CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

Urinary tract infection (UTI) is one of the most prevalent extra-intestinal bacterial infections which represents one of the most common disease encountered in medical practice among both community and hospitalized patients of all ages from neonate to the geriatric age group (Manikandan *et al*., 2011, Mekki *et al*., 2010). Worldwide, about 150 million people are diagnosed with UTI each year (Gupta *et al*., 2001) and it has been estimated that nearly 10% of human population will experience a UTI during life time (Ena *et al*., 2006). Infected urine stimulates an immunological and inflammatory response leading to renal injury and scarring, ultimately leading to end stage renal failure. Renal calculi, obstructive uropathy (posterior urethral valves), vesiculo-urethral reflux and voiding disorders can lead to urinary stasis and may predispose to the development of recurrent UTI and complications (Ramesh *et al*., 2008). More than 95% of UTI cases are caused by bacteria, among which *E. coli* is the most prevalent causative organism, which is responsible for more than 80% of infections followed by *Klebsiella* spp. (Ramesh *et al*., 2008). Other Gram negative bacteria include *Enterobacter* spp., *Pseudomonas aeruginosa, Proteus* spp., *Citrobacter* spp., *Morganella morganii*, etc. Gram positive bacteria account for 5 to 15% of UTIs and include *Enterococcus* spp., *Staphylococci,* and *Streptococci* (Akram *et al.,* 2007). Majority of UTIs are not life threatening and donot cause any irreversible damage. However, when the kidneys are involved, there is a risk of irreparable tissue damage with an increased risk of bacteremia (Manikandan *et al.,* 2011).

Drug resistance is a huge problem in treating infectious diseases like malaria, TB, diarrhoeal diseases, UTI, etc. nowadays. Drug resistance of pathogen has become a serious medical problem because of very fast arise and spread of mutant strains that are insusceptible to medical treatment. Microorganism follow varied

mechanisms to acquire drug resistance viz. horizontal gene transfer (plasmid, transposons and bacteriophage), recombination of foreign DNA in bacterial chromosome and mutations in different chromosomal locus (Klemm *et al.,* 2006). In last three decades, there have been a lot of reports on the inappropriate use of antimicrobial agent and spread of bacterial resistance among microorganisms causing UTI (Kurutepe *et al*., 2005). The changing patterns in the etiological agents of urinary tract pathogen and their sensitivities to commonly prescribed antibiotics are reported (Manikandan *et al*., 2011).

A common mechanism of bacterial resistance to beta-lactam antibiotics is the production of beta-lactamase enzymes that breakdown the beta-lactam ring of penicillin like drugs (Mirza *et al.,* 2006). The increasing use of broad spectrum cephalosporin has become one of the major factors responsible for high rate of ESBL producing microorganisms (Paterson *et al*., 2005). The production of extended-spectrum beta-lactamases (ESBLs) is an important mechanism which is responsible for the resistance to the third-generation cephalosporins. These beta-lactamases generally belongs to Ambler's molecular class A and Bush's functional class 2Be (Bush *et al.*, 1995). ESBL are plasmid mediated TEM and SHV derived enzymes which confer antibiotics resistance on certain bacteria in the family *Enterobacteriaceae* (Yan *et al.,* 2000). In recent years, there has been an increase in incidence and prevalence of ESBL producing microbial diseases (Thokar *et al.,* 2010). They are plasmid mediated and get transferred to genera of other enteric bacilli including *Proteus mirabilis, Citrobacter and Serretia* (Thomson *et al*., 1994). Global prevalence of ESBL producing bacteria varies from <1% to 74% (Phillipon *et al.,* 1989). The prevalence of multidrug resistance bacterial uropathogens is high in Nepal which is about 41.1% (Baral *et al.,* 2012) and 16% of bacterial isolates from urine were ESBL producers (Pokhrel *et al*., 2006), *E. coli* and *Klebsiella* being the most commonly isolated pathogens with high level of MDR and production of beta-lactamases. Initially the beta-lactamases produced were active against only a few beta-lactams but over the years microorganisms have learnt to elaborate newer beta-lactamases with extended substrate profile and such important group of enzymes are active against

virtually all beta-lactams except the carbapenems (Forbes *et al*., 2007). Infections due to ESBL producer range from uncomplicated urinary infections to life threatening sepsis (Rawat and Nair, 2010).

It has become very important to study the prevalence of ESBL-producing organisms because of the increasing antimicrobial resistance and decreasing number of new drugs available against such microbes (Kader *et al*, 2006). Since the initial description of ESBL, microbial isolates that are resistant to broad spectrum cephalosporins are being increasingly recognized (Itokazu *et al*., 1996).

The problems of antimicrobial resistance may be due to the fact that antibiotics can be obtained and used without medical authorization or supervision in developing countries (Pokhrel *et al*., 2006) such as Nepal. This situation has led to inappropriate usage of antibiotics with patients taking the drugs for insufficient length of time or at sub optimal dosages, which may result in antimicrobial resistance.

The presence of ESBL-producing organism in a clinical infection can cause significant treatment problems because ESBL-mediated resistance may result in treatment failure if any of the third generation cephalosporins (eg. Ceftazidime, cefotaxime and ceftriaxone) or a monobactam (aztreonam) are used (Drieux *et al*., 2008). If an ESBL producer is detected, it should always be reported as resistant to penicillins, cephalosporins and monobactams even if in vitro test results indicate susceptibility, since these may fail in treatment (CLSI, 2007; Paterson *et al.*, 2005). It is worth noting that the therapeutic options for infections caused by ESBL-producing organisms are significantly limited because the organisms are frequently resistant to other non-beta-lactam drugs such as aminoglycosides, quinolones, trimethoprim-sulfamethoxazole and tetracyclines (Pitout *et al*., 2008). This is because the plasmids bearing the genes which encode for ESBLs frequently also carry genes encoding for resistance to these classes of antibiotics (Paterson *et al*., 2005). This is making therapy of UTI difficult and promoting greater use of expensive broad spectrum antibiotics, such as carbapenems (Ejaz *et al.*, 2011).

The delay in detection and reporting ESBL-producer may lead to prolonged hospitalization of patients, increased morbidity and mortality as well as increased cost of health care (Lautenbach *et al*., 2001) and hence can be considered an economic burden to society more in context of developing country like Nepal. Thus the detection of ESBL-producers aid physician in selection of antibiotics for patients use and also helps in the planning of strategies to control spread of ESBL-producers.

The present study was conducted with an objective to find out the presence of ESBL producing *Enterobacteriaceae* and multidrug resistant strains to formulate effective antibiotic strategy to control infection and to prevent the spread of these strains.

1.2 Objectives

1.2.1 General objective

To determine the status of extended spectrum beta-lactamase producing *Enterobacteriaceae* among bacterial uropathogens.

1.2.2 Specific objectives

a) To identify the bacterial isolates causing UTI.
b) To assess the antibiotic susceptibility pattern of *Enterobacteriaceae*.
c) To determine if resistance to any of third generation cephalosporins among *Enterobacteriaceae* is due to ESBL production.
d) To compare the disk diffusion methods i.e. combination disk method and DDST for the detection of ESBLs.

CHAPTER II
LITERATURE REVIEW

2.1 Urinary Tract infection

UTI is stated as a spectrum of diseases involving microbial invasion of any of the urinary tissues extending from the renal cortex to the urethral meatus (Dhakal *et al*., 2002). Urine secreted from kidneys is sterile unless any of the organs are infected (Leigh, 1990). Since the urinary tract is open to the external environment, it is easy for pathogens to gain entry and establish infection. To prevent this, there are many innate defenses; for example, under normal circumstances, bacteria placed in the bladder are rapidly cleared, partly through the flushing and dilutional effects of voiding but also as a result of the antibacterial properties of urine and the bladder mucosa (Braunwald *et al.,* 2001). The low pH and osmolarity of urine has a bacteriostatic effect and the sphincters at the bladder end of the ureters prevent back-flow of urine into the kidneys (Mulvey *et al*., 2001). These defense mechanisms can be breached by different pathogenic microorganisms to cause urinary tract infections (UTIs), including cystitis (infection of bladder) and pyelonephritis (infection of kidney) (Braunwald *et al.,* 2001).

Infection of the urinary tract is identified by growth of a significant number of organisms of a single species in the urine, in the presence of symptoms (Kumar and Clark, 1996). Generally growth of $\geq 10^5$ CFU/ml from appropriately and aseptically collected mid-stream clean catch urine sample indicates UTI and this presence of bacteria in urine is called as bacteriuria (Pokhrel *et al*., 2006). A descending UTI occurs as a consequence of bacteremia. Blood-borne bacteria enter the kidneys through the glomerular filters, an event more likely in neonates due to immaturity of the filters. An ascending UTI occurs due to the entry of the bacteria in the vicinity of the urethral opening into the urinary tract, primarily due to fecal contamination. If left untreated, the pathogens may ascend the ureters into the kidneys establishing acute pyelonephritis. Females are vulnerable to ascending UTI in comparison to males due to their shorter urethra (Tabibian *et al.*, 2008).

During infection, bacteria undergo multiplication in urine within the urinary tract causing a condition called bacteriuria (Dhakal *et al*., 2002). They affect a variety of patients ranging from young children to the elderly and from healthy men and women to the compromised. UTI can be restricted to the bladder (essentially in females) with only superficial mucosal involvement, or it can involve a solid organ (the kidneys in both genders, the prostate in males) (Roopa and Sudha, 2010). Urinary tract infection (UTI) is one of the most important causes of morbidity in the general population, and is the second most common cause of hospital visits (Das *et al*., 2006).

2.1.1 Clinical entities of UTI

Urinary tract infection is a broad term that encompasses different clinical entities which include:

a) Asymptomatic bacteriuria: Significant bacteriuria without symptoms (Forbes *et al.,* 2007).

b) Uncomplicated urinary tract infection: Infection in a patient with a normal, unobstructed genitourinary tract with no prior instrumentation (Forbes *et al.,* 2007).

c) Complicated urinary tract infection: Infection in a patient with structural or functional abnormalities. This also includes men, pregnant women, presence of foreign body (urinary catheter, stone) (Forbes *et al.,* 2007).

d) Relapse: Recurrence of bacteriuria with the same microorganism within seven days of therapy and implies failure to eradicate infection (Forbes *et al.,* 2007).

e) Reinfection: Recurrence of bacteriuria with a new microorganism. Reinfection is difficult to differentiate from relapse when infection occurs with a microorganism of the same species as the initial infection. Approximately 80% of recurrent infections are due to reinfection (Forbes *et al.,* 2007).

f) Cystitis: Inflammatory syndrome and infection of the bladder with signs and symptoms of dysuria, frequency, urgency, and suprapubic tenderness.

g) Pyelonephritis: Bacterial infection of the kidney involving flank pain, tenderness, and fever, and often associated with dysuria, urgency, and frequency. This condition may be acute or chronic (Forbes *et al.,* 2007).

h) Urethritis: Lower urinary tract inflammation with / without bacterial infection, causing symptoms similar to those of cystitis. Most often associated with sexually transmitted diseases such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Forbes *et al.,* 2007).

i) Prostatitis: Encompasses several different clinical entities, from bacterial infection to inflammation to pain, which cause symptoms related to the prostate gland (Forbes *et al.,* 2007).

2.1.2 Symptoms and sign of UTI

The most typical symptoms of UTI are: Frequency of micturition by day and night, painful voiding, suprapubic pain and tenderness, hematuria (blood in urine), smelly urine, etc. (Kumar and Clark, 1996).

2.1.3 Factors increasing the risk of UTI

UTIs are more common in individuals with a history of UTI, incontinence, neurologic or cognitive impairment, poor nutrition, immunosuppression, or other comorbid disease states and in individuals who are frequently hospitalized, live in a nursing home or other institutionalized long term care facilities (Ficarnis *et al*., 2010). Factors increasing the risk of infection included over-distention of the bladder, vesicoureteric reflux, high pressure voiding, large post-void residuals and stones of the urinary tract (Salomon *et al.,* 2006).

2.1.4 Etiologic agents of UTI

The etiological agents of community- acquired and hospital acquired UTIs are different. Many different organisms can infect the urinary tract, the most common agents are the gram-negative bacilli mainly the family *Enterobacteriaceae* (Braunwald *et al.,* 2001). *E. coli* is the primary cause of uncomplicated infections of the urinary tract including cystitis (Gunther *et al.,* 2001). According to an International survey of the antimicrobial susceptibility of pathogens from uncomplicated UTIs, *E. coli* accounts for 77.0% of isolates (Kahlmeter, 2000). However, there is some evidence that the percentage of UTIs caused by *E. coli* is decreasing, being replaced by other members of the *Enterobacteriaceae*

(Haryniewicz *et al.*, 2001). On the other hand, another literature by Braunwald *et al*. (2001) indicated that other gram-negative rods, especially *Proteus* and *Klebsiella* and occasionally *Enterobacter,* account for a smaller proportion of uncomplicated infections. These organisms, plus *Serratia* and *Pseudomonas*, assume increasing importance in recurrent infections, associated with urologic manipulation, calculi, or obstruction (Braunwald *et al*., 2001). *Salmonella* Typhi and *Salmonella* Paratyphi can be found in the urine of about 25% of patients with enteric fever from the third week of infection (Cheesbrough, 2000).

