CORRELATION OF MULTIDRUG RESISTANT AND PLASMID PROFILE IN ESBL PRODUCING *Escherichia coli* IN UTI PATIENTS

A

DISSERTATION

SUBMITTED TO THE CENTRAL DEPARTMENT OF MICROBIOLOGY

TRIBHUVAN UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

AWARD OF MASTER OF SCIENCE IN MICROBIOLOGY

(MEDICAL)

BY

VIVEK KUMAR SINGH

CENTRAL DEPARTMENT OF MICROBIOLOGY

TRIBHUVAN UNIVERSITY

KIRTIPUR, KATHMANDU, NEPAL

2014

RECOMMENDATION

This is to certify that **Mr. Vivek Kumar Singh** has completed this dissertation work entitled "**CORRELATION OF MULTIDRUG RESISTANT AND PLASMID PROFILE IN ESBL PRODUCING *Escherichia coli* IN UTI PATIENTS**" as a partial fulfillment of M. Sc. Degree in Microbiology under our supervision. To our knowledge this thesis work has not been submitted for any other degree.

Ms. Reshma Tuladhar
Lecturer
Central Department of Microbiology
Tribhuvan University,
Kirtipur, Kathmandu, Nepal

Mr. Mahesh Kumar Chaudhary
Lecturer
Department of Microbiology
KIST MCTH
Imadole, Lalitpur, Nepal

Date: ……

CERTIFICATE OF APPROVAL

On the recommendation of **Ms. Reshma Tuladhar** and **Mr. Mahesh Kumar Chaudhary** this dissertation work by **Mr. Vivek Kumar Singh**, entitled "**CORRELATION OF MULTIDRUG RESISTANT AND PLASMID PROFILE IN ESBL PRODUCING *Escherichia coli* IN UTI PATIENTS**" has been approved for the examination and is submitted to the Tribhuvan University in partial fulfillment of the requirement for M. Sc. Degree in Microbiology.

_____

. **Prof. Dr. Anjana Singh, Ph. D.**
Head of the Department
Central Department of Microbiology,
Tribhuvan Universiy
Kirtipur, Kathmandu

Date: - _____

BOARD OF EXAMINERS

Recommended by _____

Ms. Reshma Tuladhar

Supervisor

_____

Mr. Mahesh Kumar Chaudhary

Supervisor

Approved by: _____

Prof. Dr. Anjana Singh, Ph. D.

Head of the Department

Examined by: _____

Prof. Dr. Basistha Pd. Rijal

Head of the Department, IOM

External Examiner

_____

Ms. Manita Aryal

Internal Examiner

Date: ……

ACKNOWLEDGEMENT

I am very grateful and deeply indebted to my honourable teacher and supervisor **Ms. Reshma Tuladhar**, Lecturer, Central Department of Microbiology, Tribhuvan University. It is my great pleasure to express my deepest regards and whole hearted indebtedness for her inspiring encouragement, continuous guidance, active cooperation, constant supervision, valuable suggestions, constructive criticism and help in carrying out this work successfully.

I offer my sincerest gratitude to my supervisor **Mr. Mahesh Kumar Chaudhary**, Lecturer, Department of Microbiology, KIST MCTH who has supported me thoughout my thesis with his patience and knowledge whilst allowing me the room to work in my own way. I would like to express my deepest regards and gratitude to him, for his constructive criticism in correcting this thesis with his wise advice and active cooperation in correcting the manuscript.

I am much obliged to **Dr. Anjana Singh,** Head of Department, Central Department of Microbiology for her cooperation and tremendous support during whole of this research work.

I am cordially expressing my respect to **Dr. Megha Raj Banjara**, Lecturer, Central Department of Microbiology, Tribhuvan University for his cordial cooperation & thoughtful suggestions in my thesis work.

I owe my gratitude to respected teacher **Dr. Bijendra Raj Raghubanshi**, Lecturer, Department of Microbiology, KIST MCTH for valuable advice and cordial cooperation.

I would like to convey my greatest regards and sincere thanks to Narad Pandey, laboratory technician In the KIST MCTH, Madhukar Thapa and Ramesh Khadka laboratory technician in Central Department of Microbiology, Tribhuvan

University who helped me in preserving samples, in the methods necessary to run Laminar and in preparing various solutions.

I owe special debt of profound gratitude to my father Late Mr. Madhusudan Singh and mother Mithilesh Singh without whom my master degree would not have been possible.

I remained incomplete if I do not express wholehearted thanks and gratitude to my sisters and my brother Sameer Singh for their blessings and affecstionate support.

I would also like to extend huge, warm thanks to my friends, Santosh, Rajeev, Rajesh, Surendra, Suren, Prakash, Pramod and Nabeen.

_____

Vivek Kumar Singh

Date: ……..

ABSTRACT

Urinary Tract Infection (UTI) is one of the most common infectious disease ranking next to upper respiratory tract infection, is the cause of morbidity and mortality in human. Therefore, studying bacterial pathogens causing UTI and their drug susceptibility patterns is of the highest priority. Infections caused by *Escherichia coli* have become a significant worldwide public health problem. Furthermore, the situation is worsening due to advent of increased antibiotic resistance due to the evolution of multi-resistant antibiotic plasmid genes. Extended Spectrum Beta lactmases (ESBLs) are plasmid mediated and these enzyme producing organisms exhibit co resistance to many other classes of antibiotics.

A total of 1258 urine samples were collected from May 2013 to November 2013. Bacterial isolates were identified using standard biochemical tests and antimicrobial susceptibility pattern was determined using the Kirby – Bauer's disk diffusion method following CLSI guidelines. Confirmation of the extended spectrum β-lactamase (ESBL) *E.coli* was done as guided by CLSI guidelines. Plasmid DNA isolation of all the ESBL positive strains was done by alkaline lysis method.

In the present study, out of 303 isolates, 198 isolates were of *E.coli*. These isolates were tested for antibiotic sensitivity, MDR, ESBL and plasmid profiles. The results revealed that more than 50% of the isolates exhibited multi-drug resistance. Out of 76 ESBL *E.coli* isolates, 41(53.9%) were found to possess plasmids. Some isolates possess single plasmid while other had multiple plasmids with different size ranged from 1 kb to 10 kb. The studies show good prospects for further research in the same area to explore and assign definite cause for antibiotic resistance, multi drug resistance and ESBL production.

Keywords: Prevalence; Urinary tract infection; Antimicrobial susceptibility pattern; MDR; ESBL; Plasmid Profile.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHS

LIST OF APPENDICES

ABBREVIATIONS

AmpC	Ampicillin resistant gene
ATCC	American Type Culture Collection
BA	Blood Agar
bp	Base pairs
CA	Clavulinic acid
CDC	Center for Disease Control and Prevention
Cfu	Colony forming unit
CLSI	Clinical laboratory standard institute.
CONS	Coagulase Negative Staphylococci
CTX-M	Cefotaxime resistant gene
DDST	Double disc synergy test
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
E test	Epsilon test
EDTA	Ethylene diamine tetraacetic acid
ESBL	Extended spectrum beta lactamase
ESC	Extended spectrum cephalosporins
et al	at alia (all others)
HGT	Horizontal gene transfer
IS	Insertion Sequence

kbp	Kilo base pair
LPS	lipopolysaccharide
MA	MacConkeys agar
MBL	Metalo beta lactamase
MDDDT	Modified double disc diffusion test
MDR	Multi-drug Resistant
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
MRVP	Methyl Red Voges Proskauer
NA	Nutrient Agar
NB	Nutrient Broth
PBP	Penicillin binding protein
RNA	Ribonucleic Acid
SIM	Sulfide Indole Motility
TEM	Temoniera gene
TSIA	Triple Sugar Iron Agar
UTI	Urinary Tract Infection
VGT	Vertical gene transfer
WHO	World health organization

CHAPTER-I

INTRODUCTION AND OBJECTIVES

1.1 Background

Urinary tract infections (UTIs) are among the most prevalent infectious diseases, with a substantial financial burden on society. UTIs account for more than 100,000 hospital admissions annually, most often for pyelonephritis (Foxman, 2002). Microorganisms can reach the urinary tract by haematogenous or lymphatic spread, but there is abundant clinical and experimental evidence to show that the ascent of microorganisms from the urethra is the most common pathway that leads to a UTI, especially organisms of enteric origin (e.g. *Escherichia coli* and other Enterobacteriaceae). This provides a logical explanation for the greater frequency of UTIs in women than in men, and for the increased risk of infection following bladder catheterisation or instrumentation. A single insertion of a catheter into the urinary bladder in ambulatory patients results in urinary infection in 1-2% of cases. It is thought that bacteria migrate within the mucopurulent space between the urethra and catheter, and that this leads to the development of bacteriuria in almost all patients within about 4 weeks.

Haematogenous infection of the urinary tract is restricted to a few relatively uncommon microbes, such as *Staphylococcus aureus, Candida sp., Salmonella sp.* and *Mycobacterium tuberculosis*, which cause primary infections elsewhere in the body. *Candida albicans* readily causes a clinical UTI via the haematogenous route, but is also an infrequent cause of an ascending infection if an indwelling catheter is present, or following antibiotic therapy.

The number of bacteria is considered relevant for the diagnosis of a UTI. In 1960, Kass developed the concept of significant bacteriuria ($>10^5$cfu/ml) in the context of pyelonephritis in pregnancy (Kass, 1960). Although this concept introduced quantitative microbiology into the diagnosis of infectious diseases, and is therefore still of general importance, it has recently become clear that there is no fixed bacterial count that is indicative of significant bacteriuria, which can be

applied to all kinds of UTIs and in all circumstances. The following bacterial counts are clinically relevant:

- $>10^3$cfu/ml of uropathogens in a mid-stream sample of urine (MSU) in acute uncomplicated cystitis in women.
- $>10^4$cfu/ml of uropathogens in an MSU in acute uncomplicated pyelonephritis in women.
- $>10^5$cfu/ml of uropathogens in an MSU in women, or $>10^4$cfu/mL uropathogens in an MSU in men, or in straight catheter urine in women, in a complicated UTI.

Due to the increasing threat of resistant pathogens worldwide, it has become imperative to limit the use of antibiotics, and consequently, to monitor established treatment strategies closely. It is the ambition of the present guidelines to provide both the urologist and the physician from other medical specialties with advices in their daily practice. The guidelines cover male and female UTIs, male genital infections, and special fields such as UTIs in paediatric urology, immunosuppression, renal insufficiency and kidney transplant recipients. Much attention is given to antibiotic prophylaxis, with the aim of reducing the misuse of antibiotics in conjunction with surgery. High quality clinical research is strongly encouraged.

β-Lactam antibiotics are commonly used to treat bacterial infections. The groups of antibiotics in this catagory include penicillins, cephalosporins, carbapenems & monobactams. Increased use of antibiotics, particularly the third generation of cephalosporins, has been associated with the emergence of, β-Lactamases mediated bacterial resistance, which subsequently led to the development of Extended Spectrum Beta Lactamase (ESBL) producing bacteria. ESBLs are enzymes that mediate resistance to extended spectrum e.g., third generation cephalosporins as well as monobactams such as aztreonam (CLSI, 2013). These enzymes catalyze the hydrolysis of the, β-lactam ring of antibiotic, thereby destroying the antimicrobial activity. ESBLs have been reported worldwide in many different genera of Enterobactericeae and *Pseudomonas aeroginosa* (Friedman *et al*., 2005). However, these are most

common in *Klebsiella pneumoniae* & *E. coli* (Agrawal *et al.*, 2008). ESBL producing organisms are often resistant to several other classes of antibiotics, as the plasmids with the gene encoding ESBLs often carry other resistance determinants. Initially ESBL producing organisms were isolated from nosocomial infections but these organisms are now also being isolated from community (Pitout *et al*., 2005).

The first plasmid mediated, β-lactamase in Gram-negative bacteria, TEM-1, was described in the early 1960s (Datta and Kontomichalau, 1965). Afterwards it was detected from *Klebsiella* in Europe 1980, in Germany 1983, and in France 1985 (Perez *et al*., 2007). Genetic control of beta-lactamase production resides either on plasmids or on the chromosome, while expression is either constitutive or inducible (Chiang and Liaw, 2005).

The development of extended spectrum cephalosporins in the early 1980s was regarded as a major addition to our therapeutic armamentarium in the fight against beta-lactamase mediated bacterial resistance. The emergence of *Escherichia coli* and *Klebsiella pneumoniae* resistant to ceftazidime & other cephalosporins seriously compromised the efficacy of these life saving antibiotics (Perez *et al*., 2007). The new bacterial beta-lactamases present in these common enteric bacilli (the parent TEM -1 and SHV-1 enzymes) demonstrated unique hydrolytic properties (Shobha *et al*., 2007). ESBLs are inhibited in vitro by β-lactamase inhibitors such as clavulanic acid and tazobactam. Some ESBLs are derived from earlier, broad-spectrum, β-lactamases (e.g., the TEM, SHV and OXA enzyme families) and differ from the parent enzyme by a few point mutations, which confer an extended spectrum of activity (Hawkey, 2008). Point mutations in the SHV and TEM genes that resulted in single amino acid changes, (Gly 238-ser, Glu 240-Lys arg 164-ser, arg164- His, Asp 179- Asn & Gul (Asp) 104- Lys) (Perez *et al*., 2007). More recently another family of ESBLs, the CTX-M types, has emerged and these ESBLs are becoming increasingly common (Hawkey, 2008).

There is a large reservoir of resistant genes, in bacterial genomes and in extra-chromosomal pieces of DNA (plasmids) that encode different mechanisms of

drug resistance (Soulsby, 2005). The transmission of antibiotic resistance, often to several drugs simultaneously, from one bacterium to another is attributed to plasmids. Understanding antibiotic resistance patterns and molecular characterization of plasmids is epidemiologically useful (Hanson, 2003). Although conventional antimicrobial susceptibility testing methods are useful methods for detecting resistance profiles and for selecting potentially useful therapeutic agents, they are insensitive tools for tracing the spread of individual strains within a hospital or region. Molecular methods like plasmid profiling provide powerful tools to track bacterial strains and contribute to the evaluation of nosocomial infection outbreaks, recurrent infection and clonal dissemination of specific pathogens (Sader *et al*., 1995). They are also used as a means of providing additional information, to detect and evaluate the mode of dissemination of MDR pathogens (Pfaller *et al*., 2001). Plasmid analysis has also proved a useful method for differentiating bacterial isolates (Waschmut *et al*., 1991; Dorn *et al*., 1992). The number and size of the plasmids present is used as the basis for strain identification. This strain typing technique has been used successfully for analysis of outbreaks of nosocomial infections and community acquired infections (Fornasini *et al*., 1992) caused by a variety of species of Gram negative rods.

Antimicrobial resistance among Gram-negative bacilli represents a major problem in nosocomial infection (Pitout, 1997). These organisms elaborate beta-lactamases, which can hydrolyze the amide bond in the beta-lactam ring of antibiotics. Since 1983, extended-spectrum beta-lactamase (ESBL) producing organisms have been isolated around the world (Knothe *et al*., 1983; Brun-Buisson *et al*., 1987; Rahal *et al*., 1998). ESBLs are especially dangerous because they are plasmid-associated, and the plasmids may be exchanged among a variety of bacterial species. Partly because of the difficulty to differentiate infection from colonization, a number of studies have elucidated inconsistent results concerning the risk factors and clinical significance of infections due to ESBL-producing organisms (Lautenbach et al., 2001; Schiappa et al., 1996). We therefore conducted a study of bacteriuria caused by ESBL producing *E. coli*, in order to identify the possible risk factor for infection and hospital mortality.

