CHAPTER-I
INTRODUCTION

1.1 Background

Over the past half-century, the use of antimicrobial medications has increased steadily throughout the world. Despite the continued development of newer agents, resistant strains of bacteria have challenged our ability to successfully keep infectious diseases aside. One of the most important factors in the acquisition of resistance has been the indiscriminate use of antimicrobials, especially in developing countries (Avorn *et al*., 1987; O'Brien, 1987; Slack, 1989; Kunin, 1993; Tomasz, 1994; Shann, 1995).

Antibiotic pressure is enhanced by the use of sub therapeutic doses of antimicrobial agents that are often of substandard quality. Poor infection control practices and sanitation allow Antimicrobial Resistant (AR) organisms to be spread from person to person, magnifying the effect of their selection or importation. Infections are also likely to be improperly treated or untreated in developing countries, making it likely that AR organisms, when present, will spread. The warm and humid tropical climate is conducive to propagation of bacteria (Rosas *et al*., 1997).

Antimicrobial resistance has an important impact on public health policies and it involves an increasing number of bacterial species and resistance mechanisms and it has been observed that biggest increment in antibiotic-resistance bacteria occurred in those countries where antibiotics are extensively used for prevention or treatment of microbial infections in humans as well as in veterinary medicine (Kummer, 2004; Junco-Diaz *et al*., 2006).

Unregulated dispensing by unqualified personnel is of particular concern. While most modern industrialized countries strictly limit availability of antimicrobials to the general public, many less developed countries do not have or enforce such policies. Resistance patterns which develop regionally under such conditions may then spread globally. Epidemiological studies have linked resistant *Shigella* strains in England and the US travellers recently returned from developing countries (Gross *et al*., 1981; Tauxe *et al*., 1990), and genetic analyses of pathogens in the US

have traced resistance plasmids in enterobacteriaceae to Venezuela (O'Brien *et al*., 1985) and multidrug resistant pneumococci to an endemic region of Spain (Munoz *et al*., 1991).

In Nepal, approximately 90% of drug sales occur in the private sector, predominantly through retailers not trained in pharmacy (Joshi and Khakurel, 1997). The risk of transmission of AR bacteria from one country to another grows as the "global village" shrinks. Strains can be imported into a country and disseminated before their presence is recognized, and countries vary in their capacity to detect and deal with resistant bacteria once introduced. For these reasons, the emergence of an AR bacterial strain in one location becomes a global problem. In recent Scandinavian studies, greater genetic relatedness existed between *Enterococcus faecium* and *Salmonella enterica* isolated from humans in different countries than between strains isolated from humans and animals in the same country (Seyfarth *et al*., 1997; Quednau *et al*., 1999).
Enterobacteriaceae members are broadly distributed in environment and they are etiological agents of a great number of infectious diseases (Schreckenberger *et al.,* 1999). Enterobacteriaceae that produce extended-spectrum beta-lactamases (ESBLs) are an increasing problem worldwide. Many of the outbreaks reported have been associated with cephalosporin usage and controlling third-generation cephalosporin usage appears to have been useful in limiting some outbreaks. Whereas localized outbreaks with ESBL-producing Enterobacteriaceae are well described, the overall prevalence of these organisms is unclear (Landman *et al*., 1999; Rahal *et al.*, 1998). During the past 2 decades, increased use of cephalosporins has been accompanied by the emergence of Enterobacteriaceae possessing extended-spectrum blactamases (ESBLs) (Saurina *et al.*, 2000).

Carbapenem resistance has been reported in only a small number of Enterobacteriaceae and is caused by a number of mechanisms. First, the combination of the high-level production of an AmpC beta-lactamase and loss of outer membrane proteins has been associated with carbapenem resistance in *Klebsiella pneumoniae* and *Enterobacter* species (Martinez-Martinez, 1999). Second, the finding of efficient carbapenem-hydrolyzing beta-lactamases in enterobacteriaceae remains unusual but appears to be increasing. Two distinct classes of beta-lactamases have the ability to hydrolyze carbapenems. Although class B metallobeta-lactamases are usually

associated with *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Klebsiella pneumoniae*. A small number of class A beta-lactamases have been found to possess hydrolytic activity against carbapenems (Chow and Shlaes, 1991).

Extended-spectrum beta-lactamases (ESBLs) are enzymes capable of hydrolyzing a wide range of expanded-spectrum beta-lactams, including most recent cephalosporins, but which are inactive against cephamycins and carbapenems (Livermore, 1995). To date, more than 150 different natural ESBL variants (which are detected most frequently in enterobacteria) are known, and they represent a worldwide problem in hospitalized patients (Sturenburg and Mack, 2003). Infections caused by ESBL-positive organisms often involve compromised patients, making it difficult to eradicate these pathogens in high risk wards such as intensive care units (Babini and Livermore, 2000).

The overall prevalence of ESBL-positive enterobacteria varies greatly among different geographical areas, with the highest reported value (44.9%) in Latin America (Winokur *et al*.**,** 2001). According to published reports, in Europe, ESBLs appeared to be increasing among enterobacteria in the periods 1997 through 1999 to 2001 and 2002 (Bouchillon *et al*., 2004; Nijssen**,** 2004). However, the prevalence of ESBLs differs from country to country, with the highest percentages in Greece (27.4%) and Portugal (15.5%) and the lowest in The Netherlands and Germany i.e. 2.0 and 2.6%, respectively (Bouchillon et al., 2004). *Klebsiella pneumoniae* and *Escherichia coli* are the most common ESBL-positive species, but all enterobacteria can harbor plasmid-mediated ESBL genes. In enterobacteria, classical ESBLs evolved from the TEM and SHV families (Bradford, 2001). In recent years, several new ESBLs of non-TEM, non-SHV types emerged, such as enzymes of the CTX-M, PER, VEB, and GES lineages (Jacoby and Munoz-Price**,** 2005).

Monitoring the ESBL prevalence and type in enterobacteria of clinical interest may contribute to delineating the breadth of the problem and to defining appropriate therapeutic options (Sturenburg and Mack, 2003). Production of extended-spectrum beta-lactamases (ESBLs) is the most prevalent mechanism of resistance to broad-spectrum cephalosporins among species of

enterobacteriaceae that do not carry chromosomal beta-lactamases (Jacoby and Medeiros, 1991). The proportion of ESBL producers among hospital isolates varies between geographical areas. In the United States, ESBLs are produced by more than 25% of intensive care unit *Klebsiella pneumonia* isolates (Saurina *et al.,* 2000), and in some parts of Europe, Asia, and South America they are much more common (Bell *et al.,*2002). The spread of this mechanism of resistance is therefore an emerging problem. Failure to detect and report ESBL production by gram-negative bacteria in a timely manner may result in a significant delay in appropriate antimicrobial treatment. Such delays have been shown to result in increased mortality, morbidity, and health care expenditure (Harbarth *et al.*, 2003).

In the context of Nepal, findings of many studies showed more than half of the isolates belonging to family enterobacteriaceae among gram negatives isolates with highest being *E. coli* (Gautam *et al.,* 1997; Dhakal *et al.,* 1999; Dhital *et al.,* 2000; Rai *et al.,* 2000; Chettri *et al*., 2004; Ghimire *et al.,* 2004; Manandhar *et al.,* 2005; Jha and Bapat, 2005; Bomjan, 2005; Poudyal, 2010). Thapa *et al.,* 2009 found *Citrobacter* sps. being highest followed by *Klebsiella pneumonia*e and *E. coli* among nosocomial isolates. Higher prevalence of MDR, accounting for more than half of total isolates, were found in Shrestha *et al.,* 2005; Manandhar *et al.*, 2005; Bomjan, 2005; Pokahrel *et al,* 2006; Thapa *et al*., 2009; Poudyal, 2010 while less than one forth MDR was found by Tuladhar, 2000. Poudyal, 2010 found more than half of the MDR isolates were positive for ESBL (Extended spectrum beta-lactamase) with about one forth showed ABL (AmpC beta-lactamase) and MBL (Metallo beta-lactamase) positivity while less than one forth ESBL positivity was found by Pokharel *et al.,* 2006. Baral, 2007 found cent percent ESBL positive *E. coli* among all MDR isolates with about eighty percent ABL and MBL positivity.

The emergence and spread of antimicrobial resistance among members of family enterobacteriaceae is of great concern being most frequently encountered isolates of many diseases and their pattern of AR is being changed over the time. Thus there is need for continual surveillance of resistance, rapid identification of such organisms using reliable methods since they heve potential impact on health both in the hospital and community. Hence, this study was

conducted with a aim to determine prevalence and the resistance pattern of bacteria belonging to family enterobacteriaceae.

1.2. Objectives

1.2.1 General objective

To determine the prevalence of multidrug resistant isolates obtained from different clinical samples at Nobel Medical College during January to June 2011.

1.2.2 Specific objectives

i. To identify the bacterial pathogens of family enterobacteriaceae from different clinical specimens collected from patients visiting Nobel Hospital.
ii. To assess the antimicrobial susceptibility pattern of different bacterial isolates.
iii. To assess the burden of multi-drug resistance among the isolates.

CHAPTER-II
LITERATURE REVIEW

2.1 Infections related with bacteria of family enterobacteriaceae

2.1.1 Urinary tract infection (UTI)

Urinary tract infection comprises a wide variety of clinical entities which is the result of microbial invasion of tissues lining the urinary tract extending from the renal cortex to the urethral meatus. It also refers to the presence of bacteria undergoing multiplication in urine within the urinary drainage system. Infection of the adjacent structure such as prostate and epididymis is also included in the definition of urinary tract infection. Infections may be expressed predominantly at a single site, kidney i.e. pelvis and cortex (pyelonephritis), pelvis and ureter (pyelitis), ureter (urethritis), bladder (cystitis), prostate (prostitis) and urethra (urethritis) but the entire urinary tract is always at risk of invasion by bacteria, once any one of its part is infected. As urethra is common site for urinary tract and genital tract, urethritis is also included in sexually transmitted disease especially if the infection is caused by *Niesseria gonorrhea* and *Chlamydia trachomatis.* UTI is defined as the detection of both bacteriuria (> 10^5 CFU/ml) and pyuria (≥10 leucocytes/HPF) (Goya et al, 1997). Urinary tract infection can be defined as a spectrum of disease involving microbial invasion of any of the genitourinary tissues extending from the renal cortex to urethral meatus (Singh *et al*., 1991).

Numerous microorganisms including bacteria, fungi and parasites are involved to cause UTI. The possible bacterial pathogens of family enterobacteriaceae are *E. coli, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis, P. vulgaris, Enterobacter cloacae, E. areogens, Morganella morganii, Citrobacter freundii, C. diversus, Serratia marcescens, Salmonella* Typhi, *S.* Paratyphi A. UTI is more common in women than in men, at least partially because of the short female urethra ant its proximity to the anus. The incidence of infection is highest in women, 20-50% of whom will suffer a clinical episode during their lifetime, however, most of these infections remain undiagnosed and undergo spontaneous remission (Leigh *et al*., 1990).

There are many factors related to MDR among uropathogenes. Some strains of *E. coli* must carry large plasmids longer than 200 kb that contains genes for multiple drug resistance in addition to the genes for cell invasion and cell adhesion (Snyder *et al*., 2003). Bacterial biofilms

are often associated with long term persistence of organisms in various environments. Bacteria in biofilm display dramatically increased resistance to antibiotics. The study also showed significant correlation between biofilm production and multi-drug resistance (Suman *et al*., 2007).

Although urinary tract infection is not usually thought of a disease associated with community-wide outbreaks, certain multidrug-resistant uropathogenic lineages of *E. coli* have exhibited epidemic behavior. *E. coli* O15:K52:H1 caused an outbreak of community-acquired cystitis, pyelonephritis and septicemia in South London in 1987 and 1988 and is an endemic cause of urinary tract infection in Barcelona, Spain (Manges *et al*., 2001; Phillips *et al*., 1988).