Gram- positive cocci were isolated more frequently from a hospital setting and the most common were *Enterococcus* species (Haryniewicz *et al*., 2001). *Staphylococcus saprophyticus* - novobiocin-resistant, coagulase-negative species- accounts for 10 to 15% of acute symptomatic UTIs in young females. More commonly, *Enterococci* and *Staphylococcus aureus* cause infections in patients with renal stones or previous instrumentation or surgery. Isolation of *S. aureus* from the urine should arouse suspicion of bacteremic infection of the kidney (Braunwald *et al*., 2001). Rarer infecting organisms include *Streptococcus agalactiae*, *Streptococcus milleri,* other *Streptococci* and *Gardnerella vaginalis* (Tabibian *et al.,* 2008).

2.1.4.1 *Enterobacteriaceae*

Members of the bacterial family *Enterobacteriaceae* are found in the environment but also make up part of the normal microbiota of the intestine in humans and other animals (Farmer *et al.,* 2007). They are rod shaped, gram negative, non-sporulating, facultative anaerobes that ferment different carbohydrates to obtain carbon, reduce nitrate to nitrite, and produce catalase but seldom oxidase (Cheesbrough, 2000). They may grow as mucoid colonies when cultivated on agar plates, but only *Klebsiella* spp. are truly capsulated (Abbot *et al*., 2007). *Enterobacteriaceae* can be divided into 22 genera, 69 species, and 29 biogroups or Enteric Groups (Farmer *et al.*, 1985).

Most of the *Enterobacteriaceae* can cause many different kinds of infections. Urinary tract infections (UTIs) are the most common, followed by pneumonia, wound infections, infections of bloodstream and central nervous system. Some genera are common causes of intestinal infections such as enteritis and diarrhea. They also make up an essential part of nosocomial infections, especially catheter related UTIs and ventilator associated pneumonia (Abbot *et al*., 2007, Donnenberg, 2005; Farmer *et al.,* 2007).

Table 1. Clinically important members of the family *Enterobacteriaceae* commonly causing infections (Abbot *et al*., 2007, Donnenberg, 2005; Farmer *et al.,* 2007).

Genus	Clinically important species	Common type of infections
Citrobacter	*C. freundii*	UTIs, pneumonia, meningitis, septicemia
Enterobacter	*E. aerogenes, E. cloacae*	UTIs, pneumonia, septicemia, wound infections
Escherichia	*E. coli*	UTIs, diarrhoea, septicemia, meningitis
Klebsiella	*K. pneumoniae, K. oxytoca*	UTIs, pneumonia, septicemia
Morganella	*M. morganii*	UTIs, septicemia
Plesiomonas	*P. shigelloides*	Diarrhoea, septicemia
Proteus	*P. mirabilis, P. vulgaris*	UTIs, pneumonia, septicemia, meningitis
Providencia	*P. rettgeri, P. stuartii*	UTIs
Salmonella	*S. enteritica*	Diarrhoea, typhoid fever, UTIs, osteomyelitis
Serretia	*S.marcescens, S.liquefaciens*	UTIs, pneumonia, septicemia
Shigella	*S. sonnei, S. flexneri*	Diarrhoea, dysentery
Yersinia	*Y. pestis, Y. enterocolitica*	Plaque, enteritis, diarrhoea, septicemia

2.2 Beta-lactam antibiotics

The beta-lactam antibiotics can be divided into six different groups, the penicillins, cephalosporins, carbapenems, cephamycins, monobactams, and beta-lactamase inhibitors (Smet *et al.,* 2008). Beta-lactam antibiotics contain a beta-lactam ring which is a heteroatomic ring structure consisting of three carbon atoms and one nitrogen atom. The beta-lactam ring of natural or semi-synthetic penicillins is fused with a thiazolidine ring. In cephalosporins, the beta-lactam ring is merged with a dihydrothiazine ring. In the carbapenems, the beta-lactam ring is combined with a hydroxyethyl side chain, lacking an oxygen or sulphur atom in the bicyclic nucleus. In contrast to the antibiotics, clavulanic acid, a beta-

lactamase inhibitor, is composed of a beta-lactam ring fused with an oxazolidine ring and does not possessed an amide function (Livermore, 1995; Amyes *et al.*, 2010).

In general, modifications of the R and R' groups alter the pharmacokinetic and antibacterial properties of beta-lactam antibiotics; for example, substitutions at position 7 of cephalosporins increase the penetration into the periplasmatic space and the stability against beta-lactamases, but may reduce antibiotic activity (Donowitz *et al.*, 1988).

The third generation of cephalosporins (also known as oxyimino-beta-lactams) included compounds such as cefotaxime, ceftazidime and ceftriaxone, which offered extended coverage of Gram-negative bacteria and even better beta-lactamase stability. These cephalosporins were developed because of the discovery of narrow-spectrum beta-lactamases (e.g., TEM-1) and some of them also had good oral bioavailability, as exemplified by ceftibuten, which could be used for oral treatment of pyelonephritis. The cephamycins are another group of antibiotics that were developed in the 1970s. These agents proved to have the same antimicrobial effect as the second- generation cephalosporins but were stable against class A ESBLs (Onishi *et al.*, 1974).

2.2.1 Mechanism of action of beta-lactam antibiotics

Beta-lactam antibiotics exhibit their bactericidal effects by inhibiting enzymes involved in cell wall synthesis. The integrity of the bacterial cell wall is essential to maintaining cell shape in a hypertonic and hostile environment (Massova *et al.*, 1998). Osmotic stability is preserved by a rigid cell wall comprised of alternating *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) units. These glycosidic units are linked by transglycosidases. A pentapeptide is attached to each NAM unit, and the cross-linking of two d-alanine-d-alanine NAM pentapeptides is catalyzed by PBPs, which act as transpeptidases (Goffin and Ghuysen, 1998; Sauvage *et al.*, 2008). This cross-linking of adjacent glycan strands confers the rigidity of the cell wall.

The beta-lactam ring is similar to the d-alanine-d-alanine of the NAM pentapeptide, and PBPs mistakenly use the beta-lactam as a building block during cell wall synthesis (Zapun *et al.*, 2008). This results in acylation of the PBP, which renders the enzyme unable to catalyze further transpeptidation reactions (Fisher and Mobashery, 2009). As cell wall synthesis slows to a halt, constitutive peptidoglycan autolysis continues. The breakdown of the murein sacculus leads to cell wall compromise and increased permeability. Thus, the beta-lactam-mediated inhibition of transpeptidation causes cell lysis, although the specific details of penicillin's bactericidal effects are still being unraveled (Bayles, 2000).

2.3 Antibiotic resistance

Since the majority of antibiotics have evolved in different microorganisms over years, it can be assumed that in most cases the resistance to antibiotics is no doubt just as old (Baltz *et al.*, 2006). In the battle that microbes have to fight in this context, the ability to adapt to the environment is a crucial aspect, and the same applies to other competitor organisms. Antibiotics and antibiotic resistance are a part of the eternal contest between different microorganisms. There are several mechanisms of resistance, which can be divided into subgroups as follows, according to the actions involved: decreased permeability of bacterial membranes; antibiotic efflux; altered target sites; inactivating enzymes (Opal and Pop-Vicas, 2009).

2.3.1 Mechanisms of resistance to beta-lactam antibiotics

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

i. Production of beta-lactamase enzymes is the most common and important mechanism of resistance in Gram-negative bacteria.
ii. Changes in the active site of PBPs can lower the affinity for beta-lactam antibiotics and subsequently increase resistance to these agents. Alterations of PBPs have been described in both Gram-negative and Gram-positive organisms, assuming a more important role in Gram-positive bacteria. There are several PBP-mediated mechanisms of beta-

lactam resistance, including: Point mutations altering an amino acid; the acquisition of foreign PBP resistant to beta-lactam antibiotics; recombination between susceptible PBPs and those of less susceptible species and over expression of a PBP (Fontana *et al.,* 1983).

iii. Decreased expression of outer membrane proteins (OMPs) is another mechanism of resistance. In order to access PBPs on the inner plasma membrane, beta-lactams must either diffuse through or directly traverse porin channels in the outer membrane of Gram-negative bacterial cell walls. The porins are divided into two classes: specific and non specific. Point mutations or insertion sequence in porin- encoding genes can produce proteins with decreased function and thus lower permeability to beta lactams (Doumith *et al.,* 2009). Some *Enterobacteriaceae* (e.g. *Enterobacter* spp., *Klebsiella pneumoniae,* and *E. coli*) exhibit resistance to carbapenems based on loss of these OMPs.

iv. Efflux pumps, as part of either an acquired or intrinsic resistance phenotype, are capable of exporting a wide range of substrates from the periplasm to the surrounding environment (Poole, 2004). The organism's low outer membrane permeability, can contribute to decreased susceptibility to penicillins and cephalosporins, as well as quinolones, tetracycline, and chloramphenicol (Drawz and Bonomo, 2010).

2.4 Beta lactamases and its classification

Among the many resistance mechanisms available to bacteria for circumventing antibiotics are the beta-lactamases, enzymes that target the beta-lactam ring found in penicillins, cephalosporins, monobactams, and carbapenems (Drawz and Bonomo, 2010). They are naturally found in most Gram-negative bacteria (GNB). The beta-lactamases are the collective name of enzymes that open the beta-lactam ring by adding a water molecule to the common beta-lactam bond, and this inactivates the beta- lactam antibiotic from penicillin to carbapenems. This hydrolyzation was first observed in 1940 by Abraham and Chain (penicillinase) in a strain of *E. coli* (Bush and Bradford, 2007). However, the clinical effect of such hydrolyzation was not noted until the beginning of the 1950s, when the first beta-

lactam-resistant *S. aureus* isolates appeared in hospitals (Jacoby, 2009). The beta-lactamases in *S. aureus* are found in the chromosomes and are often inducible, whereas the first plasmid-mediated beta-lactamase was detected in Gram-negative bacteria in Greece in the 1960s and was designated TEM after the name of the patient (Temoneira) who carried the pathogen (Bush and Bradford, 2007). TEM-1 is the most common beta-lactamase in Gram-negative bacteria, and it can hydrolyze penicillins (ampicillin). The beta-lactamases also quickly spread to other bacteria, and soon, after changes in only one or a few amino acids, these enzymes were able to hydrolyze narrow- spectrum cephalosporins and were found in *Enterobacteriaceae*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* (Brunton *et al.,* 1986). Compared to the TEMs, the sulphydrylvariable (SHV) beta-lactamases are similar in biochemical structure but are more common in *Klebsiella* spp. The third-generation cephalosporins were stable against hydrolysis by the original TEMs and SHVs (Bush and Bradford, 2007).

Two classification schemes for beta-lactamases are currently in use. The molecular classification is based on the amino acid sequence and divides beta-lactamases into class A, C, and D enzymes which utilize serine for beta-lactam hydrolysis and class B metalloenzymes which require divalent zinc ions for substrate hydrolysis (Bush *et al.,* 1995). The functional classification scheme takes into account substrate and inhibitor profiles in an attempt to group the enzymes in ways that can be correlated with their phenotype in clinical isolates which includes group 1 (class C) cephalosporinases; group 2 (classes A and D) broad-spectrum, inhibitor-resistant, and extended-spectrum beta-lactamases and serine carbapenemases; and group 3 metallo-beta-lactamases (Bush *et al.,* 1995) and several new subgroups of each of the major groups are described, based on specific attributes of individual enzymes.

2.5 Extended spectrum beta-lactamases (ESBLs)

Extended-spectrum beta-lactamases (ESBLs) are enzymes that can hydrolyze oxyimino- beta-lactams (e.g., cefotaxime, ceftazidime and ceftriaxone) and the monobactam (Aztreonam) resulting in resistance to these drugs. ESBLs,

predominantly derivatives of plasmid-mediated TEM or SHV beta-lactamases, arise through mutations that result in one or more amino acid substitutions that alter the configuration or binding properties of the active site, thereby expanding the hydrolytic spectrum of the enzyme (Asma *et al.*, 2006). Members of the family *Enterobacteriaceae* commonly express plasmid encoded beta–lactamases (eg TEM-1,TEM-2 and SHV-1) which confer resistant to penicillin but not to extended spectrum cephalosporins. In the mid 1980s new groups of enzyme ESBLs are detected (First detected in Germany 1983) (Knothe *et al*., 1983). According to Ambler molecular classification, with the exception of OXA type enzyme (which is class D enzyme), the ESBLs are of molecular class A similarly according to Bush-Jacoby Medeiros classification scheme, ESBLs belong to group 2be or 2d (OXA type), the latter sharing most of the fundamental properties of group of 2be enzyme though differing in being inhibitor resistant (Bush *et al.*, 1995; Ambler *et al.,* 1991). The 2be designation shows that enzyme are derived from group 2b β-lactamases (for e.g. TEM-1, TEM-2, andSHV-1); the 'e' of '2be'denotes that the β-lactamases have an extended spectrum. The ESBLs are derived from TEM-1, TEM-2 or SHV-1 differ from their progenitors by as few as one amino acid, this results change in a profound change in enzymatic activity of the ESBLs, so that they now hydrolyze the 3rd generation cephalosporins or azteonam (hence the extension of spectrum compared to the parent enzyme. (Rawal *et al.,* 2010).