1.2 OBJECTIVES

1.2.1 GENERAL OBJECTIVE

To determine prevalence of urinary tract infection and to detect plasmid genes of extended spectrum beta lactamase among *Escherichia coli*.

1.2.2 SPECIFIC OBJECTIVES

a) To isolate and identify the bacterial pathogens from mid stream urine samples.
b) To determine antibiotic susceptibility pattern of the isolates.
c) To determine the prevalence of multi drug resistant (MDR) strains among the total isolates.
d) To determine the prevalence of extended spectrum beta lactamase *Escherichia coli* (ESBL *E. coli*) strains among the total isolates.
e) To confirm ESBL by double disc diffusion test.
f) To determine the number and size of the plasmids present in each isolates by plasmid profiling.
g) To study association between the antibiotic resistance and plasmid profiles of the isolates.

CHAPTER-II
LITERATURE REVIEW

2.1 Urinary tract infection:

Urinary tract infection is one of the important causes of morbidity and mortality affecting all age groups across the life span. Anatomically, urinary tract is divided into an upper portion composed of kidneys, renal pelvis, and ureters and a lower portion made up of urinary bladder and urethra. UTI is an inflammatory response of the urothelium to bacterial invasion that is usually associated with bacteriuria and pyuria. UTI may involve only the lower urinary tract or both the upper and lower tract (Stamm and Norrby, 2001).

2.1.1 Etiologic agents:

Following microorganisms that are considered contaminants and potential pathogens (Steadman and Topley, 1998; Smith's General Urology, 2004; Stamm and Norrby, 2001; Schaeffer, 1990; Thomas, 1978).

Contaminants (commensal flora)	**Pathogens**
Bacillus species	Enterococci
Alpha and Beta hemolytic streptococci	*Corynebacterium urealyticum*
Diphtheroids	Enterobacteriaceae
Lactobacillus spp.	*Pseudomonas aeruginosa*
	Staphylococcus epidermidis
	Staphylococcus saprophyticus
	Mycoplasma
	Gardenella vaginalis
	Anaerobic bacteria
Urethra :	
E. coli	*Ureaplasma urealyticum*
Streptococcus spp.	*CONS*
Nesseria gonorrhoea	

Chlamydia trachomatis (Herzog, 1989; Drekonja and Jhonson, 2008; Roberts, 1991).

2.1.2 Methods for localization of infection of the urinary tract:

Clinical features: Suggestive of pyelonephritis, cystitis, urethritis, perinephric abscess.

- Urine analysis: Presence of cast suggests pyelonephritis and presence of bits of tissue indicates renal papillary necrosis.
- Culture: Urethral catheterisation and bladder washout methods to distinguish lower from upper urinary tract infection.
- Serology: four-fold rise or high antibody titers (against 'O' or common bacterial antigen) indicates renal involvement.
- Antibody coated bacteria: Its presence indicates invasion of kidney.
- Functional: In renal involvement, ability to concentrate urine is affected. (Thomas, 1978; Kunin, 1997).

2.2 The family Enterobacteriaceae

2.2.1 Typical characteristics

The Gram-negative bacteria belonging to Enterobacteriaceae represent a large and heterogeneous group of many genera (more than 100 species) of facultatively-anaerobic, rod-shaped (0.3-1.0 x 1.0-6.0 µm) bacteria (Eisenstein and Zalenik, 2000).

2.2.2 Natural habitat of Enterobacteria

The natural habitat of Enterobacteriaceae is soil and water, but also the human and animal intestines. Some species are insect- or plant-associated (Janda and Abbott, 1998). They are a major component of the normal human intestinal microbiota but relatively uncommon at other body sites (Balows *et al*., 1992). However, they are also common in our homes, e.g. transferred via food, bacteria can be found in the sink and on the cutting boards in the kitchen. Enterobacteriaceae represent a minority in faecal microbiota. The greatest number and the greatest species variation of bacteria are present in the colon. The total number of obligate anaerobes like Bacteroides and *Clostridium* is enormous (10^{10} - 10^{11} cells/gram of intestinal content), while facultative anarobes like

Enterobacteriaceae constitute generally less than 10^7 cells/gram of intestinal content (Guarner and Malagelada, 2003).

2.2.3 Genera belonging to the family Enterobacteriaceae

2.2.3.1 *Escherichia*

The genus *Escherichia* consists of five species; *E. blattae, E. coli*, *E. fergusonii*, *E. hermanii,* and *E. vulneris* (Bruckner *et al*., 1999). *E. coli* is the most numerous aerobic commensal inhabitant of the large intestine in humans (Janda and Abbott, 1998). In our intestines, *E. coli* helps our body to break down the food we eat as well as assist with digestion processes, waste processes, vitamin K production, and food absorption (Krieg and Holt, 2005).

E. coli is probably the most famous member of the Enterobacteriaceae group, since it is a model organism and lots of our knowledge of biochemical processes and genetics derive from this species. *E. coli* is also commonly used as an indicator of faecal contamination (Janda and Abbott, 1998).

2.3 β-lactam antibiotics

2.3.1 General structure and function

β-lactam is a generic name for all β-lactam antibiotics that contain a β-lactam ring, a heteroatomic ring structure, consisting of three carbon atoms and one nitrogen atom. The β-lactam bactericidal antibiotics consist of four main antibiotic groups. These main groups are penicillins, cephalosporins, carbapenems and monobactams. The principal classification of β-lactams is based upon the structure (Wilke *et al.,* 2005).

2.3.2 β-lactamase inhibitors

β- lactamase inhibitors are designed to inhibit or destroy the effectiveness of β-lactamase enzymes. The β-lactamase inhibitors are divided into two groups: clavulanic acid and penicillanic acid sulfones. Clavulanic acid acts synergistically with different penicillins and cephalosporins against Gram-negative bacteria that produce β-lactamases. The penicillanic acid sulfones, sulbactam and tazobactam

are structurally related and sulbactam is combined with ampicillin, while tazobactam is combined with piperacillin (Moosdeen *et al*., 1988).

2.3.3 Resistance to β-lactams

The resistance to β-lactams can be inherent or acquired. Inherent resistance in a Gram-negative bacterium is due to an outer membrane that establishes a permeability barrier against the antibiotic. For example, Gram-negative bacteria are intrinsically resistant to penicillin G by virtue of their double membrane structure which prevents the antibiotic from accessing the cell wall target. Intrinsic resistance is not considered an important clinical problem since antimicrobial agents are or were not intended for use against intrinsically resistant bacteria (Antimicrobial Resistance, 2006). It is the acquired resistance that is of clinical importance.

Acquired resistance to β-lactams operates through different mechanisms: production of β-lactamases, changes in the outer membrane permeability or alterations to the PBPs (Wilke *et al*., 2005). β-lactamases are a group of enzymes that are most likely originally targeted for synthesis of the cell wall, but have also evolved to degrade and inactivate β-lactam antibiotics. These flexible enzymes have been detected in both Gram-positive and Gram-negative bacteria, but these enzymes are especially important in Gram-negative bacteria as they are the most common cause of β-lactam resistance in this group of bacteria (Bush *et al*., 1995; Livermore, 1995).

2.4 β-lactamases

2.4.1 Classification and nomenclature of β-lactamases

Different classifications based on phenotype, gene or amino acid protein sequences and function have been attempted since the beginning of the 1970s (Richmond and Sykes, 1973; Ambler *et al*., 1991; Bush *et al*., 1995; Hall and Barlow, 2005). One of the most used classification schemes is Ambler's (Ambler *et al*., 1991) based upon amino acid sequences. By this classification the β-lactamases are divided into four molecular classes, A, B, C and D.

In addition to the Ambler classification, β-lactamases can be classified based on their substrate specificity. Penicillinases, extended-spectrum β-lactamases

(ESBLs), carbapenemases and so on are examples of classification of β-lactamases by they preferred substrate specificity. This classification does not follow the phylogeny of these enzymes. For example, ESBLs exist among several different phylogenetically defined β-lactamase groups. For this work, ESBLs are the most important group of β-lactamases. The exact definition of the ESBLs is not clear (Livermore, 2008).

The nomenclature of β-lactamases is not logical. They are named in different ways, some after their preferred substrate, like IMP (active on imipenem) and OXA (oxacillinase), others according to biochemical properties, like SHV, while others are named according to bacteria, or patient or hospitals (Bush and Jacoby, 1997).

2.4.2 Structure and action of β-lactamases

The β-lactamases are divided into two classes; serine and metallo β-lactamases that do not share sequence or structural homology. However, both β-lactamase classes hydrolyse the amide bond of the four-membered β-lactam ring. The serine β-lactamases have a serine in the middle of their structure. The covalent bond that is formed during hydrolysis between the enzyme and the β-lactam ring is typical of these enzymes. The three classes of serine ß-lactamases, A, C and D share similarity on the protein structure level, which proves that they descended from a common ancestor (Hall and Barlow, 2004). The metallo β-lactamases need a bivalent cation, usually zink, to be able to hydrolyse the β-lactam ring (Garau *et al*., 2005a).

2.5 Genetics of β-lactamases

The β-lactamases can be both chromosomal and plasmid-encoded. Most of the Enterobacteriaceae species have at least one chromosomal β-lactamase. The expression of these enzymes occurs often only at a very low level. Chromosomal β-lactamases typically confer resistance to penicillins but not to other β-lactams like extended-spectrum cephalosporins or carbapenems that plasmid-encoded β-lactamases can inactivate (Sanders, 1987).

2.5.1 Inducible or constitutive expression

The chromosomally encoded enzymes can be inducible or constitutively expressed. An inducible gene is a gene whose expression is responsive to eg., an environmental change, while a constitutively expressed gene is transcribed continually. The β-lactamase production, which can be induced by the presence of certain antibiotics, is usually encoded by the bacterial chromosome. This occurs e.g. in species expressing chromosomal *ampC*; like in *Serratia*, *Pseudomonas*, *Acinetobacter, Citrobacter* and *Enterobacter*. In *E. coli* no obvious regulators are present, and therefore the AmpC expression is uninducible (Jacobs *et al*., 1997; Schmidtke and Hanson, 2006).

2.5.2 Transferable resistance

Transferable β-lactamase genes can be spread on plasmids, transposons, insertion sequences and integrons, by conjugation, transduction or transformation.

2.5.2.1 Plasmids and Transposons

A plasmid is an extra-chromosomal double-stranded DNA molecule that occurs naturally in bacteria. Plasmids are capable of autonomous replication within a suitable host (Madigan and Martinko, 2006). Transposons are mobile genetic elements that can move around to different positions within the genome of a single cell. Transposons were first discovered in maize in 1948, and are short linear DNA segments. Class II transposons move directly from one position to another within the genome using a transposase to "cut and paste" them within the genome. Hence, class II transposons allow transfer and permanent addition of genes e.g. encoding antibiotic resistance. When the transposable elements lack additional genes, they are known as insertion sequences. Hence, insertion sequences are small, around 700 to 2500 bp in length (Madigan and Martinko, 2006). Insertion sequences participate in rearrangement of chromosomes and plasmid integration (Mahillon and Chandler, 1998). Integrons are genetic elements that contain gene cassettes that can be mobilized to other integrons or to secondary sites in the bacterial genome. The majority of the known gene cassettes encode resistance to antibiotics (Fluit and Schmitz, 1999).

Most of the β-lactamases are integrated within plasmids and transposons that enable the rapid transfer of these resistance genes between microbes. The association of insertion sequences with these β-lactamase genes are also involved in their dissemination and expression (Bennett and Hawkey, 1991; Bradford, 2001).

The β-lactamase genes are often found within integrons as part of multi-drug resistance cassettes that confer resistance to several other antibiotic classes such as aminoglycosides, macrolides, sulphonamides and chloramphenicol (Medeiros, 1997; Weldhagen, 2004; Wilke *et al*., 2005). The gene cassettes are mobile units, and each comprises a gene, normally an antibiotic resistance gene and a recombination site. Integrons are translocatable by themselves. Transfer of resistance genes occur when integron containing one or several genes is incorporated into a plasmid (Hall and Collis, 1995; Roy, 1995).

2.6 The clinically most important β-lactamases

2.6.1 Class A β-lactamases

The class A β-lactamases is a large family consisting of TEM, SHV and CTX-M β-lactamases, but also a number of other rarer enzymes that often exhibit ESBL activity. Class A β-lactamases is the most common group (Bush *et al*., 1995).

2.6.1.1 TEM β-lactamases

The TEM-1 (named after a Greek patient Temoneira) was first reported in 1965 from an *E. coli* strain and represents today one of the most prevalent β-lactamase in Enterobacteriaceae (Datta and Kontomichalou, 1965). Isolates harbouring TEM-1 ß-lactamases are resistant to ampicillin. Although TEM-type β-lactamases are most often found in *E. coli* and *K. pneumoniae*, they are also found with increasing frequency in other Gram-negative species (Bradford, 2001). The TEM ESBL phenotype is produced by single amino acid substitutions at positions 104, 164, 238, and 240, but ESBLs usually have more than a single amino acid substitution (Bradford, 2001).

2.6.1.2 SHV β-lactamases

SHV stands for sulphydryl variable and SHV-1 was first described in 1972 as Pit-2 (Pitton, 1972). Both chromosomal, e.g. *blaSHV*-1 and *blaSHV*-11, and plasmid-

mediated *blaSHV*genes are found (Livermore, 1995). The SHV ESBLs were the predominant ESBL type in Europe during the 1990s and were predominant in the United States during the beginning of the 21st century.

2.6.1.3 CTX-M β-lactamases

In 1986, a non-TEM and a non-SHV ESBL cephalosporinase was discovered in a cefotaxime-resistant *E. coli* (Matsumoto *et al*., 1988). This cefotaxime resistance was due to enzymes named cefotaximases (CTX-M), and these CTX-Ms showed a much higher degree of activity to cefotaxime than to ceftazidime (Walther-Rasmussen and Hoiby, 2004). The CTX-Ms represent plasmid acquisition of β-lactamase genes that can normally be found on the chromosome of *Kluyvera* species (Humeniuk *et al*., 2002). The CTX-M enzymes have been detected in a variety of Enterobacteriaceae species, from widely separated geographical regions. However, the CTX-M variants are mostly found in E. *coli, S.* Typhimurium, *K. pneumoniae* and *Proteus mirabilis*.

2.6.1.4 Class A carbapenemases

The molecular class A carbapenemases, including the plasmid-mediated serine β-lactamases KPC (named after *K. pneumonia* carbapenemase) and GES (Guiana extended spectrum) and the chromosomally encoded SME (named after *Serratia marcescens* enzyme) and IMI/NMC (imipenem-hydrolyzing β-lactamase/not metalloenzyme carbapenemase) enzymes, are effective carbapenemases. KPC was first detected in North Carolina 2001 (Fridkin *et al*., 1999; Yigit *et al*., 2001; Yigit *et al*., 2008). The class A carbapenems have the ability to hydrolyze a broad spectrum of antibiotics, including carbapenems, cephalosporins, penicillins, and aztreonam, and all are inhibited by clavulanic acid and tazobactam (Queenan and Bush, 2007).