2.1.2 Skin, Soft tissue and Wound infections

Skin is not only subjected to frequent trauma and frequent risk of infections but also can reflect internal disease. Skin consists of two major layers: epidermis and dermis which include glands, hairs and nails (Waugh and Grant, 2001). Approximately 15% of all patients who seek medical attention have either some skin disease or skin lesions, many of which are infectious. Many different bacteria, fungi and viruses may be involved (Forbes *et al*., 2007). Different types of skin infections may occur which are most commonly categorized as follows:

a. **Infections in and around hair follicles**

Folliculitis, furunculosis and carbuncles are localized abscesses either in or around hair follicles. For the most part, these infections are precipitated by blockage of the hair follicles with skin oils (sebum) or minor trauma. Among the etiologic agents, *S. aureus* is the most common followed by members of the family Enterobacteriaceae (Forbes *et al*., 2007).

b. **Infections in the deep layers of the epidermis and the dermis**

Most infections in the deep layers of the epidermis and dermis result from the inoculation of microorganisms by traumatic breaks in the skin.

The organisms isolated from subcutaneous abscesses depend on the site of infections e.g. anaerobes are commonly isolated from abscesses of perineal, inguinal and buttock area, whereas nonperineal infections are caused by mixed facultative anaerobic organisms. Necrotizing fasciitis is a serious infection of soft tissue involving overlaying muscle groups. Progressive bacterial synergistic gangrene is usually a chronic gangrenous condition of the skin most often encountered as postoperative complications. Organisms involved for this gangrene are

microaerobic streptococci, *Staphylococcus aureus, Proteus* species and other facultative bacteria (Goldstein, 1992).

c. **Postoperative infections**

Sources of surgical wound infections can include the patient's own normal flora or organisms present in the hospital environment that are introduced to the patients by medical procedures and/or a specific underlying disease or trauma that may interrupt a mucosal or skin surface. The nature of infecting flora depends on the underlying problem and the location of the process. Bacterial pathogens encountered are *S. aureus* followed by *Proteus* species, *Morganella* species, *E. coli* and other enterobacteriaceae (Forbes *et al*., 2007).

The possible MDR pathogens encountered with skin, soft tissues and wound infections include *S. aureus*, Enterobacteriaceae**,** *Pseudomonas aeruginosa*, etc. with highest drug resistance seen amongst the gram negative bacilli to ciprofloxacin (68.42%), gentamicin (60.41%) and beta-lactam group of antibiotics (50%). This could be due to increasing production of extended spectrum beta lactamases (ESBL), MDR efflux pump or co-resistance (Gupta *et al*., 2008).

2.1.3 Upper Respiratory Tract Infection (URTI)

The respiratory tract is arbitrarily divided at the level of the lower border of the cricoids cartilage into upper and lower parts. The nasal cavity, pharynx, epiglottis, larynx constitutes the upper respiratory tract (Forbes et al., 2007).

In the upper respiratory tract, microorganisms live primarily in the areas bathed with the secretions of the mucous membranes. The pathogenic organisms adhere to the walls of nasopharynx and oropharynx, colonize locally and elaborate a toxin that is disseminated systemically adhering preferentially central nervous system and to the muscle cells of the heart (Forbes *et al*., 2007).

The most common Gram-negative bacterial pathogens of URTI are *E. coli, Klebsiella* species, *Enterobacter* species, *Serratia* species, *Proteus* species, *Pseudomonas aeruginosa*, *Acinetobacter* species, etc. (Forbes *et al*., 2007).

There are several risk factors for both colonization and infection of MDR pathogens of URTI like severity of illness, previous exposure to antimicrobial agents, underlying diseases or conditions, invasive procedures (such as tracheostomy), repeated contact with the health care system and previous colonization by a multidrug-resistant organisms (CDC, 2000).

2.1.4 Lower Respiratory Tract Infection (LRTI)

Trachea, bronchi, bronchioles and lungs constitute the lower respiratory tract. Various predisposing factors make LRT more susceptible to colonize by pathogenic microorganisms which go on to cause disease. The aetiology and symptomatology of respiratory diseases vary with age, season, the type of population at risk and other factors (Collier *et al*., 1998).

The possible pathogens of family enterobacteriaceae that cause LRTI are *Klebsiella pneumoniae*, *Proteus* species, and others (Forbes *et al*., 2007).

Multi-drug resistance (MDR) in gram-negative bacteria associated with LRTI is on rise, but its effect on the patient's outcomes is not well established (Kwa *et al*., 2007).

2.1.5 Bloodstream Infection

Microbial invasion of the bloodstream can have serious immediate consequences, including shock, multiple organ failure, disseminated intravascular coagulation (DIC) and death. Approximately 200,000 cases of bacteraemia and fungemia occur annually, with mortality rates ranging from 20% to 50% (Collier *et al*., 1998; Forbes *et al*., 2007).

Bloodstream infections may be intravascular (those that originate within the cardiovascular system) and extravascular (those that result from bacteria entering the blood circulation through the lymphatic system from another site of infection). Intravascular infections include infective endocarditis, mycotic aneurism, suppurative thrombophlebitis and intravenous catheter associated bactaraemia. Extravascular infections include enteric fever, meningitis and epiglottitis. The most common portals of entry are the genitourinary tract (25%) and respiratory tract (25%) (Forbes *et al.,* 2007).

Common bacterial pathogens of family enterobacteriaceae that cause bloodstream infections are *E. coli, Klebsiella* species, *Enterobacter* species*, Proteus* species, *Salmonella* Typhi, etc. (WHO, 2004).

2.1.6 Other bacterial infections

There are many other bacterial infections which are equally important to diagnose in the laboratory as mentioned above like gasterointestinal tract (GIT) infections; meningitis; other central nervous system infections and infections of body fluids. The common infections of GIT are gasteroenteritis, food poisoning, diarrhea, esophagitis, gastritis and proctitis. Stool is frequent sample of GIT infections, if unavailable rectal swab and/or perianal swab may be substituted.

The routine search of bacterial agents is for *Salmonella* species, *Shigella* species, and other enterobacteriaceae (Forbes *et al*., 2007).

2.2 Antimicrobial Resistance

Antibiotics are the frontline therapeutic means for the medical intervention in infection. It is used as either prophylaxis, empiric therapy or pathogen directed therapy. The World Health Organization (WHO), the Europian Commission and the US Centres for Disease Control and Prevention (CDC) have recognised the importance of studying the emergence and determinants of resistance as well as the need for control stratagies (Oteo *et al*., 2005; WHO, 2004).

Antibiotic resistance is the acquired ability of the pathogen to withstand an antibiotic that kills off its sensitive counterparts, such resistance usually arising from random mutations in existing genes or from intact genes that already serve a similar purpose. Exposure to antibiotics and other antimicrobial products, whether in the human body, in animals, or the environment, applies selective pressure that encourages resistance to emerge favouring both 'naturally resistant strains' and which have 'acquired resistance' (ASM, 2009). It is the temporary or permanent ability of an organism and its progeny to remain viable or multiply under environmental conditions, that would otherwise destroy or inhibit other cells (Hugo and Russell, 1993).

Resistance is neither a new phenomenon nor unexpected in an environment in which potent antimicrobial agents are used. The diversity of the microbial world and the relatively specific activities of antimicrobial agents virtually ensures widespread resistance among bacteria. Resistance as a clinical entity is essentially a relative phenomenon and in reality exists as a gradient that reflects phenotypic and genotypic variations in natural microbial populations (Denyer *et al.,* 2004, Forbes *et al*., 2007; ASM, 2009).

2.2.1 Risk factors for the development of antibiotic resistance

Different factors play a role in development of antibiotic resistance but what exactly determines that some bacteria become resistant to a specific drug and not to others and what is the specific role amd the relative weight of each one of these factors in this process remains to be defined (Levi, 2001; Oteo *et al*., 2005)

i. Excessive and irrational over-utilization of antibiotics in out-patient practice and in-hospitalized patients, either therapeutically or prophylactically.
ii. Use of antibiotics in agricultural industry, particularly in the production of food.

iii. Longer survival of severely ill patients.
iv. Longer life expectancy with increased use of antibiotics in elderly.
v. Advances in medical science have resulted in the survival of many patients with severe illness and at risk of infections such as critically ill patients, immunosuppression and congenital diseases (i.e., cystic fibrosis)
vi. Lack of use of proven and effective preventive infection control measures such as hand washing, antibiotic usage restriction and proper isolation of patients with resistant infections.
vii. Increased use of invasive procedures.
viii. Increased use of prosthetic devices and foreign bodies amenable to super infection with resistant bacteria (Levi, 2001; WHO, 2004).

2.2.2 Emergence and Spread of Antimicrobial Resistance

Antibiotic resistance arises by chance through mechanisms that may represent the legacy of natural competition among microorganisms. The mechanisms, genes, and pathways of antibiotic production and resistance help microorganisms compete for niches in nature; therefore, they are fundamental components of microbial life and represent normal evolutionary phenomena. Selection for antibiotic resistance takes place anywhere an antibiotic is present: in the skin, gut, and other areas of the bodies of humans and animals and in the environment. The factors playing significant role in the increases and decreases of prevalence of resistant strains include

i. Host and clone specificity,
ii. Plasmid and clone specificity,
iii. Virulence,
iv. Interactions with other commensal flora,
v. Duration of the selection pressure, and
vi. Variable gene expression (WHO, 2004; ASM, 2009)

The emergence of antimicrobial resistance phenotypes is inevitably linked to the clinical (or other) use of antimicrobial agents against which the resistance is directed. The two major reasons for this association are:

i. Not testing for resistance to antibiotics that are not in clinical use.

ii. Nature abhors vacuum, and so when an effective antimicrobial eliminate susceptible members of the flora, resistant varieties soon fill the niche (CDC, 2002).

2.3 Mechanisms of Antimicrobial Resistance

Resistance mechanisms come in various ways resulting with partial or complete loss of antibiotic effectiveness. Such resistance may be either characteristic associated with the entire species or emerge in strains of normally susceptible species through mutation or gene transfer (Forbes *et al*., 2007).

2.3.1 Microorganism-mediated antimicrobial resistance

Microorganism mediated resistance refers to antimicrobial resistance that is due to genetically encoded traits of the microorganism and is the type of resistance that in vitro susceptibility testing methods are targeted to detect (Forbes *et al.,* 2007). This type of resistance can be divided into two major types:

A. Intrinsic resistance (inherent; natural)

Antimicrobial resistance resulting from the normal genetic, structural or physiologic state of a microorganism is referred to as intrinsic resistance. Intrinsic resistance is usually predictable in a clinical situation. Macrolide, lincosamide and streptogramin are chemically distinct inhibitors of bacterial protein synthesis and their intrinsic resistance in Gram-negative bacilli is due to low permeability of the outer membrane to these hydrophobic compounds (Forbes *et al*., 2007).

B. Acquired resistance

Antibiotic resistance that results from altered cellular physiology and structure caused by change in a microorganism's usual genetic makeup is known as acquired resistance. The presence of this type of resistance in any clinical isolate is unpredictable so it is important to detect resistance. The mechanisms that bacteria acquired to protect themselves from antibiotics can be classified into five basic types (Forbes *et al*., 2007; Denyer *et al*., 2004).

1. **Inactivation of the drug**

a. **Inactivation of -lactam antibiotics:** This inactivation of -lactam ring is catalyzed by a family of related enzymes, the -lactamases which are described in section 2.4

b. **Inactivation of Chloramphenicol by acetylation:** An enzyme, chloramphenicol acetyl transferase (CAT), which acetylates the hydroxyl groups in the side chain of antibiotic. The resulting 1,3-diacetoxychloramphenicol is inactive (Denyer *et al*., 2004).

c. **Inactivation of Aminoglycoside antibiotics:** This resistance is worldwide by more than 50 aminoglycoside modifying enzymes by following three types of inactivation reactions: i) N-acetylation of susceptible amino groups; ii) adenylation (nucleotidylation); or iii) phosphorylation of certain hydroxyl groups (Denyer *et al*., 2004).

2. **Alteration of the target**

Streptomycin resistance in *E coli* is mediated by a single amino acid replacement in either one or two specific positions of protein S12 of the 30S subunit of the ribosome (Alekshun *et al*., 2007; Denyer *et al*., 2004).

3. **Reduced cellular uptake and increased efflux**

A decrease in cellular permeability to the drug may depress the drug concentration at the target site below the inhibitory level.

i. Decreased outer membrane permeability leading to resistance to chloramphenicol has been identified in gram-negative bacteria.
ii. The Tet efflux proteins belong to the major facilitator superfamily which exchange a proton from a tetracycline-cation (usually Mg^{++}) complex reducing intracellular drug concentration and proctecting the target ribosomes leading to tetracycline resistance.