ESBLs are mutant enzymes with broader range of activity than the beta-lactamase enzymes. They hydrolyze 3rd and 4th generation cephalosporins and aztreonam but donot affect cephamycins which is second generation cephalosporin or carbapenems and remain susceptible to beta- lactamase inhibitors (Gupta, 2007). There are so many types of ESBLs like TEM, SHV, CTX, OXA, AmpC, etc. but majority of the ESBLs are derivatives of TEM or SHV enzymes and these enzymes are most often found in *E. coli* and *K. pneumoniae*. "Classical" ESBLs are derived from TEM and SHV enzymes whereas "Non Classical" ESBLs are derived from enzymes other than TEM or SHV. Upto 90% of ampicillin resistance in *E. coli* is due to production of TEM-1 (Sohei *et al.*, 2008).

Today more than 200 types of ESBL genes have been characterized. Due to high use of antibiotics their environmental release should be the matter of concern. Possibly the genes have been transferred from species to species. Several appropriate prevention and treatment guidelines may be applied to fight against ESBLs producing organisms. These may include public awareness programs especially in rural areas, combination therapy, drug cycling by making a policy, identification of new pharmacophores and good clinical practice. All ESBLs have serine at their active sites except for a small (but rapidly growing) group of metallo b-lactamases belonging to class B (They share several highly conserved amino acid sequences with penicillin binding proteins (PBPs)). Beta-lactamases attack the amide bond in the beta lactam ring of penicillins and cephalosporins, with subsequent production of pencilinoic acid and cephalosporic acid, respectively, ultimately rendering the compounds antibacterially inactive. Plasmids responsible for ESBL production tend to be large (80Kb or more in size) and carry resistance to several agents, an important limitation in the design of treatment alternatives (Paterson and Bonomo, 2005). The most frequent coresistances found in ESBL producing organisms are aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and sulfamethoxazole-trimethoprim. Since, ESBL production is usually plasmid mediated it is possible for one specimen to contain both ESBL producing and non ESBL producing cells of the same species. This suggests that for optimal detection, several colonies must be tested from a primary culture plate.

2.6 Global epidemiology of ESBLs

The epidemiology of ESBLs is quite complex. There are several different levels to consider: the wider geographical area, the country, the hospital, the community, and the host (in most cases a single patient or a healthy carrier). Furthermore, there are the bacteria (*E. coli* is more endemic, and *K. pneumoniae* is more epidemic) and their mobile genetic elements, usually plasmids. The first ESBL to be identified was found in Germany in 1983, but it was in France in 1985 and in the United States at the end of the 1980s and the beginning of the 1990s that the initial nosocomial outbreaks occurred (Rice *et al.,* 1990). It appears that the

spread of ESBL-producing bacteria is greater in developing countries than in nations with more substantial economic resources. Some plausible reasons for this difference include the following conditions that are prevalent in low- income countries: crowded hospitals, more extensive self-treatment and use of non-prescription antimicrobials, poorer hygiene in general and particularly in hospitals, as well as less effective infection control (Hawkey, 2008).

ESBLs have been reported from all parts of the world. However, prevalence varies widely even in closely related regions. The true incidence is difficult to determine because of the difficulty in detecting ESBL production and due to inconsistencies in testing and reporting (Yushau *et al*., 2010). Prevalence of ESBL in many parts of the world was (10-40%) among *E. coli* and *Klebsiella pneumoniae* (Rupp and Fey, 2003). The prevalence of ESBLs in Europe is higher than in the USA but lower than in Asia and South America (Girlich *et al.*, 2004). In 2007 at Asia pacific region ESBL was found to be 62% and 75% in *E. coli* and *Klebsiella* spp. respectively (Bell *et al*., 2007). ESBL production rate was 43%, 73.8%, 96% and 70% in *E. coli* and 60% in *Klebsiella* spp. in Asian countries (Ali 2009; Sharma *et al*., 2011). The prevalence of ESBL producing gram negative bacilli from urinary isolates in India was found to be 71.5% (Ramesh *et al.*, 2008).

There were a limited number of studies on prevalence of ESBL showing a high rate in Nepal, where *Enterobacteriaceae* were 28% to 67% in 2007 (Hammer *et al*., 2007) and at tertiary care hospital of eastern Nepal it was 14.8% in *Klebsiella spp* followed by *Proteus* spp. 12.9%, *E. coli* 53.7% in 2011 (Shrestha *et al*., 2011). In a study conducted at Tribhuvan University Teaching Hospital (TUTH), 60.40% of urinary isolates were MDR strains among which 16% of the isolates were ESBL producers (Pokhrel *et al*., 2006). In a similar study conducted at TUTH, 68.33% of the urinary isolates were MDR with 12 urinary isolates demonstrating ESBL activity (Bomjan, 2005) and 27.5% ESBL was producing *E. coli* and *Klebsiella* spp. was found in 2006 (Manandhar *et al.*, 2006). In the similar study conducted at NPHL in the urinary isolates, among all the

Enterobacteriaceae 31.57% *E. coli* was found to be ESBL producer (Thakur *et al.*, 2013). It is necessary to investigate the prevalence of ESBL positive strains in hospitals so as to formulate a policy of empirical therapy in high risk units where infections due to resistant organisms are much higher (Mathur *et al*., 2002).

2.7 Laboratory procedure for ESBL detection

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 hydrolyze different cephalosporins; and genotypic methods, which use molecular techniques to detect the gene responsible for the production of the ESBL. Clinical diagnostic laboratories use mostly phenotypic methods because these tests are easy to perform and are cost effective also. Many different techniques exist for confirming ESBL production but those utilizing similar methodology to standard susceptibility tests are the most convenient for the routine diagnostic laboratory. These all depend on detecting synergy between clavulanic acid and the indicator cephalosporin's used in the primary screening. Failure to detect ESBL production by routine disk-diffusion tests has been well documented (Paterson *et al*., 2005; Tenover *et al*., 2009).

The current CLSI recommendations (2007) for detection of ESBL's in *Klebsiella* spp. and *E. coli* includes an initial screening test with any two of the following betalactam antibiotics: cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone. Isolates exhibiting a MIC > 1μg/ml should be confirmed phenotypically using ceftazidime plus ceftazidime/clavulanic acid and cefotaxime plus cefotaxime/clavulanic acid.

2.7.1 Screening for ESBL producer

Clinical and laboratory standard institute (CLSI, 2007) has developed disk diffusion and broth microdilution screening tests for possible ESBL production.

a) **Disk Diffusion method**: The CLSI has proposed disk diffusion method for screening of ESBL production by *Klebsiella* spp., *E coli* and *Proteus mirabilis*. The possible ESBL production can be detected by noting

specific zone diameters which indicate the high level of suspicion for ESBL production. Cefpodoxime, Ceftazidime, Cefotaxime, Ceftriaxone, or Aztreonam can be used for screening; however, use of more than one of these agents for screening improves the sensitivity of detection.

b) **Dilution antimicrobial susceptibility tests:** The CLSI has proposed dilution methods for screening of ESBL production by *Klebsiella* spp. and *E coli*. Ceftazidime, Ceftriaxone, Cefotaxime, or Aztreonam can be used at screening concentration of 1μg/ml. MIC of cephalosporins at a range of ≥2μg/ml is suspicious of ESBL production and an indication for organism to be tested by phenotypic confirmatory test (CLSI, 2007).

The above mention organisms should be reported as a potential ESBL producers if the screening test results are as follows (CLSI, 2007):

Disk Diffusion	**MIC's**
Cefpodoxime≤17mm	Cefpodoxime≥2μg/ml
Ceftazidime≤22mm	Ceftazidime≥2μg/ml
Cefotaxime≤27mm	Cefotaxime≥2μg/ml
Ceftriaxone≤25mm	Ceftriaxone≥ 2μg/ml
Aztreonam≤27mm	Aztreonam≥2μg/ml

2.7.2 Phenotypic confirmatory test

The CLSI has recommended phenotypic confirmatory test for the suspected ESBL producers. Several of the phenotypic confirmatory tests include:

a) **Cephalosporin/Clavulanate combination discs:** The CLSI advocates the use of Cefotaxime (30μg), Ceftazidime (30μg) disc with and without Clavulanate (10μg) or Cefpodoxime (10μg) plus Clavulanate (1μg) for phenotypic confirmation of the presence of ESBLs. A difference of ≥5 mm between the zone diameters of either of the cephalosporin disc and their respective Cephalosporin/Clavulanate disc is taken to be phenotypic confirmation of ESBL production.

b) **Broth microdilution:** It utilizes ceftazidime (0.25 to 128μg/ml), ceftazidime plus clavulanic acid (0.25 to 128/4 μg/ml), cefotaxime (0.25 to

64μg/ml), and cefotaxime plus clavulanic acid (0.25 to 64/4 μg/ml). Both of these antibiotics should be used. Phenotypic confirmation is considered as ≥3-two fold serial – decrease in MIC of either of cephalosporin in the presence of clavulanic acid compared to its MIC when tested alone.

c) **Double disk synergy test**: This test incorporates the use of cefotaxime (30μg) and ceftazidime (30μg) disks which are placed on either side of co-amoxiclav (20+10 μg) at the distance of 20-30 mm on to a Mueller Hinton Agar plate already inoculated with test organism. ESBL production is inferred when the zone of either cephalosporin is expanded by clavulanate. Critical disk spacing is of utmost importance (Livermore and Woodford, 2003).

d) **E test for ESBLs:** These have a cephalosporin gradient at one end and a cephalosporin + clavulanate gradient at the other. ESBL production is inferred if >8-fold reduction is seen in cephalosporin MICs in the presence of clavulanate. These are accurate and precise, but more expensive than combination discs.

2.8 Treatment of patients infected with *Enterobacteriaceae* producing ESBLs

The progressive increase of extended-spectrum beta-lactamase (ESBL) - producing enteric bacteria in recent years has called for a re-evaluation of current antibiotic therapy for these infections (Garau, 2008). ESBLs are clinically significant and patients infected with ESBL-producing *Enterobacteriaceae* experience a greater likelihood of poor outcome if they are treated with inappropriate antimicrobial agents (Rupp and Fey, 2003). Another problem when treating patients with these infections is that the plasmids carrying the ESBL gene often have additional mechanisms that give rise to co-resistance to many other antibiotics (Paterson, 2006; Rupp and Fey, 2003). The clinical efficacy of the treatment does not always reflect the in vitro susceptibility to antibiotics. Until 2009, an approach related to the resistance mechanism was applied, and ESBL-producing bacteria have been reported to be resistant to all cephalosporins. Carbapenems (such as Imipenem, Meropenem, etc) are considered the first choice for treatment of patients infected with ESBL-producing *Enterobacteriaceae,*

especially in cases involving severe septicaemia or septic shock (Paterson, 2000). Treatment with a beta-lactam/beta-lactamase inhibitor might be used as a carbapenem-sparing regimen when the susceptibility results are known. Cefepime plus beta lactamase inhibitor may be an alternative to carbapenem for the treatment of moderate to severe infections as the activity of cefepime plus tazobactam was significantly better compared with that of cefoperazone+sulbactam and piperacillin+tazobactam (Sharma *et al.,* 2012). However, the emergence of CTX-M-15-producing bacteria also frequently leads to production of OXA-1- beta-lactamase, which is worrisome and renders the beta-lactam/beta-lactamase inhibitor ineffective (Rodriguez-Bano *et al.,* 2011). Cefepime, an oxyimino beta-lactam with an amino thiazolyl side chain that is often referred to as a fourth-generation cephalosporin, is active against most ESBL-producing organisms, particularly those with SHV derived enzymes (Rupp and Fey, 2003). In addition, there are some data from *in vivo* models to support the use of cefepime in the treatment of infections due to ESBL- producing *Enterobacteriaceae* (Andes *et al*., 2009). Treatment failures of ESBL producing organisms have also been observed, therefore, until more clinical data is available, clinicians should not regard cefepime as a first line therapy for ESBL-producing organisms and, if used, it should be given at high dose (≥2g every 12 hours) usually in combination with other active agents (aminoglycosides, fluoroquinolones) (Rupp and Fey, 2003). Tigecycline, a new semisynthetic glycylcycline, may be considered an alternative drug of choice for patients infected with ESBL-producing bacteria (Ku *et al*., 2008). With high susceptibilities and positive clinical outcomes from subpopulations infected with ESBL-producing *Enterobacteriaceae*, tigecycline has been increasingly utilized as a treatment option (Nicasio *et al.,* 2009).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

The materials, equipment and various reagents used in different stages of this study are listed in Appendix C.