2.6.2 Metallo β-lactamases

The metallo β-lactamases represent the class B β-lactamases and were found 40 years ago. They are able to degrade all classes of β-lactams except monobactams. Additionally, they have an efficient activity against carbapenems. The metallo β-lactamases are resistant to therapeutic β-lactamase inhibitors. The metallo β-lactamases are zinc-dependant and so called EDTA-inhibited enzymes and are

mostly found in *Pseudomonas* sp. and *Serratia* sp. (Bebrone, 2007). The metallo β-lactamases are predominantly chromosomally encoded but can also be found on plasmids.

2.6.3 Class C β-lactamases

Class C β-lactamases (AmpC) are an important group of proteins that are broadly distributed. This is the second most common β-lactamase group (Bush *et al*., 1995). AmpC is typically encoded on the chromosome of Gram-negative bacteria including *Citrobacter, Serratia* and *Enterobacter* species where its expression is usually inducible. About 20 years ago, the inducible chromosomal genes were detected on plasmids and were transferred to organisms, typically not expressing these types of β-lactamase, like *Klebsiella* spp., *E. coli*, or *Salmonella* spp. (Philippon *et al*., 2002; Hanson, 2003). AmpC β-lactamases, in contrast to ESBLs, hydrolyse broad and extended-spectrum cephalosporins but are resistant to inhibition by β-lactamase inhibitors such as clavulanic acid (Sanders, 1987; Thomson, 2001; Hanson, 2003).

2.6.4 Class D β-lactamases

The OXA β-lactamases differ from the TEM and SHV enzymes and they belong to class D according to Ambler (Livermore and Paterson, 2006). The OXA group mainly occur in *Acinetobacter* and *Pseudomonas* species. The OXA β-lactamases attack the oxyimino-cephalosporins and have a high hydrolytic activity against oxacillin and cloxacillin.

2.6.5 Other β-lactamases

In addition to the clinically most important β-lactamases mentioned above, there are hundreds of β-lactamases of which some are clinically important ESBLs. Several uncommon ESBLs can be found in Enterobacteriaceae (Naas *et al*., 2008), like BES-1 (Brazil extended-spectrum β-lactamase), (Bonnet *et al*., 2000), IBC (integron-associated β-lactamase), PER-2 (Bauernfeind *et al*., 1996b), SFO-1 (homolog to the β-lactamase of *S. fonticola*) (Matsumoto and Inoue, 1999), and TLA (Silva *et al*., 2000). These uncommon ESBLs have been found in a limited number of geographic sites. However, new gene variants are detected all the time in different Enterobacterial species.

2.7 Detection of ESBLs

Since these β-lactamase enzymes have rapidly evolved, not only a major therapeutic dilemma has raised, but also problems to detect these enzymes. Especially important is the detection of plasmid encoded ESBLs. Antibiotic resistance among bacteria can in general be detected either phenotypically or genotypically. For clinically important bacteria, diagnostic laboratories perform phenotypic-based analyses using standardised susceptibility testing methods, the disk diffusion, E-test or agar dilution method. However, for ESBLs standard MIC or disk diffusion based methods may not be enough.

The methods for detection of ESBLs can be broadly divided into two groups: phenotypic methods that use non-molecular techniques, which detect the ability of the ESBL enzymes to hydrolyse different cephalosporins; and genotypic methods, which use molecular techniques to detect the gene responsible for the production of the ESBL.

The current CLSI recommendations (2013) for detection of ESBL's in *E. coli* includes an initial screening test with beta-lactam antibiotics: cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone. The use of more than one antimicrobial agent for screening improves the sensitivity of ESBL detection.

2.7.1 Screening test for ESBLs:

2.7.1.1 Disc diffusion method:

According to the CLSIs guidelines, isolates showing inhibition zone size of ≤ 22 mmwith Ceftazidime (30 µg), ≤ 25 mm with Ceftriaxone (30 µg), ≤ 27 mm with Cefotaxime (30 µg) , ≤ 27 mm with Aztreonam (30 µg) and ≤ 22 mm with Cefpodoxime (10 µg), was identified as potential ESBL producers and short listed for confirmation of ESBL production. The use of more than one antimicrobial agent for screening improves the sensitivity of ESBL detection.)

2.7.1.2 MIC (Minimum inhibitory concentration): It is done by two test: Agar dilution method and Broth dilution method.

2.7.2 Confirmatory tests for ESBLs

2.7.2.1 Phenotypic confirmatory test with combination disk

This test requires the use of a third-generation cephalosporin antibiotic disk alone and in combination with clavulanic acid. In this study, two combinations will be used, firstly a disk of Ceftazidime (30µg) alone and a disk of Ceftazidime + Clavulanic acid (30 µg/10 µg) and secondly Cefotexime (30µg) alone and a disc of Cefotaxime+Clavulanic acid (30µg/10µg) will be used. Both the disks will be placed at least 25 mm apart, center to center, on a lawn culture of the test isolate on Mueller Hinton Agar (MHA) plate and incubated overnight at 37°C. Difference in zone diameters with and without clavulanic acid was measured.

Interpretation: When there is an increase of ≥ 5 mm in inhibition zone diameter around combination disk of Ceftazidime + Clavulanic acid versus the inhibition zone diameter around Ceftazidime disk alone, it confirms ESBL production. Control strain used to validate susceptibility tests will be *E. coli* ATCC 25922.

2.7.2.2 Vitek ESBL cards

Laboratories using conventional Vitek cards risk incorrectly reporting ESBL producing organisms as susceptible to cephalosporins when MICs are ≤ 8 µg/ml.

2.7.2.3 BD Phonex Automated Microbiology System

Bacton Dickison Biosciences (Sparks, Md) have introduced a short incubation system for bacterial identification and susceptibility testing, known as BD Phoenix. Results are usually available within 6 hours.

2.7.2.4 The E test method (Epsilon test)

Produced by AB Biodisk (Solna, Sweden) and Bio-Stat (Stockport, UK) is a plastic drug-impregnated strip, one end of which generates a stable concentration gradient of cephalosporin (i.e., ceftazidime 0.5-32 µg/ml, cefotaxime and cefepime 0.25-16 µg/ml) and the remaining end of which generates a gradient of cephalosporin (i.e., ceftazidime and cefepime 0.064-4 µg/ml, cefotaxime 0.016-1 µg/ml) plus a constant concentration of clavulanate (4 µg/ml). ESBL production is inferred if the MIC ratio for cephalosporin alone/cephalosporin plus clavulanate MIC is ≥ 8.

2.7.3 Genotypic detection

Phenotypic methods are not able to distinguish between the specific enzymes responsible for ESBL production (SHV, TEM, and CTX-M types). Several research or reference laboratories use genotypic methods for the identification of the specific gene responsible for the production of the ESBL, which have the additional ability to detect low-level resistance (i.e, can be missed by phenotypic methods). Furthermore, molecular assays also have the potential to be done directly on clinical specimens without culturing the bacteria, with subsequent reduction of detection time.

The determination of whether a specific ESBL present in a clinical isolate is related to TEM and SHV enzymes is a complicated process because point mutations around the active sites of the TEM and SHV sequences have led to amino acid changes that increase the spectrum of activity of the parent enzymes, such as in TEM-1, TEM-2, and SHV-1 (Farkosh, 2007). The molecular method commonly used is the PCR amplification of the TEM and SHV genes with oligonucleotide primers, followed by sequencing. Sequencing is essential to discriminate between the non-ESBL parent enzymes (eg, TEM-1, TEM-2, or SHV-1) and different variants of TEM or SHV ESBLs (eg, TEM-3, SHV-2, etc).

Molecular methods that do not use sequencing have been developed to characterize ESBLs and include PCR with RFLPs, PCR with single-strand conformational polymorphism, ligase chain reaction, restriction site insertion PCR, and real-time PCR. PCR amplification followed by nucleotide sequencing remains the gold standard for the identification of specific point mutation of TEM or SHV ESBL genes. This is not always straightforward and cost effective because clinical isolates often have multiple copies of ESBL genes. Sequencing is the only method for identifying CTX-M genes, which is labour intensive, time-consuming and expensive. Xu and his collegues report the development of a rapid and accurate multiplex PCR assay for simultaneous amplification of all CTX-M genes and differentiation of the five clusters (Xu *et al.*, 2005).

2.8 Plasmid profiling

The first nucleic acid-based method to be described was plasmid profiling. It was used for the first time in 1979 to study a nosocomial infection of *Klebsiella pneumoniae* (Sadowski *et al*., 1979). The advantage of this method is its simplicity and low cost, but there are several major disadvantages. Not all bacterial strains carry plasmids; they can lose the plasmids upon subculturing; and the bands obtained are relatively few and therefore the discriminatory power is low. Furthermore, a particular plasmid may appear after isolation in several bands as supercoiled, relaxed circular or linear DNA with different electrophoretic mobilities (Maslow *et al*., 1993a).

2.9. Emerging problems with extended-spectrum β-lactamases

The ESBLs are a heterogenous group. The enzymes have differencies in the activity level against the β-lactams. Additionally, the co-existence of several β-lactamases, variation of the catalytic efficiencies of the β-lactamases, as well as the penetration rates of β-lactams into bacterial cells modifies the gene expression. For example, TEM and SHV have similar activity against β-lactams, but some variants like TEM-12 have only minor ESBL activity (Livermore and Paterson, 2006). Rapid and accurate tests that identify bacterial infections, and which antimicrobial agents are suitable for treatment, are important. Even though the β-lactam antibiotics are used worldwide, the prevalence and distribution is not consistent. Today most antibiotics are used in the community in day care centers, long term facilities and local practices. Today no detection test for the presence of ESBL is completely reliable. By correct ESBL detection, prescription of unnecessary antibiotics would be avoided.

There are several risk factors or problems with organisms producing ESBLs. These organisms are important causes of nosocomial infections and limited therapeutic options are available (Dantas *et al*., 2008). For young healthy adults an ESBL infection is not life threatening, and young adults do not acquire infections as easily as older adults. With an increasing age and decreasing health the risk is increased. The length of hospital stay, severity of the illness, time in the

intensive care unit (ICU), urinary or arterial catheterization, and previous exposure to antibiotics are risk factors associated with UTI caused by ESBL-producing bacteria (Pitout *et al*., 2005a). Even though, antibiotic resistance is mostly a nosocomial problem, resistant bacteria are also spread in the community, especially in day care centers and nursing homes (Schiappa *et al*., 1996; Lautenbach *et al*., 2001; Bisson *et al*., 2002; Pena *et al*., 2006). From being most prevalent in *K. pneumoniae* strains at hospitals, the prevalence of ESBLs has changed and they are now present in community-acquired *E. coli* strains. Due to the increase of different resistance mechanisms the treatment options are narrowing against Gram-negative bacteria.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials:

The materials required for this work are listed in Appendix VII.

3.2 Methods:

The study was conducted at KIST Medical College Teaching Hospital, Imadole-6, Lalitpur from May 15, 2013 to November 15, 2013 in joint collaboration with Central Department of Microbiology, Tribhuvan University, Kirtipur.

One thousand two hundred fifty eight mid-stream urine samples from clinically UTI suspected patients were examined by routine examination, culture and antibiotic susceptibility tests of urine sample.

3.2.1 Urine sample

3.2.1.1 Specimen Collection

The patient was given a sterile, dry, wide-necked leak-proof Hi-Media container of 100ml capacity for collection of 10-20 ml of clean-catch mid-stream urine. The patient was given instructions for the collection of CC-MSU. The container labeled with date, name and lab number of the patient and the time of collection was delivered to the laboratory along with the request form as soon as possible.

3.2.2 Macroscopic examination

The specimen obtained in laboratory was observed for its color and turbidity.

3.2.3 Microscopic examination

The urine specimen was examined microscopically as a wet preparation primarily for detecting pus cells. WBCs in excess of 10^4 cells/ml (>10 cells/ml) of urine will indicate significant pyuria. 1 WBC / LPF correspond to 3 cells/µL (Cheesbrough, 2000).

3.2.4 Chemical examination

The detection of albumin and sugar in urine was performed by using uristix. The uristrix was dipped into the urine specimen for few seconds and the change in color in test area was noted after 30 seconds. The results were interpreted

according to the color change of the test area, comparing with that of the given standard color for detection of albumin and sugar.

3.2.5 Culture of specimen

The urine sample was cultured onto the MacConkey agar and Blood agar medium by the semi-quantitative culture technique using a standard loop.

A loopful of sample was touched to the centre of the plate, from which the inoculum was spread in a line across the diameter of the plate. Without flaming or re-entering urine, the loop was drawn across the entire plate, crossing the first inoculum streak numerous times to produce isolated colonies. The MacConkey and Blood agar plates were incubated aerobically at 35-37^{0}C overnight. The approximate numbers of colonies were counted and the number of bacteria, i.e. cfu/ml of urine was estimated in accordance to the volume of urine inoculated previously. For example, 100 colonies on inoculating 0.001 ml of urine would correspond to 10^5 cfu/ml.

The bacterial count was reported as

Less than 10^4/ml organisms, not significant. 10^4-10^5/ml organisms, doubtful (suggest repeat specimen). More than 10^5/ml organisms, significant bacteriuria.

However if the culture indicated the appearance of $\geq$3 organism types with no predominating organism, this was interpreted as due to possible contamination of the specimen and asked for another specimen. In addition to the previously described guidelines a pure culture of *S. aureus* was considered significant regardless of the number of cfu (Forbes *et al,* 2002).

3.2.6 Identification of the isolates

Identification of significant isolates was done by using microbiological techniques which involves morphological appearance of the colonies, staining reactions and biochemical properties (Bailey & Scotts, 1990; Cheesbrough, 1984; Mackie and McCartney, 1998).

Each of the organisms was isolated in pure form before performing biochemical and other tests. Gram staining of an isolated colony was done from primary culture. For gram negative organism a speck of single isolated colony from MacConkey agar and for gram positive the same from blood agar was

transferred into the nutrient broth and incubated at 37ºC for 4 hours. It was then subcultured on dried nutrient agar plate and incubated at 37ºC for 24 hours. Thus obtained overnight incubated culture of organism on nutrient agar was used to perform catalase, oxidase, other biochemical tests and antibiotic susceptibility test.

Details of Gram- staining procedure is mentioned in the appendix III.

Appropriate biochemical tests were performed for the confident identification of the bacterial isolates. For that, the pure colonies on the media plates were inoculated onto different biochemical media.

Gram-positive organisms were identified primarily on the basis of their response to gram's staining, catalase, oxidase, coagulase and bile esculin hydrolysis tests.

The biochemical tests used for the identification of gram-negative bacterial isolates include Catalase test, Oxidase test, Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Oxidation Fermentation test, Triple Sugar Iron (TSI) test, Motility test and gas production tests.

The composition and preparation of biochemical media and reagents used in the biochemical test are mentioned in the appendix II. The procedure for performing biochemical tests are mentioned in appendix IV.

3.2.7 Antibiotic susceptibility testing

The antibiotic sensitivity testing was performed according to the recommended Kirby-Bauer sensitivity testing method (CLSI, 2013).