(Alekshun *et al.,* 2007; Forbes *et al*., 2007; Denyer *et al*., 2004)

4. **Metabolic bypass**

Some sulphonamide resistant bacteria do not require extracellular PABA(para-amino benzoic acid), but, like mammalian cells can utilize preformed folic acid (Denyer *et al*., 2004)

5. **Any combination of mechanisms 1 through 4**

Single bacterium may exhibit several types of resistance mechanisms leading to multiple drug resistance and this is usually mediated by R factor (Forbes *et al.,* 2007; Denyer *et al*., 2004)

These basic aspects of resistance are found on following both types of microorganism-mediated antimicrobial resistance.

1. **Mutation and selection (Vertical transmission)**

   The spontaneous mutation occurs in bacterial population as 10^{-7} cells per cell division. Despite the low mutation rate, when this population is exposed to antibiotics they mutated and grow. However, the burden of drug resistance by occurrence of such rare mutation and emergence of resistant organisms in the clinical setting may not be very large, but mutated gene pose threat in clinical condition if it is found on plasmid (Alekshun *et al*., 2007; Forbes *et al*., 2007).

2. **Exchange of gene between strains and species (Horizontal transmission)**

   Drug resistant genes present on chromosome or plasmid of the bacteria can be transferred to similar strains or species or to different species. Horizontal evolution of resistance evolved from vertical evolution and is enhanced by the presence of low level of antibiotics, environmental stress and scarcity of food (Alekshun *et al*., 2007). The genetic elements responsible for drug resistance are as follows:

a. **Gene cassettes and integron:** Gene cassettes are mobile elements that can be captured due to flanking by specific DNA sequences that are recognized by integrase and genes that are apparently not expressed until they become part of an integron (Hall and Collis, 1998). Integrons arespecial case of multi-drug resistance. They are genetic elements that contain genetic determinants of a site-specific recombination system that recognizes and captures mobile gene cassettes (Alekshun *et al*., 2007; Denyer *et al.,* 2004).

   An integron may contain multiple gene cassettes and can be expressed under the control of same promoter and they seem to confer high level of multiple drug resistance to the bacteria that contains them. There are four classes of integrons, among them Class I integrons are more common. It contains at least 60 gene cassettes which encode antibiotic disinfectant resistance; resistance to aminoglycoside, penicillins, cephalosporins, trimethroprim, tetracycline, erythromycin and chloramphenicol (Alekshun *et al.,* 2007; Denyer *et al*., 2004).

b. **Insertion sequences and transposons:** The simplest transposons are called insertion sequences (IS). The IS elements are normal constituents of bacterial chromosome and plasmids. A standard strain of *E. coli* is likely to contain several (<10) copies of any one of the more common IS elements and contain antibiotic resistant genes. The transposons are well known for their capacity to carry multiple antibiotic resistance genes, but only few molecular studies of epidemiology of transposons have been published. Both these elements have two important features in common i.e. both carry genes encoding a '*transposase*'(enzyme necessary for transposition) and short *inverted terminal repeats* at the end of DNA (involved in transposition) (Alekshun *et al*., 2007; Denyer *et al.,* 2004; Snyder *et al*., 2003).

c. **Plasmid:** R-plasmid carry genes for resistance to one and often several antibiotics as described below. These genetic materials can be transferred by following three mechanisms

   1. **Conjugation:** Plasmids are genetic elements most frequently transferred by conjugation encoded by *tra* genes carried by self-transmissible plasmids. Some self-transmissible plasmids can mobilize other plasmids or portions of the chromosome for transfer. The realization of drug resistance transferred during cell conjugation came during 1950_s from a case in Japan, where it was observed that a strain of *Shigella* spp. Isolated during an epidemic of dysentery was resistant to several drugs. Most gram-negative bacilli found in soil, gut of human and animal transfer their MDR traits by conjugation (Bartoloni *et al*., 2006).
   2. **Transformation:** It is the process in which a free DNA is transformed from a donor to a recipient bacterium. The DNA released from donor cell by lysis may be absorbed by 'competent' cells and integrated into their genomes (Denyer *et al.,* 2004; Snyder *et al.*, 2003).
   3. **Transduction:** Transduction is a transfer of genetic information between bacteria by bacteriophages. It is more important in spreading resistance among Gram-positive bacteria than Gram-negative cells (Denyer *et al.*, 2004).

2.4 Multidrug Resistance

Multidrug resistance (MDR) bacterial isolates have been frequently reported from different parts of the world as an emergence of treatment problem. The World Health Organization (WHO), the European Commission (EC), and the U.S. Centers for Disease Control and Prevention (CDC) have recognized the importance of studying the emergence and determinants of multidrug resistance as well as the need for control (Alekshun *et al*., 2007; Oteo *et al.,* 2005; WHO, 2004).

2.4.1 Definition

Multidrug resistance has been defined by various researhers and organizations in different ways in different clinical settings. Some of the commonly used definitions include:

Multidrug resistance is defined as resistance to two or more classes of antimicrobial agents (CDC, 2006).

Multidrug resistance is defined as resistance to at least two antibiotics of different classes including aminoglycosides, chloramphenicol, tetracyclines and/or erythromycin (Huys *et al*., 2005).

Concurrent resistance to antimicrobials of different classes has arisen in a multitude of bacterial species complicating the therapeutic management of infections and are considered multidrug resistant if they show resistance to three or more routinely used antibiotics (Daniel *et al*., 2001).

2.4.2. Genetics of Multidrug Resistance

This is the most common method of antibiotic resistance. Bacterial antibiotic resistance can be attained through intrinsic or acquired mechanisms. Intrinsic mechanisms are those specified by naturally occurring genes found on the host's chromosome, such as, AmpC   -lactamase of gram-negative bacteria and many MDR efflux systems. Acquired mechanisms involve mutations in genes targeted by the antibiotic and the transfer of resistance determinants borne on plasmids, bacteriophages, transposons, and other mobile genetic material. This exchange is accomplished through the processes of transduction (via bacteriophages), conjugation (via plasmids and conjugative transposons), and transformation (via incorporation into the chromosome of chromosomal DNA, plasmids, and other DNAs from dying organisms) (Alekshun *et al*., 2007). Multidrug resistance in bacteria occurs by the accumulation, on resistance (R) plasmids or transposons, of genes, with each coding for resistance to a specific agent (Nikaido, 2009).

2.4.3. Multidrug Resistance and Multidrug Efflux Pumps

Multidrug resistance in many bacteria is due to the action of multidrug efflux pumps, each of which can pump out more than one drug type. The Resistant Nodulation Superfamily Pumps in gram-negative bacteria are usually coded by chromosomal genes and can be over expressed easily and some of them can easily pump out most of the antibiotics currently in use. The several multidrug efflux pumps responsible for resistance are

i. Multidrug Efflux Pumps Belonging to the Major Facilitator Superfamily
ii. Multidrug Efflux Pumps of the Small Multidrug Resistance Family
iii. The Resistance-Nodulation Division Family
iv. Multidrug Efflux Pumps Energized by Ionic Gradients
v. Multidrug Efflux Pumps of the ATP-Binding Cassette Superfamily (Nikaido, 2009)

2.4.4 Multidrug Resistance Caused by Altered Physiological States

The antibiotic susceptibility of bacterial cells is affected by their physiological states. One important consequence of this phenomenon is the occurrence of “persister” cells which is a strategy whereby bacteria naturally generate mixtures of phenotypically different populations, such that one of them can be advantageous to a changing environmental demand limiting the efficacy of antibiotic therapy (Nikaido, 2009).

2.5 - Lactams and -lactamases

2.5.1 -Lactam antibiotics

-lactam antibiotics are the most commonly used antibiotics that kill bacteria by blocking the crucial transpeptidations that lead to mechanically strong peptidoglycan through the covalent cross-links of peptide strands. These includes the penicillins, where the chemical warhead, the four-membered -lactam ring, is fused to a five-membered sulfur ring system, and the cephalosporins, where the -lactam is fused to sulfur-containing ring expanded system. Some other important members of the -lactam group antibiotics include Carbapenems; comprising a family of fused -lactam antibiotics, Monobactams; the monocyclic -lactam antibiotics, the Clavams, represented by clavulanate, not in itself an antibiotic but a mechanism based inactivator of -lactamases and the Oxacephems (Walsh *et al*., 2003; Denyer *et al*., 2004).

-lactam antibiotics are used in a wide variety of both systemic and localized infections including Respiratory tract infections, GI tract infections, CNS infections, Skin and soft tissue infections, Urinary tract infections etc. however, the clinical utility of -lactam antibiotics is under threat with the rapid dissemination and emergence of -lactamases of various types; extended spectrum -lactamases, AmpC -lactamases, and Metallo -lactamases that can easily hydrolyse these antibiotics by breaking down the -lactam ring and rendering the antibiotic harmless. Carbapenems once considered the last resort antibiotics for treating infections caused by multidrug-resistant Gram-negative bacilli, has now become the substrate of the versatile -lactamases i.e. MBL which easily hydrolyse them (Denyer *et al*., 2004; Lee *et al*., 2003; Franklin *et al*., 2006).

2.5.2 -lactamases

-lactamases are a heterogenous group of proteins with structural similarities, composed of -helices and -pleated sheets and are the members of a superfamily of active site serine proteases (Knox *et al*., 1998). lactamases (EC 3.5.2.6) have been designated by the Nomenclature Committee of the International Union of Biochemistry as "enzymes hydrolyzing amides, amidines and other C- N bonds separated on the basis of the substrates cyclic amides" (Bush *et al*., 1995) These enzymes are the major cause of bacterial resistance to -lactam antibiotics and can be either chromosome, plasmid, or transposon encoded and produced in a constitutive or inducible manner. They are secreted into the periplasmic space in gram negative bacteria or into the surrounding medium by their gram positive counterparts whereas membrane associated enzymes have been rarely reported (Livermore *et al*., 1995; Jacoby *et al*., 2005).

-Lactamases of gram negative bacteria are evolving dynamically. New developments include the production of enzymes with novel substrate profiles, reduced susceptibility to -Lactamase inhibitors, and the simultaneous production of multiple types of -Lactamases. The changes represent evolutionary upgrades which provide modern pathogens with a greater potential to resist -Lactam antibiotics and cause formidable therapeutic, infection control, and diagnostic challenges. some of the novel -Lactamases have interchangeable chromosomal and plasmid-mediated genes, making them capable of wide dissemination and conferring the potential for epidemic problems. In some pathogens relatively weak -Lactamases may be augmented by other resistance mechanisms, producing synergystic effects in which -Lactamases have greater

effect than predicted. This has created an increasing number of multiply resistant, and sometimes totally resistant pathogens (Thomson *et al*., 2000; Ahmad *et al*., 1999).

2.5.3 Classification of -Lactamases

Because of the diversity of the enzymatic characteristics of -Lactamases, many attempts have been made to categorize these enzymes by using several attributes like their hydrolytic spectrum, susceptibility to inhibitors, and whether they are encoded by plasmids or chromosome. The classification of Sawai *et al*. in 1968 was the first to get recognised. The Richmond and Sykes classification on the basis of substrate profile in 1973 and later modified by Sykes and Matthew in 1976, Mitsuhashi and Inoue classification in 1981 are some of the noted earlier classification schemes. However, the Molecular Classification scheme of Ambler proposed in 1980 and functional classification scheme proposed by Bush in 1989 and later modified in 1995 by Bush *et al*., 1995 is the most frequently used ones.

Two schemes are currently used to classify -Lactamases: the Ambler classification scheme and the Bush-Jacoby-Medeiros classification system. The Ambler classification scheme separates -lactamases into four distinct classes based on similarities in amino acid sequence. Classes A, C, and D are serine -lactamases, whereas class B are metallo -lactamases that require zinc for activity (Ambler, 1980). Bush-Jacoby-Medeiros classification system classifies -lactamases according to functional similarities i.e. substrate-inhibitor profiles. There are four categories and multiple subgroups in this classification scheme (Group 1, 2, 3 and 2a, 2c, 3a etc) (Bush *et al*., 1995). The Groups 1, 2 and 3 of Bush-Jacoby-Medeiros classification system fall respectively on the Ambler Molecular class C, A/D and B.