3.2 Methodology

This study was carried out in the Department of Microbiology, Alka Hospital, Jawalakhel, Lalitpur. A total of one thousand and fifty four urine samples were investigated during six months period beginning from May to October 2012 to find out the prevalence of ESBL-producing *Enterobacteriaceae* from in-patients and out-patients, clinically suspected of UTI. This study included paients of all age groups and both sexes suspected of urinary tract infections from whom samples are sent for routine culture and antibiotic susceptibility testing. Only those cases yielding growth of Enterobacteriaceae from the cultured urine sample was included in the study and was further tested for ESBL production. Duplicate isolates from the same patient were excluded in the study and cases which did not yield the growth of *Enterobacteriaceae*, but yielded growth of other bacteria were not included. Personnel data was collected from file records.

3.2.1 Collection and transport of urine samples

Mid-stream urine was collected from a patient with full aseptic precautions in a sterile, dry, wide mouthed, leak proof container. The sample labeled properly with demographic information of patients such as name, age, sex, hospital number, date and time of collection of specimen was accepted, otherwise a second sample was requested. When the examination and culture of specimen was not possible within 2 hours, then the urine sample was refrigerated at 4^0 c.

3.2.2 Culture of specimens

Culture of each urine sample was done by semi-quantitative method on 5% Blood agar and Mac Conkey agar plates. An inoculating loop of standard dimension was used to take up approximately fixed and a known volume (0.001ml) of mixed urine for inoculation. The plates were the incubated at 37^0C overnight. Colony count was performed so as to calculate the number of colony forming unit (CFU) per milliliter (ml) of urine and the bacterial count was reported as:

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

If the culture indicates presence of two uropathogens both showing significant growth, definitive identification and antimicrobial susceptibility testing of both were performed whereas in cases of ≥3 pathogens, it was reported as multiple bacterial morphotypes and asked for appropriate recollection with timely delivery to laboratory (Isenberg, 2004).

3.2.3 Identification of the isolates

Identification of significant isolates were done by using standard microbiological techniques which involved morphological appearance of the colonies, Gram's staining reactions, catalase test, oxidase test with other biochemical properties. The biochemical media employed were Triple Sugar Iron agar (TSI), Sulphide Indole Motility (SIM) media, Simmon's citrate media and Christensen's urease media.

3.2.4 Purity Plate

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

3.2.5 Antimicrobial susceptibility testing

The antibiotic sensitivity tests of the pathogens isolated from the clinical specimen against different antibiotics were determined by Kirby-Bauer method of disk diffusion technique as recommended by NCCLS using Mueller Hinton agar (MHA). At least three to five well isolated colonies of the same morphological types were selected from the MHA plate. The base of each colony was touched with a inoculating wire and the growth was transferred into a tube containing 5ml of nutrient broth and was incubated at 37°C (usually 2 to 6 hours) until it achieved the McFarland tube number 0.5. In case overgrowth, the broth was diluted with sterile physiological saline to match with McFarland tube number 0.5. The suspension was then adjusted to match with McFarland tube number 0.5. A sterile cotton swab was dipped into the broth and the swab was rotated several times and pressed firmly on the inner side wall of the tube above the fluid level to remove excess inoculums from the swab. Then the dried surface of a MHA plate was inoculated by streaking the swab over the entire agar surface three times, turning the plate 60^{o} between streaking. Finally the inoculum was left to dry for a few minutes at room temperature with the lid closed. Then the antimicrobial disks were placed on the surface of the prior inoculated agar plate such that there was 25mm distance from disk to disk. The commercial antibiotics used for *Enterobacteriaceae* includes amoxycillin (30 μg), ciprofloxacin (30 μg), cotrimoxazole (25 μg), gentamicin (10 μg), nitrofurantoin (300 μg), ceftriaxone (30 μg) and ceftazidime (30 μg). The disks were pressed down to ensure complete contact with the agar surface. For about 15 minutes of applying the disks, the plates were left at room temperature to allow antimicrobials to diffuse from the disk. Then they were incubated aerobically at 37°C overnight. After overnight incubation, the diameter of zone of inhibition (ZOI) of each disk was measured (including the diameter of the disk) and recorded in millimeter. It is then compared with Standard Chart developed by Kirby- Bauer to determine bacterial susceptibility towards different antimicrobial agents in terms of ‘sensitive’, ‘resistant’ and ‘moderately sensitive (intermediate)’. The measurements were made with a ruler on the under surface of the plate without opening the lid.

3.2.6 Tests for ESBL-production in *Enterobacteriaceae*

3.2.6.1 Screening of ESBL producer

The initial screen test for the production of ESBL was performed by using both ceftazidime (CAZ) (30µg) and ceftriaxone (CTR) (30µg) disks. If the zone of inhibition was less than or equal to 22mm for CAZ and/or less than or equal to 25mm for CTR, the isolate was considered a potential ESBL-producer as recommended by NCCLS.

3.2.6.2 Confirmation of ESBL producer by phenotypic method

Isolates those were suspected as ESBL- producer by screen test were tested further by double-disk synergy test (DDST) and Combination disk method (CD). In DDST method, amoxicillin- clavulanic acid (AMC) disk (20/10µg) was placed at the centre and disks containing the 30µg of CAZ, CTR, CTX and ATM were placed separately beside 20 mm distance (edge to edge), away from the central disk in a horizontal manner. Inoculated plates were incubated aerobically at 37°C for 18 - 24 h. After overnight incubation at 37°C, a clear extension of the edges of the inhibition zone of any of the antibiotics towards the disc containing clavulanic acid was regarded as a phenotypic confirmation of the presence of ESBL. Isolates with this pattern were recorded as DD positive.

Combination disk method was also used for the confirmation of ESBL-producing strains in which CAZ (30µg), alone and in combination with clavulanic acid (CA) (10µg) were used. After incubating overnight at 37°C, a $\geq$ 5 mm increase in the zone diameter for either antimicrobial agent which was tested in combination with clavulanic acid versus its zone when tested alone, was interpreted as positive for ESBL production.

3.2.7 Antibiotic susceptibility testing of ESBL producer

The susceptibility of the ESBL producing *Enterobacteriaceae* to alternative drugs such as Amikacin, cefepime, imipenem, meropenem, piperacillin/tazobactam and tigecycline was determined by the Kirby-Bauer disk diffusion method according to the Clinical Laboratory Standards Institute guidelines.

3.2.8 Quality control

Strict quality control was maintained to obtain reliable microbiological results. The quality of each agar plate prepared was maintained by incubating one plate of each batch in the incubator. Control strains of ATCC were used for the identification test and for the standardization of Kirby-Bauer test and also for correct interpretation of diameter of inhibition zones. Quality of sensitivity test was maintained by maintaining the thickness of MHA at 4mm and the pH of 7.2-7.4. Similarly antibiotics disks having correct amount as indicated was used. Strict aseptic condition was maintained while carrying out all the procedures. Purity plate for each biochemical test was maintained to ensure the pure culture inoculums used as well as to assess that the biochemical tests were undertaken in an aseptic condition.

3.2.9 Data analysis

All the data collected were analyzed using Statistical Software SPSS version 16.0. Descriptive analysis was done.

CHAPTER IV

RESULTS

The present study was carried out at the department of Microbiology, Alka hospital between May 2012 and October 2012 to look for the prevalence of ESBL strains in the *Enterobacteriaceae* isolated from urinary tract infections. 1054 mid-stream urine samples were collected from the suspected urinary tract infection patients.

4.1 Growth status of urine samples

Out of 1054 MSU samples 289 (27.4%) samples showed monomicrobial significant growth (i.e. ≥10^5 CFU/ml), 739 (70.1%) samples were sterile and 26 (2.5%) samples showed polymicrobial insignificant growth (i.e. more than two types of isolates).

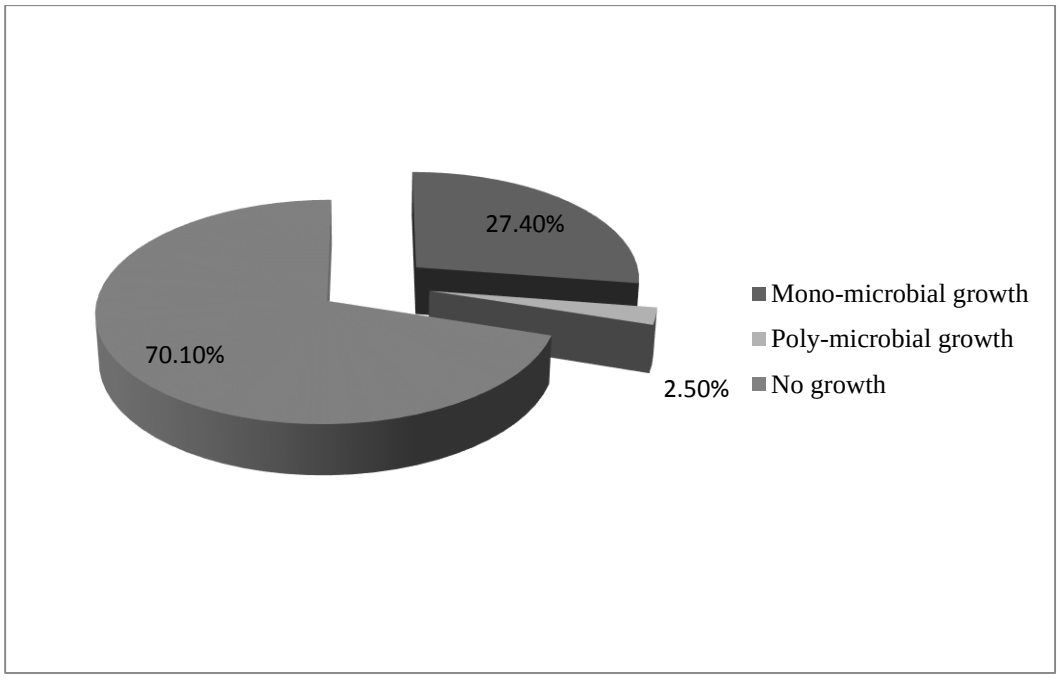

Figure 1: Growth status of urine sample

4.2 Clinical profile of the samples and growth status among ward and gender wise of patients

Among the total 1054 mid stream urine samples 854 samples were from outpatients, 244 (28.6%) showed significant growth and 200 samples were from inpatients, 71 (35.5%) showed significant growth.

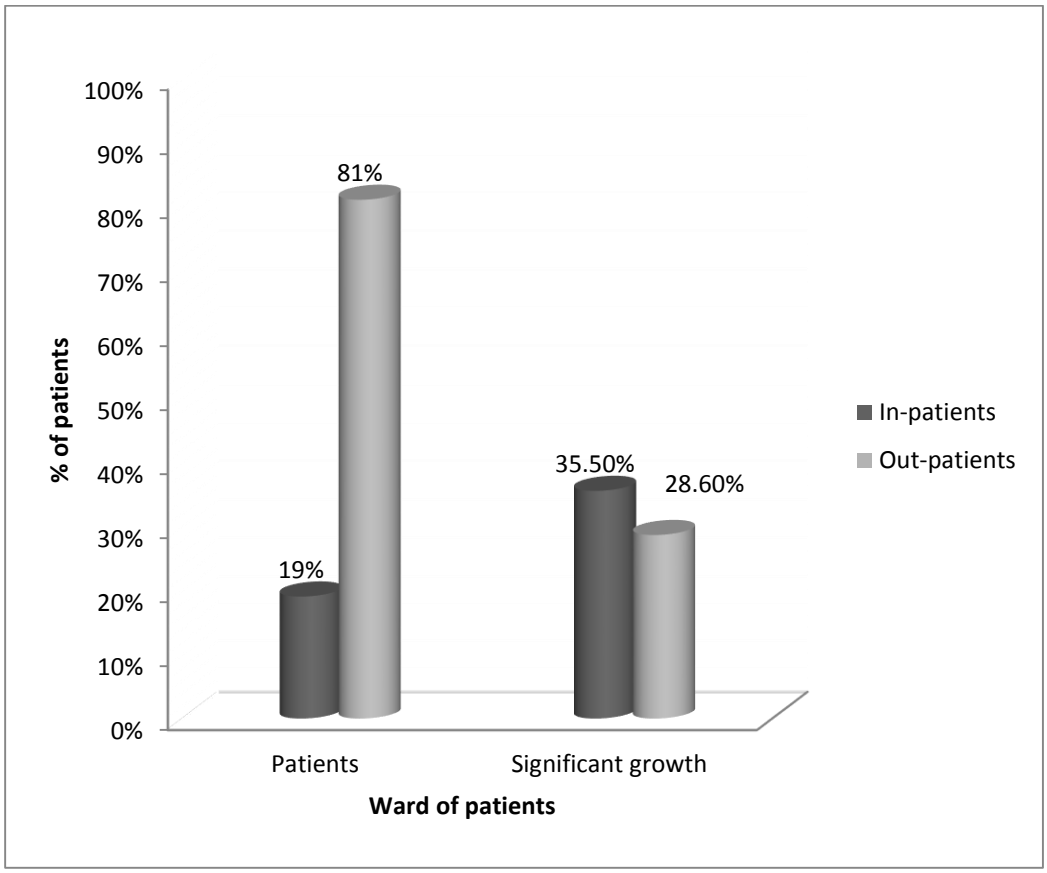

Figure 2: Ward wise distribution of patients and growth profile

Of the total samples, 355 (33.7%) samples were received from the male patient whereas 699 (66.3%) samples from female patients, the maximum number of culture request being received from age group 15-30 years; 398 (37.8%) samples and the least being received from age 6-14 years; 49 (4.6%) samples. Of the 355 samples from male, 83 (23.4%) showed signifcant growth with maximum number of growth being observed in age group 50-70 years; 24/83 (28.9%). Similarly, of the 699 samples from female, 232 (33.2%) showed significant growth with maximum no. of growth being observed in age group 15-30 years; 98/232

(42.2%). The highest prevalence of multidrug resistant isolates was observed in age group 15-30 years with 97/266 (36.5%), followed by 31-49 years with 67/266 (25.2%).