Mueller Hinton agar was prepared and sterilized as instructed by the manufacturer. The pH of the medium 7.2-7.4 and the depth of the medium at 4 mm (about 25 ml per plate) were maintained in petri dish. Using a sterile wire loop, a single isolated colony of which the sensitivity pattern is to be determined was touched and inoculated into a nutrient broth tube and was incubated for 2-4 hrs. After incubation in a good light source, the turbidity of the suspension was matched with the turbidity standard of MacFarland 0.5. Using a sterile swab, a plate of MHA was inoculated with the bacterial suspension using carpet culture technique. Using sterile forceps, appropriate antimicrobial discs (6 mm diameter) was placed, evenly distributed on the inoculated plates, not more than 5 discs

were placed on a 90 mm diameter Petri plate. Within 30 minutes of applying the discs, the plates were taken for incubation at 35^0C for 16-18 hrs. After overnight incubation, the plates were examined to ensure confluent growth and the diameter of each zone of inhibition in mm was measured and results interpreted.

3.2.8 Purity plate

The purity plate was used to ensure that the inoculation used for the biochemical tests is pure culture and also to see whether the biochemical tests are performed in an aseptic condition or not. Thus, while performing biochemical tests, the same inoculum was subcultured in respective medium and incubated. The media was then checked for the appearance of pure growth of organisms.

3.2.9 Quality control for test

Quality of each test was maintained by using standard procedures. The quality of each agar plates prepared was tested by incubating one plate of each lot on the incubator. During identification of organism, for each test ATCC control positives and control negatives was taken simultaneously. Quality of sensitivity tests was maintained by maintaining the thickness of Mueller-Hinton agar at 4mm and the pH at 7.2-7.4. Similarly antibiotic discs containing the correct amount as indicated were used. Strict aseptic conditions were maintained while carrying out all the procedures.

3.2.10 Screening test for ESBLs

Isolates were screened for ESBL production by using disc diffusion of cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CTR) and Aztreonam (AT) placed on inoculated plates containing Muller Hinton agar according to the CLSI recommendations. Isolates showing inhibition zone size of ≤22 mm with ceftazidime (30µg), ≤25 mm with ceftriaxone (30µg), ≤ 27 mm with cefotaxime (30µg) , ≤ 27 mm with Aztreonam (30 µg) were suspected for ESBL production. But both the strains were resistant as well as sensitive to 3GCs were included. *E. coli* ATCC 25922 was used as a negative control.

3.2.11 Confirmatory test for ESBLs

Phenotypic confirmatory test for ESBL producers was done by combination disc method for all the ESBL producing isolates as per CLSI 2013 guidelines as well as initially resistant to 3rd generation cephalosporins (Tenover et al. 1999; Paterson and Yu 1999).

3.2.11.1 Combination disc method

In this test a disc of ceftazidime (30µg), cefotaxime (30µg) alone and a disc of ceftazidime and cefotaxime in combination with clavulanic acid (30/10µg) were used for each isolates. Both the discs were placed 25 mm apart, centre to center, on a lawn culture of the test isolate on Muller Hinton agar plate and incubated overnight at 37^0C. A ≥5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulinic acid versus its zone when tested alone was designated as ESBL positive.

Interpretation: When there is an increase of ≥ 5 mm in inhibition zone diameter around combination disk of Ceftazidime + Clavulanic acid versus the inhibition zone diameter around Ceftazidime disk alone, it confirms ESBL production.

3.2.12 Plasmid profiling

3.2.12.1 Isolation of bacterial plasmid DNA by Alkaline-SDS Lysis

Plasmid DNA was isolated from ESBL isolates according to Sambrook & Russel (2001). *E. coli* isolates were subcultured in Luria-Bertani broth at 37^oC and 3 ml of overnight culture was subjected to plasmid DNA extraction by centrifugation at 5000 rpm for 5 min. After washing in Tris- ethylene diamine tetraacetic acid (EDTA) buffer (10mM Tris, 1mM EDTA, pH 8.0; TE), the pellet was added to the freshly prepared mixture of NaOH and SDS, to which Potassium acetate was added. The microfuge tube was inversely mixed and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred into a new microfuge tube with adding Phenol: Chloroform and centrifuged at 13,000 rpm for 10 min. The upper aqueous phase was collected in a clean microfuge tube to which Chloroform was added and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred into a new microfuge tube and cold 95% ethanol was added to precipitate bacterial

DNA. The pellet was washed with 70% ethanol and dissolved in 60 µL of TE buffer. (Detail in Appendix IX)

3.2.12.2 Preparation of agarose gel

O.8% percent agarose gel was prepared by melting 0.8 gm agarose in 100 ml of diluted TAE buffer using a microwave oven. The melted agarose was allowed to cool to about 50ºC and 50 µl ethidium bromide (0.5µg/ml) was mixed and shacked and was poured into gel tray and comb was placed. After solidification of the gel, the comb was removed. The gel was placed in a horizontal electrophoresis apparatus filled with TAE buffer.

3.2.12.3 Loading and electrophoresis of the sample

Each 5 µl of isolated Plasmid DNA was mixed with 1 µl of 6X gel loading buffer. The mixture was slowly loaded into the well using disposable micropipette tips. 1 Kbp molecular weight marker was loaded in one well to determine the size of the Plasmid DNA. Electrophoresis was carried out at 100 volts for 1 hour.

3.2.12.4 Visualization of the gel

The amplified products of the study samples were visualized by using UV trans-illuminator. The gel was photographed by a digital camera and transferred data to computer for further documentation (Sharma *et al*. 2010; Lal *et al*. 2007; Pagani *et al*. 2003).

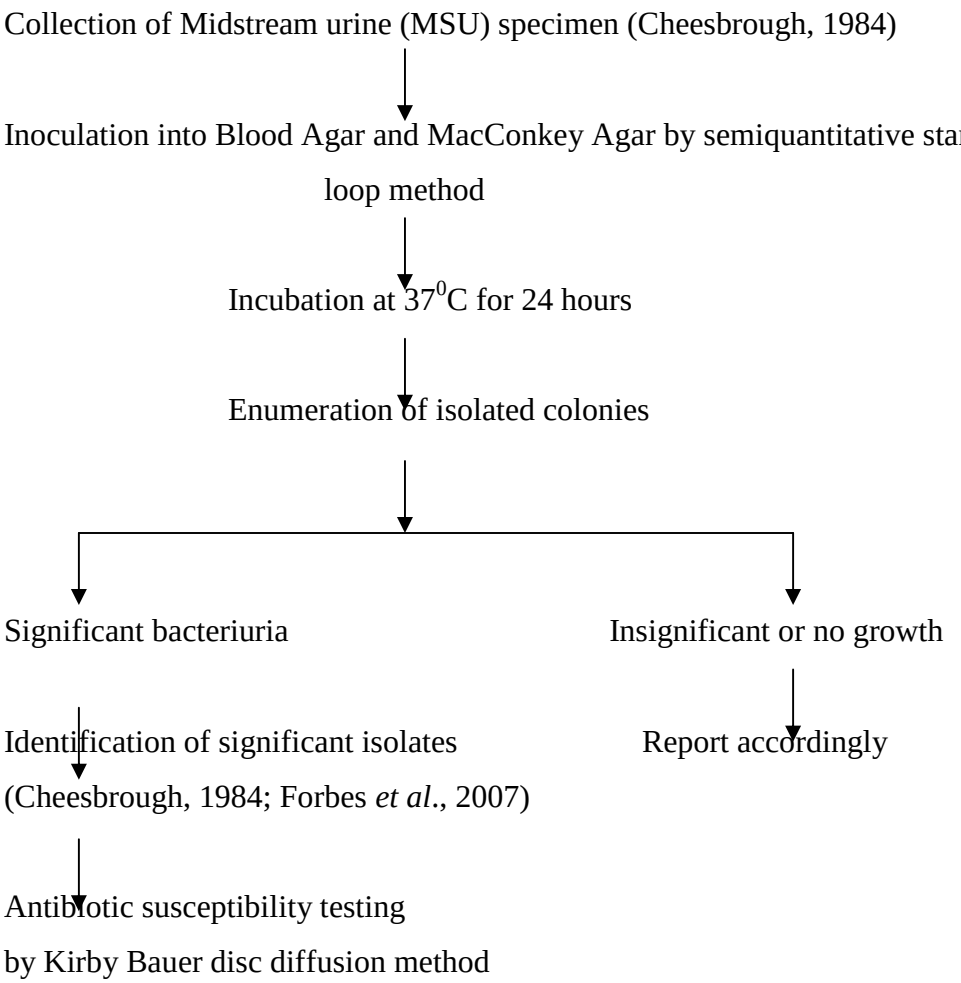


Figure 1: Flow diagram for processing urine sample

CHAPTER – IV
RESULTS

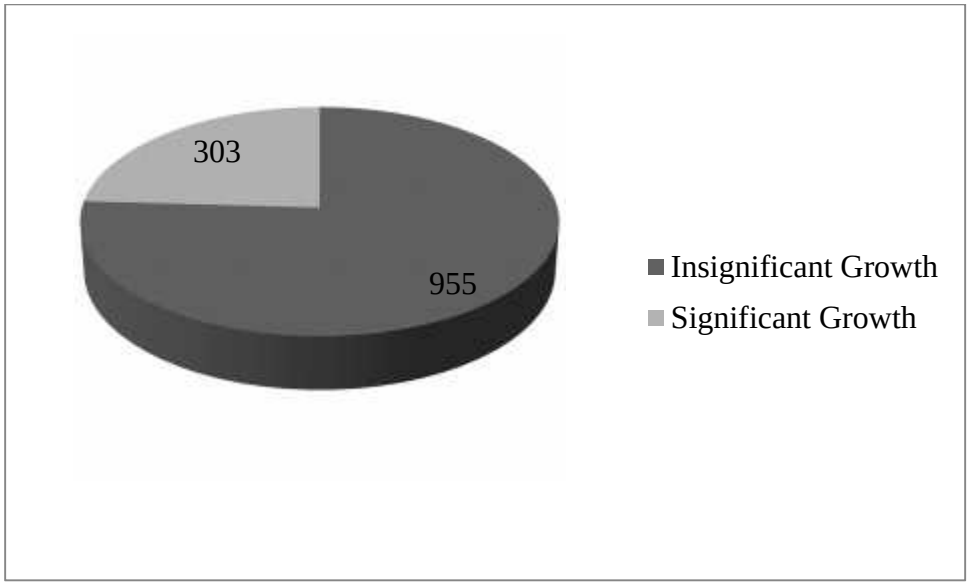
This study was conducted at the department of microbiology, KIST MCTH. A total 1258 midstream urine samples were collected from the patients suspected of urinary tract infection over the period of 6 months from May 2013 to November 2013.

4.1 Pattern of culture results

303 uropathogens recovered from urine samples of inpatients and outpatients of the hospital, with their provisional diagnosis written on their requisition form received from various wards like Medicine, Surgery, Obstetrics and Gynecology, Dermatology, Nephrology, Pediatrics, Orthopedics and Intensive care units.

Out of 1258 samples processed for culture, 303 (24.09%) samples showed significant bacteriuria (i.e.>10^5 cfu/ml), while 955 (75.91%) samples showed insignificant growth (i.e. ≤10^5 cfu/ml), growth of contaminants or no growth.

Figure 1. Pattern of culture results



4.2 Patient Demographics

Demographic parameters such as age, gender and geographic origin of specimens were retrieved from laboratory records. Among total 1258 patients 210 (69.3%) were found to be female and 93 (30.7%) were male.

Majority of the females suffering from UTI clustered in the age group of 21-40years (99/210 i.e. 47.14%) i.e child bearing age. Comparatively less number of males of same age suffered from UTI (22/93 i.e 23.66%). Among the significant bacteriuric cases, the highest number of cases i.e. 81 belonged to the age group 21-30 years.

Table 1. Age and sex wise distribution of significant bacteriuric cases

Age group	Male	Female	Total
0-10	14	20	34
11-20	7	20	27
21-30	8	73	81
31-40	14	26	40
41-50	10	23	33
51-60	9	21	30
61-70	9	8	17
71-80	13	15	28
81-90	9	2	11
91-100	0	2	2
Total	**93**	**210**	**303**

4.3 Distribution of significant bacteriuric cases in different ward

The highest number of significant bacteriuric cases was observed among inpatient department (180) followed by outpatient department (123) (Table 2).

Table 2. Ward wise distribution of significant bacteriuric cases

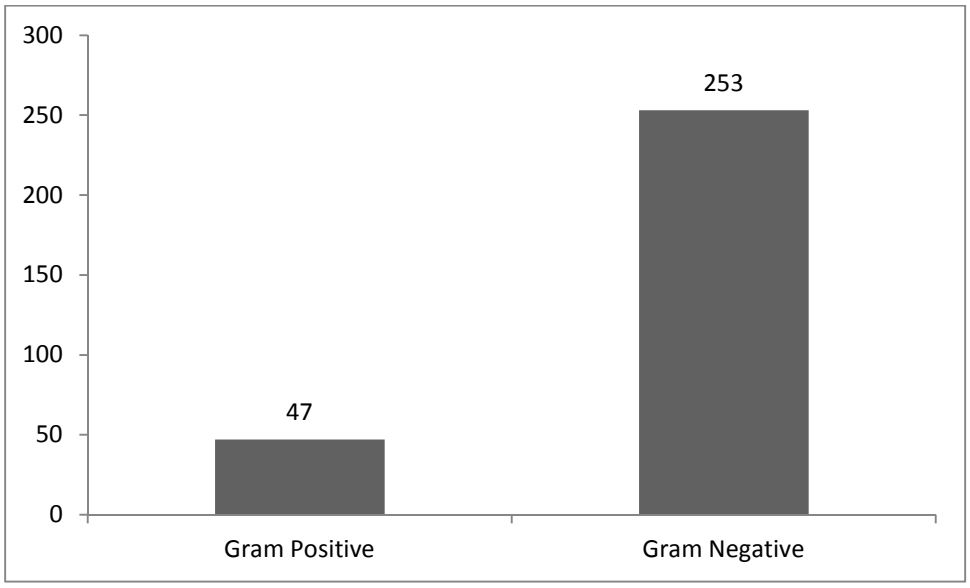
Ward	Inpatient/outpatient		Total
	Inpatient	Outpatient	
Emergency	69	14	83
Nephrology	39	21	60
Surgery	11	11	22
Paediatrics	20	11	31
Orthopedic	4	0	4
Obs/Gynae	14	45	59
Medicine	23	21	44
Total	**180**	**123**	**303**

4

4 Pattern of bacteria isolated from culture positive urine samples according to Gram's stain

Out of the total 303 isolates, maximum isolates 253 (83.49%) were found to be Gram negative bacilli, 47 isolates (15.51%) were found to be Gram positive cocci and remaining 3 were *Candida albicans* and Non *albicans Candida* species.

Figure 2. Pattern of bacteria isolated from culture according to Gram's stain



4.5 Pattern of bacterial isolates

Sixteen different species of bacteria were isolated among which *Escherichia coli* (65.3%) was found to be the predominant followed by *Staphylococcus aureus* (6.9%).

