003; 2004; 2006; Rossi *et al*., 2006). The emergence of ESBLs has been tied to the overuse and misuse of third generation cephalosporins and other antimicrobials. Several factors have been reported to increase the risk of colonization or infection with ESBL-producing bacilli (Pfller, *et al*., 2006). Risk factors associated with infection or colonization with ESBL-producing pathogens included:

1. Prolonged hospital stay,
2. Prolonged intensive care unit or neonatal intensive care unit stay,
3. Residency in long-term care facility,
4. Exposure to third-generation cephalosporins,
5. Exposure to trimethoprim-sulfamethoxazole,
6. Exposure to ciprofloxacin,
7. Total antibiotic use,
8. Delayed appropriate therapy,
9. Indwelling catheter,
10. Severity of illness,
11. Decubitus ulcer,
12. Total dependence on health care worker, and
13. Endotracheal or nasogastric tube.

1.9 Treatment Options for Infections with ESBL-Producing Organisms

Carbapenems are the drugs of choice for life-threating infection with ESBL-producing organisms. The choice between imipenem and meropenem is difficult. The clinical experience is better with imipenem but meropenem has the lower MIC. In nosocomial meningitis meropenem is regarded the drug of first choice. The combination therapy with carbapenem and antibiotic of other classes do not offer an advantage over the use of carbapenem alone. Unfortunately, carbapenem resistance has been observed in organisms that commonly encode ESBLs and hence the use of these agents should be restricted to serious life-threating infection (Segal-Maurer *et al*., 1999; Al-Jasser, 2006).

Third generation cephalosporins should not be used for treatment of infections with ESBL-producing organism; even if *in vitro* susceptibility is detected. Treatment failure may result. Cefepime should not be regarded as the first line therapy against ESBL-producing organisms because cefepime resistance is common in CTX-M-producers (Paterson *et al*., 2000; Paterson and Bonomo, 2005).

In spite of their good (*in vitro*) activity, cephamycins are not regarded as the first line therapy for ESBL-producing organism due to the probable emergence of porin-resistant mutants during the course of the treatment as observed earlier with cefoxitin (Pangon *et al*., 1989; Martínez-Martínez *et al*., 1996).

β-Lactam/β-lactamase inhibitors also are not regarded as the first line therapy for infections due to ESBL-producing organism. Activity of β-lactam/β-lactamase inhibitors depends on the inoculum size. Their effectiveness against the organisms producing multiple ESBL is less as compared to organisms encoding single ESBL. Despite all the above mentioned limitations, amoxicillin/clavulanate still considered as the second line therapy for the urinary tract infections. It is noteworthy that, the use of β-lactam/β-lactamase inhibitors has a protective role against infection and colonization with ESBL-producing *K. pneumoniae* (Bradford *et al*., 1994; Piroth *et al*., 1998; Thomson and Moland, 2001).

1.10 Hypothesis

The emergence of ESBL-producing enterobacterial members (*e.g*., *E. coli*) leading to increased morbidity and mortality as well as increased treatment failure to the most widely used antimicrobial class with the consequent increase in the cost of the health care services due to the shift to more expensive antimicrobials and to prolonged stay in the hospitals represents an extremely important clinical and public health problem all over the world.

Therefore, it was hypothesized that continuous monitoring and better understanding on the molecular mechanisms of ESBLs will help for the development of new antimicrobial agents, to stop and/or reduce the amount of horizontal transfer of antibiotic resistance markers at the intra and inter-species level among Enterobacteriaceae as well as to resolve health care problem.

1.11 The Aims and Objectives

In modern medical practise, proper diagnosis, treatment and appropriate management of urinary tract infection are serious problems for the clinicians due to lack of microbiological investigations at molecular level on the etiology and changing pattern of antibiogram of urinary tract infections. Patients admitted in the hospital take longer period to heal due to irrational use of antibiotics and in some cases the outcome becomes fatal. *E. coli*, the most common member of the family enterobacteriaceae accounts for majority of the urinary tract infections (90%). *E. coli* is widely found in the nature as ESBL. There are no significant studies on ESBL producers were detected in Bangladesh. Therefore attempts to isolate the pathogens causing urinary tract infections and to evaluate the mechanism by which resistance to antimicrobial agents develops in these pathogens which are vital for the future of antimicrobial therapy. With the view to address the present study; this investigation was undertaken with the following aims and objectives:

1. To collect and identify ESBL and non-ESBL *Escherichia coli* strains from urine samples from patients having symptoms of urinary tract infection.
2. To demonstrate antimicrobial susceptibility by disc-diffusion method as recommended by Clinical and Laboratory Standard Institute (CLSI).
3. To identify ESBL-producing *Escherichia coli* by double disc diffusion synergy test from patients having UTI.
4. To extract plasmid DNA by alkaline lysis method with some modifications described earlier.
5. To identify the genes encoding ESBL type specific by polymerase chain reaction according to the procedure as described previously.
6. To find out the clonal relationship of ESBL isolates by pulse-field gel electrophoresis (PFGE).

Chapter 2 Materials and Methods

2 Materials and Methods

2.1 Bacterial Isolates

This study was carried out at Enteric Laboratory, Laboratory Science Division (LSD), International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka. Urine samples were collected from patients having symptoms of urinary tract infections. A total of 100 samples were collected from out patients department of Sylhet Medical College and some private laboratories of Sylhet Town. Among these 25 strains were determined as ESBL producers. Pathogens associated with these samples were initially identified by Clinical Microbiology Laboratory, ICDDR,B.

A single colony of confirmed *E. coli* were grown in trpticase soy broth with 0.3% yeast and was stored at −70°C after addition of 15% glycerol for further use. *Escherichia coli* (ATCC 25922) strains lacking the ESBL-producing genes, 140 MDa invasive plasmid, virulence genes, and sensitive to all antibiotics were used as negative control in PCR for bla_{TEM}, bla_{SHV}, bla_{OXA}, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-1}}$, $bla_{\text{CTX-2}}$, $bla_{\text{CTX-8}}$, *ipaH*. *Shigella sonnei* K-436 (1,900 bp) and *Shigella sonnei* K-438 (150 bp), *Shigella sonnei* K-548 (1.37 kb) and *Shigella sonnei* K-564 (2.00 kb), *Shigella flexneri* 2a, YSH 6000 (Sasakawa *et al*., 1986) was used as positve control for both invasive plasmid antigen H (*ipaH*). *E. coli* strains PDK-9, V-517 and R1 were used as plasmid molecular weight standard *Salmonella* serotype *Braenderup* (H9812) was used as the PFGE molecular weight standard. All strains which were used as standard were collected from the Enteric Bacteriology Laboratory, ICDDR,B.

2.2 Phenotypic Characterization

2.2.1 Serogrouping

All the *E. coli* isolates (n = 30) were serologically confirmed by using commercially available antisera kit (Denka Saiken, Co. Ltd., Japan). Isolates were subcultured on tripticase soy agar (Becton-Dickinson Co., USA) plates. After about 18 hours of incubation, serological reactions were performed by the glass slide agglutination test as described by Sakazaki (1992).

2.2.2 Antimicrobial Susceptibility Test

Bacterial susceptibility to antimicrobial agents was determined by the disc diffusion method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1999) using commercial antimicrobial discs (Oxoid, Bsingstoke, UK). The antibiotic discs used in this study were ampicillin (AMP, 10 μg), amoxycillin-clavulanic acid (AMC, 20/10 μg), cefepime (FEP, 30 μg), cefotaxime (CTX, 30 μg), cefoxitin (FOX, 30 μg), ceftazidime (CAZ, 30 μg), ceftriaxone (CRO, 30 μg), imepenem (IMP, 10 μg), meropenem (MPM, 10 μg), aztreonam (ATM, 30 μg), ciproflaxacin (CIP, 5 μg), nalidixic acid (NA, 30 μg) and trimethoprim-sulfamethoxazole (SxT, 1.25/23.75 μg). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains for susceptibilty studies.

By the standard method of inoculation, an inoculating needle was used to touch to a freshly grown well-isolated colony. The inoculation needle carrying the inoculam was then used to inoculate into 2 ml of Muller-Hinton broth. The broth culture was then allowed to incubate at 37°C for 4 hour to obtain the young culture. The turbidity of actively growing broth culture was then adjusted to a McFarland 0.5 standard (3×10^8 CFU/ml). To inoculate the agar medium, a sterile, non toxic cotton swab was dipped into the standardized suspension. The excess broth was purged by pressing and rotating the swab firmly against the inside the tube above the fluid level. The swab then used to streak the surface of the agar plate to obtain a uniform inoculum. A final sweep was made of the agar rim with the cotton swab. This plate was then allowed to dry for 3 to 5 mm, before the discs were applied. Antibiotic impregnated discs were then placed to the surface of the inoculated plates with sterile forceps. All discs were gently pressed down onto the agar with forceps to ensure complete contact with agar surface.

Within 15 min after the disc were applied, the plates were examined, and the diameter of the zones of complete inhibition were measured to the nearest whole millimeter by a ruler. The zone diameters for individual antimicrobial agents was then translated into susceptible, intermediate, moderately susceptible, or resistant categories according to the interpretation table (Oxoid, England).

2.2.3 Test for ESBL Genes by Double Disc-Diffusion Synergy Method

An inoculating needle was used to transfer to a freshly grown well-isolated colony, and inoculated into 2 ml of Muller-Hinton broth. The broth cultures were then allowed to incubate at 37^{o}C for 4 hour to obtain a young culture. A Muller-Hinton agar plate was inoculated with this suspension. Disc containing the standard 30 µg of ceftazidime, or ceftriaxone, or aztreonam, cefoxitin are placed 5 mm apart (edge to edge) and from an amoxicillin-clavulanic acid disc containing 10 µg of the latter compound. Following incubation for 16-20 h at 35^{o}C any enhancement of zone of inhibition between a beta-lactam disc and that containing the beta-lactamase inhibitor is indicative of the presence of an ESBL.

2.3 Genotypic Characterization

2.3.1 Plasmid Profiling

2.3.1.1 Isolation of Plasmid DNA

Plasmid DNA was prepared according to simplified alkaline lysis method of Kado and Liu (1981).

Reagents:

1. Solution I: 40 m*M* tris-NaOAc, 2 m*M* EDTA, pH 7.4
2. Solution II (Lysing solution): 3% SDS, 50 m*M* tris, pH12.6
3. Solution III: Phenol : chloroform : isoamylalcohol (25:24:1)

Procedure:

An isolated colony of each isolate was inoculated into 1.5 ml of TSB broth containing 0.3% yeast extract (YE) and incubated overnight at 37^{o}C on a water bath-shaker. Cells were collected in a polyproplene microcentrifuge tube by centrifuging the broth culture in an Eppendorf centrifuhge (Model N.5415 C) at 14,000 rmp for 5 min. Supernatant was removed and the pellet was suspended in 100 µl of solution I by vortexing. Then 200 µl of solution II was added and was mixed gently by rapid inversion of the tube and was incubated at 55°C for 45 min in a water bath. After incubation, the tubes were taken out and equal volume of solution III (300 µl) was added and mixed well by slowly inverting the turbed until a milky white suspension was formed. Then the tubes were centrifuged at room temperature for 8 min at 14,000

rpm. It formed three layers, the upper layer was the plasmid solution, middle layer consisted of cell debris together with other proteinatious fractions, and the lower layer was the phenol. Using a Pasteur pipette the plasmid solution was carefully removed and transferred into new set Eppendorf tubes.

2.3.1.2 Separation of Plasmid DNA by Agarose Gel Electrophoresis

Reagent:

1. Agarose (Sigma Chemical Co. St. Louis, USA)
2. TBE (Tris-borate EDTA) buffer (Gibco-BRL, USA)
3. Tracking dye (10x concentration)
4. Ethidium bromoide (10 mg/ml)

Procedure:

Plasmid DNA was separated by horizontal electrophoresis in 0.7% agarose slab gels in a Trsi-borate EDTA (EDTA) buffer at room temperature using 100 volt (50 Ma) for 3 hour. Carefully, 30 µl of plasmid DNA solution was mixed with 3 µl of tracking dye (Appendix II) and was loaded into an individual well of the gel. The gel (5 mm thick) was stained with 0.5 µg/ ml of the ethidium bromide for 30 min at room temperature DNA bands were visualized and photograph was taken using an MP4 land camera with UV transilluminator.

Polaroid type 55 film was exposed through a tiffen 10 orange filter. Molecular weight of the unkown plasmid DNA was determined on the basis of its mobility through agarose gel and was compared with the mobility of the known molecular weight plasmids (Haider *et al*., 1989). Plasmid present in strains *E. coli* PDK-9 (140, 105, 2.7 and 2.1 MDa) and V5I7 (35.8, 4.8, 3.7, 3.4, 3.1, 2.0, 1.8 and 1.4 MDa) were used as molecular weight standard.

2.3.2 Detection of Specific Genes by PCR Assay

2.3.2.1 Primers and PCR Parameters

1. Detection of ESBL-Producing Genes by PCR Assay

The primers and the PCR parameters for detections of ESBL-producing genes by PCR method are presented in Table 2.1.

Table 2.1: Primers for the detection of ESBL-producing genes

Gene encoding ESBL	Primer	Oligonucleotide sequence (5′ to 3′)	T_{Ann} (ºC)	Ti_{Elong} (min)	Size of amplified product (bp)
bla$_{TEM}$	TEM-F TEM-R	5′-TCGGGGAAATGTGCGCG-3′ 5′-TGCTTAATCAGTGAGGACCC-3′	50	2.00	971
bla$_{TEM}$	SHV-F SHV-R	5′-CACTCAAGGATGTATTGTG-3′ 5′-TTA GCGTTGCCAGTGCTCG-3′	50	2.00	885
bla$_{OXA}$	OXA-F OXA-R	5′-ACCAGATTCAACTTTCAA-3′ 5′-TCTTGGCTTTTATGCTTG-3′	55	2.00	598
bla$_{CTX-M-1}$	CTX-M-1-F CTX-M-1-R	5′-GGACGTACAGCAAAAACTTGC-3′ 5′-CGGTTCGCTTTCACTTTTCTT-3′	57	2.00	200
bla$_{CTX-2}$	CTX-M-2-F CTX-M-2-R	5′-CGGYGCTTAAACAGAGCGAG-3′ 5′-CCATGAATAAGCAGCTGATTGCCC-3′	59	2.00	891
bla$_{CTX-8}$	CTX-M-8-F CTX-M-8-R	5′-ACGAACACCGCGATC-3′ 5′-CGTGGGTTCTCGGGGATA-3′	57	2.00	490
bla$_{CTX-9}$	CTX-M-9-F CTX-M-9-R	5′-GATTGACCGTATTGGGAGTTT-3′ 5′-CGGCTGGGTAAAATAGGTCA-3′	57	2.00	947

T_{Ann} is the annealing temperature and Ti_{Elong} is the elongation time used in each case.
bla$_{TEM}$ = Temoniera β-lactamase; *bla*$_{OXA}$ = Oxacillinase β-lactamase; *bla*$_{CTX}$ = Cefotaxime β-lactamase.

2. Detection of Virulence Producing Genes by PCR Assay

The primers and the PCR parameters for detection of virulence genes of *E. coli* are presented in Table 2.2.

Table 2.2: Primers for the detection of virulent genes *E. coli*

Genes encoding virulence factors	Primers	Oligonucleotide sequence (5′ to 3′)	T_{Ann} (ºC)	Ti_{Elong} (min)	Size of amplified product (bp)
ipaH	ipaH-F ipaH-R	5′-TGGAAAAACTCAGTGCCTCT-3′ 5′-CCAGTCCGTAAATTCATTCT-3′	55	2.00	423
famH	fimH-F fimH-R	5′-CTGGATGGTATGGTGAGG-3′ 5′-GGAGGCCAACAATTATTTCC-3′	60	2.00	465
pap	Pap-F Pap-R	5′-GACGGCTGTACTGCAGGGTGTGG-3′ 5′-ATATCCTTTCTGCAGGGATGCAATA-3’	65	1.50	328
afa	afa-F afa-R	5′-GCTGGGCAGCAAACTGATAACTCT-3′ 5′-CATCAAGCTGTTTGTTCGTCCGCCG-3′	65	2.0	672
hyl	Hly-F Hly-R	5′-AACAAGGATAAGCACTGTTCTGGCT-3′ 5′-ACCATATAAGCGGTCATTCCCGTCA-3′	65	1.00	556
cnf	cnf-F cnf-R	5′-GAACTTATTAAGGATAGT-3′ 5′-CATTATTTATAACGCTG-3′	50	1.00	693

T_{Ann} is the annealing temperature and Ti_{Elong} is the elongation time used in each case.
ipaH = Invasion plasmid antigen H; *fimH* = Type 1 fimbrae; *pap* = Pilus-associated pylonephritis; *afa* = Afa adhesions; *hly* = Haemolysin; *cnf* = Cytotoxic necrotizing factor.

2.3.2.2 PCR Protocol

Reagents:

1. 10x PCR buffer (Gibco-BRL, USA)
2. 50 m*M* $MgCl_2$ (Gibco-BRL, USA)
3. 2.5 m*M* dNTP (Gibco-BRL, USA)
4. *Taq* DNA polymerase (5 U/µl, Gibco-BRL, USA)
5. Primers
6. Mineral oil (Gibco-BRL, USA)
7. Filtered deionized water
8. Agarose
9. Ethidium bromide (10 mg/ml)
10. TBE (Tris-borate EDTA) buffer 9 (Gibco-BRL, USA)
11. 100 bp DNA size standard (Bio-Rad, USA)

Procedure:

Representative isolates were grown on MacConkey agar for overnight. A single colony of each isolate was suspended in 25 µl of reaction mixer containing 2.5 µl of 10x PCR buffer, 1.5 µl of 50 m*M* $MgCl_2$, 2 µl of 2.5 m*M* dNTP, 1 µl of primer (forward and reverse) together with 1 unit of *Taq* DNA polymerase (5 U/µl). Volume of the mixture was adjusted by adding filtered deionized water. The reaction mixer was overlaid with a drop of mineral oil in order to prevent condensation. PCR assay were performed in a DNA thermal cycle (model 480: Perkin-Elmer Cetus, Emeryville, USA). Each PCR test used the same basic set-up: 96^{o}C for 5 min followed by 23 cycles of 20 sec at 96°C, 20 sec at $T_{Annealing}$ (°C) and $Ti_{Elongate}$ (min) at 72^{o}C, where $T_{Annealing}$ is the specific annealing temperature and $Ti_{Elongate}$ is the specific elongation time for each reaction, with a final extension at 72^{o}C for 10 min. A reagent blank, which contained all components of the reaction mixture with the exception of the bacteria, was included in every PCR procedure. ATCC E. coli (25922) strain was used as negative control for all PCR.

Shigella flexneri 2a, YSH 6000 was used as positive control for both invasive plasmid antigen H (*ipaH*). Amplication product were subjected to horizontal gel electrophoresis in 1% agarose gel in TBE (Tris-borate EDTA) buffer at room temperature at 100 volt (50 mA) for 1h. Carefully 10 µl of amplified DNA for each

sample was mixed with 1 µl of tracking dye and loaded into an individual well of the gel (5 mm thick). DNA bands were detected by staining the gel with ethidium bromide (0.5 µg/ml) for 30 min at room temperature and photographs were taken according to the procedure as described earlier.DNA sizes of 1 kb and 100 bp were used as standard (Bio-Rad, USA) as marker to measure the molecular size of the amplified products.

2.3.3 Pulsed-Field Gel Electrophoresis

2.3.3.1 Preparation of PFGE Agarose Plugs from Cell Suspensions

Cell suspensions were made in 2 ml of cell suspension buffer (CSB) from 14-16 h blood agar cultures. The concentration of the cell suspensions were adjusted with the Dade MicroScan Turbidity Meter as described in the following steps.

1. The digital output on the instrument was tested by inserting CSB "blank" tube in the left position (position 1) of the turbidity meter and one of the other tubes of CSB in sample position (right) (position 2) of the turbidity meter. The reading should be 0.00 ± 0.01.
2. A sterile loop was used to remove growth from the agar plates into the appropriate Falcon tube which contains 2 ml of CSB. The cells were suspended by drubbing against the wall the tube gently so that the cells are evenly dispensed and formation of aerosol is minimized.
3. The tubes were inserted into the sample right position to check the reading (desired range is 0.48-0.52).
4. If the reading was greater than 0.52, additional CSB was mixed and the reading was checked again until it is within the desired range.
5. If the reading was less than 0.48 additional growths was added from the agar plate to the cell suspension until the desired range was obtained.
6. The cell suspensions were kept in ice-bath.

Each of the cell suspensions (400 µl) were transferred to labeled microcentrifuge tubes using a 1 ml pipette and trip. The microcentrifuge tubes were placed in floating rack and incubated in water at 37°C water bath for 5 min. The cell suspensions (400 µl) cell were removed in 1.5-ml tubes from water bath and 20 µl proteinase K was added to each, and mixed by closing tubes and tapping side of tubes. Four hundred

microlitre melted 1% Seakem Gold 1% SDS agarose, which was kept in 56°C water bath, was added to one of the 400 µl cell suspensions and mixed gently by pipeting. Immediately part of mixture was dispensed into appropriate well in plug molds. The plugs were allowed to solidify for 10-15 min at room temperature.

2.3.3.2 Lysis of Cell in Agarose Plugs

Twenty five microlitre proteinase K stock solution (20 mg/ml) was added per 5 ml of cell lysis buffer (50 m*M* Tris: 50 m*M* EDTA, pH 8.0, 1% sarcosine) just before use. Five milliliter of proteinase K/cell lyses buffer was dispensed to each of the 50 ml tubes. The plugs were disposed in the cell lyses buffer. The tubes were placed in a rack and incubated in a shaking water bath at 54°C for 2 h with constant agitation.

2.3.3.3 Washing of Agarose Plugs after Cell Lysis

Tubes were removed from the shaking water bath and the lysis buffer was poured off 10 ml pre-warmed (50^{o}C) sterile Type 1 was added to each tube and returned to shaking water (50^{o}C). Tubes were shaken for 10 min. Water was poured off and these wash steps were repeated. After that, the water was poured off and 10 ml pre-warmed (50^{o}C) sterile TE was added and the tubes were shaken in shaking water bath (50^{o}C) for 15 min. These steps were repeated for four times and TE was poured off each, and finally, 5 ml TE was added and plugs were stored at 4^{o}C for subsequent step(s).

2.3.3.4 Restriction Digestion of DNA in Agarose Plugs with X*ba*1

Requirements:

1. Filtered deionzed water
2. 10x H buffer
3. *Xba*1 restricrtion enzyme (10 U/ ml)

Plugs were removed from tubes containing TE with wide end of spatula and were placed in a sterile disposable petri dish. These were then cut at a 2 mm wide slice from test samples and transferred to the labeled 1.5 ml micro centrifuge tubes containing 200 µl diluted H buffer (1:10 dilution). The rest of plugs were replaced in original tubes that contained 5 ml TE buffer. Three 2-mm wide slices of *Salmonella* serotype *Braenderup* standard plugs were cut and transferred to tubes of diluted H

buffer. The tubes were incubated in a 37ºC water bath for 10-15 min. After incubation of the plug slices the H buffer was removed.

Then each of the plugs was immersed in 200 µl of reaction mixer, which was prepared according to the following calculations:

10x *Xba*l restricrtion enzymes buffer	=	20 µl
*Xbal*1 restriction enzyme (10 U/µl)	=	5 µl
Filtered deionized water	=	175 µl
Total	=	200 µl

The samples and the control tubes were incubated in a water bath at 37°C for at least 2 h.

2.3.3.5 Casting Agarose Gel and Loading Restriction Plug Slices on the Coomb

Seakem Gold (SKG) agarose (Bio-Rad, USA) (1 g) was added to 100 ml of 0.5x TBE buffer in a 500-ml Erlenmeyer flask. The slurry was heated in the microwave oven until the agarose was dissolved completely. The temperature of the slurry was equilibrated to 54-58ºC in a water bath. The casting apparatus of the PFGE was assembled according to the instruction manual (Bio-Rad, USA). The comb was put on bench top and the plugs were loaded on the bottom of the comb teeth. The *Salmonella* serotype *Braenderup* standard plug slices were put on teeth, 1, 5, and 10 and the samples were loaded on the remaining teeth. Using a Pasteur pipette, the edges of the casting platform were sealed with a small quantity of the agarose solution and allowed to set. Then the remainder of the warm agarose solution was poured into the casting stand for a thickness of approximately 5-6 mm. The gel was allowed to solidify for 30 min at room temperature.

2.3.3.6 Performance of Pulse-Field Gel Electrophoresis

Pulse-field gel electrophoresis (PFGE) was performed with the Contour Clamped Homogenous Electric Field (CHEF-DRII) apparatus from the Bio-Rad Laboratories (USA). The electrophoresis chamber was filled with approximately 2.2 litre of running buffer (0.5x TBE). The electrophoresis apparatus was prepared according to the instruction manual (Bio-Rad, USA). The gel and the platform assembly were

placed into the frame. It was ensured that the gel was covered by about 2 mm of buffer. The temperature of the running buffer was adjusted to 14ºC and the flow rate of the buffer through the electrophoresis cell was maintained approximately at 0.75 litre per min. Electrophoresis was done at 6 volts for 20 h (initial switch time 2.2 sec, final switch time 63.8 sec). After the end of run, the gel was stained with ethidium bromide (0.5 µg/ml) solution for 30 min at room temperature and then destained in sufficient distilled water for 1 h. The gel was visualized on the UV transilluminator and photographs were taken as described previously. The DNA size standard was used the *Salmonella* serotype *Braenderup* (H9812**)** ranging from 20.5 to 1,135 kb.

Chapter 3 Results

3 Results

3.1 Phenotypic Characterizations

3.1.1 Bacterial Isolates

A total of 200 urine samples were collected from UTI patients. Of these, 100 samples were found to be positive as uropathogenic bacteria. Further, of 100 samples, 23 were found to contain *Klebsiella* which were discarded. The remaining 77 isolates were confirmed as *Escherichia coli* by short biochemical reactions such as KIA, MIU and citrate utilization followed by long sugar fermentation.

3.1.2 Screening of ESBL-Producing Organisms by Double Disc-Diffusion Method (DDD)

ESBL-producers were detected by double diffusion synergy test (DDST) by the standard method as described elsewhere. Of 77 strains, only 25 isolates were identified as ESBL-producing and the remaining 52 were found to be negative in DDST test and were regarded as non-ESBL. Figure 3.1 shows the enhancement of zone of inhibition by ceftazidime (CAZ) and aztreonam (ATM) towards amoxyclavulanic acid discs. However, no such enhancement of zone of inhibition was seen in case of cefoxtin (FOX).

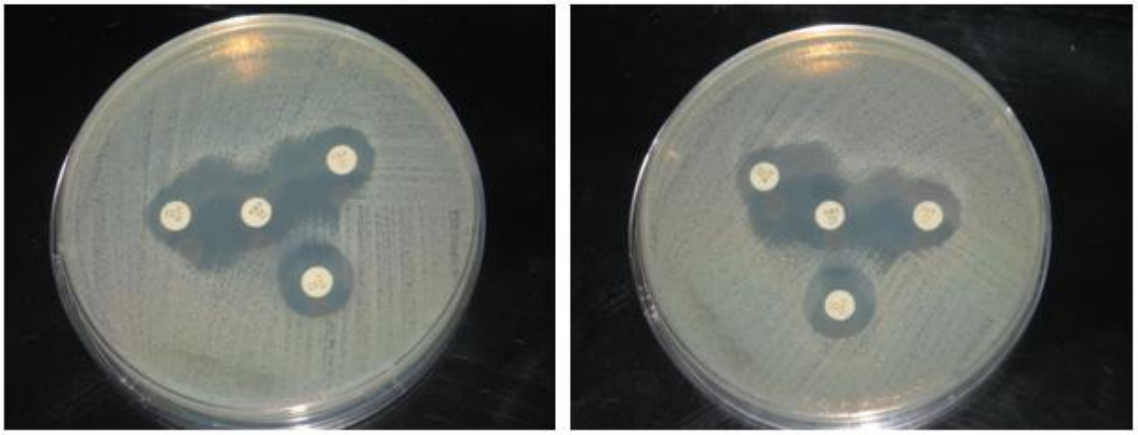

Figure 3.1: Photographs of ESBL-positive isolates of *E. coli* by double disc diffusion method showing enhancement of zone of inhibition towards amoxyclavulanic acid discs. Amoxyclavulanic acid disc is in the center, 3rd generation cephalosporin antibiotics ceftazidime (CAZ), aztreonam (ATM) discs and 2nd generation cephalosporin cefoxitin (FOX) disc are in the periphery. Enhancement of the zone of inhibition towards amoxyclavulanic acid has been observed in case of CAZ and ATM that were placed in center whereas no enhancement of the zone of inhibition was found in FOX.