2.5.4. Action of -lactamases

-lactamases catalytically disrupt the -lactam (amide) bond to form an acyl enzyme complex. A conserved serine in the active site acts as the reactive nucleophile in the acylation reaction. A critically positioned water then acts as the attacking nucleophile in the deacylation process resulting in the release of penicilloyl and cephalosporyl moiety. Penicillin-binding proteins (PBPs) have the similar mode of action, however, their structure don't allow easy acess of water such that -lactamases have hydolysis rate for -lactams upto 2-3000 times higher than PBPs (Ghuysen *et al*., 1991). The mode of action of -lactamases is shown in Figure 1.

Table1 -Lactamase Classification (Bush *et al*., 1995)

Bush-Jacoby-Medeiros system	Major subgroups	Ambler System	Main Attributes
Group 1 Cephalosporinases		C (cephalosporinases)	usually chromosomal, resistance to all -lactams except carbapenems, not inhibited by clavulanate
Group 2 Penicillinases (Clavulanic acid susceptible)	2a	A (Serine - lactamases)	Staphylococcal penicillinases
	2b	A	Broad spectrum: TEM-1, TEM-2, SHV-1
	2be	A	Extended spectrum: TEM-3??, SHV-2
	2br	A	Inhibitor resistant Tem(IRT)
	2c	A	Carbenicillin hydrolysing
	2e	A	Cephalosporinases inhibited by clavulanate
	2f	A	Carbapenemases inhibited by clavulanate
	2D	D (Oxacillin hydrolysing)	Cloxacillin hydrolysing (OXA)
Group 3 Metallo- - lactamases	3a 3b 3c	B (Metalloenzymes) B B	Zinc dependent carbapenemases
Group 4		Not classified	Miscellaneous enzymes

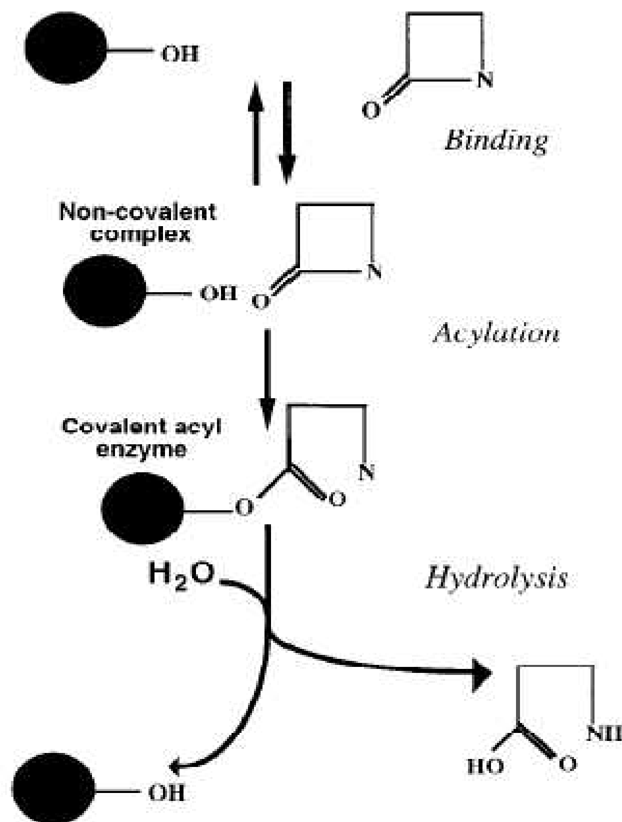

Figure 1: Action of a serine -lactamase (Waley *et al*., 1992). This mechanism desribed above is followed by -lactamases of molecular class A, C, and D whereas class B enzymes utilize zinc ion to attack the -lactam ring.

2.6 Multidrug Resistance among enterobacteriaceae: A Global Scenario

Multidrug resistance among the bacteria is one of the greatest chllenge in the field of medicine. Resistance mechanism to different classes of antibiotics such as tetracyclines, aminoglycosides and cotrimoxazole is of big issue. However, broad spectrum resistance to beta-lactams and to fluroquinolones are today of utmost significance. In early 1950s, enteric bacteria that mediated resistance to first penicillin attracted attention. The introduction of 3rd generation cephalosporins was milestone in antimicrobial chemotherapy but after few years resistance to these drug was observed in enterobacterial species (Sanders and Sanders, 1988). Beta-lactamase related resistance mechanisms were discovered in early 1990s which showed the mobilization of genes coding for enzymes with ESBL activity from environmental bacterial genus *Kluyvera* to bacteria belonging to family Enterobacteriaceae (Bonnet, 2004). The prevalence rate of beta-lactam

resistance among enterobacteraiceae is very significant in european countries especially in *E. coli* and *Klebsiella pneumoniae* (EARSS data, htpp://www.rivm.nl/earss/).

The first ESBL types were described in Central Europe and France during 1980s in *Klebsiella pneumoniae* (Paterson and Bonomo, 2005) but later on emergence of this mechanism in *E. coli* led to a complex epidemiological situation with variety of ESBL and different genetic elements carrying bla_{ESBL} genes (Jeong et al., 2004; Pitout at al., 2005). Recently different bla_{ESBL} genes were isolated from UK, France, Turkey Canada and India (Livermore et al., 2007).

According to published reports, in Europe, ESBLs appeared to be increasing among enterobacteria in the periods 1997 through 1999 to 2001 and 2002 (Bouchillon et al, 2004; Nijssen**,** 2004). However, the prevalence of ESBLs differs from country to country, with the highest percentages in Greece (27.4%) and Portugal (15.5%) and the lowest in The Netherlands and Germany (2.0 and 2.6%, respectively) (Bouchillon *et al*., 2004).

In the study conducted by Meyer et al., 2008, 4.36% *E. coli* and 7.13% *Klebsiella pneumoniae* were resistance to third generation cephalosporins. From the data of SMART program in Asia Pacific region, of 3004 gram negative bacilli collected from intra-abdominal infections during 2007, 42.2% and 35.8% of *E. coli* and *Klebsiella* species respectively were ESBL positive. Moreover, ESBL rates in India for *E. coli, Klebsiella pneumoniae* and *K. oxytoca* were 79.0%, 69.4% and 100% respectively. The rate of ESBL positivity is also higher in China and Thailand as 55.0% and 50.8% respectively (Hawser *et al*., 2009).

Apart from ESBL another important resistance mechanism that prevails is AmpC beta lactamase (ABL) that is emerging globally and is the serious threat in disease treatment. It is estimated that in United States, 3-4% of clinical *Klebsiella pneumoniae* and *K. oxytoca* isolates carry plasmid mediated AmpC enzymes (Black *et al*.,2003). In a study done in United States from 2001 to 2003, ABL were detected in 28 of 853(3.3%) isolates of *Klebsiella pneumoniae*, 5 of 137 (3.6%) *K. oxytoca*, 5 0f 359 (1.4%) *P. mirabilis* and 2 of 4 (50%) *Salmonella* isolates (Ellen *et al*., 2006).

In a study of urinary isolates in India, most isolates were resistant to 4 or more number of antibiotics with 42% of isolates producing ESBL (Akram *et al*., 2007). In contrast, in a study of 11,865 *E. coli* urinary isolates obtained from community and hospitalised patients in East London, high rates of resistance to ampicillin (55%) and trimethoprim (40%), often in

combination were observed in both sets of isolates. Although isolates exhibiting resistance to multiple drug classes were rare, resistance to cefpodoxime, indicative of extended spectrum -lactamase production, was observed in 5.7% of community and 21.6% of nosocomial isolates (Bean *et al*., 2008). In the study of 38853 urinary isolates of *E. coli* in US which was tested against ampicillin, cephalothin, ciprofloxacin, nitrofurantoin and trimethoprim-sulphamethoxazole 7.10% were resistance to three or more agents and considered multidrug resistant (Daniel *et al*., 2001). High rates of resistance to cotrimoxazole have been reported in Israel (31.0%), Spain (32.0%) and Bangladesh (60.0%). Although the prevalence of resistance to ciprofloxacin and other fluoroquinolones has generally remained low, it has reached 18.0% in Bangladesh and 4.0% in Israel. Resistance to norfloxacin is 13.0% in Spain (Gales *et al*, 2000).

In a study conducted during 1999 in US hospitals, the percentage of strains of each species exhibited an MDR phenotype were 1.7% for *E. coli*, 3.0% for *Klebsiella pneumoniae* and 7.7% for *Proteus mirabilis*. For *E. coli* and *Klebsiella pneumoniae*, the most prominent MDR phenotypes were resistance to cephalothin, cotrimoxazole and ciprofloxacin. For *Proteus mirabilis*, the prominent MDR phenotype was resistance to ciprofloxacin, cotrimoxazole and nitrofurantoin (Selman *et al.*, 2000).

A Canadian National Surveillance Study showed that ampicillin, cotrimoxazole, mecillinam, nitrofurantoin and ciprofloxacin mean resistance rates for 2,000 urinary tract isolates collected from outpatients across Canada in 1998 were 41.1%, 19.2%, 14.7%, 5.0% and 1.8% respectively. For *E. coli* isolates alone (n=1,681), comparable rates were 41.0%, 18.9%, 7.4%, 0.1% and 1.2 % respectively. The majority of *E. coli* isolates resistant to ampicillin, cotrimoxazole or ciprofloxacin were susceptible (MIC, <16µg/ml) to mecillinam (Zhanel *et al.*, 2000).

In continuous surveillance of routine samples from five Dutch laboratories to study resistance to the antibiotics most commonly prescribed for UTI in the Netherlands, namely norfloxacin, amoxicillin, trimothoprim and nitrofurantoin from 1989 to 1998 in >90000 *E. coli* isolates; it was found that resistance to norfloxacin increased from 1.3% in 1989 to 5.8% in 1998. Multiresistant, defined as resistance to norfloxacin and at least two of the other three antibiotics, increased from 0.5% in 1989 to 4.0% in 1998 (Goetsch *et al.*, 2000).

The analysis of all pertinent results in the Surveillance Network Data-base-USA from 1 January to 30 September 2000 found that 7.1% of *E. coli* was MDR. Among the MDR isolates, 97.8%

were resistant to ampicillin, 92.8% to cotrimoxazole, 86.6% to cephalothin, 38.8% to ciprofloxacin and 7.7% to nitrofurantoin. Rates of MDR were demonstrated to be higher among males (10.4%) than females (6.6%), among patients >65 years of age (8.7%) than patients <17 (6.8%) and 18 to 65 (6.1%) years of age, and among inpatients (7.6%) than outpatients (6.9%) (Sahm *et al.*, 2001).

The analysis of susceptibility testing data from the Surveillance Network Database-USA (n=286,187) from 1995 to 2001 found out that the resistance rates among *E. coli* isolates to ampicillin (range, 36.0 to 37.4% per year), cotrimoxazole (range, 14.8 to 17.0%), ciprofloxacin (range, 0.7 to 2.5%), and nitrofurantoin (range, 0.4 to 0.8%) varied only slightly over this 7-year period. It was found that in 2001, cotrimoxazole resistance among *E. coli* isolates was >10% in all nine US Bureau of the Census regions (James *et al.*, 2002).

The ECO.SENS study done at 252 community healthcare centres in 16 countries in Europe plus Canada showed that resistance in *E. coli* occurred most frequently to ampicillin (30.0%) and sulphonamides (29.0%), followed by trimethoprim (15.0%), cotrimoxazole (14.0%) and nalidixic acid (5.0%) but was low to co-amoxiclav, mecillinam, cefadroxil, nitrofurantoin, fosfomycin, gentamicin and ciprofloxacin, all at <3.0% (Kahlmeter, 2003).

A retrospective study on all of the bacterial strains isolated from the urine of outpatients who attended the Pasteur Institute of Bangui with a suspected UTI between January 2000 and April 2002 found that more than 84.0% of isolates were Enterobacteriaceae: *E. coli* (55.6%), *K. pneumoniae* (16.9%), *Citrobacter diversus* (4.2%), *Salmonella* species (3.5%) and other Enterobacteriaceae (4.2%). A high percentage of the Enterobacteriaceae were resistant to amoxicillin and cotrimoxazole although most remained susceptible to ciprofloxacin (Hadiza *et al.*, 2003).