Table 3. Pattern of bacterial isolates

Bacterial isolates	**Frequency**	**Percentage**
Escherichia coli	198	65.3
Klebsiella pneumoniae	20	6.6
Klebsiella oxitoca	1	0.3
Proteus mirabilis	6	2
Proteus vulgaris	4	1.3
Citrobacter koseri	3	1
Citrobacter freundii	2	0.7
Enterobacter species	1	0.3
Pseudomonas aeruginosa	4	1.3
Acinetobacter species	14	4.6
Staphylococus aureus	21	6.9
Coagulase negative *Staphylococcus aureus*	10	3.3
Streptococcus species	7	2.3
Enterococcus species	9	2.9
Candida albicans	1	0.3
Non *albicans Candida* species	2	0.7
Total	**303**	**100**

4

.

6

Antibiotic susceptibility pattern of the isolates

4.6.1 Antibiotic susceptibility pattern of Gram positive isolates

Among the 47 Gram positive isolates, most of them i.e. 38 (80.85%) were susceptible to Nitrofurantoin. The result is shown in table 4.

Table 4. Antibiotic susceptibility pattern of gram positive bacteria isolated from urine samples.

Antibiotic used	Sensitive	Intermediate	Resistant	Total
Ampicillin	25		22	47
Gentamicin	32		15	47
Norfloxacin	21		26	47
Nitrofurantoin	38	2	7	47
Ciprofloxacin	23	1	23	47
Cotrimoxazole	24		23	47
Ceftriaxone	24		23	47

4

6

.2 Antibiotic susceptibility pattern of Gram negative isolates

Among the common antibiotics used against all Gram negative isolates, Gentamicin was the drug of choice as 192 (75.89%) isolates was found to be susceptible to the drug followed by Nitrofurantoin with a susceptibility of 58.49.%. Most of the Gram negative isolates, i.e. 201 (79.45%) were resistant to Ampicillin. The results are shown in table 5.

Table 5. Antibiotic sensitivity pattern against gram negative bacteria isolated from urine samples.

Antibiotic used	Sensitive	Intermediate	Resistant	Total
Ampicillin	50	2	201	253
Gentamicin	192	3	58	253
Nalidixic acid	62	3	188	253
Norfloxacin	111	3	139	253
Nitrofurantoin	148	39	66	253
Ciprofloxacin	123	1	129	253
Cotrimoxazole	107	1	145	253
Ceftriaxone	129	1	123	253

Antibiotic resistance pattern of the isolates

4.7.1 Multidrug resistance pattern of the isolates from urine sample

Out of the 303 isolates, 175 (58.33%) isolates were MDR. 59.09 % *E. coli* isolated and 78.57.0% of *S. aureus* isolated were found to be MDR strains. All the isolates (100%) of C.*koseri, K.oxitoca, Enterobacter* species and *P. aeruginosa* were found to be MDR-strains. The results are shown in table 6.

Table 6. Multidrug resistance pattern of isolates from urine sample

Bacterial isolates	**MDR**	**Total**
Escherichia coli	117	198
Klebsiella pneumoniae	7	20
Klebsiella oxitoca	1	1
Proteus mirabilis	5	6
Proteus vulgaris	3	4
Citrobacter koseri	3	3
Citrobacter freundii	0	2
Enterobacter species	1	1
Pseudomonas aeruginosa	4	4
Acinetobacter species	11	14
Staphylococus aureus	6	21
Coagulase negative *Staphylococcus aureus*	5	10
Streptococcus species	4	7
Enterococcus species	8	9
Candida albicans		1
Non *albicans Candida* species		2
Total	**175**	**303**

4.8 Antibiotic susceptibility pattern of the *E. coli* isolates

The 1st line antibiotic susceptibility pattern of *E. coli* showed that 75.75% of the isolates were sensitive to Gentamicin followed by nitrofurantoin with a susceptibility of 69.19% (Table 7).

Table 7. Antibiotic susceptibility pattern of the *E. coli* isolates (n=198) with 1st line drugs

Antibiotic used	Sensitive	Intermediate	Resistant	Total
Ampicillin	45	2	151	198
Gentamicin	150	2	46	198
Nalidixic acid	41	1	156	198
Norfloxacin	75	2	121	198
Nitrofurantoin	137	32	29	198
Ciprofloxacin	84	1	113	198
Cotrimoxazole	84		114	198
Ceftriaxone	100		98	198

The 2nd line antibiotic susceptibility pattern of the MDR *E. coli* showed that 100% of the isolates were sensitive to imipenam followed by amikacin with a susceptibility of 95.74% (Table 8).

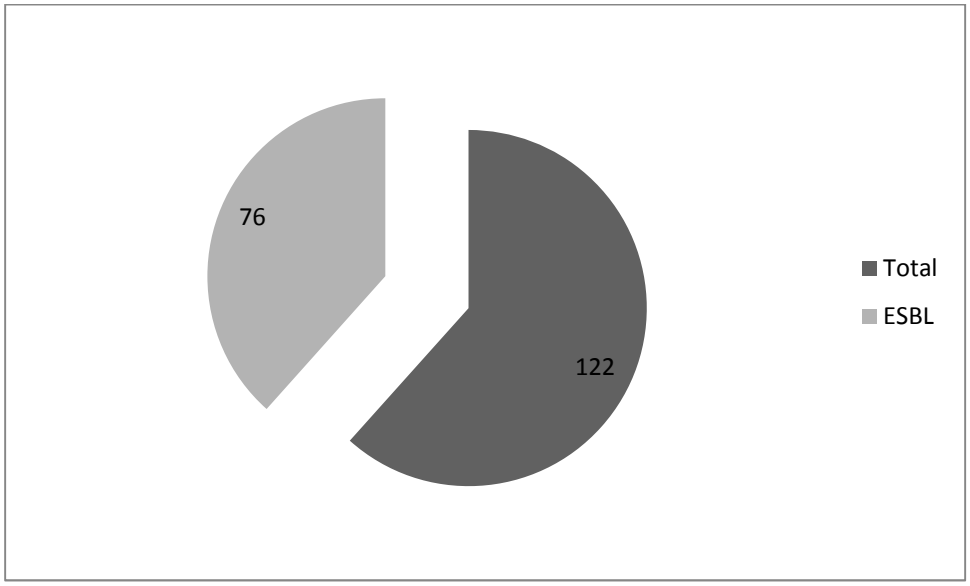
Table 8. Antibiotic susceptibility pattern of the *E. coli* isolates (n=94) with 2nd line drugs

Antibiotic used	Sensitive	Intermediate	Resistant	Total
Amikacin	90	1	3	94
Ofloxacin	10	1	83	94
Amoxycillin/Clavulanic acid	4		90	94
Ceftazidime	3	2	89	94
Imipinam	93		0	94

4.9 Rate of detection of ESBL production by Combination disc method from *E coli*

Figure 3 Showed prevalence of ESBL producing *E coli* isolates by double disc diffusion test (DDDT). Among them 76/198 (38.4 %) were ESBL positive.

Figure 3. Rate of detection of ESBL production by Combination disc method from *E coli*



4.10 Antimicrobial resistance pattern among ESBL producers

Table 9 and 10 showed antimicrobial resistance pattern among ESBL producers. All the antibiotics used were more resistant in ESBL producers. Among them Ciprofloxacin, Ampicillin and Nalidixic acid were more resistant, on the other hand Amikacin (98.43%) and Imipenam (100%) were more sensitive.

Table 9. First line Antimicrobial resistant pattern in ESBL positive strain

Name of antimicrobials	Rate of resistance to ESBL positive
Ampicillin	72
Gentamicin	28
Nalidixic acid	72
Norfloxacin	67
Nitrofurantoin	14
Ciprofloxacin	73
Cotrimoxazole	56
Ceftriaxone	76

Table 10. Second line Antimicrobial resistant pattern in ESBL positive strain

Name of antimicrobials	Rate of resistance to ESBL positive
Amikacin	1
Ofloxacin	64
Amoxicillin/Clavulanic acid	76
Ceftazidime	76
Imipenam	0

4.11 Phenotypic confirmation of ESBL positive bacteria by Combination disc method

Table 11 Showed phenotypic confirmation of ESBL positive bacteria by Combination disc method using two combinations, ceftazidime alone and with the combination of clavulinic acid (CAZ/CAZC) and cefotaxime alone and with the combination of clavulinic acid (CTX/CTXC). Most of the bacteria showed ESBL positive by both combination (CAZ/CAZC, and CTX/CTXC). *E. coli* showed maximum ESBLs production in CAZ/CAZC combination. Both the CAZ/CAZC and CTX/CTXC methods were statistically insignificant.

Table 11. Detection of ESBL by Combination Disc Method as confirmatory test (Cross tabulation between CAZ/CAZC and CTX/CTXC of ESBL detection)

	CAZ/CAZC	CTX/CTXC	Combined
ESBL positive	66	64	76
ESBL negative	10	12	
Total	76	76	76

x^2value, p >0.005, non significant

4.12 Plasmid Profiling

Seventy six isolates of *E. coli* isolated from urine samples were screened for plasmids. The overall frequency of *E. coli* isolates containing plasmids was 53.9% (41 isolates). Thirty five strains (46.1%) lacked any plasmid and in 41 strains (53.9%), 1-7 plasmids were detected. Although some strains contained plasmids of >10kb molecular mass, but mainly isolated plasmids ranged from 1 kb to 10 kb. The plasmid distributions of the isolates and antimicrobial resistance pattern were shown in Table 12, Table 13, Table 14, Table 15, Table 16, Table 17, Table 18 and Table 19 respectively. Upto 7 plasmids occurred in the isolates with sizes ranging from 1.0 kb to 10.0 kb.

Table 12. Plasmid profile of *E.coli*

Sr. No.	No. of plasmids	Plasmid sizes (kb)
1	7	1,2,3,4,7,10,>10
2	3	2,4,6
3	5	3,4,5,6,7
4	3	1,2,10
5	5	3,4,5,6,7
6	2	1,10
7	1	10
8	3	2,3,7
9	2	3,4
10	3	2,4,6
11	2	3,4
12	4	2,4,6, >10

13	2	4,6
14	1	2
15	3	5,7,10
16	3	7,8,10
17	2	2,10
18	6	1,2,3,4,7,10
19	1	1
20	1	6
21	5	1,2,3,4,7
22	4	1,3,4,10
23	2	2,10
24	2	1,2
25	1	1
26	1	1
27	2	3,4
28	1	10
29	2	1,2
30	6	1,2,3,4,8,10
31	1	10
32	7	1,2,5,6,7,8,10
33	1	10
34	1	2
35	3	1,2,10
36	2	1,10
37	3	1,2,10
38	6	1,2,3,4,10,>10
39	4	1,2,3,4
40	6	1,2,6,7,8,10
41	1	10

Table 13. Plasmid profiles and antimicrobial resistance of 11 isolates of *E. coli* harbouring 1 plasmid.

Isolates	Plasmid sizes (kb)	Drug resistance
7	10	Amp, Cot, Amc
14	2	Amp, Gen, Nit, NA, Nx, Cip, Cot, Amc, Of
19	1	Amp, Gen, Nit, NA, Cot, Amc,
20	6	Amp, Nit, NA, Cot, Amc,
25	1	Amp, NA, Nx, Cip, Cot, Amc, Of
26	1	Amp, NA, Nx, Cip, Cot, Amc, Of
28	10	Amp, Gen, Nit, NA, Nx, Cip, Cot, Amc, Of
31	10	Amp, NA, Nx, Cip, Cot, Amc, Of
33	10	Amp, Gen, Amc
34	2	Amp, NA, Nx, Cip, Cot, Amc, Of
41	10	Amp, NA, Nx, Cip, Cot, Amc, Of

Table 14. Plasmid profiles and antimicrobial resistance of 10 isolates of *E. coli* harbouring 2 plasmids.

Isolates	Plasmid sizes (kb)	Drug resistance
6	1,10	Amp, Nit, NA, Nx, Cip, Cot, Amc, Of
9	3,4	Amp, Gen, NA, Nx, Cip, Cot, Amc, Of
11	3,4	Amp, NA, Amc
13	4,6	Amp, Nit, NA, Cot, Amc
17	2,10	Amp, Nit, NA, Nx, Cip, Cot, Amc, Of
23	2,10	Amp, Gen, NA, Nx, Cip, Cot, Amc, Of
24	1,2	Amp, Nit, Gen, NA, Nx, Cip, Cot, Amc, Of
27	3,4	Amp, Nit, NA, Nx, Cip, Cot, Amc, Of
29	1,2	Amp, Gen, Nit, NA, Nx, Cip, Cot, Amc, Of
36 **T**	1,10	Amp, NA, Nx, Cip, Cot, Amc, Of

able 15. Plasmid profiles and antimicrobial resistance of 8 isolates of *E. coli* harbouring 3 plasmids.

Isolates	Plasmid sizes (kb)	Drug resistance
2	2,4,6	Amp, NA, Nx, Cip, Cot, Amc, Of
4	1,2,10	Amp, Nit, NA, Nx, Cip, , Amc, Of
8	2,3,7	Amp, Gen , NA, Nx, Cip, Cot, Amc, Of
10	2,4,6	Amp, Nit, NA, Nx, Cip, Cot, Amc, Of
15	5,7,10	A mp, NA, Amc
16	7,8,10	Amp, NA, Nx, Cip, Amc, Of
35	1,2,10	Amp, Gen, Nit, NA, Nx, Cip, Cot, Amc, Of
37	1,2,10	Amp, Gen, NA, Nx, Cip, Cot, Amc, Of

16. Plasmid profiles and antimicrobial resistance of 3 isolates of *E. coli* harbouring 4 plasmids.

Isolates	Plasmid sizes (kb)	Drug resistance
12	2,4,6, >10	Amp, NA, Nx, Cip, Cot, Amc, Of
22	1,3,4,10	Amp, NA, Nx, Cip, Cot, Amc, Of
39	1,2,3,4	Amp, NA, Nx, Cip, Amc, Of

ble 17. Plasmid profiles and antimicrobial resistance of 3 isolates of *E. coli* harbouring 5 plasmids.

Isolates	Plasmid sizes (kb)	Drug resistance
3	3,4,5,6,7	Amp, Gen, Nit, NA, Nx, Cip, Cot, Amc, Of
5	3,4,5,6,7	Amp, Gen, Nit, NA, Nx, Cip, Cot, Amc, Of
21	1,2,3,4,7	Amp, Gen, NA, Nx, Cip, Cot, Amc, Of

Table 18. Plasmid profiles and antimicrobial resistance of 4 isolates of *E. coli* harbouring 6 plasmids.