The results in the Figure 3.1 shows the disc in the centre was amoxyclavulanic acid, while those at the periphery were 3rd generation cephalosporins, ceftazidime (CAZ), aztreonam (ATM) and 2nd generation cephalosporin cefoxitin (FOX).

3.1.3 Demographic Profile of the Studied Subjects

3.1.3.1 Sex Incidence of Urinary Tract Infections

The study period was for 12 months and urine sample were collected from 100 UTI patients having clinical manifestations. The age and sex of the patients were also recorded in these groups of patients harbouring, ESBL and non-ESBL-producing *E. coli*. The incidence of female subjects were higher (72%) compared to male subjects (28%) (Figure 3.2). Confidence interval for female population is 56.2 to 82.2% (p = 0.0005).

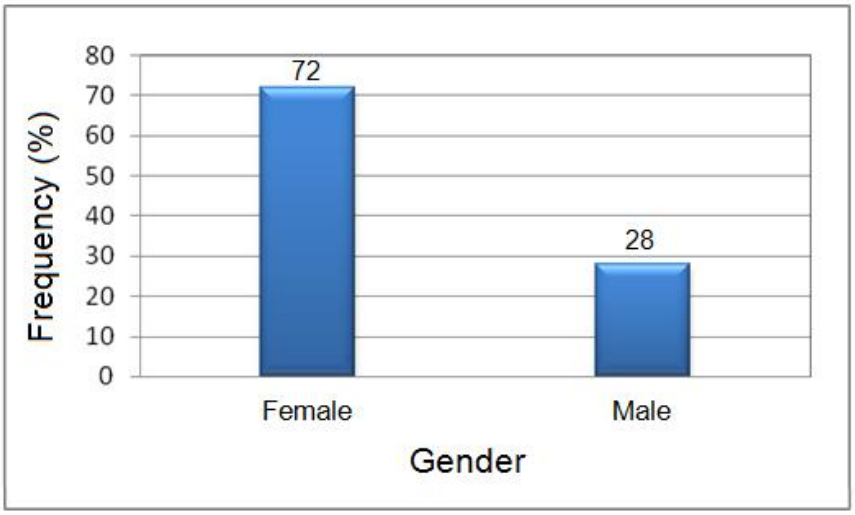

Figure 3.2: The percentage distribution of male and female populations suffering from urinary tract infections (n = 100).

3.1.3.2 Age Incidence of Urinary Tract Infections

Figure 3.3 shows the age distribution of patients that extended from 4-50 years and above. These patients were having symptomatic urinary tract infections (UTI) caused by ESBL-producing *Escherichia coli*. The highest incidence was observed in the age group 21-30 years. The next in order was from 31-40 years and above.

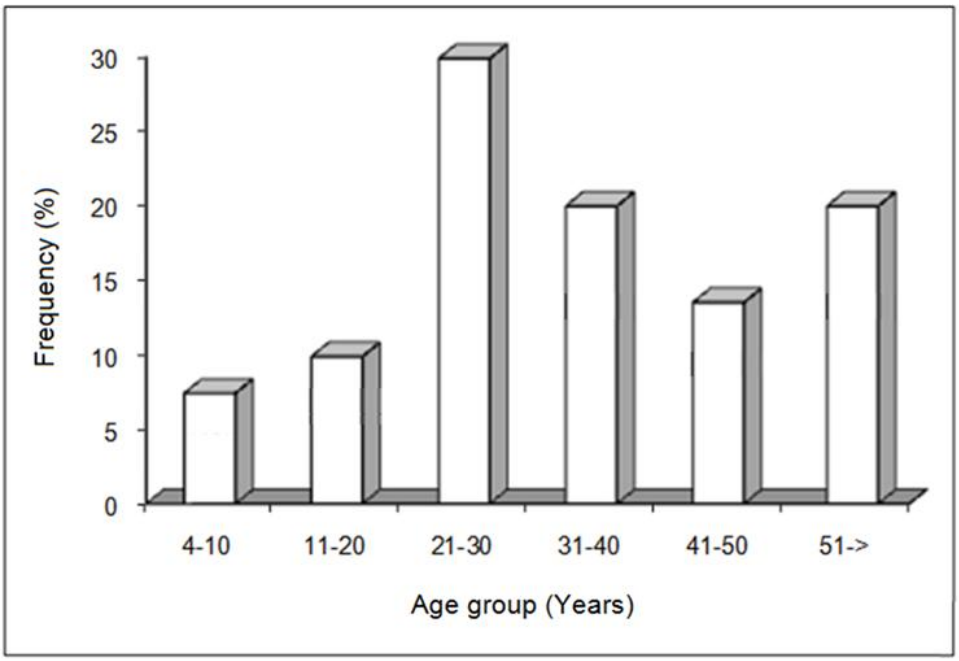

Figure 3.3: The age distribution of urinary tract infections (UTIs) patients having symptomatic UTIs caused by ESBL-producing *E. coli* (n = 100).

3.1.4 Clinical Presentation of the Urinary Tract Infection (UTI) Patients Caused by the ESBL-Producing *Escherichia coli*

3.1.4.1 Severity of Urinary Tract Infection (UTI) Patients

Figure 3.4 showes the severity of the 30 UTI patients caused by the ESBL-producing *E. coli*. The clinical presentations were broadly divided into three groups, namely, mild, moderate and severe.

Patients having bacterial urine culture ~10^5 CFU/ml associated with 5-10 microscopic urine leukocytes were designated as mild symptoms, whereas patients having bacterial culture >10^5 CFU/ml associated with microscopic urine leukocyte 10-15 were designated as moderate symptoms, and patients with bacterial culture >10^5 CFU/ml associated with microscopic leukocyte in urine numerous were grouped as severe symptomatic UTI patients. It was found that about 50% of patients were having mild form of symptoms and the remaining 40% were having moderate forms, and only 10% showed severe form of symptoms. Chi-square test (Epi Info version 5) was used to analyze the data statistically. Patients with mild symptoms were higher than patients with severe symptoms (p <0·001).

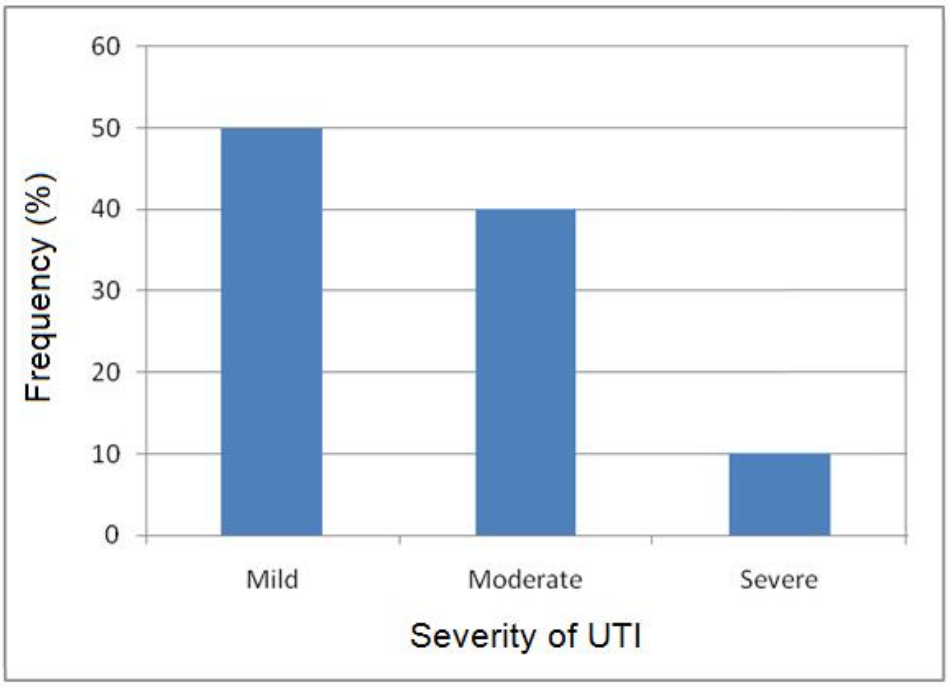

Figure 3.4: Severity of the urinary tract infection (UTI) patients caused by the ESBL-producing *E. coli* (n = 30).

3.1.4.2 Microscopic Findings and Clinical Features of UTI Patients Caused by the ESBL-Producing *Escherichia coli*

Table 3.1 shows the clinical features of patients having UTI caused by the ESBL-producing *E. coli*. Thirty patients (out of 77 patients with UTI caused by *E. coli*) revealed microscopic leukocyte in urine and they were mentioned as pus cells. Most of the patients were having fever, dysuria, and frequency of micturation. Suprapubic tenderness was negative in all patients. Six patients had plenty of pus cells.

3.1.4.3 Clinical Manifestation of UTI Patients of the Age Group 4-20 Years Caused by ESBL-Producing *Escherichia coli*

Clinical recording of patients suffering from urinary tract infection were done (Figure 3.5). Those patients who were harbouring ESBL-producing *E. coli* in their urine were taken into this study and a broad analysis was done based on clinical manifestations of UTI patients with the age group of 4-20 years. Most of the patients had fever (100%), dysuria (80%) and frequency of micturation (100%). Suprapubic tenderness was absent in all cases. Microscopic leukocytes were present in urine if their count was above 5 cells per HPF (high power field). Two (40%) patients had abundance of pus cell in their urine.

Table 3.1: Microscopic findings and clinical features of UTI patients caused by ESBL- producing *Escherichia coli* (n = 30)

Patient	Microscopic and clinical findings				
	Pus cells in urine	Fever with chill*	Dysuria	Frequency of micturation	Suprapubic tenderness
1	10-15	+	+	+	−
2	10-12	+	+	+	−
3	4-6	+	−	+	−
4	6-8	+	−	−	−
5	Plenty	+	++	+	−
6	Plenty	+	++	+	−
7	10-15	+	++	+	−
8	8-10	+	++	+	−
9	Plenty	+	++	+	−
10	Plenty	+	++	+	−
11	8-10	+	+	+	−
12	Plenty	+	++	+	−
13	5-8	+	+	+	−
14	6-8	+	+	+	−
15	6-8	+	+	+	−
16	Plenty	++	++	+	−
17	15-20	++	++	+	−
18	2-3	+	+	+	−
19	10-12	++	++	+	−
20	10-12	+	++	+	−
21	6-8	+	−	+	−
22	6-8	+	−	+	−
23	8-10	++	++	+	−
24	8-10	+	++	+	−
25	6-8	+	+	+	−
26	8-10	+	+	+	−
27	3-4	+	+	+	−
28	5-6	+	+	+	−
29	8-12	++	+	+	−
30	10-12	++	+	+	−

*Fever (+) = <100°C; Fever (++) = >100°C.

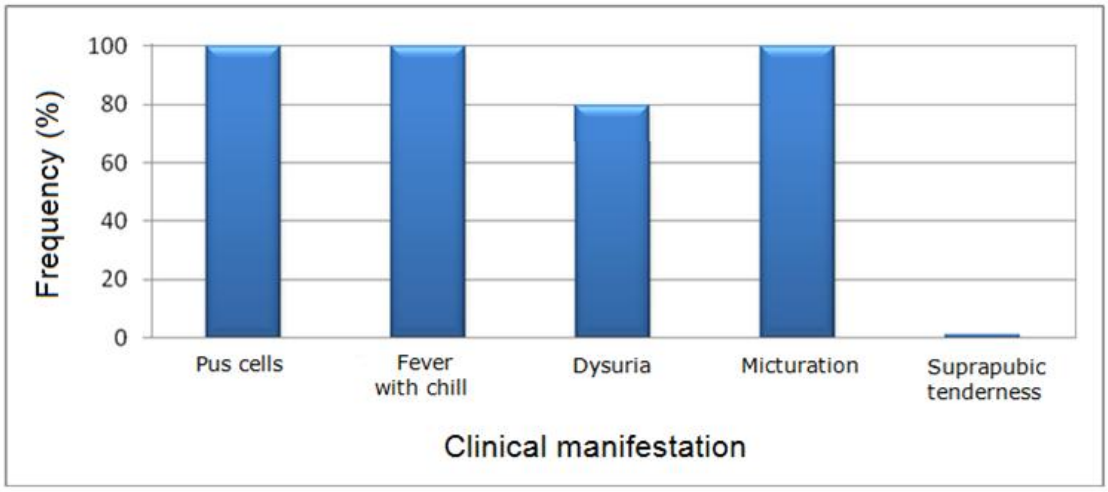

Figure 3.5: Clinical manifestation of UTI patients caused by ESBL-producing *Escherichia coli* of the age group 4-20 years (n = 5).

3.1.4.4 Clinical Manifestation of UTI Patients of the Age Group 21-40 Years Caused by ESBL-Producing *Escherichia coli*

Clinical recording of patients suffering from urinary tract infection were carried out Those patients who were harbouring ESBL- producing *E. coli* in their urine, were taken into this study and a broad analysis was done based on clinical manifestations of UTI patients between the ages 21-40 years (Figure 3.6). Most of the patients had fevers (100%), dysuria (87%) and frequency of micturation (93%). Suprapubic tenderness was not observed in any cases. Microscopic leukocytes (pus cells) were present in urine if their count was above 5 cells per HPF. Nine (60%) patients had abundance of pus cells in their urine.

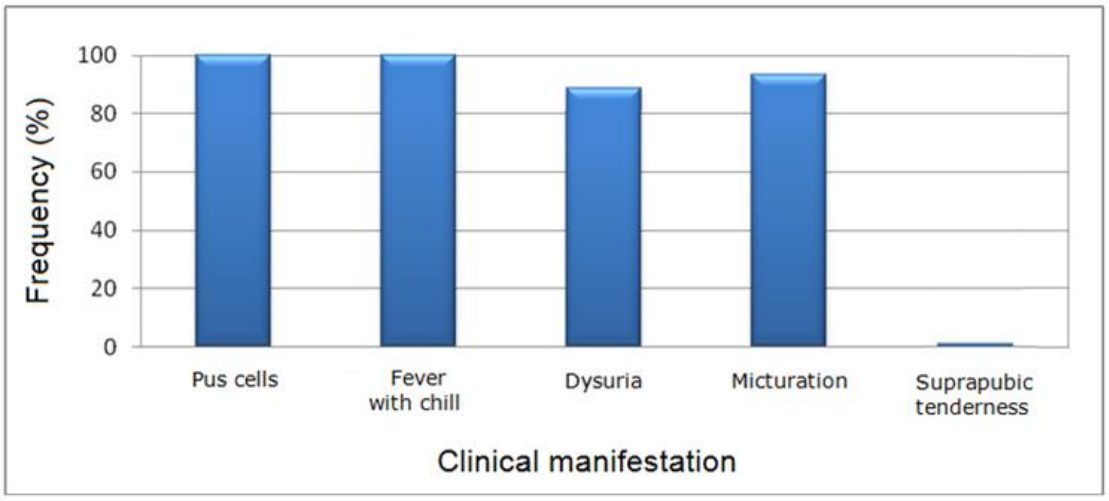

Figure 3.6: Clinical manifestation of UTI patients caused by ESBL-producing *Escherichia coli* of the age group 21-40 years (n = 15).

3.1.4.5 Clinical Manifestation of UTI Patients of the Age Group 41-60 Years Caused by ESBL-Producing *Escherichia coli*

Clinical recording of patients suffering from urinary tract infection was carried out in age groups 40-60 years. Those patients who were harbouring ESBL-producing *E. coli* in their urine were taken into this study. The results showed all patients had microscopic leukocytes (pus cells) in urine (Figure 3.7). Six (60%) patients had abundance of pus cells in urine. Frequency of micturation and fever were present in all cases. Suprapubic tenderness was absent in all cases. Dysuria was observed in 90% cases.

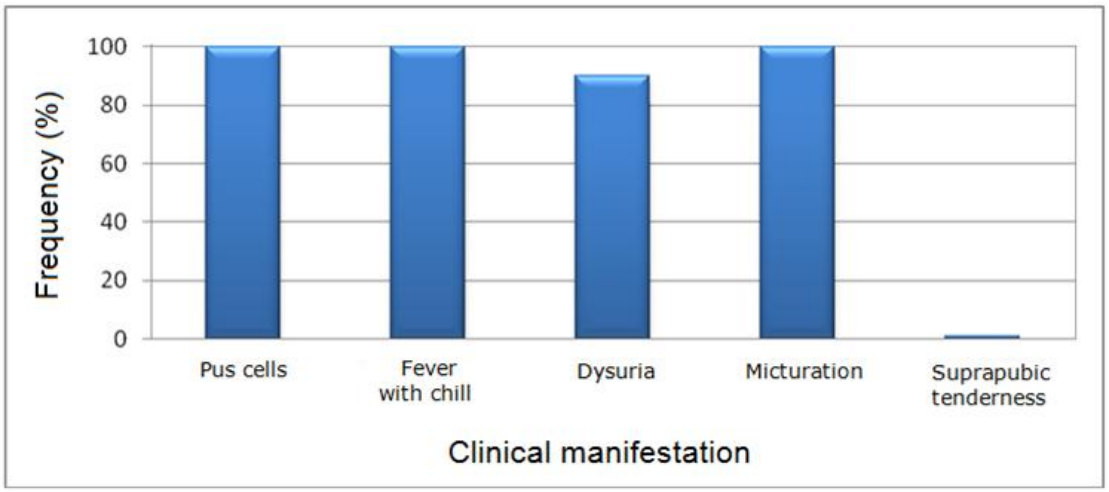

Figure 3.7: Clinical manifestation of UTI patients caused by ESBL-producing *Escherichia coli* of the age group 41-60 years (n = 10).

3.1.5 Antibiotic Susceptibility Pattern of *Escherichia coli* Isolates from Patients With Urinary Tract Infections (UTIs)

3.1.5.1 Antibiotic Susceptibility of *Escherichia coli* (Both ESBL and Non-ESBL) Isolates

Antibiotic susceptibility was done for urine isolates, which were confirmed as *E. coli*. The standard method of antibiotic susceptibility was done by Kirby-Bauer method. The isolates were grown in 2 ml of Muller-Hinton broth at 37ºC for 4 h. The young isolates were inoculated on Muller-Hinton agar. Finally, appropiate antibiotic discs were placed on the lawn having the isolates and incubated at 37ºC overnight. After 18 to 24 h, the appropiate antibiotic susceptibilities were examined. The *E. coli* isolates exhibited the highest (100%) suspectibility against carbepenem [imipenem (IPM) and meropenem (MPM)] (Figure 3.8). Amoxyclave (AMC) was effective against three-fourth (75%) of the isolates, while one-fourth (25%) isolates showed *in vitro*

resistance against the antibiotics. Ceftriaxone (CRO), ceftazidime (CAZ) and ciprofloxacin (CIP) were effective against 70, 67 and 43% of the *E. coli* isolates, respectively. Antibiotics like amoxicillin (AMC) and cotrimoxazole (SxT) were found inactive as 98-99% of the isolates exhibited resistace in disc-diffusion assays (data not shown).

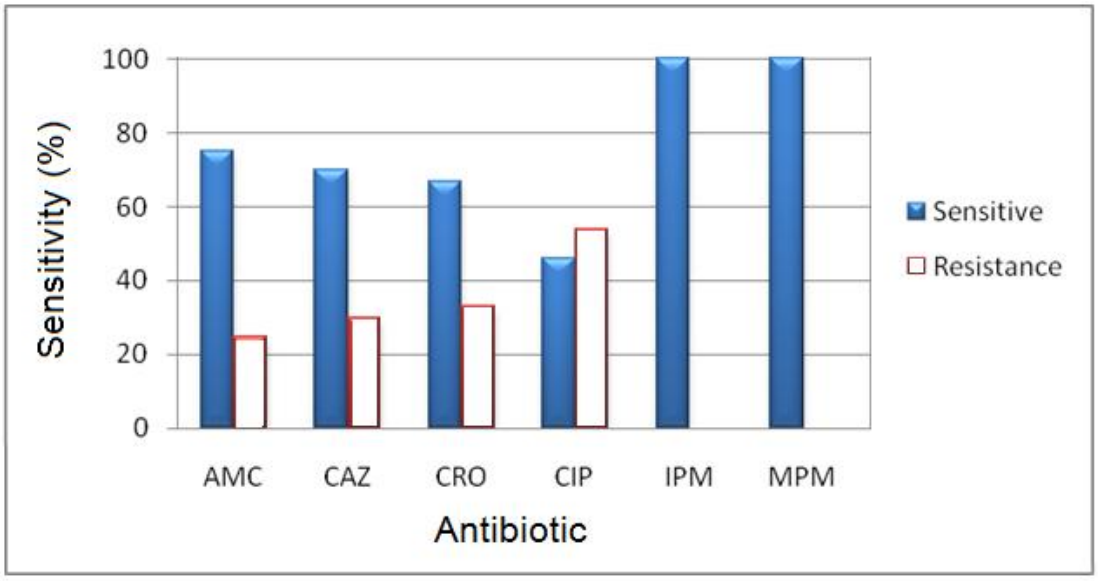

Figure 3.8: The antibiotic susceptibility to *E. coli* isolates recovered from UTI patients (n = 77).

Some ESBL isolates were sensitive to 2nd generation cephalosporins (FOX). Two ESBL isolates were also found resistant to other cephalosporin group of antibiotics (data not shown). The sensitivity patterns were higher in non-ESBL isolates as compared to ESBL isolates.

3.1.5.2 Antibiotic Susceptibility Pattern of the Non-ESBL-Producing *E. coli* Isolates obtained from the Patients with Symptomatic UTI

Twenty non-ESBL-producing E. coli isolates were tested for their resistance/sensitive pattern against two second generation and three third generation antibiotics using agar disc-diffusion method (Figure 3.9). The resistance rates among the non-ESBL isolates were 60% to ciprofloxacin (CIP), 40% to ceftazidime (CAZ), 40% to ceftriaxone (CRO), and 30% to amoxyclavulanic acid (AMC). All the isolates were susceptible to meropenem (MPM) and imepenem (IMP). Antibiotics like aztreonam (ATM) and cefepime (FEP) were also found resistant (data not shown).

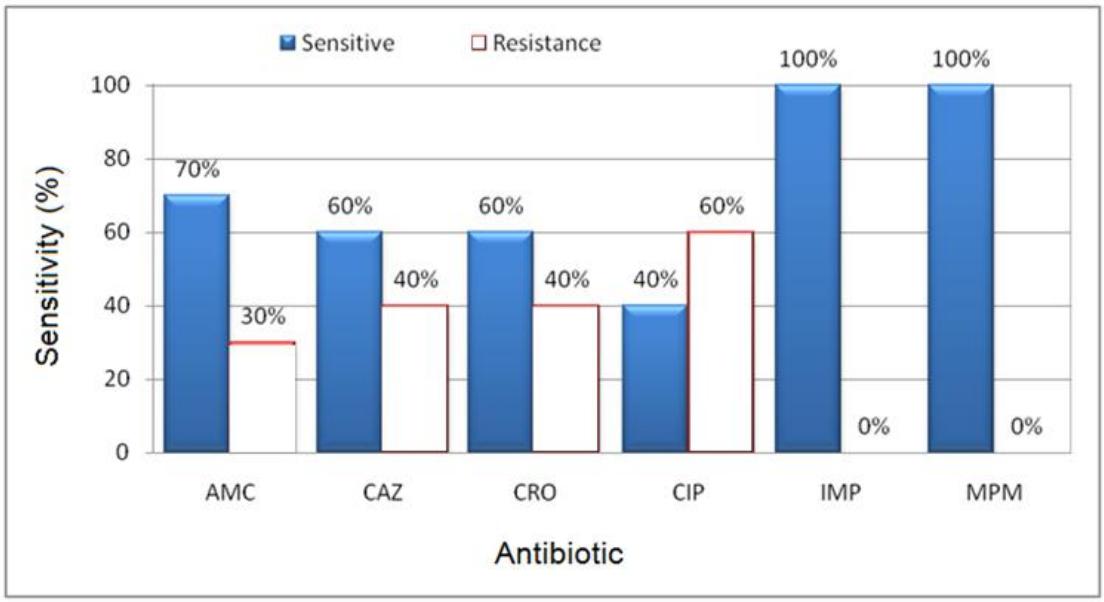

Figure 3.9: Antibiogram of the non-ESBL-producing *Escherichia coli* isolates (n = 20) obtained from urine samples of the patients with symptomatic urinary tract infection (UTI).

3.1.5.3 Antibiotic Susceptibility Pattern of the ESBL-Producing *E. coli* Isolates obtained from the Patients with Symptomatic UTI

Twenty positive ESBL-producing *E. coli* isolates were tested for their sensitivity pattern to commonly used nsecond generation and third generation antibiotics. All isolates were recovered from urine samples of the patients with symptomatic UTI. Figure 3.10 depicts the zone of inhibition and resistance pattern toward different antibiotics discs.

The results of the antibiogram are depicted in Figure 3.11. All the isolates tested exhibited susceptibility (100%) to meropenem (MPM) and imepenem (IMP). On the other hand, 80% of the isolates exhibited resistance to amoxyclavulanic acid (AMC), ceftriaxone (CRO) and ciprofloxacin (CIP). Ceftazidime (CAZ) seemed to be ineffective against more than 50% of the ESBL-isolates. Antibiotics like aztreonam (ATM), cefepime (FEP) and nalidixic acid (NA) were found to be ineffective against the ESBL-isolates (data not shown). Only few ESBL isolates, however, showed sensitive to the second generation cephalosporin (FOX) antibiotic. All the ESBL isolates were multi-drug resistant (MDR).

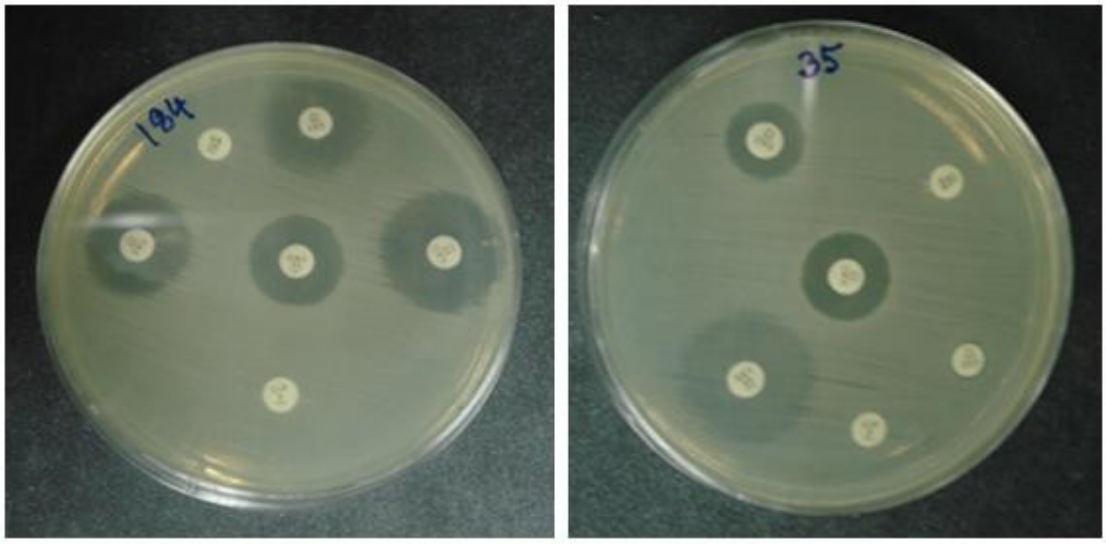

Figure 3.10: Antibiogram of representative ESBL-producing *Escherichia coli* isolates (n = 20) obtained from urine samples of the patients with symptomatic urinary tract infection (UTI). The antibiotic discs used were nalidixic acid (NA), amoxyclavulanic acid (AMC), ceftriaxone (CRO), ciprofloxacin (CIP) and trimethoprim-sulfamethoxazole (SxT).