In a prospective, multicenter study conducted between March and July 2002 in 15 microbiology laboratories located in nine autonomous regions of Spain, the most frequent pathogen found was *E. coli* (73.0%) followed by *Proteus* species (7.4%), *Klebsiella* species (6.6%) and *Enterococcus* species (4.8%). The susceptibility rates of *E. coli* were 97.9% for fosfomycin, 95.8% for cefixime, 94.3% for nitrofurantoin, 90.8% for amoxicillin-clavulanic acid and 77.2% for ciprofloxacin. Overall fluoroquinolone resistance was near 23.0%, but this rate varied

significantly according to sex, age, type of urinary infection and geographic region (Andreu e*t al.*, 2005).

The study performed with isolates from community-acquired UTIs collected from 15 centres representing six different geographic regions of Turkey showed that 38.0% of *E. coli* strains isolated from complicated UTI were found to be resistant to ciprofloxacin (Arslan *et al.,* 2005).

A study done in various geographic regions in the US and Canada revealed that the most common organisms were *E. coli* (57.5%), *K. pneumoniae* (12.4%), *Enterococcus* species (6.6%), *P. mirabilis* (5.4%), *P. aeruginosa* (2.9%), *Citrobacter* species (2.7%), *S. aureus* (2.2%), *Enterobacter cloacae* (1.9%), Coagulase-negative staphylococci (1.3%), *S. saprophyticus* (1.2%), *Klebsiella* species (1.2%), *Enterobacter aerogenes* (1.1%) and *Streptococcus agalactiae* (1.0%). Among all 1990 isolates, 45.9% were resistant to amp, 20.4% to cotrimoxazole, 14.3% to nitrofurantoin, 9.7% to ciprofloxacin and 8.1% to levofloxacin. Fluoroquinalone resistnce was highest in patients 65 years of age. For the 1142 *E. coli* isolates, resistance rates were: ampicillin (37.7%), cotrimoxazole (21.3%), ciprofloxacin (5.5%), levofloxacin (5.1%) and nitrofurantoin (1.1%). This study reported higher rates of antibiotic resistance in US versus Canada outpatient urinary isolates (Zhanel *et al.*, 2005).

An Italian study conducted during 2004 revealed that the overall prevalence of *E. coli* was 85.3%. *K. pneumoniae*, *S. saprophyticus, P. mirabilis*, *E. faecalis* and other rare species were far less represented. Determination of the antibiotic susceptibility pattern of the entire collection of *E. coli* (512 organisms) revealed that among the drugs analyzed ampicillin was the least active molecule with only 62.5% of the strains being inhibited. Amoxicillin-clavulanate and cefuroxime displayed a higher potency 87.7% and 89.2% repectively. Nitrofurantoin (96.7%) and fosfomycin (98.6%) were the most potent drugs (Fadda *et al.*, 2005).

In a study done from 1998 to 2003 in Manisa in the western part of Turkey, the range of resistance of *E. coli* to ampicillin was found to be 47.8 to 64.6% and that to cotrimoxazole was 37.1 to 44.6% during the study period. About 24.5% of isolates of *E. coli* (216 of 880) were found to be MDR. Among the MDR isolates, 100.0% were resistant to ampicillin and cotrimoxazole, 97.2% to amoxicillin-clavulanate, 87.5% to cefazolin, 80.6% to ciprofloxacin, 74.1% to gentamicin, 33.3% to nitrofurantoin and 30.6% to cefuroxime. MDR ratios were found to be

19.6% in 1998, 21.5% in 1999, 25.0% in 2000, 29.2% in 2001, 26.8% in 2002 and 27.7% in 2003 (Kurutepe *et al.*, 2005).

A prospective clinico-microbiological study including all clinically diagnosed patients with community acquired acute cystitis attending a tertiary care teaching hospital over a period of 3 years was conducted and >35.0% of the urinary *E. coli* isolates were resistant to the fluoroquinolones, which were found to be the most commonly used empirical antibiotics in acute cystitis. Resistance was minimum against nitrofurantoin (9.3%) and amikacin (11.0%). More than 80.0% of the fluoroquinolone-resistant strains were found to be sensitive to nitrofurantoin (Biswas *et al.*, 2006).

2.7 Multidrug Resistance among enterobacteriaceae in Nepal

In developing country like Nepal antibiotic resistance is one of the greatest problem that creat a number of cases of treatment failure. In Nepal, approximately 90% of drug sales occur in the private sector, predominantly through retailers not trained in pharmacy (Joshi & Khakurel, 1997). Such use of antibiotics without prescription, without benefit of guidance from a clinician or even a pharmacist and their indiscriminate usage has favoured the increasing trend of antibiotic resistance as shown by various studies.

In a study done at Maternity Hospital, Thapathali, the prevalence of *E. coli* was found to be much higher (52.5%), followed by *Klebsiella* species (40.7%) and *Proteus* species (6.8%). Among the isolated *E. coli*, 100.0%, 50.0%, 30.0%, 25.0% and 5.0% of the organisms were found to be resistant to ampicillin/amoxicillin, cephalexin, tetracycline, cotrimoxazole and ciprofloxacin respectively and 94.5%, 60.0%, 38.0%, 44.0% and 0% of the isolated *Klebsiella* species were found to be resistant to same antibiotics respectively (Ghimire *et al.*, 1994).

E. coli was found as the most predominant pathogen (57.0%) followed by *Klebsiella pneumoniae* (24.0%), *Proteus* species (10.0%), *Pseudomonas aeruginosa* (1.7%), *Salmonella typhimurium* (1.7%), *Shigella boydii (*1.7%), *Streptococcus faecalis* (1.7%) and *S. aureus* (1.7%). In vitro susceptibility test of these pathogens showed that almost all isolates were susceptible to nitrofurantoin (88.0%), followed by ciprofloxacin (81.0%), nalidixic acid (69.0%) and chloramphenicol (60.0%) whereas cotrimoxazole and amoxicillin were least effective antibiotics against these bacterial isolates (Gautam *et al.*, 1997).

E. coli (47.4%) was the most predominant bacteria followed by *Klebsiella* species (13.2%), *S. aureus* (10.5%) and *Pseudomonas aeruginosa* (7.9%). In vitro susceptibility test showed that nitrofurantoin (84.2%) was only the effective drug followed by norfloxacin (28.9%) and ampicillin (10.5%) against the bacterial isolates (Dhakal *et al.*, 1999).

E. coli was the most predominant pathogen (78.0%) followed by *K. pneumoniae* (9.0%), *Proteus mirabilis* (2.0%), *Pseudomonas aeruginosa* (2.0%), *Citrobacter* species (2.0%) and *Enterobacter* species (1.0%). With regards to antibiotic susceptibility pattern, 80.0% of the Gram negative bacteria were resistant to ampicillin, 72.0% to nalidixic acid, 70.0% to cotrimoxazole and 54.0% to chloramphenicol. Norfloxacin (73.0%) was most active quinolone; while resistance to amikacin was 29.0%. Overall resistance to ciprofloxacin, nitrofurantoin and gentamicin was 32.0% (Dhital *et al.*, 2000).

Rai *et al.,* 2000 found that *E. coli* (61.8%) was the most predominant pathogen followed by *Klebsiella pneumoniae* (12.2%) and *S. aureus* (12.2%). With regards to antibiotic susceptibility pattern, cephalexin (100.0%) was the most effective drug for Gram positive bacteria, followed by nitrofurantoin (93.8%), ciprofloxacin (85.7%), cotrimoxazole (50.0%) and norfloxacin (50.0%). Likewise, nitrofurantoin (77.3%) was the drug of choice in UTI for Gram negative bacteria, followed by gentamicin (59.1%) and cotrimoxazole (40.9%).

Tuladhar, 2000 reported that in 1947 urine specimens, culture positive were found in 517 (26.6%) of which MDR bacterial strains were detected in 122 (23.6%) cases in which *E. coli* 72 (13.1%), *Klebsiella* species 20 (3.9%) and *S. aureus* 13 (2.1%) were the predominants. Out of 1479 urine specimens of hospitalized patients, there were 230 culture positive cases of which MDR bacterial strains were detected in 81 (35.2%) cases in which the most predominants were *E. coli* 51 (22.2%), *Klebsiella* species 14 (6.1%) and *S. aureus* 5 (2.2%).

E. coli was the most common isolate accounting for 77.5% of all bacterial isolates and was followed by *Proteus* species, *Klebsiella* species *and Staphylococcus* species. Ciprofloxacin was found to be most effective antibiotic against *E. coli* followed by nalidixic acid. *Proteus* species was 100.0% susceptible to nalidixic acid and gentamicin (Chhetri *et al.*, 2004).

In a study done at NPHL, it was found that urine samples of kidney transplant patients showed 15.0% positive growth. *E. coli* (46.7%) was the most predominant bacteria causing UTI followed by *Klebsiella* species (13.3%), *Pseudomonas* species (13.3%), *S. aureus* (13.3%), *Proteus* species

(3.3%), *Citrobacte*r species (3.3%), *Streptococcus faecalis* (3.3%) and *Morganella morganii* (3.3%). Gentamicin and amikacin (100.0%) were the most effective drugs against Gram negative bacteria (Ghimire *et al.,* 2004).

Karki *et al.,* 2004 showed that five bacteria isolated were *E. coli* (33.3%), *Proteus* species (27.7%), *Klebsiella* species (16.6%), *S. aureus* (8.8%) and *P. aeruginosa* (1.1%). Nitrofurantoin was the most effective drug against all bacterial isolates.

In a study done at Kathmandu Model Hospital, it was found that the predominant bacteria causing UTI were the Gram negative isolates constituting 88.2% among them 67.9% were MDR strains (Shrestha *et al.*, 2005).

In a study done at TUTH, *E. coli* was the most common isolate accounting for 61.2% of all bacterial isolates followed by *Klebsiella* species (9.2%), *Pseudomonas aeruginosa* (7.1%), *Enterococcus faecalis* (6.1%), *Staphylococcus aureus* (6.1%) and *Proteus mirabilis* (4.1%). Gram negative bacilli showed best susceptibility towards ceftazidime (80.7%) followed by nitrofurantoin (79.5%). Multidrug resistance was observed in 68.4% of the total isolates and it was 61.7% in case of *E. coli* (Manandhar *et al.*, 2005).

In a retrospective study conducted in five hospitals of Kathmandu, the most common organisms causing UTI was found to be *E. coli* (49.0%), followed by *S. aureus* (23.0%), *Klebsiella* species (9.7%), *Proteus* species (3.6%), *Pseudomonas* species (0.8%) and *Citrobacter* species (2.8%). All the organisms causing UTI were found to be susceptible to nitrofurantoin and amoxycillin whereas ciprofloxacin was found to be most effective (Jha and Bapat, 2005).

In a study done at NPHL, *E. coli* was the most common isolate accounting for 43.3% of all bacterial isolates followed by *S. aureus* (23.3%) and *Klebsiella* species (16.6%). Amoxycillin was found to be most effective antibiotic against *E. coli* followed by nalidixic acid and nitrofurantoin. Similarly, amoxycillin and norfloxacin were equally effective against *Klebsiella* species (Jha and Bapat, 2005).

In a study conducted at Tribhuvan University Teaching Hospital (TUTH), 47.57% of the isolates from the sputum and 60.40% of urinary isolates were MDR strains among which 24.27% and 16% of the isolates from sputum and urine respectively were ESBL producers (Pokhrel *et al.*, 2004). In a study of 541 blood isolates of *Salmonella enterica* in TUTH, 5% isolates were found

to be MDR strains with 3 isolates of *Salmonella* Paratyphi A demonstrating ESBL activity (Pokhrel *et al*., 2006).

In a study of fluoroquinolone susceptibility pattern of the *Salmonella* isolates in NPHL, of the 41 *Salmonella* isolates obtained during a seven month period, 2 (4.88%) isolates of *Salmonella* Typhi were multidrug resistant (Acharya, 2008).

In a study of *Salmonella* serovars isolated from urban drinking water supply of Nepal, 35 *Salmonella* isolates were MDR and all the isolates of *Salmonella* Enteritidis and four isolates of *Salmonella* Typhimurium were resistant to ceftriaxone and indicated presence of one of the ESBL genes bla_{SHV} on PCR amplification (Bhatta *et al.,* 2007).