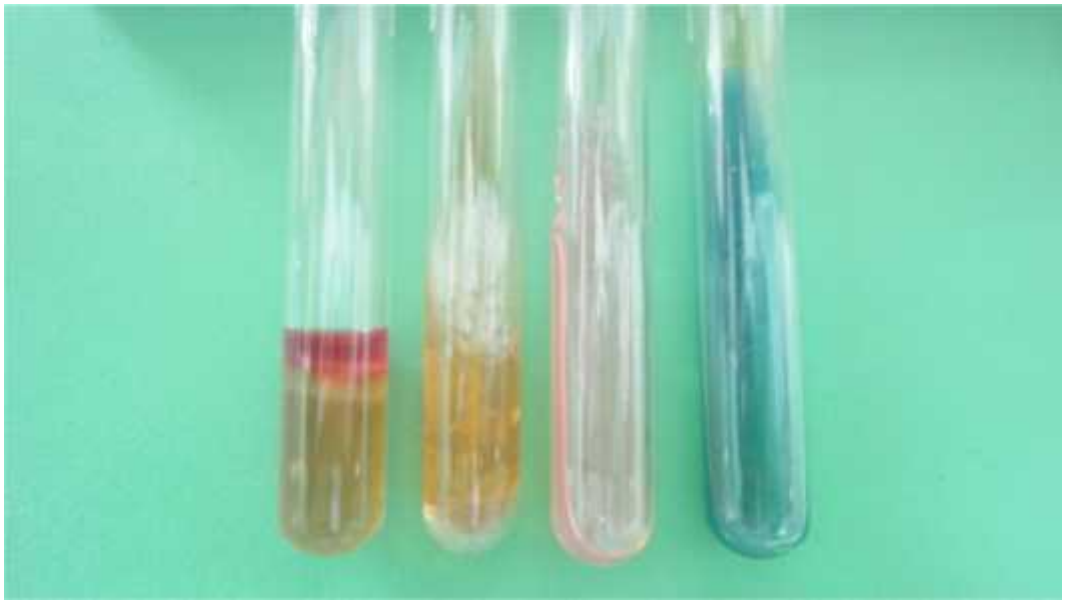
Isolates	Plasmid sizes (kb)	Drug resistance
18	1,2,3,4,7,10	Amp, NA, Nx, Cip, Cot, Amc, Of
30	1,2,3,4,8,10	Amp, Gen, Nit, NA, Nx, Cip, Cot, Amc, Of
38	1,2,3,4,10,>10	Amp, NA, Nx, Cip, Cot, Amc, Of
40	1,2,6,7,8,10	Amp, Gen, NA, Nx, Cip, Amc, Of

T able 19. Plasmid profiles and antimicrobial resistance of 2 isolates of *E. coli* harbouring 7 plasmids.

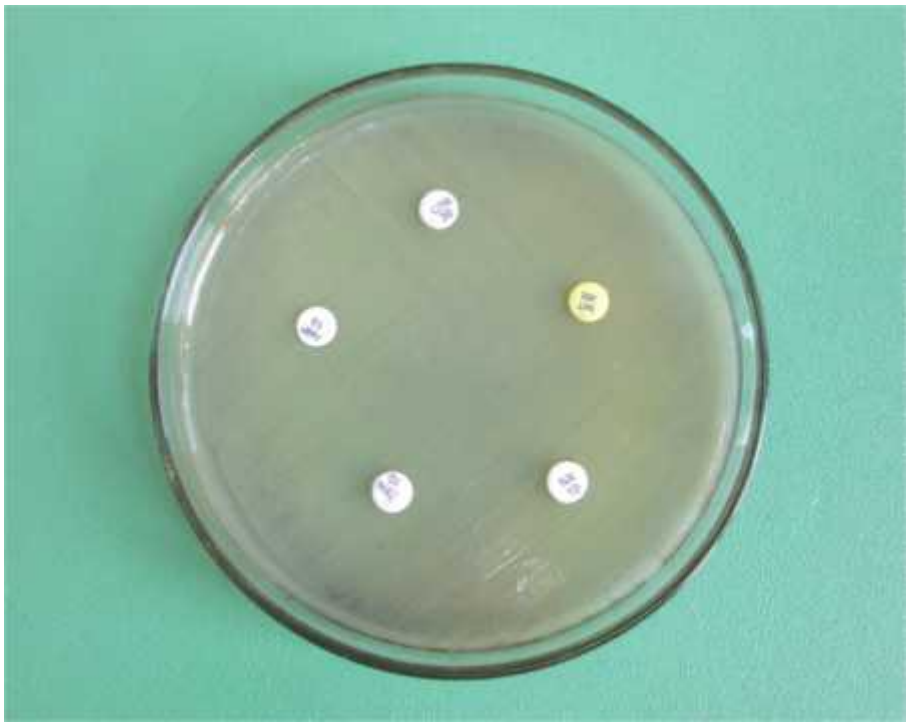
Isolates	Plasmid sizes (kb)	Drug resistance
1	1,2,3,4,7,10,>10	Amp, NA, Nx, Cip, Cot, Amc, Of
32	1,2,5,6,7,8,10	Amp, Gen, NA, Nx, Cip, Cot, Amc, Of

Photograph 1. Significant growth of *E. coli* on MacConkey agar

SIM TSI Urease Citrate

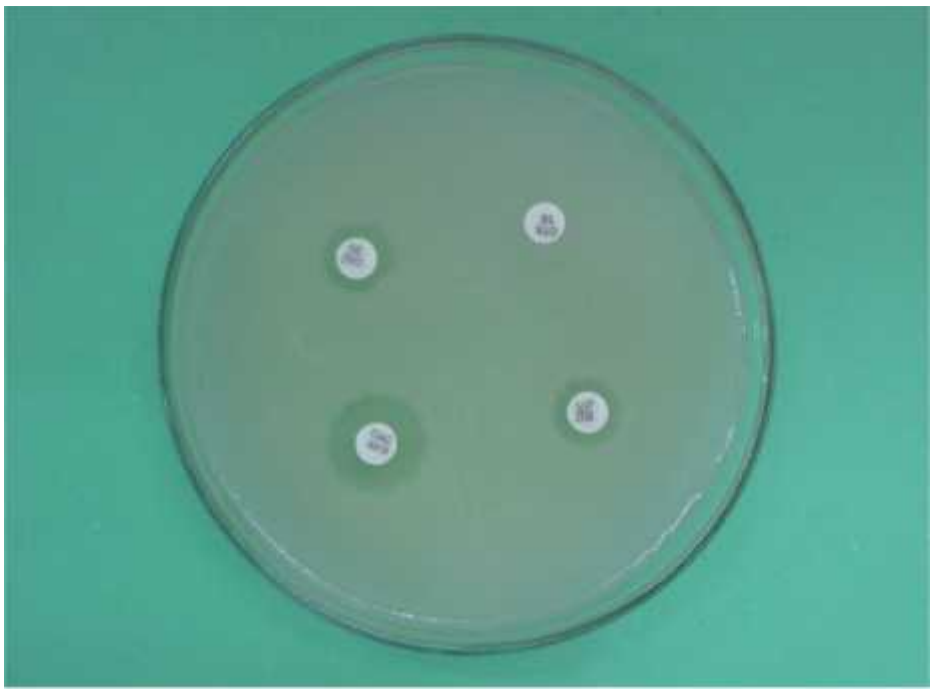


Photograph 2. Biochemical tests for *E. coli*



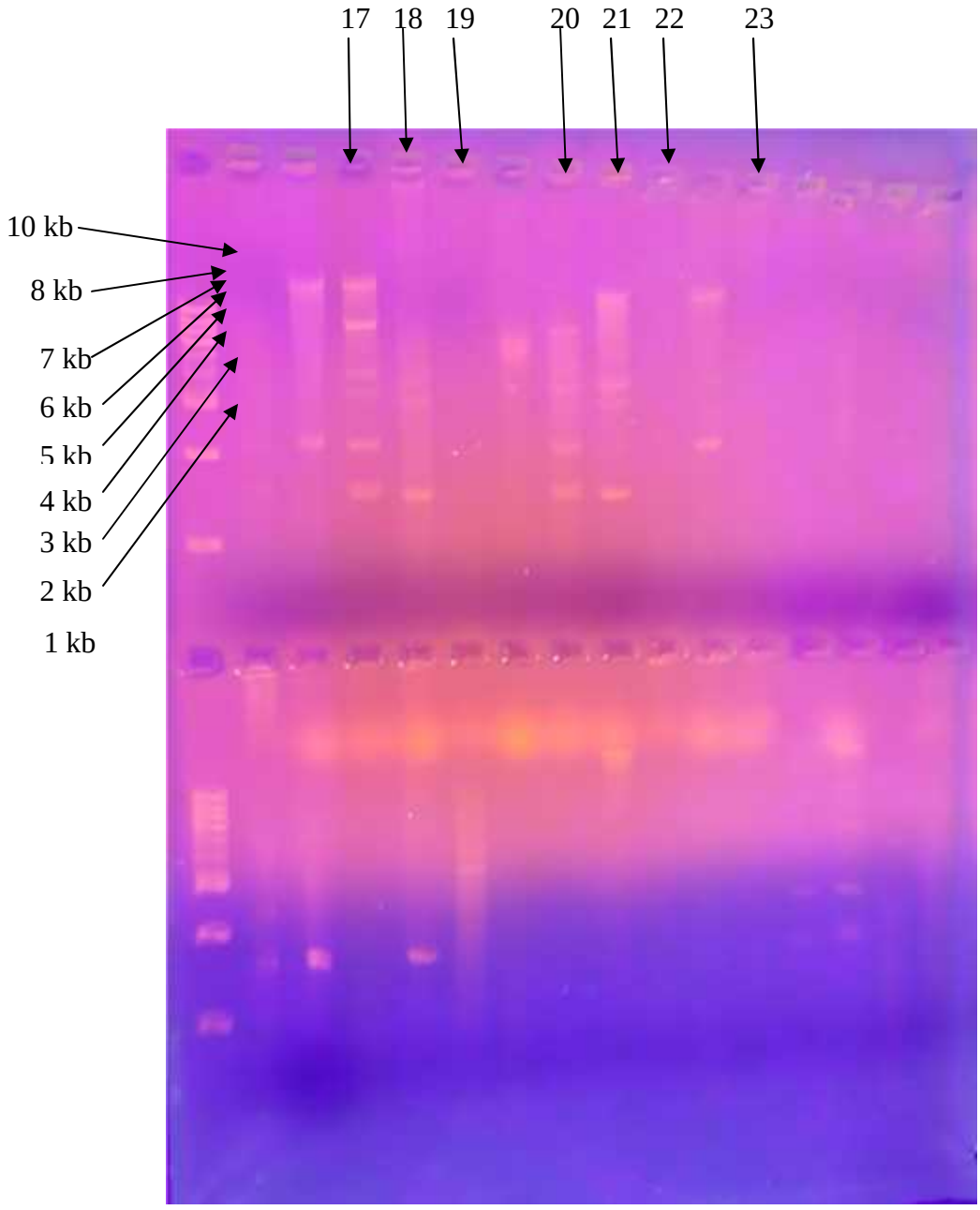
Photograph 3. Multi-drug resistant *E. coli* on Mueller- Hinton agar (AMP= Ampicillin,

CTR= Ceftriaxone, GEN= Gentamycin, NIT= Nitrofurantoin, NX=Norfloxacin)



Photograph 4. Phenotypic confirmatory test for ESBL *E. coli* (CAZ= Ceftazidime,

CAC= Ceftazidime+Clavulanic acid, CTX= Cefotaxime, CEC= Cefotaxime +Clavulanic acid



Photograph 5. Plasmid profiles of ESBL *E. coli*

CHAPTER V

DISCUSSION

Urinary tract infection is a serious health problem affecting millions of people each year. Urinary tract infections are a common problem in general practice and are usually treated empirically. Empirical treatments should be based on local data regarding common pathogens and their susceptibility to available antibiotics. This study was conducted among patients suspected of urinary tract infection, attending KIST MCTH, Lalitpur, Nepal.

One thousand two hundred fifty eight mid stream urine samples were collected and subjected to biochemical analysis and routine examination and then processed for culture and sensitivity from patients visiting the hospital.

The prevalence of UTI in the population was 24.08%. This figure is higher than the 20.2% rate recorded by Theodros Getachew Centro Nacional de Investigaciones Científicas (2010) in Cuba, and 21.8% rate recorded by Maha A. Mahmood Department of Basic Sciences, College of Dentistry, Baghdad University, Baghdad-Iraq (2011). It is lower than prevalence rate of 60% significant bacteriuria recorded by Kolawole (2009) at the Dalhatu Araf Specialist Hospital, Lafia, Nasarawa State, Nigeria and 80% by Durgesh D. Wasnik, P. M. Tumane Post Graduate Teaching Department of Microbiology, Rashtrasant Tukadoji Maharaj, Nagpur University, Nagpur (2012)– 440 033, Maharashtra (India). The high prevalence may be due to genuine population susceptibility because factors like sexual intercourse, peer group influence, pregnancy, low socio-economic status, are common among Nepali men and female.

In this study it was found that the prevalence of UTI was more in females (69.3%) than in males (30.7%). This is because of short urethra in females, injury during sexual intercourse and proximity to anus. In infants, males predominate than females in UTI.

This study demonstrated that males and females in the age group of 21-40 years suffered from UTI more commonly than the other age groups. Similar results

were seen in the study by Kolawole (2009) at the Dalhatu Araf Specialist Hospital, Nigeria. This is natural as it is the sexually active and also child bearing age group in female.

The highest percentage of significant bacteriuric cases were observed among inpatients than outpatients. The prevalence was high in emergency followed by nephrology ward.

Out of 303 cases with significant bacteriuria, 83.49 % were due to gram negative rods and 15.51% were due to gram positive cocci. In a similar study performed by Mahmood (2011), out of 100 urinary isolates, 85% constituted Gram negative isolates whereas 15% Gram Positive Isolates.

The study revealed *E. coli* and *S. aureus* to be the dominant organisms among other uropathogens. The most common organism isolated in these patients was *E. coli* (65.3%), *S. aureus* (6.9%), *K. pneumonia* (6.6%), *Acinetobacter* species (4.6%), and this finding is similar to other reports which indicate that a gram negative bacterium, particularly *E. coli*, is the commonest pathogen isolated in patients with UTI (Kolawole *et al*., 2009; Mahmood, 2011; Devanand and Saxena, 2013 and Sasirekha, 2010).

Among the common antibiotics used against all Gram negative isolates, Gentamycin was the drug of choice with susceptibility of 75.9% as 192 out of 253 isolates were found to be susceptible to the drug followed by Nitrofurantoin with a susceptibility of 58.5%. Most of the Gram negative isolates, i.e. 79.5% (201/253) were resistant to Ampicillin. Nitrofurantoin was found to be the most effective drug against urinary pathogens also in other similar studies by Gautam *et al.,* (2002); Jha and Yadav, (1992); Shrestha *et al.,* (2004).

Among the Gram positive isolates, most of them i.e. 80.9% (38/47) were susceptible to Nitrofurantoin followed by Gentamycin with the susceptibility of 68.1%. Nalidixic acid was found to be the least effective as only 7 (14.9%) isolates were sensitive to the drug.

Resistance rates among common uropathogens to many commonly used antimicrobial agents have increased over the years and these resistance rates vary from country to country. Among the 198 *E. coli* isolates, 117 (59.1%) were

isolates were MDR-strains. Similarly 100.0% of *C. freundii*, *P. aeruginosa*, *Klebsiella oxitoca*, *Enterobacter* species were MDR.

ESBLs are paradigmatic as a mechanism of resistance because of the impact they have had on the therapy of infections and the insight they have offered on the relationship between structures and function, in antibiotics and in their determinants of resistance. Of the 198 UPEC isolates, 98 (49.5%) were shown resistance to ceftriaxone and all were subjected for phenotype confirmatory test for ESBL, combination disc method (CLSI 2013). Total 76 isolates were identified as ESBL producers. Similar result was observed in the study of Paudel, 2013 in which 67 out of 222 *E. coli* isolates were ESBL producers.