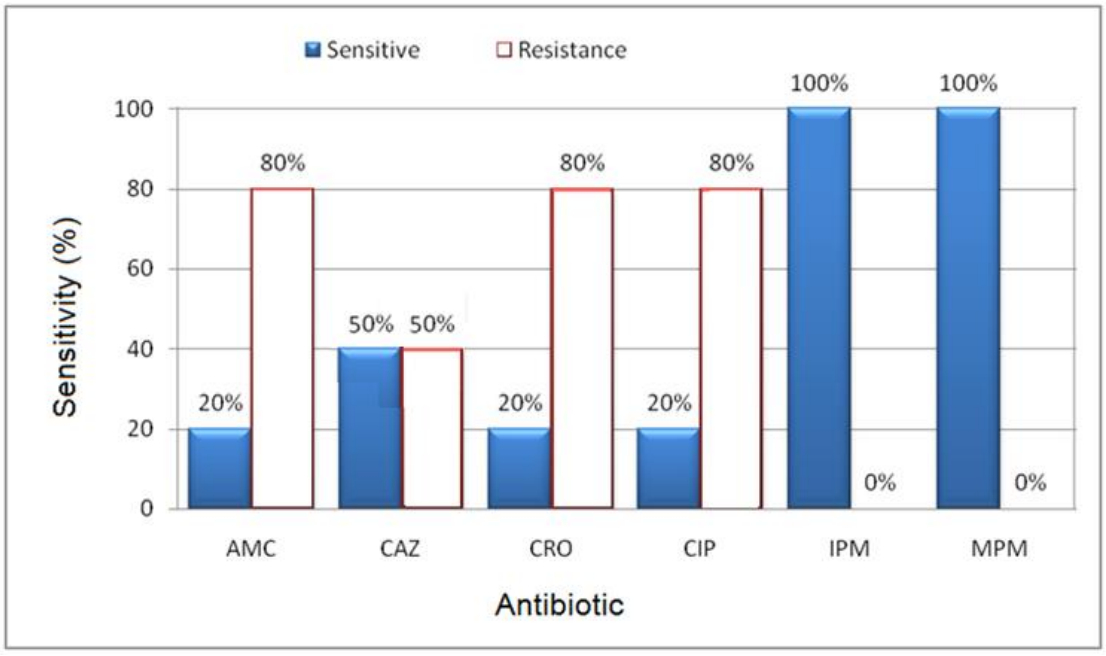

Figure 3.11: Antibiogram of the ESBL-producing *Escherichia coli* isolates (n = 20) obtained from urine samples of the patients with symptomatic urinary tract infection (UTI).

3.1.6 Serogrouping of the *Escherichia coli* Isolates

3.1.6.1 Serological Characteristics of ESBL-Producing *E. coli* Isolates

Serogroups of the tested organisms (n = 25) using the commercially available antisera (Denka Saiken Co. Ltd, Japan) as well as monovalent antibodies specific for *E. coli*

were shown in Table 3.2. The encounted serogroups included: O126, O2, O15, O1, O153, O15 and O6. These serogroups were found to be heterogenous. The most frequently encountered serotype among the ESBL isolates was O153, representing 24%. Seven (28%) of ESBL isolates could not be serogrouped and they were designated as untypable and 5 (20%) isolates showed rough reactions, which were designated as nonspecific.

Table 3.2: Serological characteristics of ESBL-producing *Escherichia coli* tested with commercially available antisera (n = 25)

ESBL *E. coli* isolate (E)	Monovalent antisera and *E. coli* serotype*									Typability
	P1	P2	P3	P4	P5	P6	P7	P8	P9	
E01	–	–	–	–	–	–	–	–	–	Nonspecific
E02	–	O126	–	–	–	–	–	–	–	Typable
E03	–	–	–	–	O2	–	–	–	–	Typable
E04	–	–	–	–	–	–	–	–	–	Untypable
E05	–	–	–	–	–	–	–	–	–	Nonspecific
E06	–	–	–	–	–	–	–	–	–	Nonspecific
E07	–	–	–	–	–	–	–	–	–	Nonspecific
E08	–	–	–	–	–	–	–	–	–	Untypable
E09	–	–	–	–	–	O15	–	–	–	Typable
E10	O1	–	–	–	–	–	–	–	–	Typable
E11	–	–	–	–	–	–	–	–	–	Untypable
E12	–	–	–	–	–	–	–	–	–	Untypable
E13	–	–	–	–	O153	–	–	–	–	Typable
E14	–	–	–	–	–	–	–	–	–	Untypable
E15	–	–	–	–	–	–	–	–	–	Untypable
E16	–	–	–	–	–	–	–	–	–	Nonspecific
E17	–	–	–	–	O153	–	–	–	–	Typable
E18	–	–	–	–	O6	–	–	–	–	Typable
E19	–	–	–	–	O153	–	–	–	–	Typable
E20	–	–	–	–	O6	–	–	–	–	Typable
E21	–	–	–	–	O153	–	–	–	–	Typable
E22	–	–	–	–	O153	–	–	–	–	Typable
E23	–	–	–	–	–	–	–	–	–	Untypable
E24	–	–	–	–	–	–	–	–	–	Typable
E25	–	–	–	–	O153	–	–	–	–	Typable

**E. coli* isolates were confirmed by serologic method using monovalent and polyvalent antisera specific for *E. coli*. P indicates as polyvalent antisera.

3.1.6.2 Serological Characteristics of Non-ESBL-Producing *E. coli* Isolates

The serological characteristics of the non-ESBL *E. coli* isolates are summarized in Table 3.3. The isolates represent a diverse or heterogeneous serogroupings. Serotype O25 monovalent predominates among the non-ESBL *E. coli* isolates.

Table 3.3: Serological characteristics of non-ESBL-producing *Escherichia coli* tested with commercially available antisera (n = 30)

Non-ESBL *E. coli* isolate (NE)	Monovalent antisera and *E. coli* serotype*									Typability
	P1	P2	P3	P4	P5	P6	P7	P8	P9	
NE01	–	–	–	–	–	–	–	–	–	Untypable
NE02	–	O146	–	–	–	–	–	–	–	Typable
NE03	–	–	–	–	O25	–	–	–	–	Typable
NE04	O86a	–	–	–	–	–	–	–	–	Typable
NE05	–	–	–	–	–	–	–	–	–	Untypable
NE06	–	O44	–	–	–	–	–	–	–	Typable
NE07	–	–	–	–	–	–	O144	–	–	Typable
NE08	–	–	–	–	–	–	–	–	–	Untypable
NE09	–	–	–	–	O25	–	–	–	–	Typable
NE10	–	–	–	–	–	–	O112ac	–	–	Typable
NE11	–	–	–	–	–	–	–	–	–	Untypable
NE12	–	–	–	–	–	–	–	O152	–	Typable
NE13	–	–	O18	–	–	–	–	–	–	Typable
NE14	–	–	–	–	–	–	–	–	O74	Typable
NE15	–	O55	–	–	–	–	–	–	–	Typable
NE16	–	–	–	–	O25	–	–	–	–	Typable
NE17	–	–	–	–	–	–	–	–	–	Untypable
NE18	–	–	–	–	–	O8	–	–	–	Typable
NE19	–	–	–	–	–	–	–	–	–	Untypable
NE20	–	–	O157	–	–	–	–	–	–	Typable
NE21	–	–	–	–	–	–	–	–	–	Untypable
NE22	O86a	–	–	–	–	–	–	–	–	Typable
NE23	O1	–	–	–	–	–	–	–	–	Typable
NE24	–	–	–	–	–	O8	–	–	–	Typable
NE25	–	–	–	–	–	–	–	–	–	Untypable
NE26	–	–	–	–	–	–	–	–	–	Untypable
NE27	–	–	–	–	O25	–	–	–	–	Typable
NE28	–	–	–	O159	–	–	–	–	–	Typable
NE29	–	–	–	–	–	–	–	–	O103	Typable
NE30	–	–	–	–	O153	–	–	–	–	Typable

**E. coli* isolates were confirmed by serologic method using monovalent and polyvalent antisera specific for *E. coli*. P indicates as polyvalent antisera.

Interesting to note that serotype of ESBL-producing *E. coli* did not match with serotypes of non-ESBL-producing *E. coli*. More than two-third (70%) non-ESBL *E. coli* isolates was typable, while the rest (30%) was untypable using the system used.

3.2 Genotypic Characterizations

3.2.1 Plasmid Profile Analysis of the ESBL-Producing *Escherichia coli* Isolates

The plasmid profile was carried out on ESBL-producing isolates from urine samples of symptomatic urinary tract infections. The investigation was done with the view to analyse the plasmid distribution of high range (≥140 MDa), middle range (30-90 MDa) and low range. As mentioned earlier that these plasmids form a unique band in agarose gel electrophoresis.

Analysis of the plasmid DNA by agarose gel electrophoresis showed that the isolates contained multiple numbers of plasmids ranging from 1.0 to 140 MDa, which formed a unique banding pattern in many of these isolates (Figure 3.12 and 3.13).

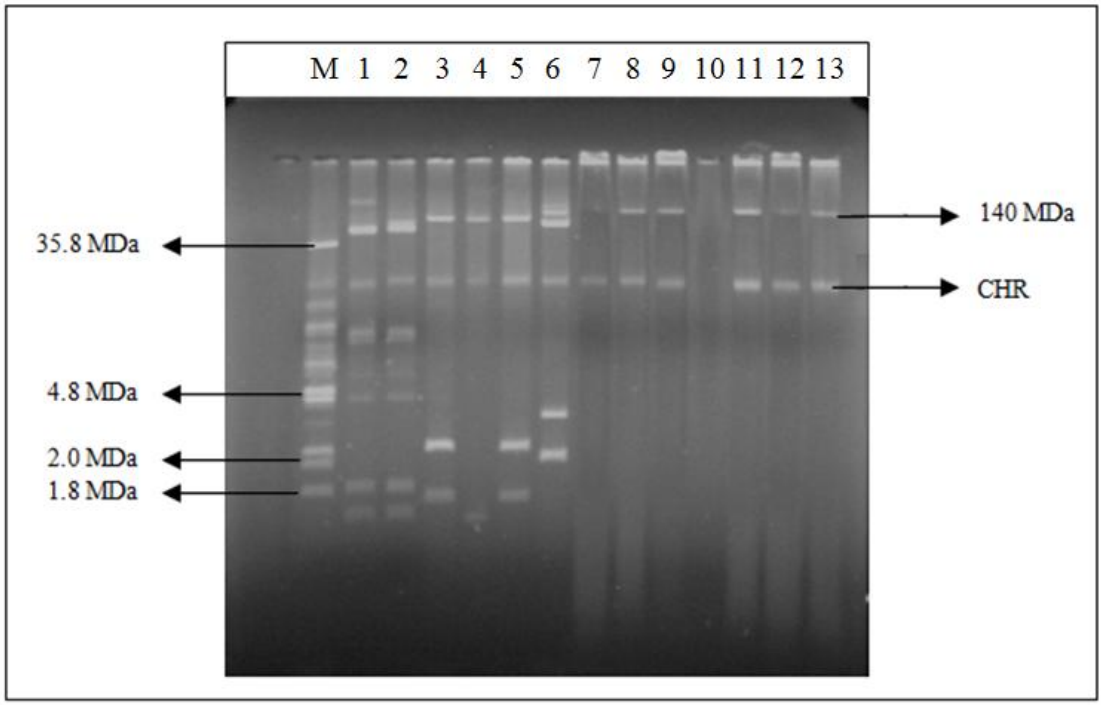

Figure 3.12: Agarose gel electrophoresis of plasmid DNA showing representative of different ESBL-producing *E. coli* isolates. Lane M: V517 (molecular marker); Lane 1: E01; Lane 2: E02; Lane 3: E03; Lane 4: E04; Lane 5: E05; Lane 6: E06; Lane 7: E07; Lane 8: E08; Lane 9: E09; Lane 10: E10; Lane 11: E11; Lane 12: E12; Lane 13: E13. CHR indicates the banding position of the chromosomal DNA.

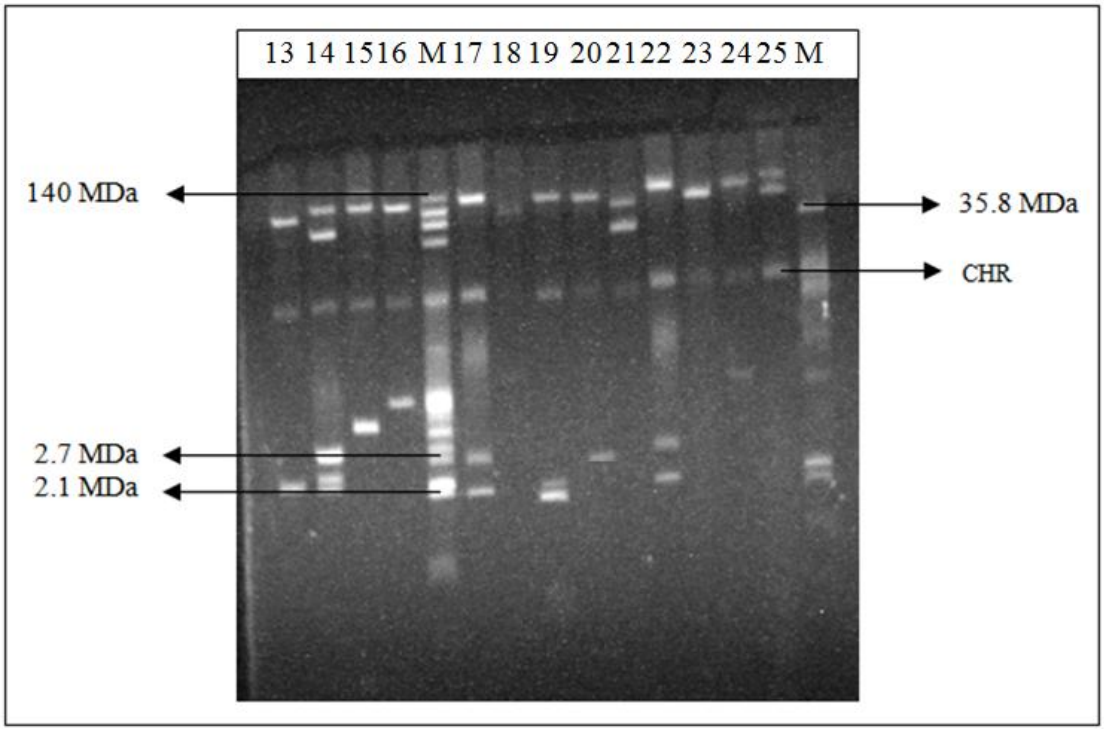

Figure 3.13: Agarose gel electrophoresis of plasmid DNA showing representative of different ESBL-producing *E. coli* isolates. Lane 13: E13; Lane 14: E14; Lane 15: E15; Lane 16: E16; Lane M: PDK9 (molcular marker); Lane 17: E17; Lane 18: E18; Lane 19: E19; Lane 20: E20; Lane 21: E21; Lane 22: E22; Lane 23: E23; Lane 24: E24; Lane 25: E25; Lane M: V517 (molcular marker). CHR indicates the banding position of the chromosomal DNA.

Plasmid patterns were grouped according to the number and molecular weights of the plasmids and each isolate belonging to a particular group. High range plasmids were designated as ≥140 MDa (PDK9 marker) and middle ranged plasmids were designated as 30 to 90 MDa (V517 marker). Low range plasmids were distributed heterogenously in the ESBL-producing strains.

Table 3.4 shows the distribution of plasmids, which ranged from 1 to 140 MDa in the ESBL group of isolates. Of the 25 ESBL isolates tested, 19 (76%) isolates showed 140 MDa and 9 isolates (36%) showed middle range plasmids (30-90 MDa). The rest (48%) of the plasmids were distributed as low range plasmids in heterogenous manner.

The plasmids distribution pattern in 25 ESBL-producing *E. coli* isolates is depicted in Figure 3.14. The results clearly showed that in the ESBL *E. coli* isolates the high range plasmids predominates over the middle range and low range plasmids.

Table 3.4: Plasmid profile analysis of the ESBL-producing *Escherichia coli*

ESBL *E. coli* isolate (E)	Plasmid molecular weight (in MDa)								
	140	30-90	20	14	7.8	5-5.9	3-3.9	2-2.9	1-1.9
E01	+	+	−	−	−	+	+	+	+
E02	−	+	−	−	−	+	+	+	+
E03	+	−	−	−	−	−	−	+	+
E04	+	−	−	−	−	−	−	−	−
E05	+	−	−	−	−	−	−	+	+
E06	+	+	−	−	−	−	+	+	−
E07	−	−	−	−	−	−	−	−	−
E08	+	−	−	−	−	−	−	−	−
E09	+	−	−	−	−	−	−	−	−
E10	−	−	−	−	−	−	−	−	−
E11	+	−	−	−	−	−	−	−	−
E12	+	−	−	−	−	−	−	−	−
E13	−	+	−	−	−	−	−	−	−
E14	+	+	−	−	−	−	−	+	+
E15	+	+	−	−	−	−	+	−	−
E16	+	−	−	−	−	+	−	−	−
E17	+	−	−	−	−	−	+	+	−
E18	+	−	−	−	−	−	−	−	−
E19	+	−	−	−	−	−	−	+	+
E20	+	−	−	−	−	−	−	+	−
E21	−	+	−	−	−	−	−	−	−
E22	+	−	−	−	−	−	+	+	−
E23	−	+	−	−	−	−	−	−	−
E24	+	−	−	−	−	−	−	−	−
E25	+	+	−	−	−	−	−	−	−

140 MDa (+) indicates the presence of large range plasmid; 30-90 MDa indicates the presence of medium range plasmids. The rest of the plasmids were distributed as low range molecular weights.

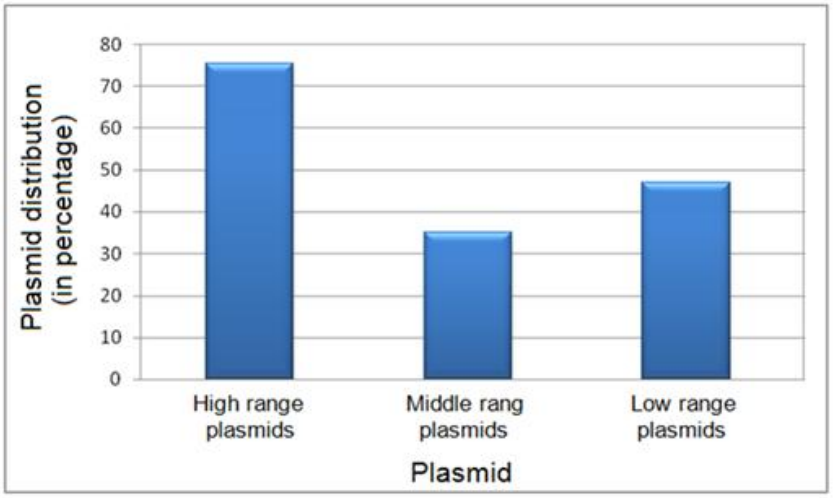

Figure 3.14: The distribution of high-, middle- and low range plasmids among the ESBL-producing *Escherichia coli* isolates.

3.2.2 Plasmid Profile Analysis of the Non-ESBL-Producing *Escherichia coli* Isolates

Analysis of the plasmid DNA by agarose gel electrophoresis showed that the isolates contained multiple plasmids ranging from 1.0 to 140 MDa, which formed a unique banding pattern in many of these isolates (Figure: 3.15 and 3.16). Plasmid patterns were grouped according to the number and molecular weights of the plasmids and each isolate belonging to a particular group (Table 3.5). Those isolate having high molecular weights 140 MDa or above were matched with the molecular marker PDK9, which had standard molecular weight 140 MDa. Of 25 isolates tested, 15 isolates revealed high molecular weight plasmids, which constituted 60% of the total plasmids. Five isolates showed the middle range plasmid (30-90 MDa) that constituted 20% of the total isolates.

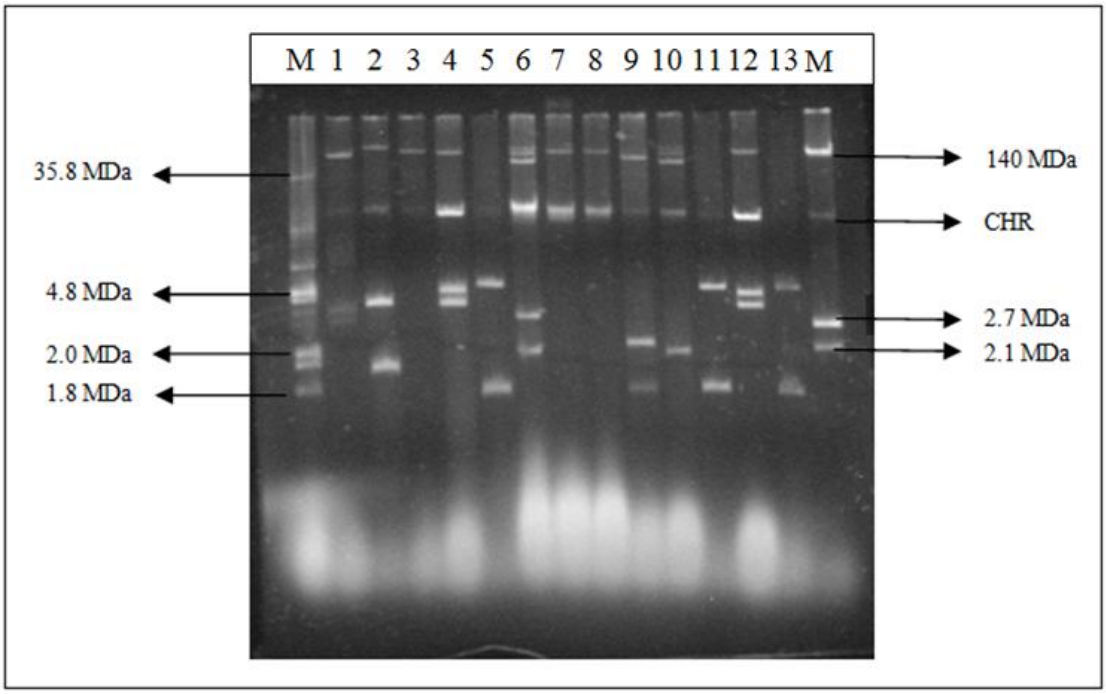

Figure 3.15: Agarose gel electrophoresis of plasmid DNA showing representative of different non-ESBL-producing *E. coli* isolates. Lane M: V517 (molecular marker); Lane 1: NE01; Lane 2: NE02; Lane 3: NE03; Lane 4: NE04; Lane 5: NE05; Lane 6: NE06; Lane 7: NE07; Lane 8: NE08; Lane 9: NE09; Lane 10: NE10; Lane 11: NE11; Lane 12: NE12; Lane 13: NE13. CHR indicates the banding position of the chromosomal DNA.

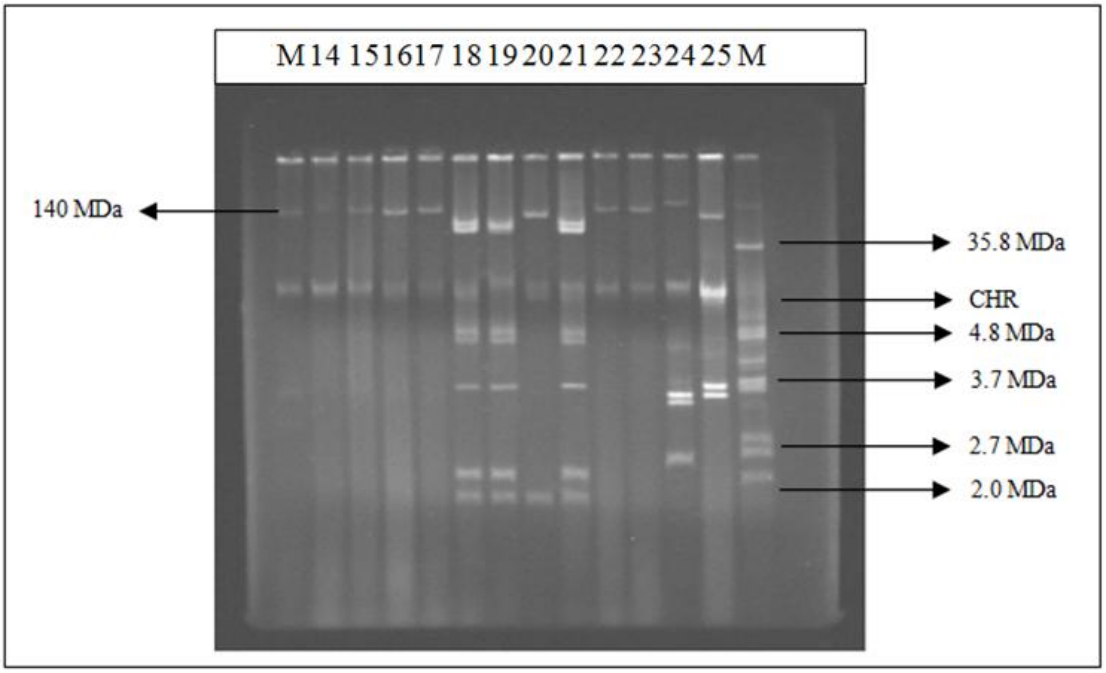

Figure 3.16: Agarose gel electrophoresis of plasmid DNA showing representative of different ESBL-producing *E. coli* isolates. Lane M: PDK9 (molcular marker); Lane 14: NE14; Lane 15: NE15; Lane 16: NE16; Lane 17: NE17; Lane 18: NE18; Lane 19: NE19; Lane 20: NE20; Lane 21: NE21; Lane 22: NE22; Lane 23: NE23; Lane 24: NE24; Lane 25: NE25; Lane M: V517 (molcular marker). CHR indicates the banding position of the chromosomal DNA.

Table 3.5 represents the results of the plasmid profile analysis of 25 non-ESBL *E. coli* isolates. A wide range of plasmids was distributed among the isolates. Of 25 isolates, 15 isolates (60%) harboured high range plasmids (140 MDa) and 5 isolates (20%) contained middle range plasmids. The remaining (55%) plasmids were distributed in heterogenous manner as low range plasmids.

The plasmids distribution pattern in 25 non-ESBL-producing *E. coli* isolates is shown in Figure 3.17. The results clearly showed that high range plasmids predominates and there was no difference between ESBL and non-ESBL isolates regarding plasmid distribution.

Table 3.5: Plasmid profile analysis of the non-ESBL-producing *Escherichia coli*

Non-ESBL *E. coli* isolate (NE)	Plasmid molecular weight (in MDa)								
	140	30-90	20	14	7.8	5-5.9	3-3.9	2-2.9	1-1.9
NE01	+	−	−	−	−	−	−	−	−
NE02	+	−	−	−	−	−	+	+	−
NE03	+	−	−	−	−	−	−	−	−
NE04	+	−	−	−	−	+	+	+	−
NE05	−	−	−	−	−	+	−	−	+
NE06	+	+	−	−	−	−	+	+	−
NE07	+	−	−	−	−	−	−	−	−
NE08	+	−	−	−	−	−	−	−	−
NE09	−	+	−	−	−	−	+	+	−
NE10	−	+	−	−	−	−	−	+	−
NE11	−	−	−	−	−	+	−	+	−
NE12	+	−	−	−	−	+	+	−	−
NE13	−	−	−	−	−	+	−	+	−
NE14	+	−	−	−	−	−	−	−	−
NE15	+	−	−	−	−	−	−	−	−
NE16	+	−	−	−	−	−	−	−	−
NE17	+	−	−	−	−	−	−	−	−
NE18	−	+	−	−	−	−	+	−	−
NE19	−	+	−	−	−	−	−	+	−
NE20	+	−	−	−	−	−	−	−	+
NE21	+	−	−	−	−	−	−	−	+
NE22	−	−	−	−	−	−	−	−	+
NE23	−	−	−	−	−	−	+	+	−
NE24	−	−	−	−	−	−	+	−	−
NE25	+	−	−	−	−	−	+	+	−

140 MDa (+) indicates the presence of large range plasmid; 30-90 MDa indicates the presence of medium range plasmids. The rest of the plasmids were distributed as low range molecular weights.