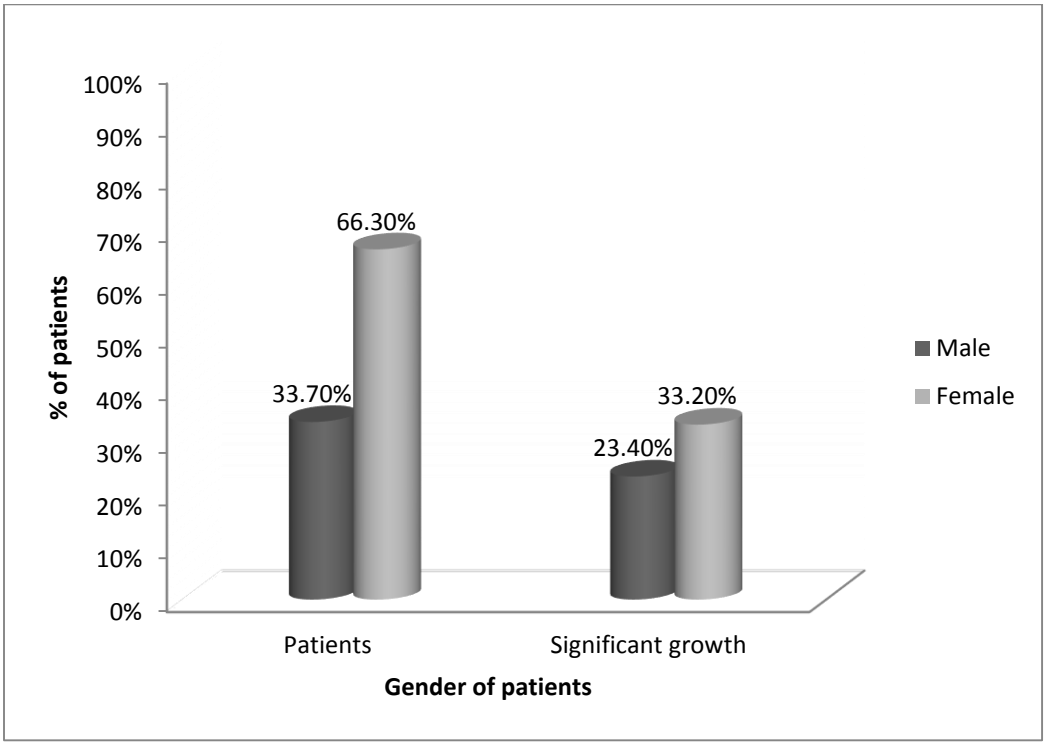

Figure 3: Gender wise distribution of patients and growth profile

4.3 Microbiological profile of urinary isolates

Out of 289 significant culture positive cases, 273 (94.5%) were due to gram negative bacteria and 16 (5.5%) were due to gram positive bacteria. The gram negative bacteria *Escherichia coli* (70.5%) was found to be the most common one followed by *Klebsiella* species (8.2%), *Proteus* species (2.6%), *Morganella morganii* (2.2%), *Citrobacter* species (1.3%), *Acinetobacter* species (1.3%) and *Pseudomonas aeruginosa* (0.6%). The gram positive bacteria isolated, includes *Staphylococcus aureus* (3.5%), *Staphylococcus saprophyticus* (1.0%) and *Enterococcus faecalis* (0.6%).

Table 2: Microbiological profile of urinary isolates

Bacterial isolates	Frequency	Percentage (%)
Escherichia coli	222	70.5
Klebsiella pneumoniae	19	6.0
Klebsiella oxytoca	7	2.2
Proteus vulgaris	5	1.6
Proteus mirabilis	3	1.0
Citrobacter spp.	4	1.3
Morganella morganii	7	2.2
Acinetobacter spp.	4	1.3
Pseudomonas spp.	2	.6
Staphylococcus aureus	11	3.5
Staphylococcus saprophyticus	3	1.0
Enterococcus faecalis	2	.6
Mixed growth	26	8.3
Total	315	100.0

4.4 Microbiological profile of *Enterobacteriaceae* and their age, gender and wardwise distribution

Out of 1054 samples 315 (29.9%) yielded the bacterial growth, out of which 267 (84.8%) yielded growth of *Enterobacteriaceae* and 22 (7%) yielded growth of other bacteria such as *Acinetobacter, Pseudomonas, Staphylococci, Enterococci*, etc., 26 (8.2%) yielded polymicrobial growth. Among those 267 samples which yielded pure growth of *Enterobacteriaceae*, 222 (83.1%) were *E.coli*, 26 (9.7%) *Klebsiella*, 8 (3%) *Proteus*, 4 (1.5%) *Citrobacter* and 7 (2.6%) *Morganella* species. The pattern of growth of *Enterobacteriaceae* is shown in the table.

Table 3: Percentage distribution of *Enterobacteriaceae* isolated from urine sample

Bacterial isolates	Number	Percentage (%)
Escherichia coli	222	83.1
Klebsiella pneumoniae	19	7.1
Klebsiella oxytoca	7	2.6
Proteus vulgaris	5	1.9
Proteus mirabilis	3	1.1
Citrobacter spp.	4	1.5
Morganella morganii	7	2.6
Total	267	100.0

Out of 267 subjects, which showed growth of *Enterobacteriaceae*, 62 (23.3%) were male patients and 204 (76.7%) were female patients. The age and sex distribution of the samples is shown in table.

Table 4: Age and sex distribution of *Enterobacteriaceae*

Age group (In years)	Sex of patients				Total	Total %
	Male	Male%	Female	Female %		
0-5	2	3.2	19	9.3	21	7.9
6-14	0	0	8	3.9	8	3
15-30	12	19.4	86	42	98	36.7
31-49	17	27.4	50	24.4	67	25.1
50-70	21	33.9	25	12.2	46	17.2
>70	10	16.1	17	8.3	27	10.1
Total	62	100	205	100	267	100

From 267 isolates of *Enterobacteriaceae*, 210 (78.7%) isolates were from outpatient department and 57 (21.3%) isolates were from inpatients. Comparison of *Enterobacteriaceae* isolates according to ward are shown in table.

Table 5: Ward wise distribution of *Enterobacteriaceae*

Bacterial isolates	Ward of patients				Total	%
	Inpatient	%	Outpatient	%		
Escherichia coli	49	22.1	173	77.9	222	100
Klebsiella pneumoniae	3	15.8	16	84.2	19	100
Klebsiella oxytoca	0	0	7	100	7	100
Proteus vulgaris	1	20	4	80	5	100
Proteus mirabilis	2	66.7	1	33.3	3	100
Citrobacter spp.	0	0	4	100	4	100
Morganella morganii	2	28.6	5	71.4	7	100
Total	57	21.3	210	78.7	267	100

4.5 Antibiotic susceptibility pattern of *Enterobacteriaceae*

Among the total *Enterobacteriaceae* 71.5% of the isolates showed resistance to Amoxycillin (Amx). All the isolates of *Klebsiella pneumoniae, Proteus vulgaris* and *Citrobacter* spp. were resistant to Amoxycillin. About 30- 50% of the isolates were resistant to Ciprofloxacin, Cotrimoxazole (Cotri) and third generation cephalosporins, Ceftriaxone (Ctr) and Ceftazidime (Caz). All the isolates of *Proteus mirabilis* was found sensitive to Cotrimoxazole, Ciprofloxacin, Ceftriaxone and Ceftazidime. Only 9.4% of the total isolates were resistant to the antibiotics, Nitrofurantoin (Nitro) and 14.2% isolates were resistant to Gentamicin (Gen). The resistance pattern of *Enterobacteriaceae* is shown in the table 6.

Table 6: Antibiotic resistance pattern of *Enterobacteriaceae*

Bacterial isolates	Antibiotics (%Resistance)						
	Amx	Cipro	Cotri	Nitro	Genta	Ctr	Caz
E. coli (n=222)	150 (67.6)	81 (36.5)	103 (46.4)	10 (4.5)	34 (15.3)	69 (31.1)	69 (31.1)
K. pneumoniae (n=19)	19 (100)	1 (5.3)	5 (26.3)	6 (31.6)	1 (5.3)	3 (15.8)	3 (15.8)
K. oxytoca (n=7)	6 (85.7)	2 (28.6)	1 (14.3)	0 (0)	1 (14.3)	1 (14.3)	1 (14.3)
P. vulgaris (n=5)	5 (100)	1 (20)	1 (20)	2 (40)	0 (0)	0 (0)	0 (0)
P. mirabilis (n=3)	2 (66.7)	0 (0)	0 (0)	2 (66.7)	0 (0)	0 (0)	0 (0)
Citrobacter spp. (n=4)	4 (100)	1 (25)	2 (50)	3 (75)	1 (25)	1 (25)	1 (25)
M. morganii (n=7)	5 (71.4)	2 (28.6)	3 (42.9)	2 (28.6)	1 (14.3)	2 (28.6)	1 (14.3)
Total (n=267)	191 (71.5)	88 (33)	115 (43.1)	25 (9.4)	38 (14.2)	76 (28.5)	75 (28.1)

4.6 Distribution of MDR isolates among *Enterobacteriaceae*

Out of the 267 isolates of *Enterobacteriaceae*, 38.6% isolates were Multi-drug resistant, which are resistant to three or more than three group of antibiotics. In this study, highest number of MDR (60%) was found in *Proteus vulgaris* followed by *Morganella morganii (*57.1%), *Citrobacter* spp. (50%), *Escherichia coli* (40.1%), *Klebsiella oxytoca* (28.6%) and *Klebsiella pneumoniae* (15.8%). No MDR strains were observed in *Proteus mirabilis*. The MDR pattern of *Enterobacteriaceae* is shown in table 7.

Table 7: Distribution of MDR *Enterobacteriaceae*

Bacterial isolates	Total	MDR	
		Number	%
Escherichia coli	222	89	40.1
Klebsiella pneumoniae	19	3	15.8
Klebsiella oxytoca	7	2	28.6
Proteus vulgaris	5	3	60
Proteus mirabilis	3	0	0
Citrobacter spp.	4	2	50
Morganella morganii	7	4	57.1
Total	267	103	38.6

High incidence of UTIs caused by MDR pathogens was observed, among male patients (59.7%, 37/62) than female patients (32.2%, 66/139) ($p < 0.05$), also in hospitalized-patients (43.9%, 25/57) than out-patients (37.1%, 78/210).

Table 8: Ward-wise distribution of MDR isolates

		Ward of patients		
		Out patients (%)	In patients (%)	Total (%)
MDR	No MDR	132 (62.9)	32 (56.1)	164 (61.4)
	MDR	78 (37.1)	25 (43.9)	103 (38.6)
	Total (%)	210 (100)	57 (100)	267 (100)

4.7 Profile of ESBL production among *Enterobacteriaceae*

Among 267 *Enterobacteriaceae*, 72 (27%) were ESBL producers. In the present study *E. coli* was the common ESBL producer. Other ESBL producers are *Klebsiella, Citrobacter and Morganella* species. No ESBL was isolated from *Proteus* species. Percentage of ESBL producers among *Enterobacteriaceae* is shown in the table 9.