In our study, resistance to third generation cephalosporins was found to coexist with resistance to two or more antibiotics like ampicillin, nalidixic acid, ciprofloxacin, Ofloxacin, Amoxicillin/Clavulanic acid as also reported by several studies indicating multidrug resistant pattern. Mechanisms of co-resistance are not clear, but one possible mechanism is the co-transmission of ESBL and résistance to other antimicrobials within the same conjugative plasmids (Levy 1982; Levy 1998; Barber 1948; Crofton & Mitchison 1948; Bradford 2001).

Above 90% ESBL positive isolates were found to be resistant to ampicillin, quinolones, fluoroquinolones, Amoxicillin/Clavulanic acid and sensitive to imipenem, Amikacin which again advocates the usage of Amikacin and carbapenem antibiotics as the therapeutic alternative to beta-lactam antibiotics as indicated in many studies (Bradford 2001; Al-Agamy *et al*., 2006). Nitrofurantoin was considered the most effective of the drugs that can be orally administered; which represent an alternative for oral empiric therapy of uncomplicated UTI and is recommended by the Infectious Disease Society of America (IDSA). Nitrofurantoin in this study demonstrates excellent activity against UPEC isolates i.e. 68 %; both in complicated and uncomplicated UTIs and also in community acquired and hospital acquired infections. This study showed good co-resistance between nitrofurantoin and fluoroquinolones and ESBL have been noted. Moreover, the production of ESBLs has been associated with decreased susceptibility to nitrofurantoin (Carattoli 2009; Laupland *et al*., 2008;

Rodriguez-Bano). As in UPEC infection, rising rates of resistance to antimicrobial drugs limits the choice of drugs that can be used to treat infections with these potent pathogens.

Aztreonam was found 100% resistant in this study, which correlates with the study done by Sasirekha *et al*., (2010). Most of the ESBL producing organisms were found to be coresistance to flouroquinolones, aminoglycosides and co-trimoxazole, which correlates with the study done by Denholm, (2009) and Jabeen, (2005). This was due to the genes encoding β-lactamases were often located on large plasmids that also encode genes for resistance to others antibiotics, including aminoglycosides, tetracycline, sulfonamides, trimethoprime and chloramphenicol (Perez *et al*., 2007). We found such associated resistance with fluroquinolones.

In this study we used two combinations with clavulanic acid (CAZ/CAZC and CTX/CTXC) and found that *E.coli* showed maximum ESBLs production in CAZ/CAZC combination, which correlates with other studies (Rahman, 2004; Thomson *et al*., 1991).

By definition, plasmids do not carry genes essential for the growth of host cells under non-stressed conditions but most plasmids confer positively selectable phenotypes including antimicrobial resistance genes among others (Carattoli, 2011). Plasmid profiling is also an important tool for epidemiological typing and has got diagnostic value as well. In view of these results, the studies on *E. coli*, focusing on the changes on molecular level, could provide valuable insights for its management. Thirty five strains (46.1%) lacked any plasmid and in 41 strains (53.9%), 1-7 plasmids were detected. Similar results were seen in the study by Sadeghi in which 1-7 plasmids were detected in 90 strains (90%). Although three strains contained plasmids of >10kb molecular mass, but mainly our isolated plasmids ranged from 1 kb to 10 kb. In this study, we found a large number of plasmids having molecular weight of 2 kb. Clinical isolates of *E. coli* are known to harbour plasmids of different molecular size ranging from 2-3 kb to 6.5 kb and maximum 26 kb. Danbara (1987) have also reported the plasmid size between 3.9kb and 50kb in *E.coli* strains isolated from patients suffering from traveler's

diarrhea. All the plasmid containing strains were resistant strains to ampicillin (100%). Majority of the isolates (92.7%) with plasmid showed multidrug resistance.

The clinical isolates of *E. coli*, along with many others are constantly exposed to the hospital environment where they gain resistance to numerous antibiotics by various mechanisms. This drug resistance increases as a function of time and their (microorganism's) exposure to many factors (antibiotics, chemicals, etc). Besides, the bacteria acquire resistance through different routes, such as natural or intrinsic resistance (inaccessibility of the target, multidrug efflux systems and drug inactivation), mutational resistance (drug target site modification, reduced permeability or uptake, metabolic by pass and derepression of multidrug efflux), extrachromosomal or acquired resistance (drug target site modification, reduced permeability or uptake, metabolic by pass and derepression of multidrug efflux). All these mechanisms of antibiotic resistance warrant a detailed investigation of multiple factors, with prioritization of the studies of molecular characterization. Multiple drug resistance among UTI isolates in USA was reported to be 7.1% in 2000 Wayne, (2005). Such multi drug resistance has serious implications for the empiric therapy of infections caused by *E.coli* and for the possible co-selection of antimicrobial resistance mediated by multi drug resistance plasmids Salm *et al*., 2001. Thus, the studies confirm the important role of plasmid numbers and plasmid size that controls the resistance characteristics in *E. coli*.

CHAPTER VI
CONCLUSION AND RECOMMENDATIONS

6.1Conclusion

Based on the findings of this study, it is concluded that UTI affected females more than males. The main organism causing UTI is *E.coli* followed by *S. aureus*. Almost all isolates are resistant to commonly prescribed antibiotics. Therefore, antibiotics should only be commenced after performing culture sensitivity test because most of the UTI patients are treated imperically with different antibiotics.

Infection-control practitioners and clinicians need the clinical laboratory to rapidly identify and characterise different types of resistant bacteria specially ESBLs efficiently to minimise the spread of these bacteria and help to select more appropriate antibiotics.

Studies of molecular epidemiology of these resistant genes can also be used for comparison with genes already isolated from other parts of the world. Further study on the isolates at molecular level may be beneficial in ruling out the cause of ESBL pattern which may help to make a positive contribution to current understanding and knowledge of the situation in UTI caused by ESBL bacterial pathogens and for the development of better treatment strategy and prevention of the disease. In view of these results, the studies on *E. coli*, focusing on the changes on molecular level, could provide valuable insights for its management.

6.2 RECOMMENDATIONS

1. A urine specimen should be obtained for culture and susceptibility testing before institution of antimicrobial therapy.
2. Empirical antimicrobial therapy should be initiated when the clinical presentation is of sufficient severity.
3. In near future, irrational use of antibiotics must be stopped, infection with that Gram negative bacteria increase the rate of resistant to drugs that are now sensitive, resulting increase morbidity and mortality.
4. It is mandatary that all the hospitals should have the provision for detection of ESBL producing strains as a routine test because these are associated with treatment failure and death may occur in many cases
5. Further studies are required to investigate MDR bacteria and ESBL from other parts of Nepal using more isolates.
6. Plasmid Profiling should be done using fresh isolates because the plasmid DNA is lost during repeated subculture.

REFERENCES

Agrawal P, Basu B, Ghosh A, Kapil K and Kumar S (2008). Prevalence of extended spectrum, lactamases among *E. coli* and *K. pneumoniae* isolates in tertiary care hospital. *Indian journal Pathology Microbiology*. **51**: 139-142.

Al-Agamy MH, Ashour ME and Wiegand I (2006). First description of CTXM β-lactamase-producing clinical *Escherichia coli* isolates from Egypt. *Int. J. Antimicrob. Agent* . **27**: 545-548.

Ambler RP, Coulson AF, Frère JM, Ghuysen JM, Joris B, Forsman M, Levesque RC, Tiraby G and Waley SG (1991). A standard numbering scheme for the class A beta-lactamases. *Biochem J*. **276**: 269–270.

Antimicrobial Resistance (2006). Implications for the Food System. An Expert Report, Funded by the IFT Foundation. *Comprehensive Reviews in Food Science and Food Safety*. **5**: 71-137.

Balows A, Trüper HG, Dworkin M, Harder W and Schleifer KH (1992). The Prokaryotes. *Springer-Verlag KG*. **1**: 12-15

Barber M (1948). Infection by penicillin resistant Staphylococci. *Lancet*. **2**: 641–644.

Bauernfeind A, Stemplinger I, Jungwirth R, Mangold P, Amann S, Akalin E, Ang O, Bal C and Casellas JM (1996b). Characterization of beta-lactamase gene blaPER-2, which encodes an extended-spectrum class A beta-lactamase. *Antimicrob Agents Chemother.* **40**: 616-620.

Bennett PM and Hawkey PM (1991). The future contribution of transposition to antimicrobial resistance. *J Hosp Infect*. 211-221.

Bercion R, Le Faou A, Mossoro-Kpinde D and Manirakiza A (2009). Increasing prevalence of antimicrobial resistance among Enterobacteriaceae uropathogens in Bangui. *Central African Republic J Infect Developing Countries*. **3**: 187-190.

Bisson G, Fishman NO, Patel JB, Edelstein PH and Lautenbach E (2002). Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella* species. Risk factors for colonization and impact of antimicrobial formulary interventions on colonization prevalence. *Infect Control Hosp Epidemiol.* **23**: 254-260.

Bonnet R, Sampaio JLM, Chanal C, Sirot D, De Champs C, Viallard JL, Labia R and Sirot J (2000). A Novel Class A Extended-Spectrum beta -Lactamase (BES-1) in *Serratia marcescens* Isolated in Brazil. *Antimicrob Agents Chemother*. **44**: 3061-3068.

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.

Bruckner DA, Colonna P and Bearson BL (1999). Nomenclature for Aerobic and Facultative Bacteria. *Clin Infect Dis*. **29**: 713-723.

Brun-Buisson C, Legrand P and Phillippon A (1987). Transferable enzymatic resistance to third generation cephalosporins during nosocomial outbreak of multiresistant *Klebsiella pneumoniae*. *Lancet*. **2**: 302–306

Bush K and Jacoby G (1997). Nomenclature of TEM β-lactamases. *J Antimicrob Chemother*. **39**: 1-3.

Bush K, Jacoby G and Medeiros A (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother*. **39**: 1211-1233.

Carattoli A (2009). Resistance plasmid families in Enterobacteriaceae. *Antimicrob Agents Chemother*. **53**: 2227–2238.

Carattoli A (2011). Plasmids in Gram negatives: molecular typing of resistance plasmids. *Int. J.Med. Microbiol*. **301**: 654–658.

Aggarwal R and Chaudhary U (2004). Extended spectrum beta lactamases (ESBL) An emerging threat to clinical therapeutics. *Indian Journal of Medical Microbiology*. **2**: 75-80.

Cheesbrough M (2000). Medical Laboratory Manual for Tropical Countries Microbiology. 1st ELBS ed. Cambridge: University Press.

CLSI (2013). Performance Standards for Antimicrobial Susceptibility Testing, Twenty-Third Informational Supplement, CLSI Document M100-S23, Wayne, PA: Clinical and Laboratory Standards Institute.

Collee JG, Fraser AG, Marmion BP and Simmons A (1999). Mackie and McCartney Practical Medical Microbiology, 14th edition, Churchill Livingstone, New York, USA.

Crofton J and Mitchison DA (1948). Streptomycin resistance in pulmonary tuberculosis. *Br. Med. J.***2**: 1009–1015.

Danbara H, Kamase IY, Shinahawa M, Arita H, Makino A and Yashikawa M (1987). *Microbial Path*. **3**: 269-278.

Dantas G, Sommer MOA, Oluwasegun RD and Church GM (2008). Bacteria Subsisting on Antibiotics. *Science*. **320**: 100-103.

Datta N and Kontomichalou P (1965). Penicillinase Synthesis Controlled By Infectious R Factors In Enterobacteriaceae. *Nature*. **208**: 239-241.

Denholm JT, Huysmans M and Spelman D (2009). Community acquisition of ESBL-producing *Escherichia coli*: a growing concern. *Medical Journal Australia*. **190**: 45-46.

Devanand P and Saxena RS (2013). Prevalence and distribution of gram negative bacteria of Enterobacteriaceae causing Urinary tract infections among hospitalized patients. *J Acad Indus Res*. **1**: 650-654.

Angrick EJ, Dorn CR and Silapanuntakul R (1992). Plasmid analysis and epidemiology of *Salmonella* enteritidis infection in three commercial layer flocks. *Avian Dis*. **36**: 844-851.

Drekonja DM and Johnson JR (2008). Urinary tract infections. *Prim Care*. **35**: 345–367.

Durgesh D, Wasnik and Tumane PM (2012). Prevalence and antibacterial susceptibility pattern of Urinary Tract Infection Causing Human Pathogenic Bacteria. *Asian Journal Of Biomedical And Pharmaceutical Sciences*. 1-3.

Eisenstein BI and Zalenik DF (2000). Enterobacteriaceae. Principles and Practice of Infectious Diseases. **5**: 133-136

Farkosh MS (2007). Extended-Spectrum beta lactamase Producing Gram Negative Bacilli. **2**: 1-5

Fluit AC and Schmitz FJ (1999). Class 1 Integrons, Gene Cassettes, Mobility, and Epidemiology. *Eur J Clin Microbiol Infect Dis*. **18**: 761-770.

Forbes BA, Sahm DF and Weissfeld AS (2002). Bailey and Scott's Diagnostic Microbiology, 11th edition. Mosby, Inc. USA.

Fornasini M, Murray BE and Reeves RR (1992). Trimethoprim resistant *Escherichia coli* in households of children attending day care centers. *J Infect Dis*. **166**: 326-330.

Foxman B (2002). Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *Am J Med*. **113**: Suppl 5S-13S.

Fridkin SK, Steward CD, Edwards JR, Pryor ER, McGowan JEJ, Archibald LK, Gaynes RP and Tenover FC (1999). Surveillance of antimicrobial use and antimicrobial resistance in United States hospitals. *Clin Infect Dis*. **29**: 245-252.

Friedman C, Callery S, Jeanes A, Piaskowski P and Scott L (2005). Best Infection Control Practices for Patients with Extended Spectrum Beta Lactamase Enterobacteriacae, *International Infection Control Council*. **23**: 155-167

Garau G, Di Guilmi AM and Hall BG (2005b). Structure-Based Phylogeny of the Metallo-β-Lactamases. *Antimicrob Agents Chemother*. **49**: 2778-2784.

Gautam P, Jha B and Manandhar SP (2002). Prevalence of Urinary tract infection in diabetic patients. A Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal.

Gerald J, Andrew G, Anthony S and Barrie PM (2006). Mackie and macCartney. Practical Medical Microbiology. 14th edition. Churchill livingstone. 367-379.

Getachew T (2010). Bacterial pathogens implicated in causing urinary tract infection (uti) and their antimicrobial susceptibility pattern in Ethiopia Revista CENIC. *Centro Nacional de Investigaciones Científicas Cuba*. **41**: 1-6.

Guarner F and Malagelada JR (2003). Gut flora in health and disease. *The Lancet*. **361**: 512-519.

Hall BG and Barlow M (2004). Evolution of the serine β-lactamases: past, present and future. *Drug Resistance Update*. **7**: 111-123.

Hall BG, and Barlow M (2005). Revised Ambler classification of β-lactamases. *J Antimicrob Chemother*. **55**: 1050-1051.

Hall RM and Collis CM (1995). Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol Microbiol*. **15**: 593-600.

Hanson ND (2003). AmpC β-lactamases: what do we need to know for the future? *J Antimicrob Chemother*. **52**: 2-4.

Hassan SH (1985). Sensitivity of *Salmonella* and *Shigella* to antibiotics and chemotherapeutic agents in Sudan. *J Trop Med Hyg*. **88**: 243 – 248.

Hawkey PM (2008). Prevalence and clonality of extended-spectrum β-lactamases in Asia. *Clinical Microbiology and Infection*. **14**: 159-165.