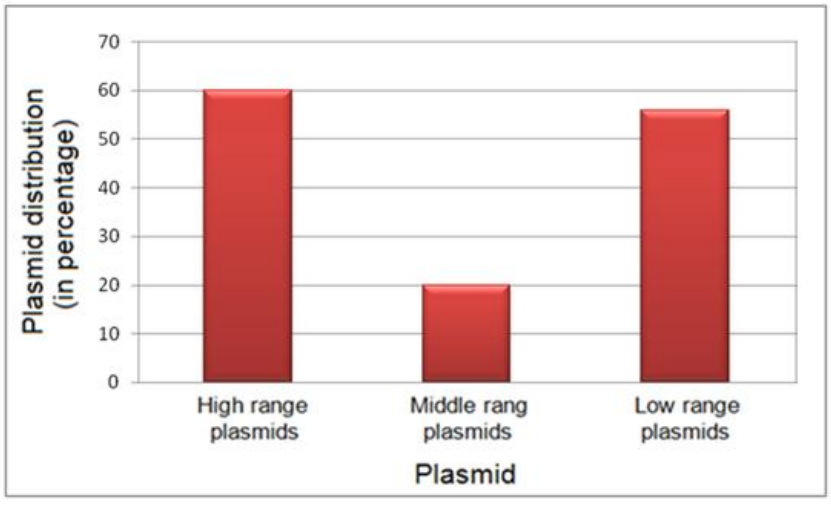

Figure 3.17: The distribution of high-, middle- and low range plasmids among the non-ESBL-producing *Escherichia coli* isolates.

3.2.3 Detection of β-Lactamase-Producing Genes in ESBL-Producing *Escherichia coli* Isolates

3.2.3.1 Detection of β-Lactamase-Producing Genes by Polymerase Chain Reaction

Detection of β-lactamase-producing genes was done by PCR. PCR analysis revealed that all the isolates produce either one or more ESBL-producing genes (Figure 3.18). The bla_{TEM} (Figure 3.19 and 3.20), $bla_{\text{CTX-M-1}}$ (Figure 3.21 and 3.22) and bla_{OXA} (Figure 3.23 and 3.24) genes were found to be present alone or together in the ESBL-producing isolates. In the PCR investigation 20 ESBL-producing isolates were used. The results showed that 45% (n = 9) of the isolates were found to contain TEM-1 gene, whereas OXA-1 gene revealed in 40% (n = 8) isolates, and 60% (n = 12) of the isolates showed CTX-M-1 gene.

Of the 20 isolates, 8 (40%) were found to harbour multiple genes encoding ESBL types. None of the isolates were positive for genes encoding SHV, CTX-M-2, CTX-M-8 and CTX-M-9 genes.

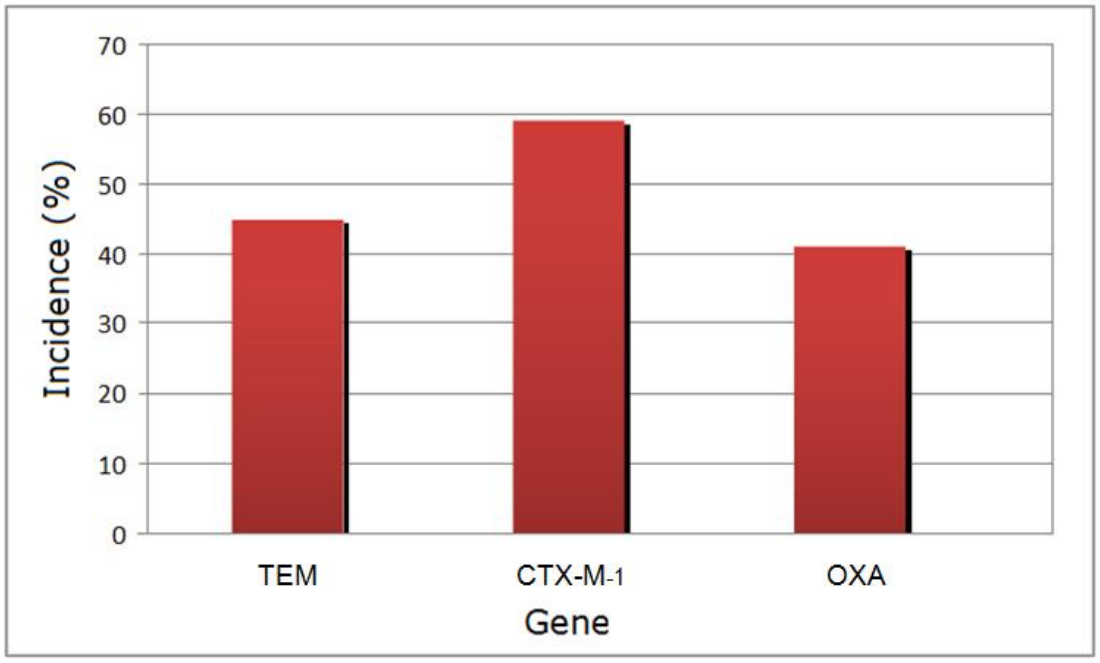

Figure 3.18: Prevalence of ESBL genotypes among the isolates of ESBL-producing *Escherichia coli*. The predominant strains were CTX-M-1, which showed the highest incidence and constituted about 60%. Statistical analysis (one tailed comparison of proportion): TEM *vs*. CTX-M-1 is $p = 0.263$; OXA *vs*. CTX-M-1 is $p = 0.17$.

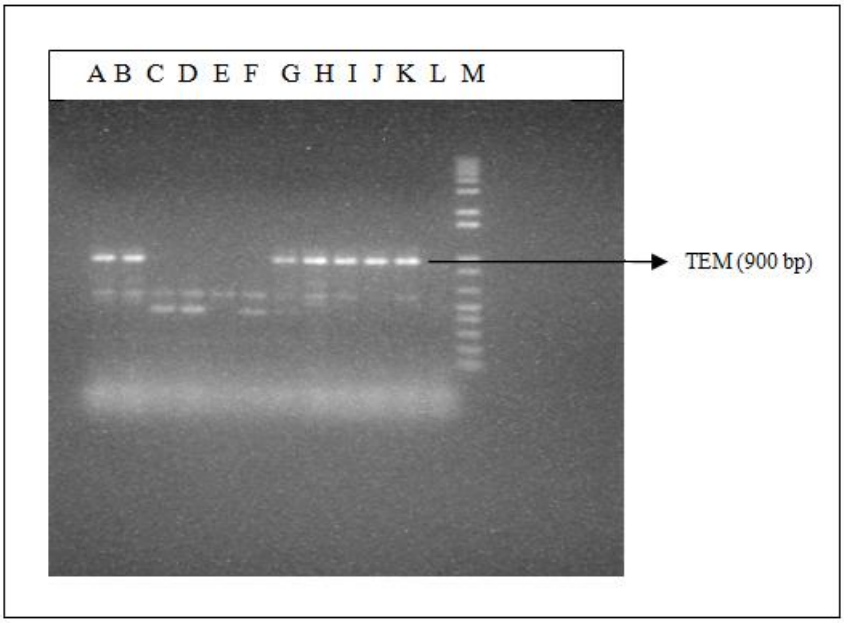

Figure 3.19: Detection of *bla*$_{\text{TEM-1}}$ gene by polymerase chain reaction (PCR). Agarose gel electrophoresis showing PCR amplification products of the *bla*$_{\text{TEM-1}}$ gene. Seven isolates were found to harbour *bla*$_{\text{TEM-1}}$ gene. Lanes A, B, G, H, I, J and K representative ESBL *Escherichia coli* isolates harbouring the *bla*$_{\text{TEM-1}}$ gene. Lane L: Positive control (ATCC *E. coli* 25922); Lane M: DNA molecular size marker (1 kb DNA ladder from Gibco-BRL, USA).

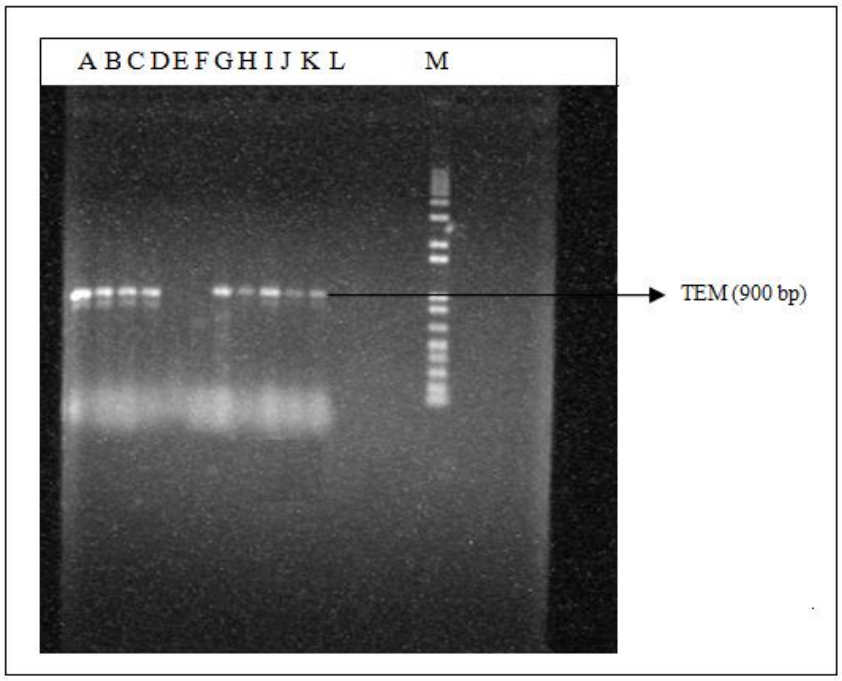

Figure 3.20: Detection of *bla*$_{\text{TEM-1}}$ gene by polymerase chain reaction (PCR). Agarose gel electrophoresis showing PCR amplification products of the *bla*$_{\text{TEM-1}}$ gene. Nine isolates were found to harbour *bla*$_{\text{TEM-1}}$ gene. Lanes A, B, C, D, G, H, I, J and K representative ESBL *Escherichia coli* isolates harbouring the *bla*$_{\text{TEM-1}}$ gene. Lane L: Positive control (ATCC *E. coli* 25922); Lane M: DNA molecular size marker (1 kb DNA ladder from Gibco-BRL, USA).

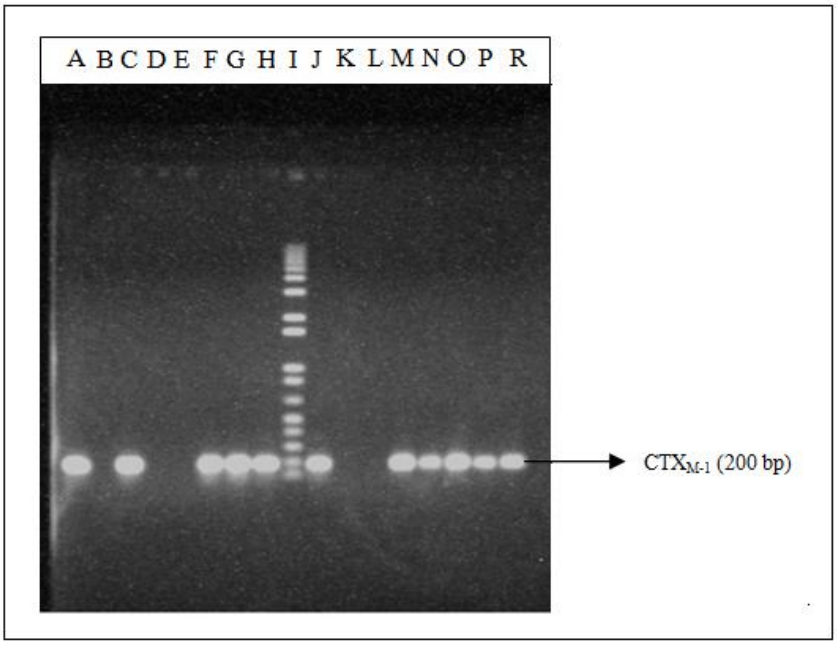

Figure 3.21: Detection of *bla*$_{CTX-1}$ gene by polymerase chain reaction (PCR). Agarose gel electrophoresis showing PCR amplification products of the *bla*$_{CTX-1}$ gene. Ten isolates were found to harbour *bla*$_{CTX-1}$ gene. Lanes A, C, F, G, H, J, M, N, O and P representative ESBL *Escherichia coli* isolates harbouring the *bla*$_{CTX-1}$ gene. Lane R: Positive control (ATCC *E. coli* 25922); Lane I: DNA molecular size marker (1 kb DNA ladder from Gibco-BRL, USA).

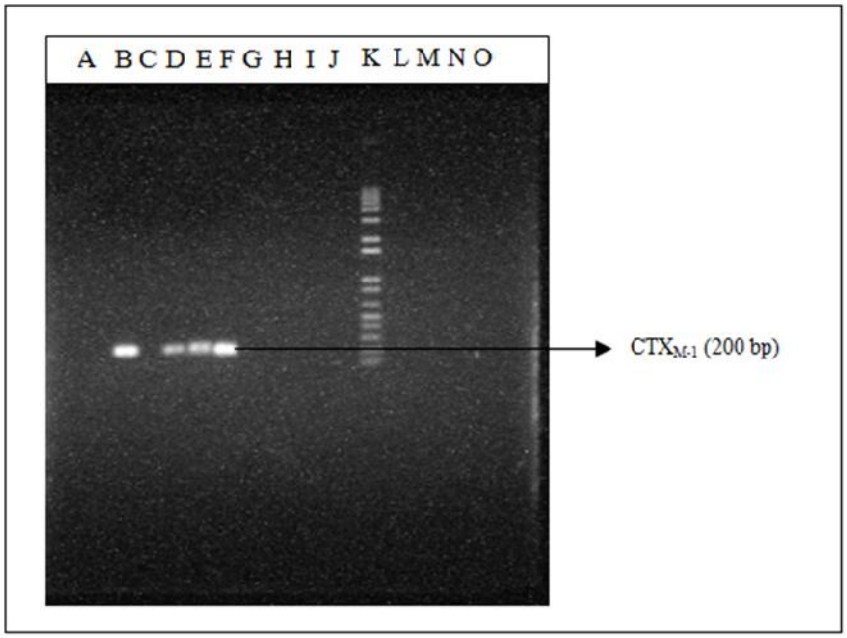

Figure 3.22: Detection of *bla*$_{CTX-1}$ gene by polymerase chain reaction (PCR). Agarose gel electrophoresis showing PCR amplification products of the *bla*$_{CTX-1}$ gene. Three isolates were found to harbour *bla*$_{CTX-1}$ gene. Lanes B, D and E representative ESBL *Escherichia coli* isolates harbouring the *bla*$_{CTX-1}$ gene. Lane F: Positive control (ATCC *E. coli* 25922); Lane K: DNA molecular size marker (1 kb DNA ladder from Gibco-BRL, USA).

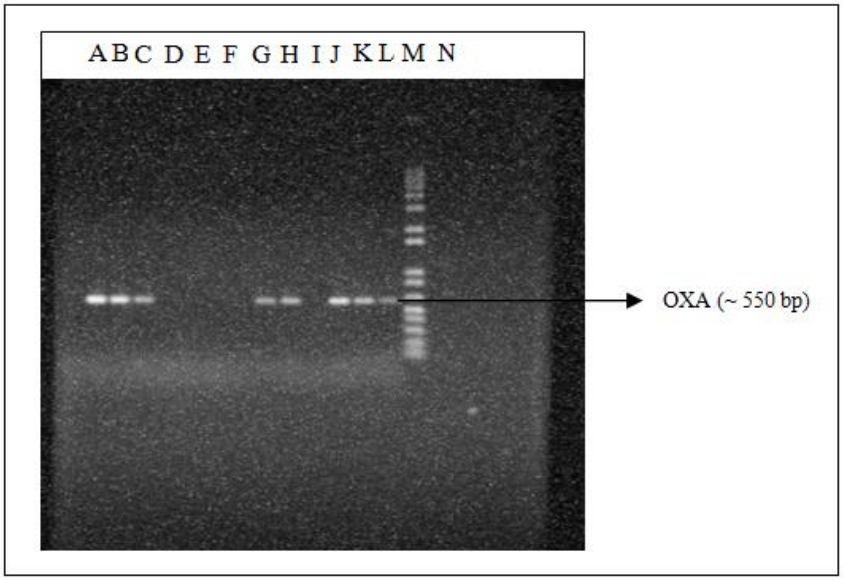

Figure 3.23: Detection of *bla*$_{OXA-1}$ gene by polymerase chain reaction (PCR). Agarose gel electrophoresis showing PCR amplification products of the *bla*$_{OXA-1}$ gene. Seven isolates were found to harbour *bla*$_{OXA-1}$ gene. Lanes A, B, C, G, H, J and K representative ESBL *Escherichia coli* isolates harbouring the *bla*$_{OXA-1}$ gene. Lane L: Positive control (ATCC *E. coli* 25922); Lane M: DNA molecular size marker (1 kb DNA ladder from Gibco-BRL, USA).

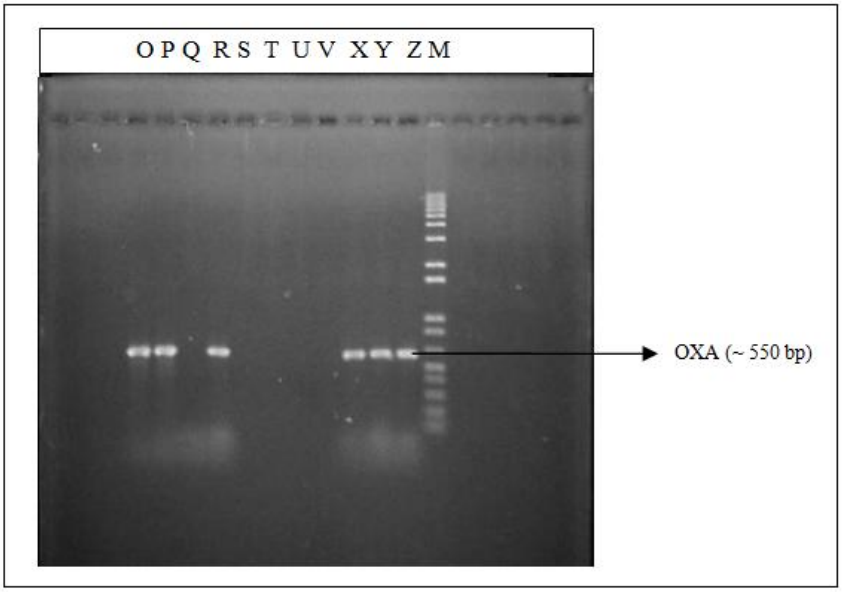

Figure 3.24: Detection of *bla*$_{OXA-1}$ gene by polymerase chain reaction (PCR). Agarose gel electrophoresis showing PCR amplification products of the *bla*$_{OXA-1}$ gene. Five isolates were found to harbour *bla*$_{OXA-1}$ gene. Lanes O, P, R, X and Y representative ESBL *Escherichia coli* isolates harbouring the *bla*$_{OXA-1}$ gene. Lane Z: Positive control (ATCC *E. coli* 25922); Lane M: DNA molecular size marker (1 kb DNA ladder from Gibco-BRL, USA).

3.2.3.2 Correlation between the Genes Encoding ESBL Type *Escherichia coli* and the Corresponding Serotypes with Clinical Manifestations

Table 3.6 shows the distribution of 20 ESBL isolates, their respective gene expressions and their specific serotypes associated with the clinical features. All the ESBL isolates having one or more β-lactamase gene(s) had no specific relation with clinical manifestations. And only one isolate having triple genes were associated with severe UTI in the present study. All the serotypes were heterogenous and had both polyvalent and monovalent serogrouping.

Table 3.6: Correlation between the genes encoding ESBL type *Escherichia coli* and their serotypes with clinical manifestations

ESBL *E. coli* isolate (E)	Presence of gene encoding ESBL type	Serotyping, polyvalent and monovalent	Mild UTI bacterial count in urine $<10^4$/ml	Moderate UTI bacterial count in urine $\sim10^4$/ml	Severe UTI bacterial count in urine $>10^5$/ml
E01	TEM	Nonspecific	Absent	Absent	Present
E02	OXA + CTX-M-1	2 (O126)	Absent	Absent	Present
E03	OXA + CTX-M-1	5 (O2)	Absent	Absent	Absent
E04	CTX-M-1	Untypable	Absent	Absent	Absent
E05	TEM	Nonspecific	Absent	Absent	Absent
E06	CTX-M-1	5 (O153)	Absent	Absent	Present
E07	TEM + OXA	Nonspecific	Present	Absent	Absent
E08	TEM	Untypable	Present	Absent	Absent
E09	CTX-M-1	6 (O15)	Absent	Absent	Present
E10	OXA	1 (O1)	Present	Absent	Absent
E11	OXA + CTX-M-1	Untypable	Present	Absent	Absent
E12	OXA + CTX-M-1	Untypable	Absent	Absent	Absent
E13	CTX-M-1	5 (O153)	Absent	Absent	Present
E14	TEM + OXA	Untypable	Present	Absent	Absent
E15	CTX-M-1	Untypable	Present	Absent	Absent
E16	TEM	Nonspecific	Present	Absent	Absent
E17	CTX-M-1	Not done	Present	Absent	Absent
E18	TEM + CTX-M-1	5 (O6)	Absent	Absent	Present
E19	TEM	5 (O153)	Absent	Absent	Present
E20	TEM + OXA + CTX-M-1	5 (O6)	Absent	Absent	Present

There was no specific correlation between ESBL gene type and their respective serotypes and with their clinical manifestations that were categorized as mild moderate and severe forms. Isolates having single genes were, E01, E04, E05, E06, E08, E09, E10, E15, E16, E17 and E19 (Table 3.6). They did not have any correlation with their specific clinical manifestations. Isolates having multiple genes were E02, E03, E07, E11, E12, E14, E18 and E20. They did not have any correlation with their specific clinical manifestations. Isolates E04, E08, E11, E12, E14 and E15 that showed untypable serotypes had no clinical correlation. Isolates E06, E13 and E19 having serotypes 5 (O153) were isolated from severe UTI patients.

3.2.4 Detection of Virulent Genes in the ESBL-Producing *Escherichia coli* Isolates

3.2.4.1 Detection of *ipaH* Genes Encoded by the ESBL-Producing *Escherichia coli* Using Polymerase Chain Reaction

Polymerase chain reaction (PCR) analysis of the gene encoding *ipaH* in several ESBL-producing *E. coli* isolates was carried out and the results are depicted in Figure 3.25 and 3.26. The *ipaH* gene could not be detected in any of the ESBL-producing *E. coli* isolates tested.

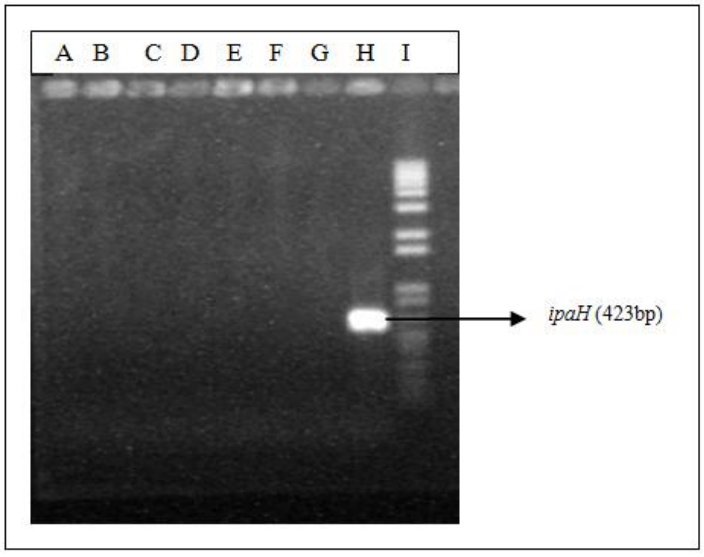

Figure 3.25: Detection of *ipaH* (invasion plasmid antigen H) gene by polymerase chain reaction (PCR). Agarose gel electrophoresis showing PCR amplification products of the *ipaH* gene. No isolate was found to harbour *ipaH* gene. Lanes A through F representative ESBL *Escherichia coli* isolates harbouring no *ipaH* gene. Lane G: Negative control (ATCC *E. coli* 25922); Lane H: Positive control (*Shigella flexneri* 2a, YSH 6000); Lane I: DNA molecular size marker (1 kb DNA ladder from Gibco-BRL, USA).

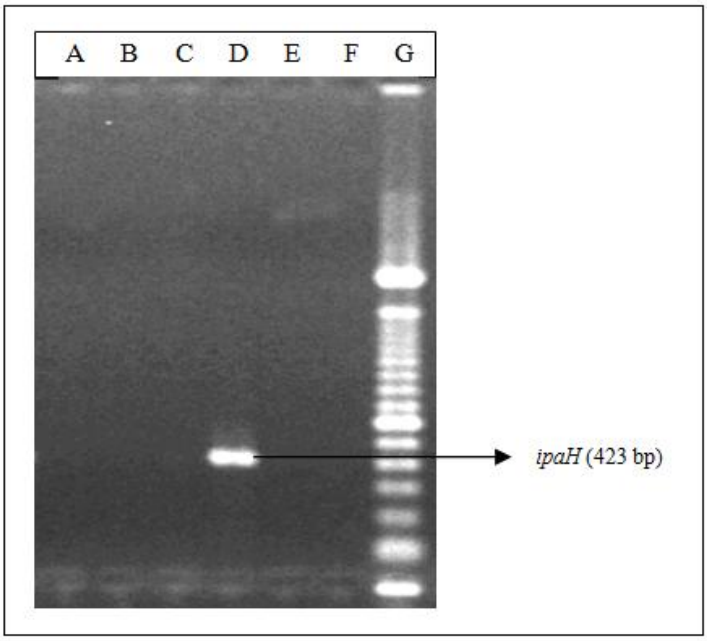

Figure 3.26: Detection of *ipaH* (invasion plasmid antigen H) gene by polymerase chain reaction (PCR). Agarose gel electrophoresis showing PCR amplification products of the *ipaH* gene. No isolate was found to harbour *ipaH* gene. Lanes A, B, C and E representative ESBL *Escherichia coli* isolates harbouring no *ipaH* gene. Lane D: Positive control (*Shigella flexneri* 2a, YSH 6000); Lane F: Negative control (ATCC *E. coli* 25922); Lane G: DNA molecular size marker (1 kb DNA ladder from Gibco-BRL, USA).

3.2.4.2 Detection of the Other Virulent Genes Encoded by the ESBL-Producing *Escherichia coli* Using Polymerase Chain Reaction

Figure 3.27 shows the evidence of virulent gene in ESBL-positive isolates. Of the 20 isolates tested, only one isolate showed both pilus-associated pylonephritis (*pap*) and afrimbial adhesin (*afa*) virulent genes, and another isolate showed only the pili-associated pylonephritis (*pap*) gene. The rest of the isolates were negative for cytotoxic necrotizing factor (*cnf*), haemolysin (*hly*) and type-1 fimbrae (*fimH*) genes.