Table 9: Percentage of ESBL producers among *Enterobacteriaceae*

Bacterial isolates	Total	MDR (%)	ESBL		MDR ESBL (%)
			Number	%	
Escherichia coli	222	89 (40.1)	67	30.2	62 (92.5)
Klebsiella pneumoniae	19	3 (15.8)	3	15.8	3 (100)
Klebsiella oxytoca	7	2 (28.6)	0	0	0 (0)
Proteus vulgaris	5	3 (60)	0	0	0 (0)
Proteus mirabilis	3	0 (0)	0	0	0 (0)
Citrobacter spp.	4	2 (50)	1	25	1 (100)
Morganella morganii	7	4 (57.1)	1	14.3	1 (100)
Total	267	103 (38.6)	72	27	67 (93.1)

4.8 Association of ESBL detection methods and ESBL producers and MDR

Out of 81 screening positive suspected ESBL producer, 72 isolates were confirmed ESBL producer by combination disk method but by double disk synergy test only 28 isolates were found to be ESBL producer.

Table 10: Association between ESBL detection by DDST and combination disk method

		Combination disk method		Total	P-value
		Increase in diameter ≥5 mm	No increase in diameter ≥5 mm		
DDST	Synergy	28	0	28	<0.05
	No synergy	44	9	53	
	Total	72	9	81	

Sensitivity of DDST test= (28/72)*100%= **38.89%** with respect to combination disk method.

ESBL producer in inpatient was higher than the outpatients which accounts for 19/53 (33.3%) and 53/210 (25.2%) respectively.

Among all the ESBL producers, 67/72 (93.1%) isolates were found to be MDR and only 5 (6.9%) isolates were non- MDR.

Table 11: Association between ESBL producers and MDR

		ESBL		Total (%)
		Negative	Positive	
MDR	No MDR	2 (28.6)	5 (71.4)	7 (100)
	MDR	7 (9.5)	67 (90.5)	72 (100)
Total (%)		9 (11.1)	72 (88.9)	81 (100)

4.9 Antibiotic susceptibility pattern of ESBL producing *Enterobacteriaceae*

In the present study all of the 72 isolates of ESBL producer were sensitive to Imipenem, 98.2% isolates were sensitive to tigecycline and piperacillin-tazobactam, 97.2% were sensitive to amikacin, 43.7% isolates were sensitive to meropenem and only 11.3% isolates were sensitive to cefepime. Along with the

beta-lactam antibiotics, ESBL producers are also associated with resistance to other group of antibiotics such as quinolones, cotrimoxazole and aminoglycosides. Among all the ESBL producer strains 76.4%, 79.2%, 37.5% are resistant to ciprofloxacin, cotrimoxazole and gentamicin resceptively. The resistance pattern of ESBLs is shown in table 13.

Table 12: Antibiotic resistance pattern of ESBL producers

Antibiotics	*E. coli* (n=67)	*K. pneumoniae* (n=3)	*Citrobacter* spp. (n=1)	*Morganella morganii* (n=1)	Total (n=72)
	% Resistance	% Resistance	% Resistanc	% Resistance	%Resistance
Amoxicillin	67 (100)	3 (100)	1 (100)	1 (100)	72 (100)
Ciprofloxacin	52 (77.6)	1 (33.3)	1 (100)	1 (100)	55 (76.4)
Cotrimoxazole	52 (77.6)	3 (100)	1 (100)	1 (100)	57 (79.2)
Nitrofurantoin	4 (6)	2 (66.7)	0 (0)	0 (0)	6 (8.3)
Gentamicin	24 (35.8)	1 (33.3)	1 (100)	1 (100)	27 (37.5)
Ceftriaxone	66 (98.5)	3 (100)	0 (0)	0 (0)	70 (97.2)
Ceftazidime	67 (100)	3 (100)	1 (100)	1 (100)	71 (98.4)
Imipenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Meropenem	30 (44.8)	1 (33.3)	0 (0)	0 (0)	31 (43.1)
Cefepime	55 (82.1)	3 (100)	1 (100)	1 (100)	59 (81.9)
Piperacillin-	1 (1.5)	0 (0)	0 (0)	0 (0)	1 (1.4)
Tigecycline	1 (1.5)	0 (0)	0 (0)	0 (0)	1 (1.4)
Amikacin	1 (1.5)	0 (0)	0 (0)	0 (0)	2 (2.8)

CHAPTER V

DISCUSSION

The present study was conducted to isolate bacteria causing UTI and determine the ESBL producing organisms among the *Enterobacteriaceae* family from the patients suspected of urinary tract infection visiting Alka Hospital, Lalitpur.

The prevalence of UTI in this study was found to be 29.9%. Out of 1054 MSU samples 289 (27.4%) samples showed monomicrobial significant growth (i.e. $\geq 10^5$ CFU/ml), 739 (70.1%) samples were sterile and 26 (2.5%) samples showed polymicrobial insignificant growth (i.e. more than two types of isolates). In the study done by Manandhar (2006) which was carried out at TUTH laboratory showed 26.4% monomicrobial significant growth and 5.7% polymicrobial growth. UTI in the vast majority of patients is caused by a single species, but occasionally there may be a mixed infection. Some microbiologists regard urine cultures with polymicrobial (mixed) growth as contaminants (Denman and Greenough, 1991). However, polymicrobial growth from mid-stream urine has been found among patients with confirmed bladder infection. Mixed infections are more likely to occur with underlying disorders that interfere with free urine flow and is frequent also patients with indwelling catheter. When mixed infection occurs and contamination is suspected, a repeat should be performed from a carefully collected mid-stream urine or preferably by catheterization or suprapubic aspiration (Denman and Greenough, 1991).

Higher rate of infections was found in female patients 232/699 (33.2%) and in male, the rate of infections was found to be 83/355 (23.4%) but there was no statistical significant difference between them ($p > 0.05$). This result is in congruous to the earlier studies by Bomjan (2005), Baral (2008) and Thakur (2013). Although everyone is susceptible to UTI, there are specific subpopulations that are at increased risk of UTI, including infants, pregnant women, and elderly patients with catheters, patients with diabetes, multiple

sclerosis or acquired immunodeficiency syndrome (AIDS) / human immunodeficiency virus (HIV) and patients with underlying urologic abnormalities. Except during the first few months of life, females are far more susceptible than males to UTI. The higher incidence of UTI in women is suggested to be due to short (about 4 cm), wide urethra, and its termination beneath the labia. This route is particularly predisposing for ascending infection by organisms colonizing the perianal area and also complicated physiology especially during pregnancy such as change in hormonal level can be the considered as reason.

The significant growth from urine sample in female was predominant (42%) in age group 15-30 which is followed by the age group 31-49 (24.4%). The reason behind maximum growth in these age groups is the female in this group are sexually active. The high rate of infection in the age groups between 15 and 30 years in females suggested that females in this age group are sexually active and sexual intercourse causes the introduction of bacteria into the bladder and is temporally associated with the onset of cystitis it thus appears to be important in the pathogenesis of UTIs. In addition, use of spermicidal coated condoms dramatically alters the normal bacterial flora and has been associated with marked increases in vaginal colonization with *E. coli* and in the risk of UTI (Braunwald *et al.*, 2001).

High prevalence of UTI (33.9%) in old age male subjects of age group (50-70 years) may be due to different conditions like prostatitis, diabetes and weak immune status. Similar study was done by Bomjan (2005), Baral (2008), Poudyal (2010) and Thakur (2013). In this study, UTI is more common in the elderly patients (aged > 50 years). Although the majority of cases (76%) spontaneously resolve, infections with urea splitting bacteria (such as *Proteu*s) are more likely to have significant squeal, including stone formation and permanent renal damage. However, symptomatic UTI among the elderly requires antimicrobial therapy (Foxman, 2002).

Gram negative isolates (94.5%) were the predominant pathogens of the urinary tract infections with *Enterobacteriaceae* (84.8%) as the major one. The gram negative bacteria *E. coli* (70.5%) was found to be the most common one followed by *Klebsiella* species (8.2%), *Proteus* species (2.6%), *M. morganii* (2.2%), *Citrobacter* species (1.3%), *Acinetobacter* species (1.3%) and *P. aeruginosa* (0.6%). These results ressembled the outcomes of previous studies by Baral (2008), Poudyal (2010) and Thakur (2013). *E. coli* have special virulent properties contributing to their being a major uropathogen throughout the world. *E. coli* can bind to the glycoconjugate receptor (Gal alpha1-4 Gal) of the uroepithelial cells of human urinary tract such that it can initiate infection itself. *E.coli* is isolated in 90% of Urinary tract infections and strains are characterized by unique virulence determinant, the p pilus (Gal-Gal) (Kot *et al.,* 2010).

This study showed that *Enterobacteriaceae* are predominant causative organisms to cause UTI. Among the Gram-negative bacteria, highest percents of resistance towards first-line antibiotics were found for amoxycillin (71.5%), co-trimoxazole (43.1%), and ciprofloxacin (33%). About 29% of the isolates were found resistant to third generation cephalosporins i.e. ceftriaxone and ceftazidime. The possible explanation for this is that most of these drugs are easily available and may be used indiscriminately, which may lead to a shift to increase prevalence of resistant organisms. However, overall nitrofurantoin and gentamicin showed a least resistance of 9.4% and 14.2% resceptively. Since nitrofurantoin has shown overall low resistance rate in both hospitalized and out-patients, it can be considered as the first-line therapy, a finding similar to other studies. Amoxycillin, ciprofloxacin and co-trimoxazole resistance rates in this report were higher, hence empirical therapy with these antibiotics seems inadequate and should be avoided.

The increasing prevalence of antibiotic resistance is a cause of serious concern and multidrug resistance among some of the most important human pathogens is increasing. Out of the total *Enterobacteriaceae*, 38.6% isolates are Multi-drug resistant. In this study, highest number of MDR (60%) was found in *P. vulgaris*

followed by *M. morganii (*57.1%), *Citrobacter* spp. (50%), *E. coli* (40.1%), *K. oxytoca* (28.6%) and *K. pneumoniae* (15.8%). The high degree of resistance could be explained by the fact that drugs are easily available without doctor's prescription from pharmacy and in developing countries like Nepal self-medication is a common practice and this might probably be a major cause of antibiotic resistance in clinical isolates. Since patient only think of going to the hospitals when they are unable to treat themselves. Expired antibiotics, self-medication counterfeit drugs, inadequate hospital control measures can as well promote the development of resistance in clinical isolates (Thakur *et al.*, 2013). These results resembled the outcomes of previous studies done by Baral (2008) and Thakur (2013). Outcome of prevalence of MDR depends on various factors, MDR criterion being the chief one followed by the types of antibiotics used in antibiogram, study isolates and study population. The emergence of MDR is clearly related to the quantity of antibiotics and how they are being used.

First described in 1983, ESBLs have contributed to dramatic increase in resistance to beta-lactamase among gram negative bacteria in recent years. All *Enterobacteriaceae* were subjected to phenotypic laboratory detection of ESBL production. In the present study out of 267 *Enterobacteriaceae* 72 (27%) were ESBL producers. It was confirmed by combined disk approximation test in which 3rd generation cephalosporins were combined with beta-lactamases inhibitor Clavulanic acid (i.e. CTX30+Clav10 or CAZ30+Clav10) in which the structural analog of beta-lactam antibiotics (Clavulanic acid i.e. inhibitor) inhibits the action of beta-lactamase and antibiotic can act on the cell wall of the bacteria, and the result confirmed by the at least or more than 5mm increase in zone of inhibition than cephalosporins alone (Rawal *et al*., 2010). The prevalence of ESBL producing *Enterobacteriaceae* varies greatly from country to country and among the hospitals within the country. Less than 1% to greater than 70% ESBLs are reported worldwide. In US ESBL producers ranges from 0 to 25%. In Asia the percentage of ESBL production in *E. coli* and *K. pneumoniae* varies among countries. 4.8% and 22% in Korea, less than 0.1% and less than 0.3% in Japan, 11% and 13% in Hong Kong. In India percentage of ESBL producers ranges from

6% to 86%. Similar prevalence rates of ESBL in Nepal were reported by the findings of Manandhar (2006). NCCLS detection methods are based on a phenotypic profile that has potential to yield false positive and false negative results. In some of the isolates, additional mechanisms of resistance, such as AmpC- beta lactamases, porin changes and inhibitor resistant TEMs (IRTs) and SHV beta-lactamases with reduced affinities for beta-lactamase inhibitors can mask CA inhibition. In addition hyperproduction of Class A 'K1' chromosomal protease by *K. oxytoca* can give positive clavulanate synergy test with cefotaxime and cefepime (never ceftazidime), so the producers are confused with ESBL producers.

In the present study the prevalence of ESBL among the bacteria of family *Enterobacteriaceae* was higher in isolates obtained from inpatients (19 out of 57 i.e. 33.3%) as compared to out-patients isolates (53 out of 210 i.e. 25.2%). The high prevalence of ESBL-producing isolates described in this study was probably due to the long term hospital stay with large amount of third-generation cephalosporins consumed.