Helfand MS and Bonomo RA (2005). Extended spectrum β-lactamases in Multidrug Resistant *Escherichia coli*: Changing the therapy for Hospital-Acquired and Community Acquired Infections. *Oxford Journals*. **43**: 1415-1416.

Herzog LW (1989). Urinary tract infections and circumcision. *Am J Dis Child*. **143**: 348.

Humeniuk CG, Arlet, Gautier V, Grimont P, Labia R and Philippon A (2002). β-Lactamases of *Kluyvera ascorbata*, Probable Progenitors of Some Plasmid-Encoded CTX-M Types. *Antimicrob Agents Chemother*. **46**: 3045-3049.

Jabeen K, Zafar A and Hasan R (2005). Frequency and sensitivity pattern of extended spectrum beta lactamase producing isolates in a tertiary care hospital laboratory of Pakistan. *Journal Pakistan Medical Association*. **55**: 436-439.

Jacobs C, Frère JM and Normark S (1997). Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in gram-negative bacteria. *Cell (Cambridge, Mass).* **88**: 823-832.

Janda MJ and Abbott SL (1998). The Enterobacteria. Lippincott-Raven Publishers, Philadelphia Jaurin, B. and T. Grundstrom (1981). ampC Cephalosporinase of *Escherichia coli* K-12 Has a Different Evolutionary Origin from That of β-lactamases of the Penicillinase Type. *Proceedings of the National Academy of Sciences*. **78**: 4897-4901.

Jha VC and Yadav JN (1992). Bacterial species isolated from urine of UTI patients and their sensitivity to commonly available antibiotic. *J Nep Med Assoc*. **30**: 222-225

Kass EH (1960). Bacteriuria and pyelonephritis of pregnancy. *Arch Intern Med.* **105**: 194-198.

Knothe H, Shah P and Kremery V (1983). Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. *Infection*. **11**: 315–317

Kolawole AS, Kolawole OM, Kandaki-Olukemi YT, Babatunde SK, Durowade KA and Kolawole CF (2009). Prevalence of urinary tract infections (UTI) among patients attending Dalhatu Araf Specialist Hospital. *International Journal of Medicine and Medical Sciences*. **1**: 163-167.

Krieg NR and Holt JG (2005). Facultatively anaerobic gram-negative rods. Family I. Enterobacteriaceae. Bergey's Manual of Systematic Bacteriology. Baltimore, Williams & Wilkins. **1**: 408–420.

Kunin CM (1997). Care of urinary catheter urinary tract infections. Detection, prevention and management, 5thedition. Baltimore: Williams and Wilkins. 227-299.

Lal P, Kapil A, Das BK and Sood S (2007). Occurence of TEM and SHV gene in extended spectrum beta lactamases (ESBLs) producing *Klebsiella* spp. isolated from a tertiary care hospital. *Indian Journal Medica*. **125**: 173-178.

Laupland KB, Church DL, Vidakovich J, Mucenski M and Pitout JD (2008). Community-onset extended-spectrum β-lactamase (ESBL) producing *Escherichia coli*: importance of international travel. *J Infect*. **57**: 441–448.

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**: 1162-71.

Levy SB (1982). Microbial resistance to antibiotics. An evolving and persistent problem. *Lancet.* **2**: 83–88.

Levy SB (1998). The challenge of antibiotic resistance. *Sci. Am*. **278:** 46–53.

Livermore DM (1995). β-Lactamases in Laboratory and Clinical Resistance. *Clin Microbiol Rev*. **8**: 557-584.

Livermore DM (2008). Defining an extended-spectrum β-lactamase. *Clin Microbiol Infect*. **14**: 3-10.

Livermore DM and Paterson DL (2006). Pocket guide to Extended-Spectrum β-Lactamases in Resistance. Current Medicine Group Ltd, London. **4**: 15-27

Madigan MT and Martinko JM (2006). Biology of the Microorganims. Pearson Prentice Hall, Upper Saddle River.

Mahmood MA (2011). Department of Basic Sciences, College of Dentistry, Baghdad University, Baghdad-Iraq. **4**: 66-68

Mahillon J and Chandler M (1998). Insertion Sequences. *Microbiol Mol Biol Rev*. **62**: 725-774.

Maslow JN, Slutsky AM and Arbeit RD (1993). Application of Pulsed-Field Gel Electrophoresis to molecular epidemiology. In: Diagnostic Molecular Microbiology: Principles and Applications. **44**: 183-198

Matsumoto Y and Inoue M (1999). Characterization of SFO-1, a Plasmid-Mediated Inducible Class A beta -Lactamase from *Enterobacter cloacae*. *Antimicrob Agents Chemother*. **43**: 307-313.

Matsumoto Y, Ikeda F, Kamimura T, Yokota Y and Mine Y (1988). Novel plasmid-mediated beta-lactamase from *Escherichia coli* that inactivates oxyimino-cephalosporins. *Antimicrob Agents Chemother*. **32** :1243-1246.

Medeiros AA (1997). Evolution and dissemination of β-lactamases accelerated by generations of beta-lactam antibiotics. *Clin Infect DisSupplemental* **1**: 19-45.

Moosdeen F, Williams JD and Yamabe S (1988). Antibacterial characteristics of YTR 830, a sulfone beta-lactamase inhibitor, compared with those of clavulanic acid and sulbactam. *Antimicrob Agents Chemother.* **32**: 925-927.

Naas T, Poirel L and Nordmann P (2008). Minor extended-spectrum β-lactamases. *Clin Microbiol Infect*. **14**: 42-52.

Pagani L, Amico ED, Migliavacca R, DíAndrea MM, Giacobone E, Amicosante G, Romero E and Rossolini GM (2003). Multiple CTX-M-Type Extended-Spectrum, β-Lactamases in Nosocomial Isolates of Enterobacteriaceae from a Hospital in Northern Italy. *Journal of Clinical Microbiology*. **41**: 4264-4269.

Paterson DL and Yu VL (1999). Extended spectrum beta lactamases: a call for improved detection and control. *Clin Infect Dis*. **29**: 1419-1422.

Pena C, Gudiol C, Tubau F, Saballs M, Pujol M, Dominguez MA, Calatayud L, Ariza J and Gudiol F (2006). Risk-factors for acquisition of extended-spectrum beta-lactamase producing *Escherichia coli* among hospitalised patients. *Clin Microbiol Infect.* **12**: 279-284.

Perez F, Endimiani A, Hujer KM and Bonomo RA (2007). The continuing challenge of ESBLs. *Current Opinion in Pharmacology*. **7**: 459-469.

Persing DH, Smith TF, Tenover FC and White TJ (Ed.) Washington, D.C. *ASM Press*. **5**: 63-572.

Pfaller MA, Ehrhardt AF and Jones RN (2001). Frequency of pathogen occurrence and antimicrobial susceptibility among community-acquired respiratory tract infections in the respiratory surveillance program study: Microbiology from the medical office practice environment. *Am J Med*. **111**: 4S-12S.

Philippon A, Arlet G and Jacoby GA (2002). Plasmid-Determined AmpC-Type β-Lactamases. *Antimicrob Agents Chemother*. **46**: 1-11.

Pitout JDD, Gregson DB, Church DL, Elsayed S and Laupland KB (2005a). Community-Wide Outbreaks of Clonally Related CTX-M-14 Beta-Lactamase-Producing *Escherichia coli* Strains in the Calgary Health Region. *J Clin Microbiol*. **43**: 2844-2849.

Pitout JDD, Sanders C and Snaders WE Jr (1997). Antimicrobial resistance with focus on beta-lactam resistance in gram-negative bacilli. *Am J Med* **103**: 51–59

Pitton JS (1972). Mechanism of bacterial resistance to antibiotics. *Rev Physiol Biochem Pharmacol*. **65**: 15-93.

Poudel S (2013). Status of extended spectrum beta lactamase producing Enterobacteriaceae among bacterial uropathogens. Central Department Of Microbiology, TU, Kirtipur, Nepal.

Queenan AM and Bush K (2007). Carbapenemases: the Versatile β-Lactamases. *Clin Microbiol Rev*. **20**: 440-458.

Rahman MM, Haque JA, Hossain MA, Sultana R, Islam F and Islam S (2004). Prevalence of extended spectrum beta lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in an urban hospital in Dhaka Bangladesh. *International journal of antimicrobial agents*. **24**: 508-510.

Rahal JJ, Urban C, Horn D and Freeman K (1998). Class restriction of cepha-losporin use to control total cephalosporin resistance in nosocomial *Klebsiella*. *JAMA*. **280**: 1233–1237

Rasmussen BA and Bush K (1997). Carbapenem-hydrolyzing beta-lactamases. *Antimicrob Agents Chemother*. **41**: 223-232.

Richmond MH and Sykes RB (1973). The β-lactamases of gram-negative bacteria and their possible physiological role. *Adv Microb Physiol*. **9**: 31-88.

Roberts JA (1991). Etiology and pathophysiology of pyelonephritis. *Am J Kidney Dis*. **17**: 1–9.

Rodriguez-Bano J and Navarro MD (2008). Extended-spectrum β-lactamases in ambulatory care: a clinical perspective. *Clin Microbiol Infect*. **14**:104–110.

Roy PH (1995). Integrons: novel mobile genetic elements mediating antibiotic resistance in Enterobacteria and *Pseudomonas*. *APUA Newsletter*. **13**: 4-6.

Sader HS, Pfaller MA and Hollis RJ (1995). The use of molecular techniques in the epidemiology and control of hospital infectious. *Clin Lab Med*. **15**: 407-431.

Sadowski PL, Peterson BC, Gerding DN and Cleary PP (1979). Physical characterization of ten R plasmids obtained from an outbreak of nosocomial *Klebsiella pneumoniae* infections. *Antimicrob. Agents Chemother*. **15**: 616-624.

Salm DF, Thornsbery C, May Field DC, Jones ME and Karlowsky JA (2001). *Antimicro. Agent chemother*. **41**: 15-22.

Sambrook J and Russell DW (2001). Molecular cloning. A Laboratory Manual. 3rd edition. CSHL press.

Sanders CC (1987). Chromosomal Cephalosporinases Responsible for Multiple Resistance to Newer β-Lactam Antibiotics. *Annu Rev Microbiol*. **41**: 573-594.

Sasirekha B, Manasa R, Ramya P and Sneha R (2010). Frequency and Antimicrobial Sensitivity Pattern Of Extended Spectrum β-Lactamases Producing *E. coli* and *Klebsiella pneumoniae* Isolated In A Tertiary Care Hospital. *Al Ameen journal Medical science*. **3**: 265-271.

Schaeffer JA (1990). Infection of the urinary tract. In: Walsh, Retike, Vaghen Wein. Cambell's Urology, 7th edition Philadelphia. **1**: 534-611.

Schiappa DA, Hayden MK, Matushek MG, Hashemi FN, Sullivan J, Smith KY, Miyashiro D, Quinn JP, R. Weinstein RA and Trenholme GM (1996). Ceftazidime-resistant *Klebsiella pneumoniae* and *Escherichia coli* bloodstream infection: a case-control and molecular epidemiologic investigation. *J Infect Dis*. **174**: 529-36.

Schmidtke AJ and Hanson ND (2006). Model System To Evaluate the Effect of ampD Mutations on AmpC-Mediated β-Lactam Resistance. *Antimicrob Agents Chemother*. **50**: 2030-2037.

Sharma J, Sharma M and Roy P (2010). Detection of TEM and SHV genes in *Escherichia coli* and *Klebsiella pneumoniae* isolates in a tertiary care hospital from India. *Indian J Med*. **132**: 332-336.

Shobha KL, Gowrish RS, Sreeja CK and Sugandhi R (2007). Prevalence of ExtendedSpectrum β- Lactamases in Urinary Isolates of *Escherichia coli*, *Klebsiella* and *Citrobacter* Species and their Antimicrobial Susceptibility Pattern in tertiary care hospital. *Indian journal for the Practicing Doctor*. **3**: 1-2.

Silva J, Aguilar C, Ayala G, Estrada MA, Garza-Ramos U, Lara-Lemus R and Ledezma L (2000). TLA-1: a New Plasmid-Mediated Extended-Spectrum beta -Lactamase from *Escherichia coli*. *Antimicrob Agents Chemother*. **44**: 997-1003.

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

Stamm WE and Norrby SR (2001). Urinary Tract Infections: Disease Panorama and Challenges. *J Infect Dis*. **183**: 1-4.

Steadman R and Topley N (1998). The virulence of *Escherichia coli* in Urinary tract. Chapter 3. In: Brumfit W, Jercmy MT, Hamilteon Miller Eds. Urinary tract infections. 1st ed. Chapman and Hall Publication, London. 37-41.

Tanagho and McAninch (2004). Smith's General Urology 16th edition., eds. Chapter 13. Bacterial Infections of the Urinary Tract. *Nguyen, Hiep*. 203-227.

Tenover FC, Mohammed MJ, Gorton TS and Dembek ZF (1999). Detection and reporting of organisms producing extended spectrum beta-lactamases: survey of laboratories in Connecticut. *Journal Clinical Microbiology*. **37**: 4065-4070.

Thomas AC (1978). Mycoplasma in human pyelonephritis demonstration of antibodies in serum and urine. *J Clin Microbiol*. **8**: 197.

Thomson KS, Sanders CC and Washington JA (1991). High-level resistance to cefotaxime and ceftazidime in *Klebsiella pneumoniae* isolates from C level and, Ohio. *Antimicrob Agents Chemother*. **35**: 1001-1003.

Thomson KS (2001). Controversies about extended-spectrum and AmpC β-lactamases. *Emerg Infect Dis*. **7**: 333-6.

Walther-Rasmussen J and Hoiby N (2004). Cefotaximases (CTX-M-ases), an expanding family of extended-spectrum β-lactamases. *Can J Microbiol*. **50**: 137-165.

Waschmut IK, Griffin PM and Wells JG (1991). *Escherichia coli* O157:H7, a cause of hemorrhagic colitis and hemolytic uremic syndrome. *Acta Paediatr Jpn*. **33**: 603-612.

Watanabe T (1963). Infective heredity of multidrug resistance in bacteria. *Bacteriol Rev*. **27**: 87–115.

Wayne PA, Coque TM, Baquero F and Canton R (2008). Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. *Euro Surveill.* **13**: 1-11.

Weldhagen GF (2004). Integrons and β-lactamases-a novel perspective on resistance. *Int J Antimicrob Agents*. **23**: 556-562.

Wilke MS, Lovering AL and Strynadka NCJ (2005). β-Lactam antibiotic resistance: a current structural perspective. *Curr Opin Microbiol*. **8**: 525-533.

Xu L, Ensor V, Gossain S, Nye K and Hawkey P (2005). Rapid and simple detection of CTX-M genes by multiplex PCR assay. *Journal of Medical Microbiology*. **54**: 1183-1187.

Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CW, Alberti S, Bush K and Tenover FC (2001). Novel Carbapenem-Hydrolyzing β-Lactamase, KPC-1, from a Carbapenem-Resistant Strain of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother*. **45**: 1151-1161.

Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CW, Alberti S, Bush K and Tenover FC (2008). Novel Carbapenem-Hydrolyzing β-Lactamase, KPC-1, from a Carbapenem-Resistant Strain of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother*. **52**: 809-812.