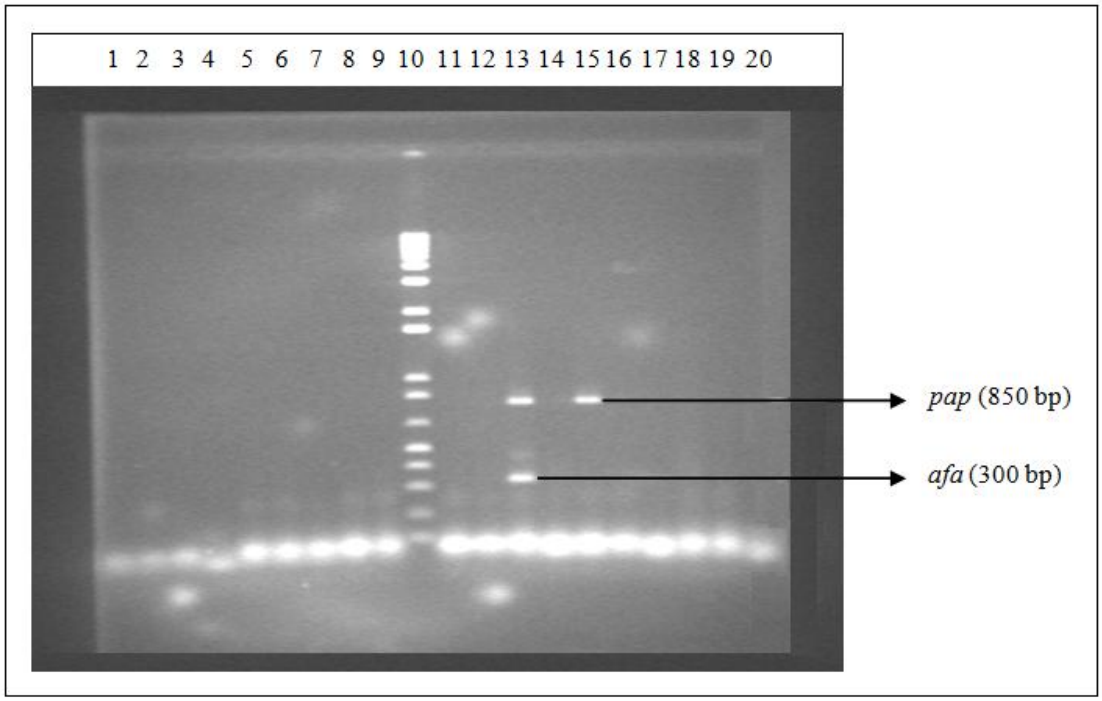

Figure 3.27: Detection of virulent genes by polymerase chain reaction (PCR). Agarose gel electrophoresis showing PCR amplification products of the pilus-associated pylonephritis (*pap*) and afrimbial adhesin (*afa*) virulent genes. No isolate was found to harbour cytotoxic necrotizing factor (*cnf*), haemolysin (*hly*) and type-1 fimbrae (*fimH*) genes. Lanes 1-9 and 11-20 representative ESBL *Escherichia coli* isolates; only two isolates (Lane 13 and 15) showed gene *pap* and/or *afa*. Lane 10: DNA molecular size marker (1 kb DNA ladder from Gibco-BRL, USA).

3.2.5 Correlation among the Serotype, Plasmid Profile, Antibiogram and Genes Encoding ESBL Type *Escherichia coli*

The genes encoding ESBL type with their respective serogroupings, plasmid profile and antibiogram of the isolates used in this study are summarized in Table 3.7. Serotypes of the ESBL isolates were heterogenous in distribution and they contained both single and multiple plasmids. Serogrouping of the isolates E01, E05, E06, E07 and E16 showed nonspecific reactions. The ESBL isolates, namely, E02, E03, E09, E10, E13, E17, E18, E19 and E20 showed polyvalent and their respective monovalent serogroupings. Isolates E04, E08, E11, E12, E14 and E15 could not be assigned into any serogroups and were designated as untypable. Most of the ESBL isolates (n = 20) contained 140 MDa plasmids (15/20, 75%) and showed multidrug resistance (16/20, 80%) excepting in four isolates (4/20, 20%). Isolates E01, E02, E03, E05, E06, E14, E15, E16, E17, E19 and E20 showed multiple plasmids (Table 3.7). The antibiogram clearly demonstrates that most the isolates were resistant to all the antibiotics.

Table 3.7: Correlation among the serotype, plasmid profile, antibiogram and PCR analysis for β-lactamase genes encoded by the ESBL-producing *Escherichia coli* isolates (n = 20) recovered from urine samples of the patients with urinary tract infections (UTIs)

ESBL *E. coli* isolate (E)	Serotype	Plasmid (MDa)	Antibiogram				β-Lactamase (*bla*) gene (PCR)		
			AMC	CRO	CAZ	CIP	TEM	OXA	CTX-M
E01	Nonspecific	140, 62, 6.0, 4.3, 2.0	R	R	R	R	+	−	−
E02	Poly 2 (O126)	62, 35.5, 4.3, 2.0	R	R	R	R	−	+	+
E03	Poly 5 (O2)	140, 2.0	R	R	R	R	−	+	+
E04	Untypable	140	R	R	R	R	−	−	+
E05	Nonspecific	140, 2.7, 2.0	R	R	R	R	+	−	−
E06	Nonspecific	140, 62, 2.7, 2.0	R	R	R	R	−	−	+
E07	Nonspecific	Absent	S	S	R	R	+	+	−
E08	Untypable	140	R	R	R	R	+	−	−
E09	Poly 6 (O15)	140	R	R	R	R	−	−	+
E10	Poly 1 (O1)	Absent	S	S	R	R	−	+	−
E11	Untypable	140	R	R	R	R	−	+	+
E12	Untypable	140	R	R	R	R	−	+	+
E13	Poly 5 (O153)	35.5	R	R	R	R	−	−	+
E14	Untypable	140, 35.5, 2.7, 2.0	R	R	R	R	+	+	−
E15	Untypable	140, 35.5, 4.3	R	R	R	R	−	−	+
E16	Nonspecific	140, 5.0	R	R	R	R	+	−	−
E17	Poly 5 (O153)	140, 2.7, 2.0	R	R	R	R	−	−	+
E18	Poly 5 (O6)	140	R	R	R	R	+	−	+
E19	Poly 5 (O153)	140, 2.0, 1.4	S	S	R	R	+	−	−
E20	Poly 5 (O6)	140, 2.0	S	S	R	R	+	+	+

AMC = Amoxyclavulanic acid; CRO = Ceftriaxone; CAZ = Ceftazidime; CIP = Ciprofloxacin; TEM = Temoniera; OXA = Oxacillinase; CTX-M = Cefotaxime-M.

PCR analyses further showed that all of the ESBL isolates contained single or multiple genes encoding ESBL types (Table 3.7). Single gene, TEM, OXA or CTX-M-1, was present in 12 isolates that constituted 60% of the ESBL isolates tested. Seven ESBL isolates, on the other hand, 7 (35%) isolates were found to harbour two genes in various combinations, *i.e.*, OXA + CTX-M-1, TEM + OXA, and TEM + CTX-M-1. Only one isolate (5%) showed triple gene (TEM + OXA + CTX-M-1). It also was noticed that there is no consistency between the presence of ESBL type genes (single or multiple) and the occurance of the 140 MDa plasmids. Two isolates (E07 and E10) devoid of plasmid but still retained at least one ESBL type gene. Two other isolates (E02 and E13) showed middle range plasmid and lacking 140 MDa plasmids exhibited one or two ESBL type genes.

3.2.6 Pulsed-Field Gel Electrophoresis (PFGE) Analyses

3.2.6.1 PFGE Analyses of ESBL-Producing *Escherichia coli* Isolates

PFGE analysis of *Xba*I digested chromosomal DNA of the ESBL-producing *E. coli* isolates yielded 21 to 23 reproducible DNA fragments ranging in size approximately from 20 to 600 kb (Figure 3.28).

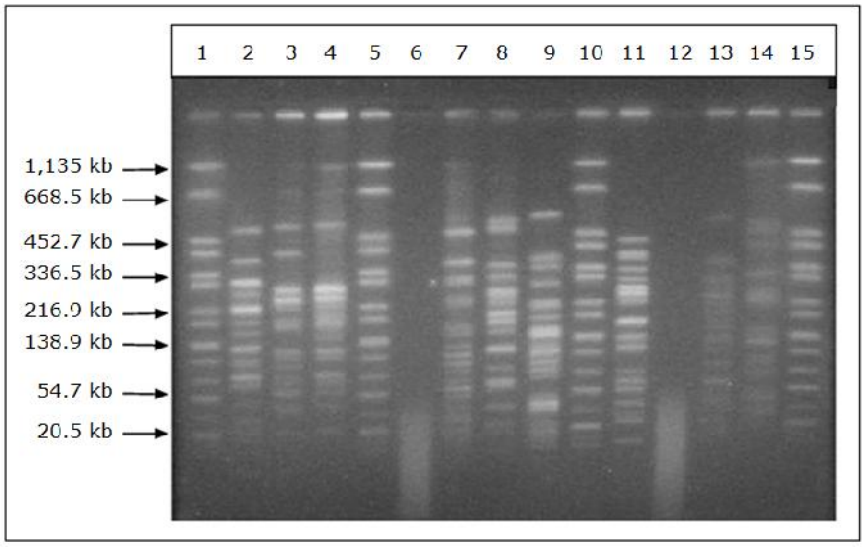

Figure 3.28: Pulsed-field gel electrophoresis (PFGE) banding patterns of *Xba*I digested chromosomal DNA of some representative ESBL-producing *Escherichia coli* isolates. Lane 1: *Salmonella* Branderup (H9812 molecular weigth marker); Lane 2: E01; Lane 3: E02; Lane 4: E03; Lane 5: *Salmonella* Branderup (H9812 molecular weigth marker); Lane 6: E05; Lane 7: E06; Lane 8: E07; Lane 9: E08; Lane 10: *Salmonella* Branderup (H9812 molecular weigth marker); Lane 11: E10; Lane 12: E11; Lane 13: E12; Lane 14: E13; Lane 15: *Salmonella* Branderup (H9812 molecular weigth marker).

Banding profile analysis of the chromosomal DNA from ESBL-producing *E. coli* suggested that all the strains were heterogeneous in their PFGE banding pattern. Of the 20 ESBL-producing isolates, two isolates were untypable using PFGE.

3.2.6.2 PFGE Analyses of Non-ESBL-Producing *Escherichia coli* Isolates

PFGE analysis of *Xba*I digested chromosomal DNA of the non-ESBL *E. coli* isolates yielded 21 to 23 reproducible DNA fragments ranging in size approximately from 20 to 600 kb. Banding profile analysis of the chromosomal DNA from non-ESBL *E. coli* also suggested that all the isolates were completely different in their PFGE banding pattern. Therefore, they are not clonally related. It is also interesting to note that none of the banding pattern obtained from ESBL-producing *E. coli* matches with that of non-ESBL type.

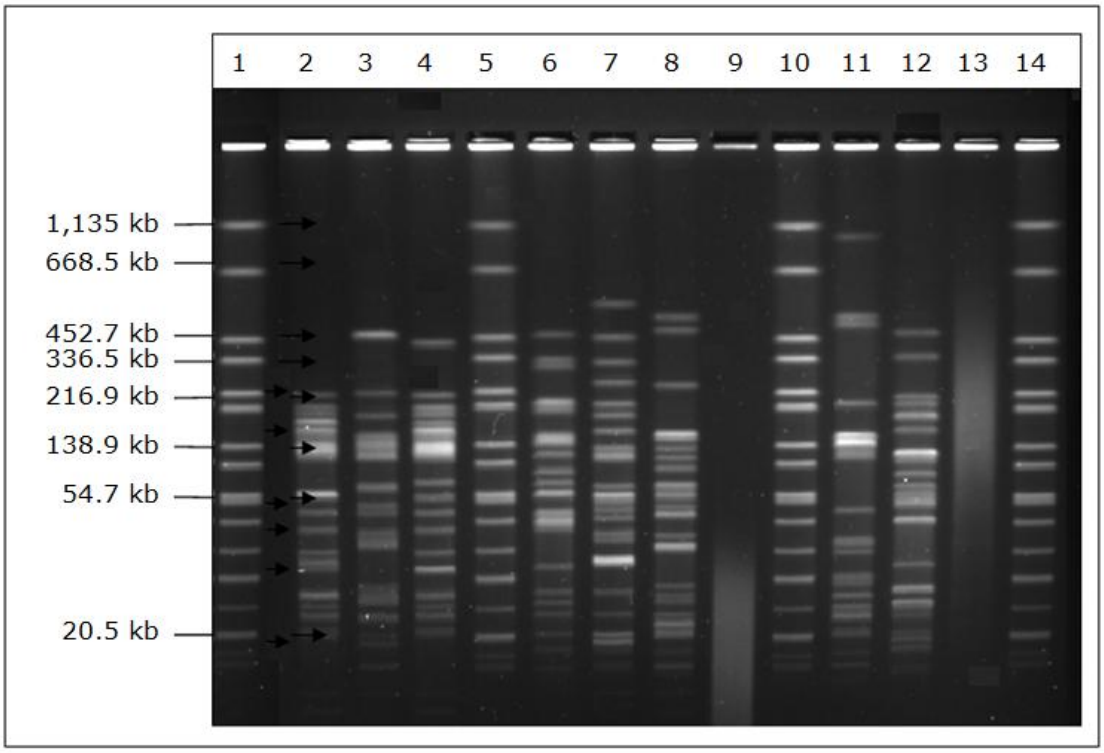

Figure 3.29: Pulsed-field gel electrophoresis (PFGE) banding patterns of *Xba*I digested chromosomal DNA of some representative non-ESBL-producing *Escherichia coli* isolates. Lane 1: *Salmonella* Branderup (H9812 molecular weigth marker); Lane 2: E01; Lane 3: E02; Lane 4: E03; Lane 5: *Salmonella* Branderup (H9812 molecular weigth marker); Lane 6: E05; Lane 7: E06; Lane 8: E07; Lane 9: E08; Lane 10: *Salmonella* Branderup (H9812 molecular weigth marker); Lane 11: E10; Lane 12: E11; Lane 13: E12; Lane 14: *Salmonella* Branderup (H9812 molecular weigth marker).

Chapter 4 Discussion

4 Discussion

4.1 Clinical Significance of Extended-Spectrum β-Lactamase (ESBL)-Producing *Escherichia coli*

Urinary tract infections (UTIs) are a serious health problem affecting millions of people each year. UTIs have been reported to affect up to 150 million individuals annually world wide (Stamm *et al.,* 1999). They are the most common bacterial infection in women and account for significant morbidity and health care cost (Karlowsky *et al.,* 2002).

Infections of the urinary tract are the second most common type of infection in humans and are mainly caused by uropathogenic *Escherichia coli* (UPEC). The UPECs are responsible for between 70 to 90 % of community-acquired UTI's (Nicolle, 2001; Huang and Stafford, 2002; Li *et al*, 2010; Søraas *et al.,* 2013). They contribute largely (38-40%) to nosocomial infections (Struelens *et al.,* 2004; Isikgoz Tasbakan *et al.,* 2013). The severity of UTI depends on both the virulence of the bacteria and the susceptibility of the host (Fowler *et al.,* 1977; Sharma *et al*., 2007; Jadhav *et al*., 2011). Antibiotics are the typical treatment for urinary tract infections (Calbo *et al*., 2006). Women are at most the risk of developing a UTI. In fact, half of all women will develop a UTI during their lifetimes. The reason for this is that they have short urethra, menstrual bleeding and faecal contamination from the perinium. It has been extensively reported that adult women have higher prevalence of UTI than men, principally owing to anatomic and physical factors (Gales *et al*., 2001; Kumar *et al*., 2006).

Over the last 20 years, many new β-lactam antibiotics have been developed that were specifically designed to be resistant to the hydrolytic action of β-lactamases. However, with each new class that has been used to treat patients, new β-lactamases emerged that caused resistance to that class of drug (Canton *et al*., 2003; Habeeb *et al.,* 2013).

It was described in 1983 (Knothe *et al.,* 1983) that extended-spectrum β-lactamases (ESBL) have contributed to the dramatic increase in resistance to β-lactam agents among Gram-negative bacteria in recent years (Coudron *et al.,* 1977; Bradford, 2001;

Bush, 2001; Abreu *et al*., 2013). There has been limited study on ESBL-producing organisms in Bangladesh. The first reported study was conducted by Rahman *et al.* (2004a). It was reported that samples cultured were 43.2% *E. coli* and 39.5% *Klebsiella pneumoniae*. Samples were collected from various sources such as urine sputum, pus, throat swab, etc. Urine samples showed the highest incidence of ESBL production (Rahman *et al*., 2004a).

Other past studies had been shown in Bangladesh by Alim (2009). Moreover disc-diffusion synergy test for ESBL-producing *E. coli* and *Klebsiella* spp. isolated from urinary tract infections, throat swabs and wounds have been documented (Rahman *et al*., 2010). Occurrence and characterization of multidrug-resistant New Delhi metallo-β-lactamase-1-producing bacteria isolates were also investigated in Bangladesh (Islam *et al*., 2010).

The present investigation was undertaken with the view to highlight the clinical significance of urinary tract infection patients and its relationship with extended spectrum of β-lactamases producing *Escherichia coli* at molecular level/genetic level. This study was carried out with 77 isolates including 25 ESBL and 52 non-ESBL isolates. These isolates were collected from symptomatic UTI patients residing in different regions of Sylhet District (community-acquired infection). These patients reported in the private laboratories located at the city center. Clinical data and urine samples were collected from these private labs. All the isolates were from the female populations because the present study showed 72% were female and 28% male and these findings were consistent with other studies (Hoque *et al.,* 1976; Usein *et al.,* 2001). The female population showed significantly higher incidence of UTI, because vagina is in close proximity with anal canal and the length of urethrae is short. Sexual contacts in young women and menstrual cycle can cause ascending infections (Hoque *et al*., 1976).

The present study was carried out on uncomplicated UTI patients having clinical manifestations. These patients had diverse clinical symptoms such as frequency of micturation, dysuria, flank pain, fever and suprapubic tenderness. These findings were based on specific clinical lab reports such as presence of ‘numerous pus cells in urine’. All the patients in the present study had the clinical picture of typical urinary

tract infections (UTI). The patients were between 4 to 50 years and above and the age group between 21-30 years showed the highest incidence (28%). The next in order were from 31-50 years (20%). These results suggested that patients of younger age are more susceptible to urinary tract infections because they were newly married women and the frequent sexual contacts, have increased the incidence of bacteriuria. Menstrual cycles may also enhance the ascending infections (Hoque *et al*., 1976).

The clinical presentations of these patients were divided into three groups mild, moderate and severe. The patients having bacterial urine cultures ~10^5 CFU/ml associated with 5-10 microscopic urine leukocytes were designated as mild symptoms. Patients having bacterial culture >10^5 CFU/ml, associated with microscopic urine leukocyte 10 to 15 were designated as moderate symptoms respectively. And patients having bacterial culture >10^5 CFU/ml, associated with microscopic leukocyte in urine numerous, were grouped as severe symptomatic UTI patient.

The results paradoxically showed that patients having mild symptoms have higher prevalence as compared to patients having severe symptoms. These results indicate that in a community-acquired infection the majority of people are vulnerable to pathogens of low virulence. These findings were explained by other studies which stated that UPEC strains of low virulence are less capable of causing urinary tract infection compared with susceptible strains (Kawamura-Sato *et al*., 2010). There has been no such study on ESBL-producing *E. coli* isolates showing low virulence factors in Bangladesh.

4.2 Phenotypic Characterization of ESBL *Escherichia coli* Isolates

The present investigation was further carried out for the isolation and confirmation of uropathogenic *Escherichia coli* (*E. coli*). The *E. coli* isolates were subcultured and identified primarily by employing short biochemical methods. These biochemical tests used were Kliglar's iron agar (KIA), motility indole urea (MIU) and citrate utilization. These methods were followed according to the standard microbiological/ biochemical methods at enteric laboratory of International Centre for Diarrhoeal Disease Research, Bangladesh, (ICDDR,B), Dhaka. Majority of isolates were

confirmed as *E. coli* and those strains which showed doubtful reactions were discarded.

Detection of the ESBL-producing *E. coli* was done by disc-diffusion synergy test in which an augmentin disc (amoxicillin plus clavulanic acid) was placed in the center of a plate and cefotaxime, ceftazidime, aztreonam and ceftriaxone were placed 30 mm from the augmentin disc. Enhancement of the zone of inhibition of any one of the four drug disc towards the disc containing clavulanic acid suggested the presence of an extended-spectrum β-lactamases (Jarlier *et al*., 1988; Bradford *et al*., 2001; Rahman *et al.,* 2004a).

The present study confirmed 25 *E. coli* isolates as ESBL-producing *E. coli* and they were used in rest of the investigations. In a previous study carried by Rahman *et al*. (2004a), it was observed that there were different rates of isolation of the ESBL in the different combination of 3rd generation cephalosporin (ceftriaxone, cefotaxime and ceftazidime) discs with augmentin disc. In that study, the highest rate of ESBL-positivity was observed with ceftazidime and augmentin combination. Ceftazidime with amoxyclavulanic acid also showed the highest rate of ESBL-positivity in the present study. Simultaneous use of four cephalosporin discs with an augmentin disc was recommended in screening for ESBL-producing organisms (Rawat and Nair, 2010). These ESBL-producing strains were collected from the community. Therefore the serogrouping were carried out for epidemiological purpose. Serological classification scheme is based on antigenic differences in highly variable bacterial surface molecules (Salyers *et al*., 2005). ESBL-producing isolates were subjected to standard serological methods using commercially available antisera (Denka Saiken Co. Ltd., Japan).

In the present study, the most prevalent ESBL serogroups were O1, O2, O6, O15, O126, and O15. The predominant serogroup was O153 in the present study. Importantly, a particular clone detected by multilocus sequence typing (MLST) in this serogroup (O15:K52-H1) of ESBL-producing *E. coli* had disseminated in many countries as a uropathogenic clone (Prats *et al.,* 2000). Past studies have identified ST131 in ESBL-producing *E. coli* in urine by multilocus sequence typing (MLST) (Peirano *et al.,* 2011). The MLST was not done in the present study due to limitations in the laboratory.

The non-ESBL isolates showed different serogroups and the results did not match with the serogroups of ESBL isolates. Nine ESBL isolates could not be grouped into any particular serogroup and they were designated as untypeable. Four ESBL isolates were rough type in reactions.

However, a complete serogrouping scheme was not possible because of lack of H-antigen specific antisera. Further study is underway to complete the serotyping scheme. These studies suggest that the strains do not have similar serogrouping with previous studies and therefore they are heterogenous. Olesen *et al*. (2013) report the characteristics of 115 extended-spectrum beta-lactamase (ESBL)-producing *E. coli* clinical isolates, from 115 unique Danish patients, over a 1-year study interval. Forty-four (38%) of the ESBL isolates represented sequence type 131 (ST13)1, from phylogenetic group B2. The remaining 71 isolates were from phylogenetic groups D (27%), A (22%), B1 (10%), and B2 (3%). Serogroup O25 ST131 isolates (n = 42; 95% of ST131) comprised 7 different K antigens, whereas two ST131 isolates were O16:K100:H5 (Olesen *et al*., 2013). Other studies showed, serogroup O4 haemolysin production was the most prevalent of 39 ESBLs isolates (Marijan *et al.,* 2007).

Past studies have also mentioned that most frequently detected specific serogroups of uropathogenic *E. coli* by phenotypic method correlated with the presence of urovirulence genes by genotypic method as shown by PCR (Blanco *et al*., 1997; Yamamoto *et al*., 2007).

Studies have also reported that O-serogrouping of *E. coli* has been an invaluable typing method for epidemiological investigations (Bertschinger, 1999). Because when there is an out break of infectious disease; then it is easy to trace the pathogen responsible for that particular disease. For example multiplex PCR method can be used for detection of clinical strains (Morin and Hopkins, 2002; Li *et al*., 2010). These strains were serologically characterized as O1, O2, O18, and O75 (Li *et al*., 2010).

Moreover, there are clinical studies which have shown that the majority ESBL-producers in UTI belonged to serotype O25 (Jadhav *et al*., 2011). This particular serotype O25 was expressed as O antigen and was predominantly isolated from patients with pyelonephritis, prostatitis, cystitis, simple UTI, septicaemia and pyrexia of unknown origin (PUO) (Jadhav *et al.,* 2011). Thus the foregoing studies support

the hypothesis that serotyping could be a useful index to identify uropathogenic *E. coli* (Yamamoto *et al*., 1995).

Recently, serotype O25:H4-ST131 has gained much importance as uropathogenic clone in ESBL-producing isolates (Nicolas-Chanoine *et al*., 2008). The clonal spread of the ST131 epidemic *E. coli* strain is also linked to other ESBLs types (Lau *et al*., 2008; Oteo *et al*., 2010; Peirano *et al*., 2011).

The foregoing study was further carried out on antibiotic susceptibility. Because antibiotic resistance is a major phenotypic trait, particularly for the clinical isolates as it has a potential interest in exploring the characteristics of these ESBL-producing *E. coli.* In the present study, all *E. coli* strains were collected from patients residing in the community having symptomatic urinary tract infections. These patients had a history of antibiotic therapy both for non-ESBLs and ESBLs. Therefore, antibiotic susceptibility of non-ESBL isolates showed that *E. coli* isolated from UTI patients had wide range antibiotic susceptibility; the highest incidence of sensitivity (100%) observed with imipenem and meropenem. Amoxyclavulanic acid showed 70.6% sensitivity and resistance by 29.4%. Ceftriaxone, ceftazidime and ciprofloxacin were sensitive by 65.1, 60.1 and 40.2%, respectively. Drugs like amoxicillin and cotrimoxazole were resistant by 98% (data not shown). Previous studies showed similar range of antibiotic susceptibility (Mahmood *et al.,* 2008).

These results suggested that UTI patients having *E. coli* are frequently exposed to antibiotics and develop multi-drug resistance. The majority of the patients had history of antibiotic intake. Therefore, it was speculated that these isolates may have chromosomes or plasmid mediated resistance. Chromosomes mediated resistance was not done in the present study.

The *E. coli* isolates that were confirmed as ESBLs strains phenotypically were subjected to antibiotic susceptibility tests. Twenty ESBL-producing isolates were used for antibiotic susceptibility and it showed resistance percentage higher than sensitivity percentage (amoxyclavulanic acid 80%, ceftazidime 55%, ceftriaxone 80%, ciproflaxacin 80%). In contrast, the non-ESBL isolates showed higher sensitivity (amoxyclavulanic acid 70%, ceftriaxone 65%, ceftazidime 60%, ciproflaxacin 40%) than resistance pattern. These results explain the fact that ESBL isolates can cause

wide range of antibiotic resistance in the community (Dewar *et al.,* 2014). The susceptibility test results showed that all the ESBL-producing isolates were resistant to 3rd generation cephalosporins (ceftriaxone, cefotaxime, and ceftazidime). This reflects the relationship between the ESBL and resistance to 3rd generation cephalosporins. Increased resistance might be due to extensive use of 3rd generation cephalosporins and other β-lactam drugs. All the ESBL-producing isolates were also resistant to 4th generation cephalosporins (cefepem) and monobactams (aztreonam) in the present investigation (data not shown).

Recently, ESBL isolates have been treated with carbapenems such as imipenem and meropenem (Matsumoto *et al*., 2004; Rahman *et al*., 2004a; Takahashi *et al*., 2009; Marković *et al*., 2013). In the present study both the ESBL and non-ESBL isolates were 100% sensitive to the carbapenems. So far no resistant strains to carbapenems have been detected in Bangladesh.