Of the 81 bacterial isolates tested for the ESBL production, 72 (88.9%) isolates tested positive for ESBL production. The majority consisted of *E. coli* i.e. 67/72 (93.1%) followed by *K. pneumoniae* 3/72 (4.2%). one isolates of *Citrobacter* spp. and one isolates of *M. morganii* showed ESBL production. Similar pattern of results were seen in the study carried out by Baral (2008) who showed the presence of 28.12% ESBL producers out of 96 MDR isolates, Bomjan (2005) who found the presence of 28.3% ESBL producers among various clinical isolates and Sharma (2004) who found 8% *K. pneumoniae*, 12.5% *E. coli*, 12.5% *C. freundii*, 25% *Acinetobacter calcoaceticus* and 5% *P. aeruginosa* as ESBL-producing strains.

As the two tests i.e. double disk synergy test and combination disk method were applied to confirm the ESBL producing strains, only 28 test positive was shown by double disk synergy test out of 72 ESBL producer which was shown positive

by combination disk method which is statistically significant. This indicates the double disk synergy test is less sensitive for the detection of ESBL producing strains which was in agreement to the result shown by Pokhrel (2006). The sensitivity of DDST varies with the distance between the discs (Ho *et al., 2000*). Zali (2000) reported that the clinical strains producing SHV-6 ESBL and Amp C type β-lactamase producers would not be detected by double disc diffusion tests. In the presence of AmpC along with ESBL in Gram negative organisms, the DDST may not show positivity as Amp C type beta lactamase inhibits the action of clavulanate and hence obscures the synergistic effect of clavulanic acid and third generation cephalosporins.

In the present study all the ESBL producers were resistant to Amoxycillin. This is due to the enzyme beta-lactamase that inactivates the antibiotics and renders them ineffective. Similar resistance pattern was observed in the study carried out by Manandhar (2006), Pokhrel (2006) and Poudyal (2010).

ESBL producers showed wide resistance to the non-beta-lactam antibiotics. In the present study, 76.4% of strains showed resistance to ciprofloxacin (p-value <0.05), 79.2% to cotrimoxazole and 8.3% to nitrofurantoin. The high level of drug resistance seen among *E. coli* is mediated by beta-lactamases, which hydrolyze the beta-lactam ring inactivating the antibiotic, the classical TEM-1, TEM-2, and SHV-1 enzymes are the predominant plasmid-mediated beta-lactamases of Gam-negative rods (Livermore, 1995). Mutations at the target site i.e. *gyrA,* which is a gyrase subunit gene, and *parC,* which encodes a topoisomerase subunit, confer resistance to fluoroquinolones (Ozeki *et al.,* 1997). In addition to this mechanism, there are more than seven efflux systems in *Escherichia coli* that can export structurally unrelated antibiotics; these multidrug resistance efflux pump **(**MDR pump) systems contribute to intrinsic resistance for toxic compounds such as antibiotics, antiseptics, detergents, and dyes (Sulavik *et al*., 2001).

Similarly higher level of drug resistance seen among *K. pneumoniae* is mediated by the production of different kind of β-lactamases primarily ESBL, AmpC and

Metallo β-lactamases. The fact that the carriage of resistance trait for quinolones and aminoglycoside in the plasmid along with the gene for β-lactamases have had a great impact on the drug resistance character shown by these pathogenic bacteria (Lee *et al*., 2003; Picao *et al*., 2008 and Walsh *et al*., 2005). The *acr*R and *ram*A genes are involved in expression of the MDR phenotype in strains of *Klebsiella pneumoniae* (Denyer *et al*., 2004). Moreover, various clinical isolates show alteration of nonspecific porins associated with the presence of active drug efflux in these bacteria; both processes maintain a very low intracellular concentration of drugs and contribute to a high resistance level for structurally unrelated molecules including β-lactam antibiotics, quinolones, tetracyclines, and chloramphenicol (Martinez-Martinez *et al*. 2002).

There are many mechanisms whereby *Proteus* spp. confer resistance to the drugs including intrinsic impermeability and acquired resistance as plasmids, transposons and mutations (Gutmann, 1985). He reported in a study on a wide spread of plasmids resistance genes among *Proteus* species that 44% of antibiotic resistance were plasmid mediated, 32% by chromosome, while 24% of the resistance pattern to antibiotics could not be ascertained. Transferable resistance has been identified for some antibiotic groups as β-lactams, aminoglycosides, macrolides, sulphonamides, tetracyclins, chloramphenicol, etc. However the production of plasmid or chromosomal encoded β-lactamase enzymes is the most common mechanism of resistance in gram negative bacteria causing clinical significant infection (Bush *et.al.*, 1995).

For ESBL producing *Enterobacteriaceae,* the imipenem was found to be effective against all the isolates indicating that they are the drugs of choice for treating serious infections caused by ESBL-producing microorganisms, however the other carbapenem agent, meropenem was not found to be as effective as imipenem. Also the data indicate that a valuable option for treatment is represented by Tigecycline (98.6%) and Amikacin, a bactericidal drug effective against 97.2% of ESBL producing strains. In addition, beta-lactam–beta-lactamase inhibitor combinations i.e. Piperacillin-Tazobactam remained quite active against most

isolates (98.6%). This also corresponds to the results reported by other investigators Baral (2008) and Dahal (2013).

Generally most gram negative pathogens exhibit resistant due to presence of enzymes beta lactamase mainly extended spectrum beta lactamase which has capability to hydrolyze most of the penicillin's and 3rd generation cephalosporin and monobactams but not carbapenems and are inhibited by beta lactamases inhibitors such as clavulanic acid and tazobactam.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The prevalence of UTI was comparatively found higher in female patients than in male patients and higher among age group 15-30, the major causative agent being *E. coli* among the gram negative bacteria.

The prevalence of ESBL producing *Enterobacteriaceae* are increasing. In the present study, the increasing pattern of the drug resistance was seen among ESBL producers. ESBL-producing strains are creating significant therapeutic problems since these pathogens are resistant to a wide range of beta-lactams, including third generation cephalosporins as well as have potential for plasmid mediated quinolone resistance which is creating significant therapeutic problems. Combination disk method was found more sensitive than DDST method for the detection of ESBL producers. Imipenem, tigecyclines, amikacin, piperacillin-tazobactam and nitrofurantoin were found to be the most effective anti-microbial agents to use for the ESBLs producing isolates associated infection.

6.2 Recommendations

1. If the patient is not responding to the third generation cephalosporin antimicrobials, the clinician should think for the ESBL-producing organism and request for its test.
2. For the detection of ESBL combination disk method should be used instead of double disk synergy test (DDST) as it yields low sensitivity i.e. 38.89% with respect to combination disk method.
3. Imipenems, tigecyclines, amikacin, piperacillin-tazobactam and nitrofurantoin can be used for the treatment of ESBL producing *Enterobacteriaceae.*

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APPENDIX-A

INFORMED CONSENT FORM

Consent form for Urine sample testing in patients

This is to inform that I have been counseled about the urine sample test to be conducted on me and have been explained about the implications of the test result. All details pertaining to Bacterial pathogens, its transmission, testing procedure, its limitations and interpretations of results have been explained to me in a manner that I can understand. I have been given the return date of my test results (if not same day service). I also understand that I am free to refuse the test and still get the help I need from this centre without being discriminated against.

I hereby give my consent for the test to be conducted in order for me to know my status of urinary tract infection.

Code no. of the participant: … … … … … … … … … … …

Signature of the participant: … … … … … … … … … …

Date: … … … … … … … … … … …

APPENDIX B

CLINICAL AND MICROBIOLOGICAL PROFILE OF PATIENT

Clinical Profile:

Name: … … … … … … … … Lab No: … ……

Age/Sex: … … … … … … …. Date: … … ……

Brief Clinical History: Dysuria……; Frequency…….; Hematuria…….; Fever……..; Abdominal pain……; Others……

Microbiological Profile:

Day 1 (… …/… …/… … … …)

Direct Microscopic Examination (if necessary):

(1) … … … … … … … …

Incubation temperature/Time:…… ……

Culture on: 1) … … … 2) … … 3) … …

Day 2 (… …/… …/… … …. …)

Reading of culture plates:

Media Used	Features	Shape	Size	Color	Texture	Opacity	Consistency

Gram staining result: … … … … …

Catalase: … … … … … Oxidase: … … … … … …

Coagulase: … … … … … Others: … … … … … … …

Provisional Identification of Organism: … … … … … … … … … … … … …

Inoculation on: 1) … … … … … … 2) … … … … … … 3) … … … … … …

Day 3 (… …/… …/… … … …)

BIOCHEMICAL TEST

TSI: … … … … … … SIM: … … … … … …

Citrate: … … … … … … Urea: … … … … … …

Others: … … … … … …

Organism identified as: … … … … … …

Antibiotic sensitivity test method: Kirby-Bauer method

Antibiotics used	***Zone of inhibition (mm)***	***Intrepretation***

Screening agent used	***Zone of inhibition (mm)***	***Intrepretation***

Combination disk used	***Increase in size of zone of inhibition (mm)***	***Intrepretation***

Production of ESBL: Positive…… Negative……..

APPENDIX-C

LIST OF EQUIPMENTS AND MATERIALS

A. EQUIPMENT:

Autoclave, Incubator, Hot air oven, Microscope, Refrigerator, Weighing machine, Water bath, Gas burners, Glass wares, Inoculating wire and loops

B. MICROBIOLOGICAL MEDIA:

Blood Agar	Hugh and Leifson Media
Chocolate Agar	Sulphur Indole Motility Media
Mac conkey Agar	Mueller Hinton Agar
Triple Sugar Iron Agar	Urea Agar Base
Mueller Hinton Broth	Simmon's Citrate Agar

C. CHEMICALS AND REAGENTS:

Catalase Reagent (3% H_2O_2), Oxidase Reagent (1% Tetramethyl p-phenylene diamine dihydrochloride), Kovac's Reagent, Barritt's Reagent (40% KOH, 5% α-napthol in a ratio of 1:3), Barium Chloride, Conc. H_2SO_4, Gram's reagent, etc.

D. ANTIBIOTIC DISKS

The antibiotics that will be required for the susceptibility tests are as follows: Amikacin (30µg), Amoxycillin (10µg), Aztreonam(30µg) , Bacitracin, Cefotaxime (30µg), Ceftazidime (30µg), Ceftriaxone (30µg), Cotrimoxazole (25µg), Ciprofloxacin (5µg), Cefepime (30µg), Gentamycin (10µg), Imipenem (10µg), Meropenem (10µg), Nitrofurantoin (300µg), Piperacillin/Tazobactam, Tigecycline.

APPENDIX-D

I. Composition and Preparation of Different Culture Media

The culture media used were from two companies

a. Hi-Media Laboratories Pvt. Limited, Bombay, India.
b. Oxoid Unipath Ltd. Basingstoke, Hampshire, England.
c. Mast Diagnostics, Mast house, Derby Road, Bootle.

(All compositions are given in grams per liter and at 25^0C temperature)

1. Blood agar (BA)

Blood agar base (infusion agar) + 5-10% sheep blood

Ingredients	gm/liter
Beef heart infusion	500.0
Tryptose	10.0
Sodium Chloride	5.0
Agar	15.0

Final pH (at 25^0C) 7.3±0.2

42.5 grams of the blood agar base medium was suspended in 1000 ml distilled water and sterilized by autoclaving at 121^0C (15lbs pressure) for 15 minutes. After cooling to 40-50^0C, 50 ml sterile defibrinated sheep blood was added aseptically and mixed well before pouring.

2. MacConkey Agar (MA)

(Without sodium taurocholate, without salt and crystal violet)

Ingredients	gm/liter
Peptone	20.0
Lactose	10.0
Sodium taurocholate	5.0
Sodium chloride	5.0
Neutral Red	0.04
Agar	20.0

Final pH (at 25^0C) 7.4±0.2

55 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

3. Mueller Hinton Agar (MHA)

Ingredients	gm/liter
Beef, Infusion form	300.0

Casein Acid Hyrolysate	17.5
Starch	1.5
Agar	17.0

Final pH (at 25^0C) 7.4±0.2

38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve. 10 ml was distributed in test tubes and sterilized by boiling in water bath for 10 minutes.

4. Nutrient Agar (NA)

Ingredients	gm/litre
Peptone	10.0
Sodium Chloride	5
Beef Extract	10.0
Yeast Extract	1.5
Agar	12.0

Final pH (at 25^0C) 7.4±0.2

37 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

5. Nutrient Broth (NB)

Ingredients	gm/litre
Peptone	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5

Final pH (at 25^0C) 7.4±0.2

13 grams of the medium was dissolved in 1000 ml distilled water and autoclaved at 121^0C for 15 minutes.