In a study of nosocomial isolates in Kathmandu Medical College (KMC), *Citrobacter* species was accounted as the most frequently isolated nosocomial pathogen with high prevalence of MDR strain followed by *K. pneumoniae* and *E. coli* (Thapa *et al*., 2009)

In a study of prevalence of multidrug resistance clinical isolates in Kathmandu Model Hospital, 41.07% of the clinical isolates were found to be MDR with *E. coli* (46.12%) being the most predominant MDR strain. Of the MDR *E. coli* 100%, 81.03% and 75.75% strains respectively demonstrated ESBL, ABL and MBL activity (Baral, 2008).

In a similar study conducted at TUTH, 68.33% of the urinary and 71.43% of the sputum isolates were MDR with 12 urinary isolates and 3 isolates from sputum demonstrating ESBL activity (Bomjan, 2005).

In the study carried out by Poudyal 2010, of 130 *E. coli* isolates 82(63.08%) were multidrug resistant followed by *Klebsiella pneumoniae* (83.33%). Among 110 bacterial isolates 69 (62.72%) were positive for ESBL production. Majority were *E. coli* (86.96%) followed by *K. pneumoniae* (5.8%), *K. oxytoca*, *P. mirabilis*, *Acinetobacter* species and *Citrobacter freundii* among them 31.82% *E. coli* and 18.18% *K. pneumoniae* showed positivity for ABL production.

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 II.

3.2 Methodology

This study was conducted at Nobel Medical College Teaching Hospital and Research Centre, Biratnagar during January to June 2011. Samples taken from patients of all age groups and both sexes were subjected to routine culture and antibiotic susceptibility testing following standard methodology (Vandepitte *et al*., 2003; Forbes *et al*., 2007).

3.2.1 Sample size and sample types

A total of 2454 different samples including urine (1265), pus (611), body fluids (74), blood (404), stool (32), CSF (27) and sputum (41) were collected in a clean, leak proof plastic container with no visible signs of contamination and labeled properly.

3.3 Collection and transportation of specimen

3.3.1 Urine samples

Patients were asked to collect 10-20 ml of clean voided (clean catch) first morning mid stream urine in a sterile, dry, wide necked, leakproof container, instructing the patient not to halt and restart the urinary system for a midstream urine collection but preferably move the container into the path of the already voiding urine. The container was then labelled properly and immediately delivered to the laboratory with the request form as soon as possible for further processing. When immediate delivery was not possible, the specimen was refrigerated at 4-6^{0}C, and when a delay in delivery of more than 2 hours was anticipated, boric acid (1.8% w/v) was added as preservative to the urine (Vandepitte *et al.,* 2003; Forbes *et al*., 2007).

3.3.2 Sputum samples

The sputum sample was collected in a sterile, leakproof, disposable container under the close supervision of health care worker. The patient was asked not to rinse or gargle the mouth with non-sterile water or mouthwash prior to sample collection and also instructed to collect specimen resulting from deep cough not the saliva or post-nasal discharge. The container was then labelled properly and immediately delivered to the laboratory as soon as possible for further processing (Vandepitte *et al.,* 2003; Forbes *et al*., 2007).

3.3.3 Pus samples

Pus samples were usually obtained from wounds or abscesses that are clinically infected or deteriorating or that fail to heal over a long period. 2% Cholorohexidine followed by an iodine solution was used for disinfecting closed wound and aspirates whereas it was debrided then rinsed thoroughly with sterile saline for open wound prior to collection of sample. Exudate or pus samples was collected from the deepest portion of the lesion. For swab collections, sterile cotton wool swab was gently rolled over the surface of the wound approximately five times where there is evidence of pus or inflamed tissue. The pus swabs thus collected were labelled properly with demographic information, type of specimen and anatomic location and tarnsported to the laboratory. For fluid needle aspiration, the syringe was properly capped and labelled and immediately dispatched to the laboratory (Vandepitte *et al.,* 2003; Forbes *et al*., 2007).

3.3.4 Body fluid specimens (Pleural, Peritoneal and synovial fluid)

These speimens were obtained with the help of trained physician by percutaneous aspiration using care to avoid contamination with commensal microbiota. The needle puncture site was cleaned with alcohol and disinfected with iodine solution to prevent introduction of specimen contamination or infection of patient. About 1-5 ml of the sample was drawn and transported to laboratory, properly labelled, in a sterile tube or vial for further processing (Vandepitte *et al.,* 2003; Forbes *et al*., 2007).

3.3.5 Cerebrospinal Fluid

This procedure was performed under the supervision of physician. CSF samples were collected either by lumbar puncture or ventricular shunt. The puncture site was disinfected with the help of antiseptic solution and alcohol prior to collection then the CSF sample was slowly drained into sterile leakproof tubes, properly labelled, and dispatched to laboratory as soon as possible (Vandepitte *et al.,* 2003; Forbes *et al*., 2007).

3.3.6 Stool

Patients were provided with two small wooden sticks and a clean leakproof plastic container. The patients were instructed to collect the stool specimen on a piece of toilet tissue or old newspaper and transferred it to the container, using the two sticks. At least 5 g of faeces and, if present, those parts that contain blood, mucus or pus were collected avoding the contamination

with urine and sealed with lid. The samples were labeled properly and delivered to the laboratory immediately after collection (Vandepitte *et al.,* 2003; Forbes *et al*., 2007).

3.3.7 Semen

The patients were given sterile, clean, dry, leak proof plastic container and requested to collect the semen following 3-7 days of sexual abstinence. The samples were labeled properly and dispatched to the laboratory as soon as possible. The semen samples collected by the method of coitus interruptus were not generally accepted (Vandepitte *et al.,* 2003; Forbes *et al*., 2007).

3.4 Macroscopic examination of specimens

The urine sample obtained was observed for its color and turbidity and reported accordingly. Similarly, the sputum sample was macroscopically examined to see whether it consisted of only saliva or real sputum. In case if it was found only to be watery, it was reported as 'unsuitable for microbiological examination' and another specimen was requested. The stool sample was obeseved for its color, consistency and presence of any abnormal components (e.g. mucus or blood). Body fluids, CSF were observed for turbidity, blood and stains (Vandepitte *et al.,* 2003; Forbes *et al*., 2007).

3.5 Culture of Specimens

3.5.1 Urine culture

The urine samples were cultured onto the Cysteine Lactose Electrolyte Deficient (CLED) agar plates by the semi-quantitative culture technique using a standard calibrated loop.

i. A calibrated loop was immersed vertically just below the surface of well-mixed uncentrifuged urine specimen.
ii. A loopful of urine was then streaked on the plate to make straight line inoculum down the center of the plate and the urine was streaked by making series of passes at 90^0 angle throughout the inoculum.
iii. The plates were the incubated at 37^0C overnight.
iv. Colony count was performed so as to calculate the number of CFU per ml of urine and the bacterial count was reported according to the Kass, Marple and Sandford criteria as:
    1) Less than 10^4/ml organisms: **not significant**
    2) 10^4-10^5/ml organisms: **doubtful significance** (suggest repeat specimen)
    3) 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 (Forbes *et al*., 2007).

3.5.2 Sputum

The sputum samples were inoculated into the Blood agar and MacConkey agar plates and were incubated at 37^0C in an aerobic condition (Vandepitte *et al.,* 2003; Forbes *et al*., 2007).

3.5.3 Pus, Body Fluids, CSF and Semen

These samples were inoculated into Blood agar and MacConkey agar plates and were incubated aerobically at 37^0C overnight. Additionally, these samples were inoculated into Robertson's Cooked Meat Medium (RCM) Broth for enrichment and incubated aerobically at 37^0C overnight. In case no growth was observed from primary inoculation, the RCM thus inoculated was used to re-inoculate the plates (Vandepitte *et al.,* 2003; Forbes *et al*., 2007).

3.5.4 Stool

About 1 gram of samples were transferred to Selenite-F broth for enrichment and incubated aerobically at 37^0C for 24 hours. They were then subcultured on Blood Agar, Mac Conkey Agar, Xylose–lysine–deoxycholate (XLD) agar and Deoxycholate citrate agar (DCA) and incubated aerobically at 37^0C for 24 hours (Vandepitte *et al.,* 2003; Forbes *et al*., 2007).

3.6 Identificaion of the isolates

3.6.1 Identification of isolates of family enterobacteriaceae

The identification of various gram negative isolates was done by using standard microbiological techniques as described in Bergey's Manual of systemic bacteriology which comprises of studying the colonial morphology, staining reactions and various biochemical properties. Isolated colonies from the pure culture were identified by performing the standard conventional biochemical tests (Catalase, Oxidase, Indole, Methyl Red, Voges-Proskauer, Citrate, Triple Sugar Iron and Urease tests) (Appendix VI).

3.6.2 Antimicrobial susceptibility testing

Susceptibility tests of the different clinical isolates towards various antibiotics were performed by modified Kirby-Bauer disk diffusion method. The procedure for antibiotic susceptibility testing is as follows:

1) Mueller Hinton agar (MHA) was prepared and sterilized as instructed by the manufacturer.
2) The pH of the medium was adjusted to 7.2-7.4 and the depth of the medium at 4mm (about 25 ml per plate) was maintained in petri-dish.
3) Using a sterile wire loop, a single isolated colony whose susceptibility pattern is to be determined was touched and inoculated into peptone water tube and was incubated at 37^0C for 2-4 hrs.
4) After incubation, the turbidity of the suspension was matched with the turbidity standard of 0.5 MacFarland tube.
5) Using sterile swab, the plate of MHA was inoculated with the bacterial suspension using carpet culture technique. The plate was left for about 5 minutes to let the agar surface dry.
6) Using sterile forceps, appropriate antimicrobial discs (6 mm diameter) was placed, evenly distributed on the inoculated plates; no more than 6 discs were placed on a 90 mm diameter Petri-dish.
7) After overnight incubation, the plate was examined to ensure confluent growth and the diameter of each zone of inhibition in mm was measured and compared with standard zone interpretative chart provided by the company (Vandepitte *et al*., 2003).

The preparation and composition of Mueller Hinton agar medium is mentioned in the Appendix I.

3.6.3 Quality control during antimicrobial susceptibility testing

Mueller Hinton agar and the antibiotic discs were checked for their lot number, manufacture and expiry date, and proper storage. The culture and biochemical media tubes and plates were checked for final pH testing, sterility testing and performance testing (by *E. coli* ATCC 25922). For the standardization of Kirby-Bauer test and for performance testing of antibiotics and MHA,

control strains of *E. coli* (ATCC 25922). Quality of sensitivity tests was maintained by maintaining the thickness of Mueller-Hinton agar at 4 mm and the pH at 7.2-7.4.

3.6.4 Data analysis

Chi-square test was used to determine significant association of dependable variables like bacterial infections, MDR etc. to different independent variables (gender, age, type of patients etc.) . The deatiled of data analysis is shown in Appendix VIII.

CHAPTER-IV
RESULTS

A total of two thousand four hundred and fifty four different non-repeated clinical samples were collected and processed for culture and antibiotic susceptibility test.

4.1 Growth patterns

Of total 2454 different non-repeated clinical samples processed, the total growth was 25.35% (n=622). Among them gram positive and gram negative bacteria accounted for 48.0% (n=298) and 52.0% (n=324) respectively. Of total gram negatives isolated, 92.60% (n=300) belonged to family enterobacteriaceae and 7.40% (n-=24) were gram negatives other than enterobacteriaceae.