Recent studies were carried out with ESBL-producing *E. coli*, which have been observed in outpatient settings. In that study, antimicrobial susceptibility showed fosfomycin, nitrofurantoin, ertapenem and pivmecillinam all are highly sensitive and can be considered for oral treatment in ESBL-producing *E. coli* (Graninger, 2003; Nicolle and Mulvey, 2007; Auer *et al*., 2010; Falagus *et al*., 2010; Beuk *et al*., 2013; Dewar *et al*., 2014; Jansåker *et al.,*2014). Quinolones, in particular, have been found to be an independant risk factor in many studies (Colodner *et al*., 2004; Sobel and Kaye*,* 2005; Rodríguez-Baño *et al*., 2008; Yagi *et al*., 2010). One of the major problems that concerns the ESBL-positive isolates is the high rate of resistance to non-β-lactam antibiotics particularly quinolones, trimethoprim-sulphamethoxazole, and aminoglycosides (Dewar *et al*., 2014). In the present study, ESBL isolates showed 80.2% resistance and 19.8% sensitivity with quinolone antibiotics (ciprofloxacin). In non-ESBL group, the isolates have revealed 60 % resistance and 40 % sensitivity with ciprofloxacin. The resistance prevalence was higher in ESBL group (80.2% ESBL isolates resistance *vs*. 60.0% non-ESBL isolates resistance). These findings suggest there has been wide range of indiscriminate and inappropriate use of antibiotics in Bangladesh.

Previous studies have focused on the characteristics of quinolone and flouroquinolone resistant uropathogenic *E. coli* (UPEC) strains and they documented that *E. coli* strains harbour fewer virulence factors than susceptible strains (Piatti *et al.*, 2008; Kumiko *et al*., 2010). Other studies have shown that high percentages of ESBL-producing *E. coli* were responsible for resistance to ciprofloxacin (Dewar *et al*., 2014). It is also suggested that it may be resistant to other fluoroquinolones − the drug of choice for the treatment of infections caused by ESBL-producing strains (Kariuki *et al*., 2007; Jadhav *et al*., 2011).

4.3 Genotypic Characterization of ESBL *Escherichia coli* Isolates

Analysis of plasmid profile was carried out in the present study because the ESBLs are most often encoded on plasmids and they are responsible for transfer of antibiotic resistance gene to other organisms in hospital and community. Previous studies 60-90% of plasmids (R-factor) were responsible for drug resistance (Choudhury *et al*., 1994). Moreover, many antibiotic resistance genes in these strains are carried in plasmids which have been shown to vary within and between different lineages (Karisik *et al.,* 2006; Lau *et al.,* 2008).

A number of studies reported that the ESBL marker also transferred through plasmid. *E. coli* J53 ($Azide^R$) strain was used successfully for transfer of ceftazidime resistance in different studies (Hernandez *et al*., 2005; Ryoo *et al*., 2005). The conjugation experiment had not been done due to inavailability of *E. coli* J53 strain. The *E. coli* K12 strain available in the laboratory exhibited nalidixic acid resistance. Since all the *E. coli* isolates included in this study showed resistance to nalidixic acid, the conjugation experiment was not performed with K12 strain.

Analysis of plasmid profiles is a useful tool with which to document the appearance of plasmid associated with important phenotypic characteristics, most importantly the drug resistance. In the present study, analysis of the plasmid DNA of ESBL *E. coli* isolates had shown that all isolates contained multiple numbers of plasmid ranging from 1.0 to140 MDa. Middle ranged plasmids for ESBL isolates (30 MDa to 90 MDa) were found to be present in 35% isolates and high ranged plasmids (140 MDa and above) were present in 68% isolates. Similar results were seen in non-ESBL isolates;

75% for high range and 25% for low range plasmid. As estimated by agarose gel electrophoresis, plasmids determining extended-spectrum enzymes ranged in size from 53 to 200 MDa (80 to 300 kb) (Jacoby and Sutton, 1991). The foregoing results clearly revealed that majority of ESBL and non-ESBL isolates harboured high molecular wt plasmids (molecular weight ≥140 MDa). Therefore, these size-ranged plasmids should not be used as a molecular marker for the ESBLs and non-ESBLs identification. The data also suggested that there can be plasmid transmission between the isolates (Ahmed and Salam, 2002). Plasmids of high molecular weight are generally said to be transmissible between the organisms (Levinson, 2010).

There were speculations about the relationship between diarrhoeagenic *E. coli* and uropathogenic *E. coli*. Because majority of enteroinvasive *E. coli* and enteropathogenic *E. coli* harboured high range and middle range plasmids. One possible explanation is that; uropathogenic *E. coli* may originate from enteropathogenic/enteroinvasive *E. coli* due to faecal contamination. Because extraintestinal pathogenic *E. coli* strains cause UTIs are called uropathogenic *E. coli* (Li *et al*., 2010).

Other studies have mentioned that *E. coli* isolated from urine during an active infection may be among the host's vaginal or rectal flora, serving as a source for recurrent infection (Kunin *et al*., 1997; Mitsumori *et al*., 1997). These results indicated that these isolates that synthesized ESBLs, were encoded by the genes that are typically plasmid-borne and those isolates which do not synthesize ESBLs may also have similar plasmid distribution, because plasmids of high molecular weight are transmissible between isolates (Levinson, 2010).

Correlation of plasmid characteristics with antibiotic susceptibility of ESBL isolates were observed in the present study. The results clearly showed that there was no specific correlation among the parameters such as plasmid molecular weight, serotypes, amoxyclavulanic acids, ceftazidime, ceftriaxone and ciprofluxacin. This correlation was analysed with the view that if there were any ESBL-specific plasmid and ESBL-specific serotype isolates which were responsible for multi-drug resistance to commonly used antibiotics.

The serotypes were heterogenous and they did not correlate with antibiotic susceptibility. Seven isolates (55%) that were untypeable and they also contained respective 140 MDa plasmids but there was no specific relationship with antibiotic susceptibility. Other studies have mentioned that certain serotypes were more often associated with a particular virulent genotype (Yamamoto *et al*., 1995; Blanco *et al*., 1996; 1997). The present study did not show any specific correlation and further investigations are required with large sample population from the community.

The non-ESBL isolates which were confirmed by phenotypic method double-disc synergy test (DDST) also showed similar negative results by genotypic PCR method. These isolates were discarded in the present study.

The foregoing study was further carried out on the identification of genes responsible for ESBL production. The isolates with ESBL phenotypes were examined for the presence of bla_{TEM}, bla_{SHV}, bla_{OXA}, and $bla_{\text{CTX-M}}$ genes by PCR. The results indicated that the phenotypic method can be consistent with genotypic methods. Past studies have documented these findings (High *et al*., 1988; Yamamoto *et al*., 1995; Blanco *et al*., 1997).

The total numbers of representative strains were 20 in PCR study and these isolates showed 45% of gene encoding TEM-1 type and 41% of gene encoding OXA-1 type and 60% of gene encoding CTX-M-1. Of the 20 isolates, 8 (40%) isolates showed multiple genes encoding ESBL. None of the isolates were positive for gene encoding SHV, *IpaH* or CTX-M-2, CTX-M-8 and CTX-M-9 group specific PCR.

It appeared that in our district Sylhet, the CTX-M-1 group-specific ESBL might be the predominant type. Moreover, CTX-M-producers among ESBL *E. coli*, have been found out to be 64 % in a study carried out in Chicago (Qi *et al*., 2010), 80% in Brazil, and 90% in Turkey (Azap *et al*., 2010; Abreu *et al.,* 2013). These results were consistent with the present study, which showed CTX-M-1 60% as compared to TEM and OXA. The clinical significance of these findings were that doctors should be more aware of the risk of CTX-M-producing *E. coli* and the selection of appropiate antibiotics such as ertapenem, fosfomycin and nitrofurantoin (Qi *et al*., 2010; Qiao *et al*., 2013).

The CTX-M type β-lactamases represent a rapidly emerging group, which have been found predominantly in Enterobacteriaceae, particularly in *E. coli, K. pneumoniae, Proteus mirabilis* and *Salmonella typhimurium* (Tzouvelekis *et al*., 2000; Bradford *et al*., 2001; Bonnet *et al*., 2004). In some countries, CTX-M type enzymes were the ESBL's most frequently isolated from *E. coli* strains (Pitout *et al*., 2004; Lau *et al*., 2008, Peirano *et al*., 2011). In recent times, it has been reported that the CTX-M-type ESBLs are rapidly disseminating because they are cause major out breaks in Europe (Stobberingh *et al*., 1999; Canton and Coque, 2006) and most of East Asia (Munday *et al*., 2004; Habeeb *et al.,* 2013).

Previous studies have also shown that ESBL-producing, *E. coli* encoded by CTX-M-15 isolates contained gene *aac (6')-Ib-cr* (Fihman *et al.,* 2008; Pitout *et al.,* 2009). It is to be mentioned that the recently described gene *aac(6')-Ib-cr* reduces the activity of aminoglycosides and fluoroquinolone and they appear to be associated with CTX-M-15 and OXA-1 producing isolates (Machado *et al*., 2006; Lau *et al*., 2008; Pitout *et al*., 2009 ; Yumuk *et el*., 2008; Peirano *et al*., 2011; Jadhav *et al*., 2011). These findings were supported by a community based study in Turkey that 90% of the uropathogenic *E. coli* (UPEC) isolates were harbouring CTX-M-15 ESBL type (Azap *et al*., 2010). Although these investigations were not done in the present study; it should therefore be interesting to explore the possibilities of CTX-M-15 strains habouring gene *aac (6')-Ib-cr* in UPEC isolates of our community.

Clinical manifestations of UTI have important signs for the preliminary diagnosis of UTI patients in the community, particularly pyuria and bacteriuria (Yamamoto *et al*., 1995). Previous studies have shown that pyuria and bacteriuria were important signs, indicative of UTI (Yamamoto *et al*., 1995). Therefore, a correlation was examined in the present study for genes encoding ESBL type *E. coli* with their corresponding serotypes and their clinical manifestations. No significant correlation was found between the genotype and severity of pyuria and bacteriuria. The reasons for no correlation were based on the fact that clinical manifestations of UTI patients are variable from patient to patient and the majority of their serotypes were untypeable. About 30% of the isolates were not typeable in the present study. Other studies have documented this finding in urine containing ESBL *E. coli* (Yamamoto *et al*., 1995).

The representative ESBL strains were examined for correlation of serotypes plasmid profile, antibiogram and PCR results. The PCR results showed that all the isolates contained single and multiple genes encoding ESBL type. Single gene either TEM-1 OXA-1 or CTX-M-1 were present in 12 isolates that constituted for 60% and 7 isolates were having double genes that account for 35%. Only one isolates showed triple gene, *e.g.*, TEM-1, OXA-1 and CTX-M-1, representing 5% of the total isolates. The existence of multiple genes in ESBL uropathogenic *E. coli* (UPEC) has been documented. That study had shown 95% of isolates were CTX-M-15 and co-produced TEM-1 and OXA-1 β-lactamases (Peirano *et al*., 2011; Habeeb *et al.,* 2013).

The foregoing results suggested very rapid emergence of multidrug resistant ESBL-producing *E. coli* posing a very serious threat in the treatment of nosocomial and community acquired infections.

Interestingly, it was seen in present study that isolates having single genes were harbouring 140 MDa. The clinical significance can not be concluded from this preliminary study. However other studies have stated that ESBL production is usually plasmid mediated, therefore specific genes are responsible for ESBL production in *E. coli* (Jacoby and Sutton *et al*., 1991; Sharma *et al*., 2007).

The present study was further analysed for correlation of gene encoding ESBL type isolates with their corresponding plasmid molecular weights and antibiograms. It was noted that isolates No. E07 and E10 did not have any plasmids and they were sensitive to specific antibiotics. Presumably, the gene encoding ESBL types were integrated into the chromosomes because these four isolates did not have plasmids (Jacoby and Sutton 1991).

The present study was further tested for the identification of virulent genes in ESBL *E. coli* genotype. Because it was proposed that genetic detection of virulence factors would be useful for rapid diagnosis of cystitis, especially in patients with severe pyuria or bacteriuria (Yamamoto *et al.,* 1995). Therefore, the presence of virulent genes was detected by PCR in ESBL isolates from patients having pyuria and bacteriuria in the present study.

There is limited evidence of virulent gene in ESBL-positive isolates in the present study. Of the 20 isolates, only 2 isolates showed both pilus-associated pylonephritis (*pap*) and afrimbial adhesin (*afa*) and only one isolate showed the pilus-associated pylonephritis (*pap*). The remaining isolates were negative for cytotoxic necrotizing factor (*cnf*) haemolysin (*hly*), and type-1 fimbrae (*fimH*). All the other virulent genes were absent suggesting that the *E. coli* isolates were not capable of producing heat-labile toxin (*lt*), heat stable toxin (*st*), attaching and effacing phenotype (*eae*), invasion-associated locus (*ial*), shiga toxin 1 (*stx1*), shiga toxin 2 (*stx2*) and aggregative property (e_{Agg}) respectively. These virulence factors are normally present in *E. coli* causing gastrointestinal infection (Salyers *et al*., 2005). One of the explanations is that the *E. coli* isolates were collected from urine of UTI patients; therefore they did not have other virulent genes.

A recent class of *E. coli* named, uropathogenic *E. coli* strains (UPEC), has been recommended in various studies (Blanco *et al*., 1997; Silveira *et al*., 2001; Naveen *et al.*, 2005, Cheryl-Lynn *et al.,* 2008; Lloyd *et al*., 2009) and the biological characteristics of UPEC includes virulent genes such as hemolysin (*hly*) and aerobactin (*aer*) production, expression of P fimbriae, type-1 serum resistance, cytotoxic necrotizing factor type 1 (CNF), pyelonephritis-associated pili (*pap*), S-fimbriae (*sfa*) and afimbrial adhesion (*afa*).

It has been discussed earlier that certain serotypes, such as O1, O2, O4, O6, O7, O8, O15, O16, O18, O21, O22, O25, O75 and O83, are commonly associated with UTI (Yamamoto *et al*., 1995; Li *et al*., 2010) and each virulent factor was significantly associated with UTI and correlated with their serotypes (Yamamoto *et al*., 1995; Blanco *et al*., 1997). In the current study, a correlation was not possible because only two isolates showed virulent genes. Nevertheless, the present study suggested that ESBL isolates can also harbour the virulent genes and they have a role in the pathogenesis of ESBLs infection in the community. Both the ESBLs and non-ESBLs isolates were collected from the community, therefore a molecular epidemiological typing "pulsed-field gel electrophoresis" is considered as the gold standard (Minarini *et al*., 2007). The PFGE has been used for the determination of clonal relationships among the *E. coli* isolates. In this method, restriction endonucleases are used to digest chromosomal DNA that generates large fragments (Chung *et. al*., 2000). The

restriction fragments are resolved into a pattern of discrete bands in an agarose gel in which the orientation of the electric field across the gel is changed periodically ('pulsed') rather than kept constant as in conventional agarose gel electrophoresis. The DNA restriction patterns of the isolates are than compared with one another to determine their relativity.

Molecular typing of the ESBL-producing *E. coli* is important in clinical settings because helps to track the pathogen responsible for the outbreak of disease in community and hospital (Nemoy *et al*., 2005). In this case, *Xba*I restriction enzyme was used as it was recommended by the PulseNet program (Hunter *et al*., 2005). It gave the best discrimination of the strains, since it has a long ranged DNA cutting site and cut the DNA infrequently. According to criteria published by Tenover *et al*. (1995), the isolates were analyzed. A difference of more than 6 bands differences between two strains have been considered as different PFGE type. In present study, 11 *E. coli* isolates showed heterogeneous banding pattern and their corresponding serogrouping were also heterogenous. The PFGE analysis in the present study clearly indicates that there is no clonal relationship among *E. coli* isolates. These findings are consistent with previous study (Valverde *et al*., 2004; Minarini *et al*., 2007).

4.4 Conclusion

The emergence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* in the community and in hospital had caused multidrug resistance. As these organisms are growing in number, therefore they have limited the therapeutic options. The present study had showed that ESBL *E. coli* had diverse phenotypic and genotypic properties. The results distinctly showed that ESBL strains are emerging in a district like Sylhet. The clinical manifestation of classical UTI patients made no significant difference with ESBL UTI patients. Moreover, the phenotypic experiments such as disc-diffusion synergy test, serogroupings and antibiograms revealed the results for ESBL-producing isolates. These findings were not consistent with non-ESBL isolates. Similarly, the genotypic studies revealed the plasmid profile results and the PCR results (plasmid-mediated genes and virulent genes). These findings indicate that plasmid-mediated genes (TEM-1, CTX-M-1 and OXA-1) and virulent genes [*e.g*., pyelonephritis-associated pili (*pap*) and afrimbrial adhesion (*afa*)] are

present in the ESBL isolates only. Perhaps newer antimicrobial agents can be anticipated, which inhibits the replication of plasmids at genetic level. Finally, the PFGE both for ESBL and non-ESBL showed heterogenous bands, which suggested that there is no clonal relationship among the isolates and they have acquired the genes from multiple source of infections. The foregoing results clearly indicated that epidemiological investigations are anticipated in different districts of Bangladesh such as multilocus sequence typing (MLST) or PFGE of ESBL-producing *E. coli* with reference to clinical significance.

Chapter 5 References

5 References

Abreu AG, Marques SG, Monteiro-Neto V, Gonçalves AG and Marques SG. 2013. Extended-spectrum β-lactamase-producing enterobacteriaceae in community-acquired urinary tract infections in São Luís, Brazil. *Braz J Microbiol*. **44**(2): 469-471.

Ahmed I and Salam A. 2002. Extended spectrum β-lactamases and bacterial resistance. *Pakistan J Med Sci*. **18**: 151-155.

Alim R, Amin R and Saleh AB. 2009. Detection of extended spectrum β-lactamase (ESBL) producing bacteria in urban areas of Bangladesh. *Bangladesh J Med Microbiol*. **13**(1): 5-9.

Al-Jasser AM. 2006. Extended-spectrum β-Lactamases (ESBLs): A global problem. *Kuwait Med J.* **38**(3): 171-185.

Ambler RP. 1980. The structure of β-lactamases. *Phil Trans R Soc Lond Biol*. **289**: 321-331.

Arlet G, Brami G, Décrè D, Flippo A, Gaillot O, Lagrange PH and Philippon A. 1995. Molecular characterisation by PCR-restriction fragment length polymorphism of TEM beta-lactamases. FEMS Microbiol Lett. **134**(2-3): 203-208.

Auer S, Wojna A and Hell M. 2010. Oral treatment options for ambulatory patients with urinary tract infections caused by extended-spectrum-β-lactamase-producing *Escherichia coli*. *Antimicrob Agents Chemother*. **54**(9): 4006-4008.

Azap OK, Arslan H, Serefhanoğlu K, Colakoğlu S, Erdoğan H, Timurkaynak F and Senger SS. 2010. Risk factors for extended-spectrum beta-lactamase positivity in uropathogenic *Escherichia coli* isolated from community-acquired urinary tract infections. *Clin Microbiol Infect*. **16**(2): 147-151.

Bachmann BJ. 1990. Linkage map of *Escherichia coli* K-12, 8th edn. *Microbiol Rev*. **54**: 130-197.

Barthélémy M, Péduzzi J, Bernard H, Tancrède C and Labia R. 1992. Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum

beta-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoca*. *Biochim Biophys Acta*. **1122**(1): 15-22.

Bauernfeind A, Casellas JM, Goldberg M, Holley M, Jungwirth R, Mangold P, Röhnisch T, Schweighart S and Wilhelm R. 1992. A new plasmidic cefotaximase from patients infected with *Salmonella typhimurium*. *Infection*. **20**(3): 158-163.

Bauernfeind A, Grimm H and Schweighart S. 1990. A new plasmidic cefotaximase in a clinical isolate of Escherichia coli. *Infection*. **18**(5): 294-298.

Bauernfeind A, Stemplinger I, Jungwirth R, Ernst S and Casellas JM. 1996. Sequences of beta-lactamase genes encoding CTX-M-1 (MEN-1) and CTX-M-2 and relationship of their amino acid sequences with those of other beta-lactamases. *Antimicrob Agents Chemother*. **40**(2): 509-513.

Belaaouaj A, Lapoumeroulie C, Caniça MM, Vedel G, Névot P, Krishnamoorthy R and Paul G. 1994. Nucleotide sequences of the genes coding for the TEM-like beta-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). *FEMS Microbiol Lett*. **120**(1-2): 75-80.

Bennett K and Grinsted D. 1984. *Methods in Microbiology (Plasmid Technology)*, Vol. 17, pp. 1-27. Academic Press, London.

Bergan T. 1984. Classification of enterobacteriaceae. *Methods in Microbiology*, 1st edn. Vol. 14, pp. 1-41. Academic Press, London.

Bertschinger HU. 1999. Postweaning *Escherichia coli* diarrhea and edema disease. In *Diseases of Swine* (Straw BE, D'Allaire S, Mengeling WL and Taylor DJ eds.), pp. 441-454. Iowa State University Press, Ames, Iowa.

Beuk C, Hill C, Whitehead S, Blondel-Hill E, Wagner K and Cheeptham N. 2013. Determination of susceptibility to fosfomycin and tigecycline of Enterobacteriaceae, particularly *Escherichia coli* isolates, producing extended-spectrum β-lactamases from multiple regional Canadian hospitals. *Can J Infect Dis Med Microbiol*. **24**(3): e80-82.

Blanco M, Blanco JE, Alonso MP and Blanco J. 1996. Virulence factors and O groups of *Escherichia coli* isolates from patients with acute pyelonephritis, cystitis and asymptomatic bacteriuria. *Eur J Epidemiol*. **12**(2): 191-198.

Blanco M, Blanco JE, Rodríguez E, Abalia I, Alonso MP and Blanco J. 1997. Detection of virulence genes in uropathogenic *Escherichia coli* by polymerase chain reaction (PCR): Comparison with results obtained using phenotypic methods. *J Microbiol Methods*. **31**(1-2): 37-43.

Blazquez J, Morosini MI, Negri MC and Baquero F. 2000. Selection of naturally occurring extended-spectrum TEM beta-lactamase variants by fluctuating beta-lactam pressure. Antimicrob Agents Chemother. **44**(8): 2182-2184.

Bloch CA and Rode CK. 1996. Pathogenicity island evaluation in *Escherichia coli* K1 by crossing with laboratory strain K-12. *Infect Immun*. **64**(8): 3218-3223.

Bonnet R. 2004. Growing group of extended-spectrum β-lactamases: The CTX-M enzymes. *Antimicrob Agents Chemother*. **48**: 1-14.

Bonnet R, De Champs C, Sirot D, Chanal C, Labia R and Sirot J. 1999. Diversity of TEM mutants in *Proteus mirabilis*. *Antimicrob Agents Chemother*. **43**(11): 2671-2677.

Bouza E and Cercenado E. 2002. *Klebsiella* and *Enterobacter*: Antibiotic resistance and treatment implications. *Semin Respir Infect*. **17**: 215-230.

Boyd EF, Nelson K, Wang F-S, Whittam TS and Selander RK. 1994. Molecular genetic basis of allelic polymorphism in malate dehydrogenase (*mdh*) in natural populations of *Escherichia coli* and *Salmonella enterica*. *Proc Natl Acad Sci USA*. **91**: 1280-1284.

Bradford PA. 2001. Extended spectrum β-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev*. **14**: 933-951.

Bradford PA, Cherubin CE, Idemyor V, Rasmussen BA and Bush K. 1994. Multiply resistant *Klebsiella pneumoniae* strains from two Chicago hospitals: Identification of the extended-spectrum TEM-12 and TEM-10 ceftazidime-hydrolyzing-β-lactamases in a single isolate. *Antimicrob Agents Chemother.* **38**: 761-766.

Branger C, Lesimple AL, Bruneau B, Berry P and Lambert-Zechovsky N. 1998. Long-term investigation of the clonal dissemination of *Klebsiella pneumoniae* isolates producing extended-spectrum beta-lactamases in a university hospital. *J Med Microbiol.* **47**(3): 201-209.

Brenner DJ. 1984. Family enterobacteriaceae. In *Bergey's Manual of Systematic Bacteriology* (Krieg NR and Malt JG eds.), Vol. 1, pp. 408-420. Williams and Wilkins, Baltimore.

Brown TA. 2010. *Gene Cloning and DNA Analysis: An Introduction*, 10th edn., pp. 13-17. Blackwell Science Ltd., Oxford.

Bush K. 2001. New β-lactamases in Gram-negative bacteria: Diversity and impact on the selection of antimicrobial therapy. *Clin Infect Dis*. **32**: 1085-1089.

Bush K, Jacoby GA and Medeiros AA. 1995. A functional classification scheme for βlactamases and its correlation with molecular structure. *Antimicrob Agents Chemother*. **39**: 1211-1233.

Calbo E, Romaní V, Xercavins M, Gómez L, Vidal CG, Quintana S, Vila J and Garau J. 2006. Risk factors for community-onset urinary tract infections due to *Escherichia coli* harbouring extended-spectrum beta-lactamases. *J Antimicrob Chemother*. **57**(4): 780-783.

Canton R and Coque TM. 2006. The CTX-M beta-lactamases pandemic. *Curr Opin Microbial*. **9**: 466-475.

Canton R, Coque TM and Baqueero F. 2003. Multi-resistant Gram-negative bacilli: From epidemics to endemics. *Curro Opin Infect Dis*. **16**: 315-325.

Choudhury MR 1999. *Modern Medical Microbiology*, 5th edn., p. 348. Bishwa Parichaya, Dhaka.

Chowdhury MA, Rahman KM, Miah MR and Haq JA. 1994. Transferable drug resistance (R-factor) among the enterobacteriaceae in urinary tract infections: A study at an urban hospital in Bangladesh. *J Trop Med Hyg*. **97**(3): 161-166.

Chung M, de Lencastre H, Matthews P, Tomasz A, Adamsson I, Aires de Sousa M, Camou T, Cocuzza C, Corso A, Couto I, Dominguez A, Gniadkowski M, Goering R, Gomes A, Kikuchi K, Marchese A, Mato R, Melter O, Oliveira D, Palacio R, Sá-Leão R, Santos Sanches I, Song JH, Tassios PT, Villari P; Multilaboratory Project Collaborators. Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: Comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. *Microb Drug Resist*. **6**(3): 189-198.

Colodner R, Rock W, Chazan B, Keller N, Guy N, Sakran W and Raz R. 2004. Risk factors for the development of extended-spectrum beta-lactamase-producing bacteria in nonhospitalized patients. *Eur J Clin Microbiol Infect Dis*. **23**(3): 163-167.

Coque TM, Oliver A, Pérez-Díaz JC, Baquero F and Cantón R. 2002. Genes encoding TEM-4, SHV-2, and CTX-M-10 extended-spectrum beta-lactamases are carried by multiple *Klebsiella pneumoniae* clones in a single hospital (Madrid, 1989 to 2000). *Antimicrob Agents Chemother*. **46**(2): 500-510.

Corkill JE, Anson JJ and Hart CA. 2005. High prevalence of the plasmid-mediated quinolones resistance determinant qnrA in multigrug-resistant Enterobacteriaceae from blood cultures in Liverpool, UK. *J Antimicrob Chemother*. **56**(6): 1115-1117.

Coudron PE, Moland ES and Sanders CC. 1997. Occurrence and detection of extended-spectrum beta-lactamases in members of the family Enterobacteriaceae at a veterans medical center: Seek and you may find. *J Clin Microbiol*. **35**(10): 2593-2597.

Dewar S, Reed LC and Koerner RJ. 2014. Emerging clinical role of pivmecillinam in the treatment of urinary tract infection in the context of multidrug-resistant bacteria. *J Antimicrob Chemother*. **69**(2): 303-308.

Donenberg MS and Welch RA. 1996. Virulence determinants of uropathogenic *Escherichia coli*. In *Urinary Tract Infections: Molecular Pathogenesis and Clinical Management* (Mobley HLT and Warren JW eds.), pp. 135-174. American Society for Microbiology, Washington DC.

Drieux L, Brossier F, Sougakoff W and Jarlier V. 2008. Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae: Review and bench guide. *Clin Microbiol Infect.* **14**(Suppl. 1): 90-103.

Dromigny JA, Nabeth P, Juergens-Behr A and Perrier-Gros-Claude JD. 2005. Risk factors for antibiotic-resistant *Escherichia coli* isolated from community-acquired urinary tract infections in Dakar, Senegal. *J Antimicrob Chemother*. **56**(1): 236-239.