6. Mueller Hinton Broth

Ingredients	gm/litre
Beef	300.00
Casein Hydroxylate	17.50
Starch	1.50
Calcium	0.003665
Magnesium	6.29

Final pH (at 25^0C) 7.3±0.1

21 gram of the media was added to 1 litre of distil water, mixed well to dissolve and sterilized by autoclaving at 121^{0}C for 15 minutes.

7. Tryptic Soy broth+ 20% Glycerol

Ingredients		gm/litre
Pancreatic Digest of Casein		15.0
Enzymatic Digest of Soybean Meal	5.0	
Sodium Chloride	5.0	
Glycerol	200ml	

40 gram of the media was suspended in 1 litre of distilled water containing 200ml glycerol and mixed thoroughly. It was boiled completely and autoclaved at 121^{0}C for 15 minutes.

II. Biochemical Test Media

1. MR-VP Medium

Ingredients	gm/litre
Buffered Peptone	7.0
Dextrose	5.0
Dipotassium Phosphate 5.0	

Final pH (at 25^{0}C) 6.9±0.2

17 grams was dissolved in 1000 ml distilled water. 3 ml of medium was distributed in each test tube and autoclaved at 121^{0}C for 15 minutes.

2. Hugh and Leifson's Medium

Ingredients	gm/litre
Tryptone	2.0
Sodium Chloride	5.0
Dipotassium Phosphate	0.3
Bromothymol Blue	0.08
Agar	2.0

Final pH (at 25^{0}C) 6.8±0.2

9.4 grams of the medium was rehydrated in 1000 ml cold distilled water and then heated to boiling to dissolve completely. The medium was distributed in 100 ml amounts and sterilized in the autoclave for 15 minutes at 15 lbs pressure (121^{0}C). To 100 ml sterile medium aseptically added

10ml of sterile Dextrose and mixed thoroughly and dispensed in 5 ml quantities into sterile culture tubes.

3. Sulphide Indole Motility (SIM) medium

Ingredients	gm/litre
Beef Extract	3.0
Peptone	30.0
Peptonized Iron	0.2
Sodium Thiosulphate	0.025
Agar	3.0

Final pH (at 25^0C) 7.3±0.2

36 grams of the medium was suspended in 1000 ml distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized.

4. Simmon Citrate Agar

Ingredients	gm/litre
Magnesium Sulfate	0.2
Mono-ammonium Phosphate	1.0
Dipotassium Phosphate	1.0
Sodium Citrate	2.0
Sodium Chloride	5.0
Agar	15.0
Bromothymol Blue	0.08

Final pH (at 25^0C) 6.8±0.2

24.2 grams of the medium was dissolved in 1000ml distilled water. 3ml medium was distributed in test tubes and sterilized by autoclaving at 121^0C for 15 minutes. After autoclaving tubes containing medium were tilted to form slant.

5. Triple Sugar Iron (TSI) Agar

Ingredients	gm/litre
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Beef Extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0

Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0

Final pH (at 25^0C) 7.4±0.2

65 grams of the medium was dissolved in 1000ml of distilled water and sterilized by autoclaving at 15 lbs (121^0C) pressure for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch of thickness.

6. Christensen Urea Agar

Ingredients	gm/litre
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Dipotassium Phosphate	1.2
Mono-potassium Phosphate	0.8
Phenol Red	0.012
Agar	15.0

Final pH (at 25^0C) 7.4±0.2

24 grams of the medium was suspended in 950 ml distilled water and sterilized by autoclaving at 121^0C for 15 minutes. After cooling to about 45^0C, 50 ml of 40% urea was added and mixed well. Then 5 ml was dispensed in test tube and set at slant position.

III. Staining and Test Reagents

1. For Gram's Stain

(a) Crystal Violet solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml

Distilled Water (D/W) to make 1 litre

Preparation: In a clean piece of paper, 20 gm of crystal violet was weighed and transferred to a clean brown bottle. Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Finally the volume was made 1 litre by adding D/W.

(b) Lugol's Iodine

Potassium Iodide	20.0 g

Iodine	10.0 g
Distilled Water	1000 ml

Preparation: To 250 ml of D/W, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1 litre by adding D/W.

(c) Acetone-Alcohol Decoloriser

Acetone	500 ml
Ethanol (Absolute)	475 ml
Distilled Water	25 ml

Preparation: To 25 ml D/W, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

(d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

Preparation: In a clean piece of paper, 10 gm of safranin was weighed and transferred to a clean bottle. Then 1 litre D/W was added to the bottle and mixed well until safranin dissolved completely.

3. Normal saline

Sodium Cholride	0.85 g
Distilled Water	100 ml

Preparation: The sodium chloride was weighed and transferred to a leak-proof bottle premarked to hold 100 ml. Distilled water was added to the 100 ml mark, and mixed until the salt was fully dissolved. The bottle was labeled and stored at room temperature.

4. Test Reagents

a. For Catalase test

Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

Preparation: To 97 ml of D/W, 3 ml of hydrogen peroxide was added and mixed well.

b. For Oxidase Test

Oxidase Reagent (impregnated in Whatman's No. 1 filter paper)

Tetramethyl *p*-phenylene diamine dihydrochloride (TPD) 1 gm

Distilled Water	100 ml

Preparation: This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. To that solution strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

c. For Indole Test

Kovac's Indole Reagent

Isoamyl alcohol	30 ml
p-dimethyl aminobenzaldehyde	2.0 g
Hydrochloric acid	10 ml

Preparation: In 30 ml of isoamylalcohol, 2 g of *p*-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of conc. HCl was added and mixed well.

d. For Methyl Red Test

Methyl Red Solution

Methyl red	0.05 g
Ethyl alcohol (absolute)	28 ml
Distilled Water	22 ml

Preparation: To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml D/W was added to that bottle and mixed well.

e. For Voges-Proskauer Test (Barritt's Reagent)

Solution A

α-Napthol	5.0 g
Ethyl alcohol (absolute)	100 ml

Preparation: To 25 ml D/W, 5 g of α-Napthol was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

Solution B

Potassium hydroxide	40.0 g
Distilled Water	1000 ml

Preparation: To 25 ml D/W, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

Appendix-E

Gram-staining Procedure

First devised by Hans Christian Gram during the late 19th century, the Gram-stain can be used effectively to divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal dye to was out easily with the decolorizer alcohol or acetone (Gram-negative). The following steps are involved in Gram-stain:

1. A thin film of the material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 10-30 seconds.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
8. The slide was flooded with counter stain (safranin) for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 1000X.

APPENDIX-F

BIOCHEMICAL TESTS FOR IDENTIFICATION OF BACTERIA

Catalase test

During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. Catalase enzyme breaks down hydrogen peroxide into water and oxygen. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* sp.

A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g., Blood Agar) or if an iron wire loop is used.

Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test.

A piece of filter paper was soaked with few drops of oxidase reagent. Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

Oxidation-Fermentation test

This test is done to determine the oxidative or fermentative metabolism of carbohydrate resulting in production of various organic acids as end product. Some bacteria are capable of metabolizing carbohydrates (as exhibited by acid production) only under aerobic conditions, while others produce acid both aerobically and anaerobically. Most medical bacteria are facultative anaerobes.

The test organism was stabbed into the bottom of two sets of tubes with Hugh and Leifson's media, bromothymol blue being the pH indicator. The inoculated medium in one of the tubes was covered with a 10 mm deep layer of sterile paraffin oil. The tubes were then incubated at 37°C for 24 hours. After incubation the tubes were examined for carbohydrate utilization as shown by acid production.

Fermentative organism utilizes the carbohydrate in both the open and sealed tubes as shown by a change in colour of the medium from green to yellow. Oxidative organisms, however, are able to use the carbohydrate only in the open tube.

Indole Production test

This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatole (methyl indole) and indoleacetic acid.

A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37°C for 24 hours. After 24 hours incubation, 0.5 ml of Kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

Methyl Red test

This test is performed to test the ability of an organism to produce sufficient acid from the fermentation of glucose to give a red color with the indicator methyl red (denotes changes in degree of acidity by color reactions over a pH range of 4.4-6.0).

A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red color, indicating acidity.

Voges Proskauer (VP) test

This test is employed to detect the production of acetyl methyl carbinol (a neutral end product) or its reduction product 2, 3-butanidiol during fermentation of carbohydrates.

A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of Barritt's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red colour.

Citrate Utilization test

This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. Organisms capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

Motility test

The motility media used for motility test are semisolid, making motility interpretations macroscopic. Motile organisms migrate from the stabline and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stabline, and the surrounding media remains colorless and clear.

Triple Sugar Iron (TSI) Agar

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium).

The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours. Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. Phenol red is the pH indicator which gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

Urea Hydrolysis test

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator incorporated in the medium.

The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37°C overnight. Positive organism shows pink red color due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in colour of the indicator to pink.

APPENDIX-G

Table: Distinguishing reactions of the commoner and pathogenic Enterobacteriaceae

Species	Test/ substrate											
	lac	mot	gas	ind	VP	cit	PDA	ure	lys	H_2S	inos	ONPG
E. coli	+	+	+	+	-	-	-	-	+	-	-	+
Shigella groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-
Sh. sonnei	-	-	-	-	-	-	-	-	-	-	-	+
Salmonella (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-
S. typhi	-	+	-	-	-	-	-	-	+	+	-	-
S. paratyphi A	-	+	+	-	-	-	-	-	-	-	-	-
C. freundii	±	+	+	-	-	+	-	±	-	±	-	+
C. koseri	±	+	+	+	-	+	-	±	-	-	-	+
K. pneumoniae	+	-	++	-	+	+	-	+	+	-	+	+
K. oxytoca	+	-	++	+	+	+	-	+	+	-	+	+
E. aerogenes	+	+	++	-	+	+	-	-	+	-	+	+
E. cloacae	+	+	+	-	+	+	-	±	-	-	-	+
Hafnia alvei	-	+	+	-	+	-	-	-	+	-	-	+
Serratia marcescens[b]	-	+	±	-	+	+	-	-	+	-	±	+
P.mirabilis	-	+	+	-	±	±	+	++	-	+	-	-
P. vulgaris	-	+	+	+	-	-	+	++	-	+	-	-
M. morganii	-	+	+	+	-	-	+	++	-	±	-	-
Providencia rettgeri	-	+	-	+	-	+	+	++	-	-	+	-
P. stuartii	-	+	-	+	-	+	+	±	-	-	+	-
P. alcalifaciens	-	+	+	+	-	+	+	-	-	-	-	-
Yersinia enterocolitica[c]	-	-	-	±	-	-	-	±	-	-	±	+
Y. pestis	-	-	-	-	-	-	-	-	-	-	-	±
Y.pseudotuberc ul-osis	-	-	-	-	-	-	-	+	-	-	-	±

[a] lac, inos, fermentation of lactose, inositol; mot, motility; gas, gas from glucose; ind, indole production; VP, Voges-Proskauer; cit, Citrate utilization (Simmons'); PDA, phenylalanine deaminase; ure, urease; lys, lysine decarboxylase; H_2S, H_2S produced in TSI agar; ONPG, metabolism of *o*-nitrophenyl-β-D-galactopyranoside.

[b] Some strains of Serratia marcescens may produce a red pigment

[c] *Yersinia* are motile at 22°C.

{Key: +, ≥85% of strains positive; -, ≥ 85% of strains negative; 16-84% of strains are positive after 24-48 hour at 36°C}

(Source: Collee *et al.* 1996)

APPENDIX-H

ZONE SIZE INTERPRETATIVE CHART OF ANTIBIOTICS USED

Antimicrobial Agents used	**Symbol**	**Disc Content**	**Resistant (mm or less)**	**Intermediate (mm)**	**Susceptible (mm or more)**
Amoxicilin	Amx	10 µg	13	14-16	17
Ceftriaxone	Ctr	30 µg	13	14-20	21
Ciprofloxacin	Cip	5 µg	15	16-20	21
Cefotaxime	Ctx	30 mcg	12	13-14	15
Cotrimoxazole	Cot	1.25/ 23.75µg	10	11-15	16
Nalidixic acid	NA	30 mcg	13	14-18	19
Gentamicin	G	10 µg	12	13-14	15
Nitrofurantoin	Nit	300µg	14	15-16	17
Norfloxacin	Nx	10 µg	12	13-16	17
Ofloxacin	Of	5 µg	12	13-15	16
Ceftazidime	Caz	30 mcg	14	15-17	18
Amikacin	Ak	30 mcg	14	15-16	17
Cefepime	Cpm	30 mcg	14	15-17	18
Imipenem	Imp	10 mcg	13	14-15	16
Meropenem	Mrp	10 mcg	13	14-15	16
Piperacillin-Tazobactam	Pit	100/10 mcg	17	18-20	21
Tigecycline	Tgc	15 mcg			

(Source: Product Information Guide, Hi-Media Laboratories Pvt. Limited, Bombay, India)