The most predominant was urine 1265 (51.55%) followed by pus and blood samples that accounted for 611 (24.90%) and 404 (16.46%) respectively. Of total samples, 12.22 % (300/2454) bacterial growth was found for enterobacteriaceae with highest isolates being obtained from urine samples 177/300(59.0%) followed by pus samples 108/300(36.0%). Regarding growth rate, highest growth rate was found in pus samples followed by urine samples of 17.67% and 14.00% respectively. No isolates of family enterobacteriaceae were obtained from fluid, CSF and stool samples. (Table 2)

Table 2: **Distribution of different clinical samples and growth pattern**

Specimens	**No. of specimens**		**Growth**	
	No.	**%**	**No.**	**%**
Urine	1265	51.55	177	14.00
Pus	611	24.90	108	17.67
Blood	404	16.46	14	3.46
Fluid(pleural and peritoneal fluid)	74	3.00	0	0.00
Sputum	41	1.67	1	2.44
Stool	32	1.30	0	0.00
CSF	27	1.10	0	0.00
Total	**2454**		**300**	**12.22**

4.2 Distribution of samples and patient types

Out of 2454 samples, most predominant were constituted from outdoor patients with 1507(61.41%) cases. Of the total outdoor samples, 10.55% (159/1507) samples showed growth

with higher in females (7.63%) than in males (2.92%). Highest growth was found from indoor samples 14.89 % (141/947) with females and males of 7.92% and 6.97% respectively. (Table 3)

Table 3: Distribution of the samples on patient types

Type of Patients	Male		Female		TOTAL (Isolate %) (Isolate)
	No. (%)	Isolates (%)	No. (%)	Isolates (%)	
Outdoor	490 (19.96)	44 (2.92)	1017 (41.44)	115 (7.63)	1507 (10.55) 159
Indoor	484 (19.72)	66 (6.97)	463 (18.86)	75 (7.92)	947 (14.89) 141
Total	**974 (39.68)**	**110 (4.55)**	**1480 (60.30)**	**190 (7.74)**	**2454 (12.22) 300**

Out of 2454 clinical samples, predominant number of samples 1480(60.31%) with highest growth of 13% (191/1480) were found in female patients of all age groups. The age group 21-30 years was found highest of 536 (21.84%) for culture. Age group 81-90 years and over 90 years were least with only 20 (0.81%) cultures. Maximum isolates were found from age group of 41-50, 18.52 % (n=243) whereas least isolates were found from age group over 80 (n=20). (Table 4)

Table 4: Age and gender wise distribution of patients for culture and their growth pattern

Age group (in years)	Outdoor patients		Indoor patients		TOTAL (Isolates)	(Isolate%)
	Male (Isolates)	Female (Isolates)	Male (Isolates)	Female (Isolates)		
0-10	123(7)	186(5)	190(14)	133(5)	632(31)	4.90
11-20	79(5)	181(21)	74(8)	45(6)	379(40)	10.55
21-30	135(10)	239(49)	91(15)	71(14)	536(88)	16.42
31-40	51(2)	140(10)	54(11)	74(14)	319(37)	11.60
41-50	23(1)	130(18)	14(5)	76(21)	243(45)	18.52
51-60	31(7)	82(9)	35(7)	33(9)	181(32)	17.67
61-70	22(7)	44(2)	12(4)	16(2)	94(15)	15.95
71-80	18(4)	10(0)	11(2)	11(3)	50(9)	18.0
>81	8(1)	5(1)	3(0)	4(1)	20(3)	15.0
TOTAL	**490(44)**	**1017(115)**	**484(66)**	**463(76)**	**2454(300)**	**12.22**

4.3 Pattern of bacterial isolates from different samples

4.3.1 Microbiological profile of urine samples

Of the 1265 urine samples, 286 (22.60%) samples were received from the male patient whereas 979 (77.40%) samples from female patients, the maximum number of culture request being received from age group 21-30 yrs; 326 (25.77%) samples and the least being received from age >80 yrs; 15 (1.185%) samples. Of the 286 samples from male, 47 (16.43%) showed signifcant growth with maximum no. of growth being observed in age group 21-30 yrs. and 61-70 yrs; with each of 9/47 (19.15%) respectively. Similarly, of the 979 samples from female, 130 (13.28%) showed significant growth with maximum no. of growth being observed in age group 21-30 yrs; 54/130 (41.54%). The highest prevalence of multidrug resistant isolates was observed in age group 21-30 yrs with 31/98 (31.63%), followed by 51-60 yrs with 11/98 (11.22%). (Appendix VII)

Of 177 isolates, *E. coli* were predominant constituting 163/177 (92.10%) of the total isolates followed by *K. pneumoniae and Citrobacter* species with 7(3.95%) each. (Table 5)

Table 5: Microbiological profile of Urinary isolates and their genderwise distribution

Organisms	Male		Female		Total(%)
	No.	%	No.	%	
E. coli	44	26.99	119	73.01	163(92.10)
K. pneumoniae	2	28.57	5	71.43	7(3.95)
Citrobacter species	2	28.57	5	71.43	7(3.95)

4.3.2 Microbiological profile of blood samples

Of 404 blood samples, 249(61.63%) were from male and 155(38.37%) were from female. Among 249 samples 11(4.42%) and 155 samples 3(1.93%) showed significant growth in male and female patients respectively with highest isolates obtained from age group below 10 yrs (Appendix VII).

Of 14 isolates, *Citrobacter* species were most predominant, 8/14 (57.14%) followed by *E. coli* (28.57%), *K. pneumoniae* (7.14%) and *Enterobacter* species (7.14%). (Table 6)

Table 6: Microbiological profile of blood isolates and their genderwise distribution

Organisms	Male		Female		Total (%)
	No.	%	No.	%	
Citrobacter spp.	6	75.00	2	25.00	8(57.14)
E. coli	3	75.00	1	25.00	4(28.57)
*Enterobacte*r spp.	1	100.00	0	0.00	1(7.14)
K. pneumoniae	1	100.00	0	0.00	1(7.14)

4.3.3 Microbiological profile of pus samples

Of 611 (328 from male and 283 from female) processed pus samples, 108 (17.67%) samples i.e. 51/328 (15.55%) and 57/283 (20.14%) from male and female patients respectively showed growth with highest growth isolated from age group 41-50 yrs. (Appendix VII)

Of 108 isolates, *E. coli* were predominant constituting 78/108 (72.22%) of the total isolates followed by *Citrobacter* species (19.44%), *K. pneumoniae* (6.48%), *P. mirabilis* (0.925%) and *M. morganii* (0.925%). (Table 7)

Table 7: Microbiological profile of pus isolates and their genderwise distribution

Organisms	Male		Female		Total (%)
	No.	%	No.	%	
E. coli	38	48.72	40	51.28	78(72.22)
Citrobacter spp.	8	38.10	13	61.90	21(19.44)
K. pneumoniae	3	42.86	4	57.14	7(6.84)
P. mirabilis	1	100.00	0	0.00	1(0.92)
M. morganii	1	100.00	0	0.00	1(0.92)

4.3.4 Microbiological profile of sputum samples

Of 41 (21 from male and 20 from female) processed sputum samples, 1 (2.44%) sample i.e. 1/21 (4.76%) and 0/20 (0.00%) from male and female patients respectively showed growth. (Appendix VII)

Of the 41 samples only single isolate of *K. pneumoniae* was isolated. (Table 8)

Table 8: Microbiological profile of sputum isolates and their genderwise distribution

Organisms	Male		Female		Total (%)
	No.	%	No.	%	
K. pneumoniae	1	100.00	0	0.00	1(100.00)

4.3.5 Microbiological profile of CSF and body fluid samples

Out of the 27 (20 from male and 7 from female) and 74(50 from male and 24 female) processed CSF and Body fluids samples respectively, no growth of bacteria of family enterobacteriaceae obtained. (Appendix VII)

4.3.6 Microbiological profile of stool samples

Out of the 32 (20 from male and 12 from female) processed stool samples no growth of bacteria of family enterobacteriaceae obtained. (Appendix VII)

4.4 Antibiotic susceptibility pattern of the isolates

Antibiotics were applied according to the guideline of CLSI, 2010 for gram negative bacteria. Of the 15 different antibiotics used to study the sensitivity of the isolates, imipenem was found to be the drug of choice with a susceptibility of 98.11% (52/53) followed by Amikacin with 89.55% and meropenem with 89.47% susceptibility. Among others Chloramphenicol, Piperacillin+Tazobactam, Gentamicin, Ciprofloxacin, Cefotaxime and Ceftazidime were found to be more effective with 78.35%, 74.64%, 69.18%, 53.50%, 52.90 and 42.34% susceptibility, whereas; nalidixic acid, amoxycillin+clavulanic acid and amoxycillin were found to be the least effective drugs with susceptibility of 29.10%, 7.547% and 0.00% respectively. (Table 9)

4.4.1 Antibiotic susceptibility pattern of *E. coli* isolated from different samples (n=237)

The antibiotic susceptibility pattern of *E. coli* showed that imipenem, amikacin, nitrofurantoin and meropenem were drug of choice with susceptibility 100%(38), 93.22%, 94.97% and 87.50% respectively. Ceftazidime and cefotaxime showed the susceptibility of 43.48% and 54.33% respectively. (Table 10)

Table 9: Antibiotic sensitivity pattern of the isolates from different samples

Antibiotic used	Sensitive		Intermediate		Resistant		Total
	No.	%	No.	%	No.	%	
Amoxycillin	0	0.00	0	0.00	138	**100.0**	138
Amoxycillin+clavulanic acid	4	7.547	--	--	147	**92.45**	151
Cefotaxime	137	52.90	5	1.93	117	45.17	259
Ceftazidime	83	42.34	5	2.55	108	55.10	196
Piperacillin+Tazobactam	103	74.64	10	7.25	25	18.11	138
Gentamicin	202	69.18	9	3.08	81	27.74	292
Amikacin	257	**89.55**	5	1.74	25	8.71	287
Nalidixic acid	71	29.10	0	0.00	173	**70.90**	244
Norfloxacin	76	45.24	0	0.00	92	54.76	168
Ciprofloxacin	145	53.50	9	3.32	117	43.18	271
Imipenem	52	**98.11**	0	0.00	1	1.89	53
Meropenem	170	**89.47**	0	0.00	20	10.53	190
Chloramphenicol	76	78.35	1	1.03	21	21.65	97
Co-trimoxazole	23	40.35	0	0.00	34	59.65	57
Nitrofurantoin	158	**96.34**	7	4.27	6	3.66	164

Table 10: Antibiotic susceptibility pattern of *E. coli* isolated from different samples

Antibiotic used	Sensitive		Intermediate		Resistant		Total
	No.	%	No.	%	No.	%	
Amoxycillin	0	0.00	0	0.00	130	**100.0**	130
Amoxicillin+clavulanic acid	4	10.53	--	--	132	**89.47**	136
Cefotaxime	113	54.33	5	2.40	90	43.27	208
Ceftazidime	70	43.48	5	3.10	86	53.42	161
Piperacillin+Tazobactam	81	75.70	9	8.41	17	15.88	107
Gentamicin	176	73.64	9	3.76	54	22.60	239
Amikacin	220	**93.22**	5	2.12	11	4.66	236
Nalidixic acid	63	27.15	0	0.00	169	**72.84**	232
Norfloxacin	66	42.30	0	0.00	90	57.70	156
Ciprofloxacin	106	48.00	9	4.07	106	48.00	121
Imipenem	38	**100.0**	0	0.00	0	0.00	38
Meropenem	167	**87.50**	0	0.00	20	12.50	187
Chloramphenicol	56	82.35	1	1.47	11	16.17	68
Co-trimoxazole	21	42.00	0	0.00	29	58.00	50
Nitrofurantoin	151	**94.97**	4	2.51	4	2.51	159

4.4.2 Antibiotic susceptibility pattern of *Citrobacter* species isolated from different samples (n=36)

The antibiotic susceptibility pattern of *Citrobacter* species showed that imipenem, meropenem, Norfloxacin, Ciprofloxacin, Chloramphenicol and Amikacin were drug of choice with susceptibility 100%, 100%, 80.0%, 79.40%, 73.68% and 73.53% respectively. Amoxycillin, Amoxycillin+clavulanic acid, Ofloxacin and Co-trimoxazole were least effective with susceptibility of 0.00%. Cefotaxime and Ceftazidime showed the susceptibility of 35.53% and 35.30% respectively. (Table 11)

Table 11: Antibiotic susceptibility pattern of *Citrobacter* species isolated from different samples

Antibiotic used	Sensitive		Intermediate		Resistant		Total
	No.	%	No.	%	No.	%	
Amoxycillin	0	0.00	0	0.00	5	**100.0**	**5**
Amoxicillin+clavulanic acid	0	0.00	--	--	12	**100.0**	**12**
Cefotaxime	12	35.30	0	0.00	22	64.70	**34**
Ceftazidime	9	34.62	0	0.00	17	65.38	**26**
Piperacillin+Tazobactam	15	65.22	1	4.35	7	30.43	**23**
Gentamicin	13	38.24	0	0.00	21	61.76	**34**
Amikacin	25	73.53	0	0.00	9	26.47	**34**
Nalidixic acid	2	40.00	0	0.00	3	60.00	**5**
Norfloxacin	4	80.00	0	0.00	1	20.00	**5**
Ciprofloxacin	27	79.40	0	0.00	7	20.60	**34**
Imipenem	1	**100.0**	0	0.00	0	0.00	**1**
Meropenem	1	**100.0**	0	0.00	0	0.00	**1**
Chloramphenicol	14	73.68	0	0.00	5	26.32	**19**
Co-trimoxazole	0	0.00	0	0.00	1	**100.0**	**1**
Nitrofurantoin	2	40.00	2	40.00	1	20.00	**5**

4.5 Antibiotic resistance pattern of the isolates

4.5.1 Distribution of MDR isolates among different samples (n=178)

Of total isolates, *E. coli* was the most prediminant (79.0%) followed by *Citrobacter species* (12.0%), *K. pneumoniae* (5.33%) and *Enterobacter species*, *P. mirabilis* and *M. morganii* with 0.33% each. Of total *E. coli* isolates, (80.90%) were MDR isolates followed by *Citrobacter*

species and *K. pneumoniae* with 14.04% and 3.4% respectively (n=178). *P. mirabilis*, *M. morganii* and *Enterobacter* species showed 100% MDR of total isolated. (Table 12).