Eckert CV, Gautier M and Arlet G. 2006. DNA sequence analysis of the genetic environment of various $bla_{\text{CTX-M}}$ genes. *J Antimicrob Chemother*. **57**(1): 14-23.

Eckert C, Gautier V, Saladin-Allard M, Hidri N, Verdet C, Ould-Hocine Z, Barnaud G, Delisle F, Rossier A, Lambert T, Philippon A and Arlet G. 2004. Dissemination of CTX-M-type beta-lactamases among clinical isolates of Enterobacteriaceae in Paris, France. *Antimicrob Agents Chemother*. **48**(4): 1249-1255.

Edelstein M, Pimkin M, Palagin I, Edelstein I and Stratchounski L. 2003. Prevalence and molecular epidemiology of CTX-M extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals. *Antimicrob Agents Chemother*. **47**(12): 3724-3732.

Edwards PR and Ewing WH. 1972. *Identification of Enterobacteriaceae*, pp. 137-166. Burgess Publishing Company, Minnesota.

Elwell LP, Saunders JR, Richmond MH and Falkow S. 1977. Relationships among some R plasmids found in *Haemophilus influenzae*. *J Bacteriol*. **131**(1): 356-362.

Essack SYL, Hall M, Pillay DG, Mcfadyen ML and Livermore DM. 2001. Complexity and diversity *of Klebsiella pneumoniae* strains with extended spectrum β-lactamases isolated in 1994 and 1996 at a teaching hospital in Durrban, South Africa. *Antimicrob Agents Chemother*. **45**: 88-95.

Everett MJY, Jin F, Ricci V and Piddock LJ. 1996. Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrob Agents Chemother*. **40**: 2380-2386.

Falagas ME, Kastoris AC, Kapaskelis AM and Karageorgopoulos DE. 2010. Fosfomycin for the treatment of multidrug-resistant, including extended-spectrum beta-lactamase producing, Enterobacteriaceae infections: A systematic review. *Lancet Infect Dis*. **10**(1): 43-50.

Farmer JJ 3rd, Davis BR, Hickman-Brenner FW, McWhorter A, Huntley-Carter GP, Asbury MA, Riddle C, Wathen-Grady HG, Elias C and Fanning GR. 1985. Biochemical identification of new species and biogroups of Enterobacteriaceae isolated from clinical specimens. *J Clin Microbiol*. **21**(1): 46-76.

Fihman V, Lartigue MF, Jacquier H, Meunier F, Schnepf N, Raskine L, Riahi J, Sanson-le Pors MJ and Berçot B. 2008. Appearance of aac(6')-Ib-cr gene among extended-spectrum beta-lactamase-producing Enterobacteriaceae in a French hospital. *J Infect*. **56**(6): 454-459.

Fowler JE Jr and Stamey TA. 1976. Studies of introital colonization in women with recurrent urinary infections. VII. The role of bacterial adherence. *Trans Am Assoc Genitourin Surg*. **68**: 78-82.

Gales AC, Jones RN, Turnidge J, Rennie R and Ramphal R. 2001. Characterization of *Pseudomonas aeruginosa* isolates: Occurrence rates, antimicrobial susceptibility patterns, and molecular typing in the global SENTRY Antimicrobial Surveillance Program, 1997-1999. *Clin Infect Dis*. **32**(Suppl. 2): S146-S155.

Gangoué-Piéboji J, Bedenic B, Koulla-Shiro S, Randegger C, Adiogo D, Ngassam P, Ndumbe P and Hächler H. 2005. Extended-spectrum-beta-lactamase-producing Enterobacteriaceae in Yaounde, Cameroon. *J Clin Microbiol*. **43**(7): 3273-3277.

Gniadkowski M. 2001. Evolution and epidemiology of extended-spectrum beta-lactamases (ESBLs) and ESBL-producing microorganisms. *Clin Microbiol Infect*. **7**(11): 597-608.

Graninger W. 2003. Pivmecillinam−therapy of choice for lower urinary tract infection. *Int J Antimicrob Agents*. **22**(Suppl. 2): 73-78.

Gupta K. 2002. Addressing antibiotic resistance. *Am J Med*. **113**(Suppl. 1A): 29S-34S.

Habeeb MA, Sarwar Y, Ali A, Salman M and Haque A. 2013. Rapid emergence of ESBL producers in *E. coli* causing urinary and wound infections in Pakistan. *Pak J Med Sci*. **29**(2): 540-544.

Haider K, Huq MI, Talukder KA and Ahmed QS. 1989. Electropherotyping of plasmid DNA of different serotypes of *Shigella flexneri* isolated in Bangladesh. *Epidemiol Infect*. **102**: 421-428.

Hales BA, Hart CA, Batt RM and Saunders JR. 1992. The large plasmids found in enterohemorrhagic and enteropathogenic *Escherichia coli* constitute a related series of transfer-defective Inc F-IIA replicons. *Plasmid*. **28**: 183-193.

Hardy K. 1986. *Bacterial Plasmids*, 2nd edn., pp. 82-87. American Society for Microbiology, Washington DC.

Hata M, Suzuki M, Matsumoto M, Takahshi MI and Sato S. 2005. Cloning of a novel gene for quinolones resistance from a transferable plasmid in *Shigella flexneri* 2b. *Antimicrob Agents Chemother*. **49**: 801-803.

Henquell C, Chanal C, Sirot D, Labia R and Sirot R. 1995. Molecular characterization of nine different types of mutants among 107 inhibitor-resistant TEM β-Iactamases from clinical isolates of *Escherichia coli. Antimicrob Agents Chemother*. **39**: 427-430.

Hernández JR, Martínez-Martínez L, Cantón R, Coque TM, Pascual A; Spanish Group for Nosocomial Infections (GEIH). 2005. Nationwide study of *Escherichia coli*

and *Klebsiella pneumoniae* producing extended-spectrum beta-lactamases in Spain. *Antimicrob Agents Chemother*. **49**(5): 2122-2125.

High NJ, Hales BA, Jann K and Boulnois GJ. 1988. A block of urovirulence genes encoding multiple fimbrae and haemolysin in *Escherichia coli* O4:K12:H. *Infect Immun*. **56**: 513-517.

Hinnebush J and Tilly K. 1993. Linear plasmids and chromosomes in bacteria. *Mol Microbiol*. **10**: 917-922.

Ho PL, Tsang DN, Que TL, Ho M and Yuen KY. 2000. Comparison of screening methods for detection of extended-spectrum beta-lactamases and their prevalence among *Escherichia coli* and *Klebsiella* species in Hong Kong. *APMIS*. **108**(3): 237-240.

Hoque N, Kibria G and Begum SN. 1976. Prevalence of urinary tract infection in adult women. *Bangladesh Med Res Counc Bull*. **2**(2): 75-85.

Huang ES and Stafford RS. 2002. National patterns in the treatment of urinary tract infections in women by ambulatory care physicians. *Arch Intern Med*. **162**: 41-47.

Hunter SB, Vauterin P, Lambert-Fair MA, Van Duyne MS, Kubota K, Graves L, Wrigley D, Barrett T and Ribot E. 2005. Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: Converting the National Databases to the new size standard. *J Clin Microbiol*. **43**(3): 1045-1050.

Huovinen S, Huovinen P and Jacoby GA. 1988. Detection of plasmid-mediated β-lactamases with DNA probes. *Antimicrob Agents Chemother*. **32**: 175-179.

Isikgoz Tasbakan M, Durusoy R, Pullukcu H, Sipahi OR, Ulusoy S; Turkish Nosocomial Urinary Tract Infection Study Group. 2011. Hospital-acquired urinary tract infection point prevalence in Turkey: Differences in risk factors among patient groups. *Ann Clin Microbiol Antimicrob*. **12**: 31.

Islam MA, Huq H, Nabi A, Talukdar PK, Ahmed D, Talukder KA, Cravioto A and Endtz HP. 2013. Occurrence and characterization of multidrug-resistant New Delhi metallo-β-lactamase-1-producing bacteria isolated between 2003 and 2010 in Bangladesh. *J Med Microbiol*. **62**(Pt. 1): 62-68.

Jacoby GA and Medeiros AA. 1991. More extended spectrum β-lactamases. *Antimicrob Agents Chemother*. **35**: 1697-1704.

Jacoby GA and Sutton L. 1991. Properties of plasmids responsible for production of extended-spectrum β-lactamases. *Antimicrob Agents Chemother*. **35**: 164-169.

Jadhav S, Hussien A, Devi S, Kumar A, Parveen S, Gandham N, Wieler LH, Ewers C and Ahmed N. 2011. Virulence characteristics and genetic affinities of multiple drug resistant uropathogenic *Escherichia coli* from a semi urban locality in India. *Plos One*. **6**(3): e18063.

Jansåker F, Frimodt-Møller N, Sjögren I and Dahl Knudsen. 2014. Clinical and bacteriological effects of pivmecillinam for ESBL-producing *Escherichia coli* or *Klebsiella pneumoniae* in urinary tract infections. *J Antimicrob Chemother*. **69**(3): 769-772.

Jarlier V, Nicolas MH, Fournier G, Philippon A. 1988. Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in Enterobacteriaceae: Hospital prevalence and susceptibility patterns. *Rev Infect Dis*. **10**(4): 867-878.

Jeong SH, Bae IK, Lee JH, Sohn SG, Kang GH, Jeon GJ, Kim YH, Jeong BC and Lee SH. 2004. Molecular characterization of extended-spectrum beta-lactamases produced by clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* from a Korean nationwide survey. *J Clin Microbiol*. **42**(7): 2902-2906.

Jones D, Bichler K, Hartung D, Spitzmuller B and Daschner FD. 2005. Plasmid mediated quinolones resistance in isolates obtained in German intensive care units. *Antimicrob Agents Chemother*. **49**: 773-775.

Karisik E, Ellington MJ, Pike R, Warren RE, Livermore DM and Woodford N. 2006. Molecular characterization of plasmids encoding CTX-M-15 beta-lactamases from Escherichia coli strains in the United Kingdom. *J Antimicrob Chemother*. **58**(3): 665-668.

Kariuki S, Revathi G, Corkill J, Kiiru J, Mwituria J, Mirza N and Hart CA. 2007. *Escherichia coli* from community-acquired urinary tract infections resistant to fluoroquinolones and extended-spectrum beta-lactams. *J Infect Dev Ctries*. **1**(3): 257-262.

Karlowsky JA, Kelly LJ, Thornsberry C, Jones ME and Sahm DF. 2002. Trends in antimicrobial resistance among urinary tract infection isolates of *Escherichia coli* from female outpatients in the United States. *Antimicrob Agents Chemother*. **46**(8): 2540-2545.

Kawamura-Sato K, Risa Y, Keigo S and Michio O. 2010. Virulence genes, quinolone and fluoroquinolone resistance, and phylogenetic background of uropathogenic *Escherichia coli* strains isolated in Japan. *Jpn J Infect Dis*. **63**: 113-115.

Kim J and Lee HJ. 2000. Rapid discriminatory detection of genes coding for SHV β-lactamases by ligase chain reaction. *Antimicrob Agents Chemother*. **44**: 1860-1864.

Komatsu M, Aihara M, Shimakawa K, Iwasaki M, Nagasaka Y, Fukuda S, Matsuo S and Iwatani Y. 2003. Evaluation of MicroScan ESBL confirmation panel for Enterobacteriaceae-producing, extended-spectrum beta-lactamases isolated in Japan. *Diagn Microbiol Infect Dis*. **46**(2): 125-130.

Knothe H, Shah P, Krcmery V, Antal M and Mitsuhashi S. 1983. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. *Infection*. **11**(6): 315-317.

Kumar MS, Lakshmi V and Rajagopalan R. 2006. Occurrence of extended spectrum beta-lactamases among Enterobacteriaceae spp. isolated at a tertiary care institute. *Indian J Med Microbiol*. **24**(3): 208-211.

Kunin CM. 2009. Catheter-associated urinary tract infections: A syllogism compounded by a questionable dichotomy. *Clin Infect Dis*. **48**(9): 1189-1190.

Lau SH, Kaufmann ME, Livermore DM, Woodford N, Willshaw GA, Cheasty T, Stamper K, Reddy S, Cheesbrough J, Bolton FJ, Fox AJ and Upton M. 2008. UK epidemic *Escherichia coli* strains A-E, with CTX-M-15 beta-lactamase, all belong to the international O25:H4-ST131 clone. *J Antimicrob Chemother*. **62**(6):1241-1244.

Lauerman LH. 2004. Advances in PCR technology. *Anim Health Res Rev*. **5**(2): 247-248.

Lautenbach E, Patel JB, Bilker WB, Edelstein PH and Fishman NO. 2001. Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*: Risk factors for infection and impact of resistance on outcomes. *Clin Infect Dis*. **32**(8): 1162-1171.

Lederberg J. 1952. Cell genetics and hereditary symbiosis. *Physiol Rev.* **32**(4): 403-430.

Leflon-Guibout V, Speldooren V, Heym B and Nicolas-Chanoine MH. 2000. Epidemiological survey of amoxicillin-clavulanate resistance and corresponding molecular mechanisms in Escherichia coli isolates in France: New genetic features of bla_{TEM} genes. *Antimicrob Agents Chemother*. **44**: 2709-2714.

Levine MM. 1988. Characterization of enteroadherent aggregative *Escherichia coli,* putative agent of diarrhoeal diseases. *J Infect Dis*. **158**: 75-79.

Levinson W. 2010. *Review of Medical Microbiology and Immunology*, 11th edn., p. 9. McGraw-Hill Companies, Inc., New York.

Li D, Liu B, Chen M, Guo D, Guo X, Liu F, Feng L and Wang L. 2010. A multiplex PCR method to detect 14 *Escherichia coli* serogroups associated with urinary tract infections. *J Microbiol Methods*. **82**(1): 71-77.

Lior H. 1996. Classification of *Escherichia coli*. In *Escherichia coli in Domestic Animals and Humans* (Gyles CL ed.), pp. 31-72. CAB International, Wallingford.

Livermore DM. 1995. β-Lactamases in laboratory and clinical resistance. *Clin Microbiol Rev*. **8**: 557-584.

Lloyd AL, Rasko DA and Mobley HL. 2007. Defining genomic islands and uropathogen-specific genes in uropathogenic *Escherichia coli*. *J Bacteriol*. **189**(9): 3532-3546.

Lloyd AL, Henderson TA, Vigil PD and Mobley HLT. 2009. Genomic islands of uropathogenic *Escherichia coli* contribute to virulence. *J Bacteriol*. **191**: 3469-3481.

Mabilat C and Courvalin P. 1990. Development of "oligotyping" for characterization and molecular epidemiology of TEM β-lactamases in members of the family Enterobacteriaceae. *Antimicrob Agents Chemother*. **34**: 2210-2216.

Machado E, Coque TM, Cantón R, Baquero F, Sousa JC, Peixe L; Portuguese Resistance Study Group. 2006. Dissemination in Portugal of CTX-M-15-, OXA-1-, and TEM-1-producing Enterobacteriaceae strains containing the aac(6')-Ib-cr gene, which encodes an aminoglycoside- and fluoroquinolone-modifying enzyme. *Antimicrob Agents Chemother*. **50**(9): 3220-3221.

Maheswari UB, Palvai S, Anuradha PR and Kammili N. 2013. Hemagglutination and biofilm formation as virulence markers of uropathogenic *Escherichia coli* in acute urinary tract infections and urolithiasis as virulence markers of uropathogenic. *Indian J Urol*. **29**(4):277-281.

Mahmood B, Ullah ME, Talukder KA, Gomes DJ, Chowdhury MAR and Roy SR. 2008. Analysis of plasmid profile in ESBL *Escherichia coli* isolated from urinary tract infections in Sylhet. *North East Med Coll J*. **3**(1): 20-23.

Marchandin H, Carriere C, Sirot D, Jean-Pierre H and Darbas H. 1999. TEM-24 produced by four different species of Enterobacteriaceae, including *Providencia rettgeri*, in a single patient. *Antimicrob Agents Chemother*. **43**(8): 2069-2073.

Marijan T, Vranes J, Bedenić B, Mlinarić-Dzepina A, Plecko V and Kalenić S. 2007. Emergence of uropathogenic extended-spectrum beta lactamases-producing *Escherichia coli* strains in the community. *Coll Antropol.* **31**(1): 227-233.

Marković T, Jeinić L, Smitran A and Petković M. 2013. Prevalence and antimicrobial susceptibility of extended-spectrum beta-lactamase producing urinary isolates of *Escherichia coli* in outpatients. *Srp Arh Celok Lek*. **141**(11-12): 775-779.

Martínez-Martínez L, Hernández-Allés S, Albertí S, Tomás JM, Benedi VJ and Jacoby GA. 1996. *In vivo* selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded-spectrum-cephalosporins. *Antimicrob Agents Chemother*. **40**(2): 342-348.

Matsumoto T and Muratani T. 2004. Newer carbapenems for urinary tract infections. *Int J Antimicrob Agents*. **24**(Suppl. 1): S35-S38.

Miró E, Navarro F, Mirelis B, Sabaté M, Rivera A, Coll P and Prats G. 2002. Prevalence of clinical isolates of *Escherichia coli* producing inhibitor-resistant β-lactamases at a University Hospital in Barcelona, Spain, over a 3-year period. *Antimicrob Agents Chemother*. **46**(12): 3991-3994.

McDaniel TK and Kaper JB. 1997. A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. *Mol Microbiol*. **23**: 399-407.

Medeiros AA, Cohenford M and Jacoby GA. 1985. Five novel plasmid-determined beta-lactamases. *Antimicrob Agents Chemother*. **27**(5): 715-719.

Medeiros AA, Hedges RW and Jacoby GA. 1982. Spread of a "*Pseudomonas*-specific" beta-lactamase to plasmids of enterobacteria. *J Bacteriol*. **149**(2): 700-707.

Minarini LAR, Camargo IIiana LBC, Pitondo-Silva A and Darini Ana LC. 2007. Multilocus sequence typing of uropathogenic ESBL-producing *Escherichia coli* isolated in a Brazilian community. *Curr Microbiol*. **55**: 524-529.

Mitsumori K, Terai A, Yamamoto S and Yoshida O. 1997. Virulence characteristics and DNA fingerprints of *Escherichia coli* isolated from women with acute uncomplicated pyelonephritis. *J Urol*. **158**(6): 2329-2332.

Morin MD and Hopkins WJ. 2002. Identification of virulence genes in uropathogenic *Escherichia coli* by multiplex polymerase chain reaction and their association with infectivity in mice. *Urology*. **60**(3): 537-541.

Morosini MI, Canton R, Martinez-Beltran J, Negri MC, Perez-Diaz JC, Baquero F and Blazquez J. 1995. New extended-spectrum TEM-type beta-lactamase from *Salmonella enterica* subsp. *enterica* isolated in a nosocomial outbreak. *Antimicrob Agents Chemother*. **39**(2): 458-461.

Munday CJ, Xiong J, Li C, Shen D and Hawkey PM. 2004. Dissemination of CTX-M type beta-lactamases in Enterobacteriaceae isolates in the People's Republic of China. *Int J Antimicrob Agents*. **23**(2): 175-180.

Nataro JP, Maher KO, Mackie P and Kaper JB. 1987. Characterization of plasmids encoding the adherence factor of enteropathogenic *Escherichia coli*. *Infect Immun*. **55**(10): 2370-2377.

Nathisuwan SD, Burgess S and Lewis JS. 2001. ESBLs: Epidemiology, detection and treatment. *Pharmacotherapy*. **21**: 920-928.

Naveen R and Mathai E. 2005. Some virulence characteristics of uropathogenic *Escherichia coli* in different patient group. *Indian J Med Res*. **122**: 143-147.

NCCLS. 2004. *Performance Standards for Antimicrobial Susceptibility Testing*, 14th edn. National Committee for Clinical Laboratory Standard (NCCLS), NCCLS document M100-S14. Wayne, Pennsylvania.

Nemoy LL, Kotetishvili M, Tigno J, Keefer-Noris AD, Harris EN and Perencevich JA. 2005. Mutilocus sequence typing versus pulse-field gel electrophoresis for characterization of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates. *J Clin Microbiol*. **43**: 1776-1781.

Nicolas-Chanoine MH, Blanco J, Leflon-Guibout V, Demarty R, Alonso MP, Caniça MM, Park YJ, Lavigne JP, Pitout J and Johnson JR. 2008. Intercontinental emergence of *Escherichia coli* clone O25:H4-ST131 producing CTX-M-15. *J Antimicrob Chemother*. **61**(2): 273-281.

Nicolle LE. 2001. Epidemiology of urinary tract infection. *Infect Med*. **18**: 153-162.

Nicolle LE. 2005. Catheter-related urinary tract infection. *Drugs Aging*. **22**(8): 627-639.

Nicolle LE and Mulvey MR. 2007. Successful treatment of ctx-m ESBL producing *Escherichia coli* relapsing pyelonephritis with long term pivmecillinam. *Scand J Infect Dis*. **39**(8): 748-749.

Nüesch-Inderbinen MT, Hächler H and Kayser FH. 1996. Detection of genes coding for extended-spectrum SHV beta-lactamases in clinical isolates by a molecular genetic method, and comparison with the E test. *Eur J Clin Microbiol Infect Dis*. **15**(5): 398-402.

O'Brien AD, Tesh VL, Donohue-Rolfe A, Jackson MP, Olsnes S, Sandvig K, Lindberg AA and Keusch GT. 1992. Shiga toxin: Biochemistry, genetics, mode of action, and role in pathogenesis. *Curr Top Microbiol Immunol*. **180**: 65-94.

Olesen B, Hansen DS, Nilsson F, Frimodt-Møller J, Leihof RF, Struve C, Scheutz F, Johnston B, Krogfelt KA and Johnson JR. 2013. Prevalence and characteristics of the epidemic multiresistant *Escherichia coli* ST131 clonal group among extended-spectrum beta-lactamase-producing *E. coli* isolates in Copenhagen, Denmark. *J Clin Microbiol*. **51**(6): 1779-1785.

Ong CL, Ulett GC, Mabbett AN, Beatson SA, Webb RI, Monaghan W, Nimmo GR, Looke DF, McEwan AG and Schembri MA. 2008. Identification of type 3 fimbriae in uropathogenic *Escherichia coli* reveals a role in biofilm formation. *J Bacteriol*. **190**(3): 1054-1063.

Oteo J, Pérez-Vázquez M and Campos J. 2010. Extended-spectrum [beta]-lactamase producing *Escherichia coli*: Changing epidemiology and clinical impact. *Curr Opin Infect Dis*. **23**(4): 320-326.

Ouellette M, Paul GC, Philippon AM and Roy PH. 1988. Oligonucleotide probes (TEM-1, OXA-1) versus isoelectric focusing in beta-lactamase characterization of 114 resistant strains. *Antimicrob Agents Chemother*. **32**(3): 397-399.

Palzkill T, Thomson KS, Sanders CC, Moland ES, Huang W and Milligan TW. 1995. New variant of TEM-10 beta-lactamase gene produced by a clinical isolate of *Proteus mirabilis*. *Antimicrob Agents Chemother*. **39**(5): 1199-1200.

Pangon B, Bizet C, Buré A, Pichon F, Philippon A, Regnier B and Gutmann L. 1989. *In vivo* selection of a cephamycin-resistant, porin-deficient mutant of *Klebsiella pneumoniae* producing a TEM-3 beta-lactamase. *J Infect Dis*. **159**(5): 1005-1006.

Paterson DL. 2006. Resistance in Gram-negative bacteria: Enterobacteriaceae. *Am J Med*. **119**(6 Suppl. 1): S20-S28.

Paterson DL and Bonomo RA. 2005. Extended-spectrum beta-lactamases: A clinical update. *Clin Microbiol Rev*. **18**(4): 657-686.

Paterson DL, Hujer KM, Hujer AM, Yeiser B, Bonomo MD, Rice LB, Bonomo RA; International *Klebsiella* Study Group. 2003. Extended-spectrum beta-lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: Dominance and widespread prevalence of SHV- and CTX-M-type beta-lactamases. *Antimicrob Agents Chemother*. **47**(11): 3554-3560.

Paterson DL, Ko WC, Von Gottberg A, Mohapatra S, Casellas JM and Goossens H. 2004. Antibiotic therapy for *Klebsiella pneumoniae* bacteremia: Implications of production of extended-spectrum beta-lactamases. *Clin Infect Dis*. **39**: 31-37.

Paterson DL, Mulazimoglu L, Casellas JM, Ko WC, Goossens H, Von Gottberg A, Mohapatra S, Trenholme GM, Klugman KP, McCormack JG and Yu VL. 2000. Epidemiology of ciprofloxacin resistance and its relationship to extended-spectrum beta-lactamase production in *Klebsiella pneumoniae* isolates causing bacteremia. *Clin Infect Dis*. **30**(3): 473-478.

Peirano G, van Greune CH and Pitout JD. 2011. Characteristics of infections caused by extended-spectrum β-lactamase-producing *Escherichia coli* from community hospitals in South Africa. *Diagn Microbiol Infect Dis*. **69**(4): 449-453.

Perez F, Endimiani A, Hujer KM and Bonomo RA. 2007. The continuing challenge of ESBLs. *Curr Opin Pharmacol*. **7**(5): 459-469.

Perilli M, Felici A, Franceschini N, De Santis A, Pagani L, Luzzaro F, Oratore A, Rossolini GM, Knox JR and Amicosante G. 1997. Characterization of a new TEM-derived beta-lactamase produced in a *Serratia marcescens* strain. *Antimicrob Agents Chemother*. **41**(11): 2374-2382.

Perilli M, Segatore B, de Massis MR, Riccio ML, Bianchi C, Zollo A, Rossolini GM and Amicosante G. 2000. TEM-72, a new extended-spectrum beta-lactamase detected in *Proteus mirabilis* and *Morganella morganii* in Italy. *Antimicrob Agents Chemother*. **44**(9): 2537-2539.

Petrosino JF and Palzkill T. 1996. Systematic mutagenesis of the active site omega loop of TEM-1 beta-lactamase. *J Bacteriol*. **178**(7): 1821-1828.

Pfaller MA and Segreti J. 2006. Overview of the epidemiological profile and laboratory detection of extended-spectrum β-lactamases. *Clin Infect Dis*. **42**: S153-S163.

Piroth L, Aubé H, Doise JM and Vincent-Martin M. 1998. Spread of extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae*: Are beta-lactamase inhibitors of therapeutic value? *Clin Infect Dis*. **27**(1): 76-80.

Pitout JD, Hanson ND, Church DL and Laupland KB. 2004. Population-based laboratory surveillance for *Escherichia coli*-producing extended-spectrum beta-lactamases: Importance of community isolates with $bla_{\text{CTX-M}}$ genes. *Clin Infect Dis*. **38**(12): 1736-1741.

Pitout JD, Wei Y, Church DL and Gregson DB. 2008. Surveillance for plasmid-mediated quinolone resistance determinants in Enterobacteriaceae within the Calgary Health Region, Canada: The emergence of aac(6')-Ib-cr. *J Antimicrob Chemother*. **61**(5): 999-1002.

Prats G, Navarro F, Mirelis B, Dalmau D, Margall N, Coll P, Stell A and Johnson JR. 2000. *Escherichia coli* serotype O15:K52:H1 as a uropathogenic clone. *J Clin Microbiol*. **38**(1): 201-209.

Qi C, Pilla V, Yu JH and Reed K. 2010. Changing prevalence of *Escherichia coli* with CTX-M-type extended-spectrum beta-lactamases in outpatient urinary *E. coli* between 2003 and 2008. *Diagn Microbiol Infect Dis*. **67**(1): 87-91.

Qiao LD, Chen S, Yang Y, Zhang K, Zheng B, Guo HF, Yang B, Niu YJ, Wang Y, Shi BK, Yang WM, Zhao XK, Gao XF, Chen M and Tian Y. 2013. Characteristics of urinary tract infection pathogens and their *in vitro* susceptibility to antimicrobial agents in China: Data from a multicenter study. *BMJ Open*. **3**(12): e004152.

Radice M, Power P, Di Conza J and Gutkind G. 2002. Early dissemination of CTX-M-derived enzymes in South America. *Antimicrob Agents Chemother*. **46**(2): 602-604.

Raksha R, Srinivasa H and Macaden RS. 2003. Occurrence and characterisation of uropathogenic *Escherichia coli* in urinary tract infections. *Indian J Med Microbiol*. **21**(2): 102-107.

Rahman MM, Haq JA, Hossain MA, Sultana R, Islam F and Islam AH. 2004a. Prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in an urban hospital in Dhaka, Bangladesh. *Int J Antimicrob Agents*. **24**(5): 508-510.

Rahman M, Shoma S, Rashid H, Siddique AK, Nair GB and Sack DA. 2004b. Extended-spectrum beta-lactamase-mediated third-generation cephalosporin resistance in *Shigella* isolates in Bangladesh. *J Antimicrob Chemother*. **54**(4): 846-847.

Rahman NMW, Lutfor AB, Jhora ST, Yasmin M and Haq JA. 2010. Detection of CTX-M gene in extended spectrum beta-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella species* of different hospitals. *Bangladesh J Med Microbiol*. **4**(2): 28-31.

Ramphal R and Ambrose PG. 2006. Extended spectrum β-lactamases and clinical outcomes: Current data. *Clin Infect Dis*. **42**: S164-S172.

Ramphal R and Ambrose PG. 2006. Extended-spectrum beta-lactamases and clinical outcomes: Current data. *Clin Infect Dis*. **42**(Suppl. 4): S164-S172.

Rawat D and Nair D. 2010. Extended-spectrum β-lactamases in Gram-negative bacteria. *J Glob Infect Dis.* **2**(3): 263-274.

Revathi G and Singh S. 1997. Detection of extended-spectrum cephalosporin resistance due to inducible lactamases in hospital isolates. *Indian J Med Micribiol*. **15**: 113-115.

Rodríguez-Baño J, Alcalá JC, Cisneros JM, Grill F, Oliver A, Horcajada JP, Tórtola T, Mirelis B, Navarro G, Cuenca M, Esteve M, Peña C, Llanos AC, Cantón R and Pascual A. 2008. Community infections caused by extended-spectrum beta-lactamase-producing *Escherichia coli*. *Arch Intern Med*. **168**(17): 1897-1902.

Rossi F, Baquero F, Hsueh PR, Paterson DL, Bochicchio GV, Snyder TA, Satishchandran V, McCarroll K, DiNubile MJ and Chow JW. 2006. *In vitro* susceptibilities of aerobic and facultatively anaerobic Gram-negative bacilli isolated from patients with intra-abdominal infections worldwide: 2004 results from SMART (Study for Monitoring Antimicrobial Resistance Trends). *J Antimicrob Chemother.* **58**(1): 205-210.

Rowe-Magnus DA and Mazel D. 2002. The role of integrons in antibiotic resistance gene capture. *Int J Med Microbiol*. **292**(2): 115-125.

Ryoo NH, Kim EC, Hong SG, Park YJ, Lee K, Bae IK, Song EH and Jeong SH. 2005. Dissemination of SHV-12 and CTX-M-type extended-spectrum beta-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* and emergence of GES-3 in Korea. *J Antimicrob Chemother*. **56**(4): 698-702.

Saiki RK, Arnheim N and Erlich HA. 1985. A novel method for the detection of polymorphic restriction sites by cleavage of oligonucleotide probes: Application to sickle-cell anemia. *Nature Biotechnol*. **3**: 1008-1012.

Sakazaki R. 1992. Bacteriology of *Vibrio cholerae* and related organisms. In *Cholera* (Barua D and Greenough WB III eds.), pp. 37-55. Plenum Medical Book Company, New York.

Salyers AA and Whitt DD. 2005. *Escherichia coli* gastrointestinal infections. In *Bacterial Pathogenesis: A Molecular Approach* (Salyers AA and Whitt DD eds.), pp. 191-192. American Society for Microbiology, Washington DC.

Sambrook J, Fritsch EF and Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanders CC, Barry AL, Washington JA, Shubert C, Moland ES, Traczewski MM, Knapp C and Mulder R. 1996. Detection of extended-spectrum-beta-lactamase-producing members of the family Enterobacteriaceae with Vitek ESBL test. *J Clin Microbiol*. **34**(12): 2997-3001.

Sasakawa C, Kamata K, Sakai T, Murayama SY, Makino S and Yoshikawa M. 1986. Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and Congo red binding activity in *Shigella flexneri*. *Infect Immun*. **51**(2): 470-475.

Schaechter M and Neidhandt FC. 1987. Introduction. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Schaechter M and Umbarger HE eds., pp. 1-2. ASM Press, Washington DC.

Schappert SM. 1999. Ambulatory care visits to physician offices, hospital outpatient departments, and emergency departments: United States, 1997. *Vital Health Stat 13*. **143**(i-iv): 1-39.

Schwartz DC and Cantor CR. 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell*. **37**: 67-75.

Segal-Maurer S, Mariano N, Qavi A, Urban C and Rahal JJ Jr. 1999. Successful treatment of ceftazidime-resistant *Klebsiella pneumoniae* ventriculitis with

intravenous meropenem and intraventricular polymyxin B: Case report and review. *Clin Infect Dis*. **28**(5): 1134-1138.

Sensakovic JW and Smith LG. 1995. Beta-lactamase inhibitor combinations. *Med Clin North Am*. **79**: 695-704.

Shapiro JA. 1999. Views about evolution are evolving. *Am Soc Microbiol News*. **65**: 201-207.

Sharma S, Bhat GK and Shenoy S. 2007. Virulence factors and drug resistance in *Escherichia coli* isolated from extraintestinal infections. *Indian J Med Microbiol*. **25**(4): 369-373.

Silveira WD, Benetti F, Lancellotti M, Ferreira A, Solferini VN and Brocchi M. 2001. Biological and genetic characteristics of uropathogenic *Escherichia coli* strains. *Rev Inst Med Trop Sao Paulo*. **43**(6): 303-310.

Smith-Keary PF. 1988. *Genetic Elements in Escherichia coli*. p. 216. MacMillan Education Ltd., London.

So M and McCarthy BJ. 1980. Nucleotide sequence of the bacterial transposon Tn1681 encoding a heat-stable (ST) toxin and its identification in enterotoxigenic *Escherichia coli* strains. *Proc Natl Acad Sci USA*. **77**(7): 4011-4015.

Sobel JD and Kaye D. 2005. Urinary tract infections. In *Principles and Practise of Infectious Diseases* (Mandell GL, Bennett JE and Dolin R eds.), pp. 875-905. Churchill Livingstone, Philadelphia.

Søraas A, Sundsfjord A, Sandven I, Brunborg C and Jenum PA. 2013. Risk factors for community-acquired urinary tract infections caused by ESBL-producing enterobacteriaceae − A case-control study in a low prevalence country. *PLoS One*. **8**(7): e69581.

Sougakoff W, Goussard S and Courvalin P. 1988. The TEM-3 β-lactamase, which hydrolyzes broad-spectrum cephalosporins, is derived from the TEM-2 penicillinase by two amino acid substitutions. *FEMS Microbiol Lett*. **56**: 343-348.

Soulsby EJ. 2005. Resistance to antimicrobials in humans and animals. *Br Med J*. **331**: 1219-1220.

Spratt BG. 1994. Resistance to antibiotics mediated by target alterations. *Science*. **264**(5157): 388-393.

Stamm WE. 1999. The epidemiology of urinary tract infections: Risks factors reconsidered. *Inter Sci Conf Antimicrob Agents Chemother*. **39**: 769.

Stobberingh EE, Arends J, Hoogkamp-Korstanje JA, Goessens WH, Visser MR, Buiting AG, Debets-Ossenkopp YJ, van Ketel RJ, van Ogtrop ML, Sabbe LJ, Voorn GP, Winter HL and van Zeijl JH. 1999. Occurrence of extended-spectrum beta-lactamases (ESBL) in Dutch hospitals. *Infection*. **27**(6): 348-354.

Struelens MJ, Denis O and Rodriguez-Villalobos H. 2004. Microbiology of nosocomial infections: Progress and challenges. *Microbes Infect*. **6**(11): 1043-1048.

Takahashi S, Kurimura Y, Takeyama K, Hashimoto K, Miyamoto S, Ichihara K, Igarashi M, Hashimoto J, Furuya R, Hotta H, Uchida K, Miyao N, Yanase M, Takagi Y, Tachiki H, Taguchi K and Tsukamoto T. 2009. Efficacy of treatment with carbapenems and third-generation cephalosporins for patients with febrile complicated pyelonephritis. *J Infect Chemother*. **15**(6): 390-395.

Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH and Swaminathan B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. *J Clin Microbiol*. **33**(9): 2233-2239.

Thomas CGA. 1988. Gram-negative bacilli. In *Medical Microbiology*, 6th edn., pp. 273-274. Bailliere Tindall, London.

Thomson CJ. 1999. The global epidemiology of resistance to ciprofloxacin and the changing nature of antibiotic resistance: A 10 year perspective. *J Antimicrob Chemother*. **43**(Suppl. A): 31-40.

Thomson KS. 2001. Controversies about extended-spectrum and AmpC beta-lactamases. *Emerg Infect Dis*. **7**(2): 333-336.

Thomson KS and Moland ES. 2001. Cefepime, piperacillin-tazobactam, and the inoculum effect in tests with extended-spectrum-β-lactamase-producing Enterobacteriaceae. *Antimicrob Agents Chemother.* **45**: 3548-3554.

Thomson KS and Sanders CC. 1992. Detection of extended-spectrum beta-lactamases in members of the family Enterobacteriaceae: Comparison of the double-disk and three-dimensional tests. *Antimicrob Agents Chemother*. **36**(9): 1877-1882.

Tschäpe H. 1994. The spread of plasmids as a function of bacterial adaptability. *FEMS Microbiol Ecol*. **15**(1-2): 23-31.

Tzouvelekis LS and Bonomo RA. 1999. SHV-type beta-lactamases. *Curr Pharm Des*. **5**(11): 847-864.

Tzouvelekis LS, Tzelepi E, Tassios PT, Legakis NJ. 2000. CTX-M-type beta-lactamases: An emerging group of extended-spectrum enzymes. *Int J Antimicrob Agents*. **14**(2): 137-142.

Usein CR, Damian M, Tatu-Chitoiu D, Capusa C, Fagaras R, Tudorache D, Nica M and Le Bouguénec C. 2001. Prevalence of virulence genes in *Escherichia coli* strains isolated from Romanian adult urinary tract infection cases. *J Cell Mol Med*. **5**(3): 303-310.

Vahaboglu H, Ozturk R, Akbal H, Saribas S, Tansel O and Coşkunkan F. 1998. Practical approach for detection and identification of OXA-10-derived ceftazidime-hydrolyzing extended-spectrum beta-lactamases. *J Clin Microbiol*. **36**(3): 827-829.

Valverde A, Coque TM, Sánchez-Moreno MP, Rollán A, Baquero F and Cantón R. 2004. Dramatic increase in prevalence of fecal carriage of extended-spectrum beta-lactamase-producing Enterobacteriaceae during nonoutbreak situations in Spain. *J Clin Microbiol*. **42**(10): 4769-4775.

Velasco M, Horcajada JP, Mensa J, Moreno-Martinez A, Vila J, Martinez JA, Ruiz J, Barranco M, Roig G and Soriano E. 2001. Decreased invasive capacity of quinolone-resistant *Escherichia coli* in patients with urinary tract infections. *Clin Infect Dis*. **33**(10): 1682-1686.

Vercauteren E, Descheemaeker P, Ieven M, Sanders CC and Goossens H. 1997. Comparison of screening methods for detection of extended-spectrum beta-lactamases and their prevalence among blood isolates of *Escherichia coli* and *Klebsiella* spp. in a Belgian teaching hospital. *J Clin Microbiol*. **35**(9): 2191-2197.

Vila J, Ruiz J, Goñi P and De Anta MT. 1996. Detection of mutations in parC in quinolone-resistant clinical isolates of *Escherichia coli*. *Antimicrob Agents Chemother*. **40**(2): 491-493.

Vila J, Simon K, Ruiz J, Horcajada JP, Velasco M, Barranco M, Moreno A and Mensa J. 2002. Are quinolone-resistant uropathogenic *Escherichia coli* less virulent? *J Infect Dis*. **186**(7): 1039-1042.

Villa L, Pezzella C, Tosini F, Visca P, Petrucca A and Carattoli A. 2000. Multiple-antibiotic resistance mediated by structurally related IncL/M plasmids carrying an extended-spectrum beta-lactamase gene and a class 1 integron. *Antimicrob Agents Chemother*. **44**(10): 2911-2914.

Wang M, Sahm DF, Jacoby GA and Hooper DC. 2004. Emerging plasmid-mediated quinolone resistance associated with the qnr gene in Klebsiella pneumoniae clinical isolates in the United States. *Antimicrob Agents Chemother*. **48**(4): 1295-1299.

Whittam TS, Wolfe ML, Wachsmuth IK, Orskov F, Orskov I and Wilson RA. 1993. Clonal relationships among *Escherichia coli* strains that cause hemorrhagic colitis and infantile diarrhea. *Infect Immun*. **61**(5): 1619-1629.

Wilson ML and Gaido L. 2004. Laboratory diagnosis of urinary tract infections in adult patients. *Clin Infect Dis*. **38**(8): 1150-1158.

Yagi T, Kurokawa H, Shibata N, Shibayama K and Arakawa Y. 2000. A preliminary survey of extended-spectrum beta-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. *FEMS Microbiol Lett*. **184**(1): 53-56.

Yamamoto S. 2007. Molecular epidemiology of uropathogenic *Escherichia coli*. *J Infect Chemother*. **13**(2): 68-73.

Yamamoto S, Tsukamoto T, Terai A, Kurazono H, Takeda Y and Yoshida O. 1995. Distribution of virulence factors in *Escherichia coli* isolated from urine of cystitis patients. *Microbiol Immunol*. **39**(6): 401-404.

Yu WL, Jones RN, Hollis RJ, Messer SA, Biedenbach DJ, Deshpande LM and Pfaller MA. 2002. Molecular epidemiology of extended-spectrum beta-lactamase-producing, fluoroquinolone-resistant isolates of *Klebsiella pneumoniae* in Taiwan. *J Clin Microbiol*. **40**(12): 4666-4669.

Yumuk Z, Afacan G, Nicolas-Chanoine MH, Sotto A and Lavigne JP. 2008. Turkey: A further country concerned by community-acquired *Escherichia coli* clone O25-ST131 producing CTX-M-15. *J Antimicrob Chemother*. **62**(2): 284-288.

Zhou XY, Bordon F, Sirot D, Kitzis MD and Gutmann L. 1994. Emergence of clinical isolates of *Escherichia coli* producing TEM-1 derivatives or an OXA-1 beta-lactamase conferring resistance to beta-lactamase inhibitors. *Antimicrob Agents Chemother*. **38**(5): 1085-1089.

Appendices

Appendix 1: Culture Media

Sterilization of all tubed media is accomplished at 15 lb pressure per square inch (121°C) for 15 minutes unless otherwise specified. Longer sterilization times would be required for large volumes of media. Most of the media are available commercially in powdered form, with specific instructions for their preparation and sterilization.

Kligler Iron Agar (pH 7.4)

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	15.0 g
Proteose peptone	5.0 g
Lactose	10.0 g
Dextrose	1.0 g
Ferrous sulfate	0.2 g
Sodium chloride	5.0 g
Sodium thiosulfate	0.3 g
Agar	12.0 g
Phenol red	0.024 g
Distilled water	1,000.0 ml

Luria-Bertani Broth Medium (pH 7.5)

Bacto tryptone	10.0 g
Sodium chloride	10.0 g
Bacto yeast extract	5.0 g

MacConkey's Agar (pH 7.1)

Bacto peptone	17.0 g
Proteose peptone	3.0 g
Lactose	10.0 g
Bile salts mixture	1.5 g
Sodium chloride	5.0 g
Agar	13.5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Distilled water	1,000.0 ml

Motility Test Medium (pH 7.2)

Tryptose	10.0 g
Sodium chloride	5.0 g
Agar	5.0 g
Distilled water	1,000.0 ml

MR-VP Broth (pH 6.9)

Peptone	7.0 g
Dextrose	5.0 g
Potassium phosphate	5.0 g
Distilled water	1,000.0 ml

Mueller-Hinton Agar (pH 7.4)

Beef, infusion	300.0 g
Casamino acids	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	1,000.0 ml

Mueller-Hinton Broth (pH 7.4)

Beef, infusion	300.0 g
Casamino acids	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	1,000.0 ml

Nutrient Agar (pH 7.0)

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
Distilled water	1,000.0 ml

Nutrient Broth (pH 7.0)

Peptone	5.0 g
Beef extract	3.0 g
Distilled water	1,000.0 ml

Simmons Citrate Agar (pH 6.9)

Ammonium dihydrogen phosphate	1.0 g
Dipotassium phosphate	1.0 g
Sodium chloride	5.0 g
Sodium citrate	2.0 g
Magnesium sulfate	0.2 g
Agar	15.0 g
Bromothymol blue	0.08 g
Distilled water	1,000.0 ml

Trypticase (Tryptic) Soy Agar (pH 7.3)

Trypticase (tryptone)	15.0	g
Phytone (soytone)	5.0	g
Sodium chloride	5.0	g
Agar	15.0	g
Distilled water	1,000.0	ml

Trypticase (Tryptic) Soy Broth (pH 7.3)

Tryptone	17.0	g
Soytone	3.0	g
Dextrose	2.5	g
Sodium chloride	5.0	g
Dipotassium phosphate	2.5	g
Distilled water	1,000.0	ml

Appendix 2: Stock Solutions

All the working solutions used in the work were prepared from the stock solutions.

Acridine Orange Dye Solution

It was prepared by adding 0.01 g amount in 80 ml distilled water (filtered) and vigorously vortexed. The volume was made upto 100 ml and covered with aluminum foil to protect light reaction. It was stored at 4°C.

0.5 *M* EDTA

186.1 g of $Na_2EDTA.2H_2O$ (disodium ethylene diamine tetra-acetic acid) and 20 g of NaOH pellets were added to 800 ml distilled water and dissolved by stirring on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH and final volume was made up to 1l with distilled water. The solution was sterilized by autoclaving and stored at room temperature (RT).

Ethidium Bromide Solution

Ethidium bromide was dissolved in distilled water at a concentration of 10 mg/ml and stored at 4°C in the dark.

Gel Loading Buffer

6% concentrated loading buffer consisted of 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (type 400; Pharmacia), 0.5 μg/ml Rnase in water. It was stored at 4°C in 1 ml aliquot.

Gel Tracking Dye Solution

2.5% Ficoll 400

11 m*M* EDTA

3.3 m*M* Tris-HCl

0.017% SDS

0.15% Bromophenol Blue

This solution is designed for use as a visual indicator of electrophoresis. It is a pre-mixed loading buffer with a tracking dye for agarose gel electrophoresis.

McFarland 0.5 Standard

0.05 ml of 1.0% barium chloride ($BaCl_2$)

9.95 ml 1.0% sulfuric acid (H_2SO_4)

The reagents are mixed well to provide the corresponding cell density of approximately 1.5 x 10^8 CFU/ml with % transmittance of 74.3 and absorbance (OD) value of 0.08 to 0.1 at wavelength of 600 nm.

Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 2 g of KH_2PO_4 in 800 ml of distilled water. pH was adjusted to 7.4 with HCl. The final volume was adjusted to one litre by distilled water. The solution was sterilized by autoclaving for 20 minutes and stored at room temperature (RT).

3 *M* Sodium Acetate

40.81 g of $Na_2(CH_3COOH).H_2O$ was dissolved in 80 ml of distilled water. The pH was adjusted to 5.2 with glacial acetic acid. The final volume was adjusted to 100 ml with distilled water and the solution was sterilized by autoclaving. It was stored at 4°C.

3 *M* Sodium Chloride (NaCl)

175.3 g of NaCl was dissolved in distilled water to a final volume of one litre. The solution was autoclaved and stored at room temperature (RT).

10% Sodium Dodecyl Sulfate (SDS)

10 g of SDS (sodium dodecyl sulfate) (Sigma, USA) was added to 80 ml of distilled water and dissolved by stirring on a magnetic stirrer slowly to avoid foaming. The final volume was adjusted to 100 ml with distilled water and stored at room temperature (RT).

10 *M* Sodium Hydroxide (NaOH)

40 g of NaOH pellet was dissolved in distilled water to final volume of 100 ml. The solution was stored in an airtight bottle at room temperature (RT).

20x SSC (Standard Saline Citrate)

175.3 g of NaCl and 88.2 g of trisodium citrate was dissolved in distilled water. The pH was adjusted to 7.0 and the final volume was made upto one litre the buffer was sterilized by autoclaving at RT.

TBE Buffer (Gibco-BRL, USA)

The total content of a bag having the formula of 100 mM tris, 90 mM Boric acid, 1.0 mM EDTA was mixed with 0.99 l of distilled water to make the 1x concentrated TBE buffer. The buffer was stored at RT.

TE Buffer (pH 8.0)

TE [10 m*M* Tris-Cl (pH 8.0), 1 m*M* EDTA] was prepared by diluting concentrated stocks of 1 *M* Tris-Cl (pH 8.0) and 0.5 *M* EDTA. The buffer was stored at 4°C.

1 *M* Tris-HCl

121.1 g tris-base was dissolved in 800 ml of distilled water. The pH was adjusted to the desired value by adding concentrated HCl, and the final volume was made up to one litre with distilled water. The solution was sterilized by autoclaving and stored at room temperature (RT).

Washing Solution

100 m*M* sodium acetate

50 m*M* Tris-HCl

The ingredients were mix and dissolved and the final pH was adjusted to 8.0.

Appendix 3: Commonly Used Reagents

Alkaline Lysis Buffers for Plasmid Preparation

Solution I

50 m*M* glucose (4.504 g/500 ml)

25 m*M* Tris-HCl, pH 8.0 (1.5137 g)

10 m*M* EDTA, pH 8.0 (1.8612 g)

This solution can be prepared in batches of approximately 500 ml, autoclaved for 15 minutes, and stored at 4°C.

Solution II

0.2 *N* NaOH (freshly diluted from 10 *N* stock)

1% SDS (from 20% stock)

Prepare fresh prior to use.

Solution III

60 ml 5 *M* potassium acetate

11.5 ml Glacial acetic acid

28.5 ml dH_2O

This solution is 3 *M* with respect to potassium and 5 *M* with respect to acetate.

TE Buffer (Tris-EDTA): 100x

10 m*M* Tris-Cl, pH 8.0 (60.55 g/500 ml)

1 m*M* EDTA, pH 8.0 (18.612 g/500 ml)

Dissolve the ingredients in approximately 300 ml distilled water; pH of the solution to 8.0 with concentrated HCl. Bring the final volume to 500 ml.

Tris-Acetate EDTA Buffer: 50x

1 *M* Tris-HCl (121 g/500 ml)

28.55 ml Glacial acetic acid

0.5 *M* EDTA, pH 8.0 (>50 ml)

Add ingredients to approximately 300 ml deionized water. Adjust the pH to 7.5 with glacial acetic acid. Bring the final volume to 500 ml.

Tris Borate EDTA Buffer (TBE)

121.1 g Tris

7.4 g EDTA

55 g Boric Acid

Dissolve the ingredients in approximately 800 ml deionized water; pH the solution to 8.3 with dry boric acid. Filter and make 1 liter.

Phenol:Chloroform:Isopropyl Alcohol: 25:24:1

25 ml Phenol

24 ml Chloroform

1 ml Isopropyl alcohol

Add solvent to make 50 ml solution.

Reagents for Pulsed-Field Gel Electrophoresis

Name of the reagent	Composition	Source
Cell suspension buffer	100 m*M* Tris:100 m*M* EDTA, pH 8.0	Bio-Rad, USA
Tris:EDTA buffer	TE; 10 m*M* Tris: 1 m*M* EDTA, pH 8.0	Bio-Rad, USA
Cell lysis buffer	50 m*M* Tris: 50 m*M* EDTA, pH 8.0 + 1% sarcosine	Bio-Rad, USA
1% SeaKem Gold agarose	1% SKG in 0.5 x TBE	Bio-Rad, USA
Lysozyme solution	10 m*M* Tris, pH 7.2; 50 m*M* NaCl; 0.2% sodium deoxycholate; 0.5% sodium lauryl sarcosine; 1 mg/ml lysozyme and 20 μg/ml RNase	Bio-Rad, USA
1x Wash buffer	20 m*M* Tris, pH 8.0; 50 m*M* EDTA	Bio-Rad, USA
Proteinase K solution	100 m*M* EDTA, pH 8.0; 0.2% sodium deoxycholate; 1% sodium lauryl sarcosine; 1 mg/ml Proteinase K	Bio-Rad, USA
PMSF, 100 m*M*	Phenylmethanesulfonylfluoride	Bio-Rad, USA

Appendix 4: The 20 Amino Acids and Their Official Codes

No.	1-Letter code	3-Letter code	Name
1	A	Ala	Alanine
2	R	Arg	Arginine
3	N	Asn	Asparagine
4	D	Asp	Aspartic acid
5	C	Csy	Cystein
6	Q	Gln	Glutamine
7	E	Glu	Glutamic acid
8	G	Gly	Glycine
9	H	His	Histidine
10	I	IIe	Isoleucine
11	L	Leu	Leucine
12	K	Lys	Lysine
13	M	Met	Methionine
14	F	Phe	Phenylalanine
15	P	Pro	Proline
16	S	Ser	Serine
17	T	Thr	Threonine
18	W	Trp	Tryptophan
19	Y	Tyr	Tyrosine
20	V	Val	Valine

Appendix 5: Antibiotic Susceptibility Interpretation

Zone Diameter Interpretive Standards and Equivalent Minimum Inhibitory Concentration (MIC) Breakpoints for Enterobactreriaceae

Testing Conditions

Medium: Mueller-Hinton agar

Inoculum: Growth method or direct colony suspension, equivalent to a 0.5 McFarland standard

Incubation: 35 ± 2°C; ambient air; 16 to 18 hours

Minimum QC Recommendations

Escherichia coli ATCC 25922

Escherichia coli ATCC 35218 (for βlactam/β-lactamase inhibitor combinations)

Antimicrobial agent	Disc content	Zone diameter (Nearest whole mm)			Equivalent MIC Breakpoints (μg/ml)	
		R	I	S	R	S
Penicillins						
Ampicillin (AMP)	10 μg	≤ 13	14-16	≥ 17	≥ 32	≤ 8
β-Lactam/β-lactamase inhibitor combinations						
Amoxycillin-Clavulanic acid (AMC)	20/10 μg	≤ 13	14-17	≥ 18	≥ 32/16	≤ 8/4
Cephems						
Cefepime (FEP)	30 μg	≤ 14	15-17	≥ 18	≥ 32	≤ 8
Cefotaxime (CTX)	30 μg	≤ 14	15-22	≥ 23	≥ 64	≤ 8
Cefoxitin (FOX)	30 μg	≤ 14	15-17	≥ 18	≥ 32	≤ 8
Ceftazidime (CAZ)	30 μg	≤ 13	14-16	≥ 17	≥ 32	≤ 8
Ceftriaxone (CRO)	30 μg	≤ 14	15-17	≥ 18	≥ 32	≤ 8
Carbapenems						
Imepenem (IMP)	10 μg	≤ 13	14-15	≥ 16	≥ 16	≤ 4
Meropenem (MPM)	10 μg	≤ 13	14-15	≥ 16	≥ 16	≤ 4
Monobactams						
Aztreonam (ATM)	30 μg	≤ 15	16-21	≥ 22	≥ 32	≤ 8
Fluoroquinolone						
Ciprofloxacin	5 μg	≤ 15	16-20	≥ 21	≥ 4	≤ 1
Quinolones						
Nalidixic acid (NA)	30 μg	≤ 13	14-18	≥ 19	≥ 32	≤ 8
Folate pathway inhibitors						
Trimethoprim/ Sulfamethoxazole 1:19 (SxT)	1.25/ 23.75 μg)	≤ 10	11-15	≥ 16	≥ 8/152	≤ 2/38