Table 12: Multipledrug resistant profile of isolates from different samples

Sample	Urine(MDR) %	Pus(MDR) %	Blood(MDR) %	Sputum(MDR) %	Total(MDR) %
E. coli	155(93)60.00	78(50)64.10	4(1)25.00	--	237(144)60.76
C. spp.	7(4)57.14	21(15)71.43	8(6)75.00	--	36(25)69.44
K. pneumoniae	7(1)14.28	7(5)71.42	1(0)0.00	1(0)0.00	16(6)37.50
P. mirabilis	--	1(1)100.00	--	--	1(1)100.00
M. morganii	--	1(1)100.00	--	--	1(1)100.00
Enterobacter spp.	--		1(1)100.00	--	1(1)100.00

4.5.2 Distribution of MDR among gender and type of patients

Out of total 300 drug resistant isolates from different samples, MDR isolates were found as 59.0% (177/300) strains, among these, majority strains were isolated from female outpatients with 31.64% (56/177) MDR strains. Similarly out of 300 drug resistant isolates, non-MDR isolates were found as 41% (123/300) strains, among these, most strains 48% (59/123) were isolated from female outpatients. (Table 13)

Table 13: **Distribution of MDR among gender and type of patients**

Pattern of drug resistance	Male		Female		Total
	Out-patient	In-patient	Out-patient	In-patient	
Multidrug resistance (MDR)	30	42	56	49	**177**
Non-multidrug resistance (Non-MDR)	14	24	59	26	**123**
Total	**44**	**66**	**115**	**75**	**300**

4.5.3 Pattern of MDR strains and Non-MDR strains from different samples among age of patients

Among total 177 MDR isolates from different samples, predominant MDR isolates (43/177) was belonged from the patients of age group 21-30 years, among these 31 MDR

isolates were found from urine samples whereas 12 MDR isolates were obtained from pus samples. Out of 123 Non-MDR isolates from different samples, predominant non-MDR isolates (87/123) was belonged from the patients of age group 21-30 years, among these 63 non-MDR isolates were found from urine, 23 from pus and 1 from sputum. The least number of MDR isolates were found from the patients of age group of over 80 years with 2 MDR strains; among these all strains were isolated from urine samples. (Appendix VII)

CHAPTER-V
DISCUSSION

This study was conducted during January to June 2011 in Nobel Medical College Teaching Hospital and Research Centre, Biratnagar to determine the status of multiple drug resistance among bacteria of family enterobacteriaceae.

Out of 2454 different clinical samples (Urine, Sputum, Pus, Body fluids, Blood, CSF and Stool) major portion was contributed by urine with 1265 (51.55%) followed by pus and blood samples with 611 (24.9%) and 404 (16.46%). Similar pattern of sample distribution was shown by Poudyal, (2010); Baral, (2008); Dhungel, (2001). Among total samples, 1480 (60.30%) were from female and 974 (39.68%) from male. Similarly, 1507 (61.41%) samples were from outdoor patients and 947 (38.59%) from indoor patients. Similar findings were obtained from work of Poydyal, (2010); Baral, (2008); Bomjon, (2005).

Age group of 0-10 years and 21-30 years had maximum samples of 632 (25.75%) and 536 (21.84%) for culture of specimen respectively. Out of total samples, 300 (12.22%) showed positive for enterobacteriaceae among which 63.33% were from female whereas 36.67% from male. Similar studies were carried out by Poudyal, (2010); Baral, (2008); Dhakal, (1999) with growth positivity of 19.61%, 22.35% and 25.16% respectively.

Out of 1265 urine samples only 177 (14.00%) samples showed significant growth. The low growth positivity was observed in similar studies carried out by Poudyal, (2010); Baral, (2008); Shrestha, (2007); Bomjan, (2005); Chettri *et al*., (2001); Dhakal, (1999). The low growth rate might be due to inclusion of every patients requesting for urine culture regardless of their symptoms and illness or prior use of antibiotics or it might be due to presence of fastidious organisms that we are not be able to grow on routine culture media (Manandhar, 1996). However, very low growth positivity (4.6%) had also been reported from elsewhere (Tuladhar, 1990).

Large number of samples for culture was from females (74.60%) than male (22.60%) with maximum number of culture being received from age group 21-30 years (25.77%) having highest percentage of positivity. Similar findings were observed in the earlier studies by Poudyal, (2010); Baral, (2008); Shrestha, (2007); Bomjan, (2005); Rajbhandari and Shrestha, (2002); Chhetri *et al*., (2001). Contrary to the earlier studies with high rate of positivity among female

this study showed higher rate of positivity in male (16.43%) than female (13.28%). This might be a result of nosocomial infections.

Out of total 404 blood samples, 14 (3.46%) showed positive growth. This was similar from the findings of Poudyal, (2010); Baral, (2008); Shakya, (2001); Shrestha, (1996). Out of 611 pus samples, 108 (17.67%) showed growth positivity. Similar findings were observed in the work of Poudyal, (2010); Baral, (2008); Dhungel, (2001); Rai *et al.,* (2000). However, no isolates of family enterobacteriaceae were recovered from body fluids, CSF and stool samples. No recovery of pathogenic isolates from stool sample might be due to inappropriate timing of collection or prior exposure to antibiotics.

Out of 41 sputum samples 1 (2.44%) showed positive growth. This was similar from findings of Poudyal, 2010 with recovery of 3.66% isolates belonging to enterobacteriaceae family. Such low rate of recovery might be due to the factors such as prior exposure to antibiotics or infection of respiratory tracts by viruses and other organisms like *Mycoplasma* species, Chlamydiae, Fungi, *Legionella* species etc. (Smith and Easmon, 1990).

The growth pattern of pathogens from different samples was increased from the age group 31-40 years to 81-90 years with highest positivity rate of 18.92%. This pattern indicates that the chances of bacterial infection increases with the age which supports the fact of lowered immunity with age. Although the number of samples was high in outdoor samples, the growth positivity was found to be higher in case of indoor samples with 14.89%. This supports the fact of nosocomial infections and severity of infections in case of indoor patients (Podyal, 2010; Baral, 2008).

Of total urine samples, majority of samples were from female patients (77.40%) with higher being reported from age group 21-30 years (25.77%). Similar findings were reported from the study of Steenberg *et al*., (1985); Manandhar *et al*., (1996); Rajbhandari and Shrestha, (2002); Regmi *et al*., (2003); Shrestha *et al*., (2005); Jha and Bapat , (2005); Shrestha, (2007). The significant growth among females was found to be 13.28% with maximum number of growth being observed in age group 21-30 years (41.54%). The females of this age group are sexually active and are of child bearing age. A number of studies suggest that sexual activity is important factor in the pathogenesis of UTI in women (Kunin and McCamack, 1968; Leigh, 1990; Forbes *et al.,* 2002).

Among the uropathogens, *E. coli* (90.10%) was found to be most predominant organism followed by *Klebsiella pneumoniae* and *Citrobacter* species (3.95%) for both. This data resembles with the studies of Chettri *et al.,* (2001); Sharma *et al*., (1983); Tuladhar *et al*., (1989); Jha and Yadav, (1992); Manandhar *et al*., (1996); Dhakal *et al*., (1999); Shrestha, (2007); Baral, (2008); Poudyal, (2010) in Nepal and Steenberg *et al.,* (1969); Kalhmeter, (2003); Leigh, (1990); Fowler, (1990); Kosakai, (1990); Farrel *et al.,* (2003); Gales *et al.,* (2002) in the international context.

E. coli infection is higher in females as compared to male. In this study also, *E coli* infection was found to be 73.10% in females whereas 26.99% in males (out of total *E. coli* isolates). Similar results were found by Shrestha, (2007); Dhakal *et al*., (1999); Gautam *et al*., (1998); Kosakai *et al*., (1990); Vorland *et al*., (1985). *E. coli* have special virulent properties to cause UTI, being the major uropathogen throughout the world. *E. coli* can bind to glyco-conjugate receptor of the uroepithelial cells of human urinary tract so it can initiates infection itself. *E. coli* is isolated in 90.0% of infections and strains are characterized by unique virulence determinant, the p pilus (Gal-Gal receptor) (Johnson. 1991). *E. coli* is the most predominant organism to colonize the urethral meatus (Schaeffer and Chamiel, 1983) and perineum (Leigh, 1990) before ascending to bladder.

Klebsiella pneumoniae and *Citrobacter* species were isolated as the second most common pathogen causing UTI each being 3.95%. Similar pattern were observed in the studies of Poudyal, (2010); Baral, (2008); Shrestha, (2007); Puri, (2006); Das *et al*., (2006); Astal *et al.,* (2002); Gautam *et al.,* (1998); Ghimire *et al*., (1995); Hadzia *et al*., (2003); Kumari *et al.,* (2005); Manandhar *et al.,* (2005), Sharma, (1983); Zhanel *et al*., (2005). This might suggests the nosocomial infections. All *Klebsiella pneumoniae* were isolated from female patients only that resembles with the study of Shrestha, (2007).

Maximum number of blood samples were collected from age group <10 years and among them 14 (3.46%) showed the growth. Among them the most predominant was *Citrobacter* species (57.14%) followed by *E. coli* (28.57%), *K. pneumoniae* and *Enterobacter* species with 7.14% each. This suggests that neonatal infections were mostly associated by bacteria of family enterobacteriaceae (CDC, 1996; Richards *et. al.,* 1999; Roberts, 1980).

Of 611 pus samples 108 (17.67%) showed the growth with highest prevalence of *E. coli* (72.22%) followed by *Citrobacter* species, *K. pneumoniae* and *Proteus* species suggesting high rate of infection due to enterobacteriaceae according to the site of infection and their prominences in environment (Forbes *et al.,* 2007). These findings matched with the study of Poudyal, (2010); Baral, (2008); Joshi, (1997).

Of 41 sputum samples only a single isolates of *Klebsiella pneumoniae* (2.44%) isolated. This result matched with the study of Poudyal, (2010) which showed the positivity of 3.65% for *K. pneumoniae*.

Similarly no growth was observed in case of body fluids and CSF. Also no stool samples showed the growth of gasterointestinal pathogenic microorganisms belonging to family enterobacteriaceae. Similar findings were observed in the study of Baral, (2008).

The growth pattern of the pathogens from different samples was increased with the age group 31-40 years to 81-90 years with highest isolates being 18.52%. This finding suggests the higher chances of acquiring infections with increasing age that might be due to deterioration of immunity with the age. Higher isolation of bacterial pathogens was found from indoor patients with 14.99% compared to the outdoor patients with 10.55% isolates suggesting the nosocomial infections and degree of severity. Similar finding is being observed in the study of Baral, (2008).

Of total 300 isolates, major pathogens were isolated from age group 14-50 years with *E. coli* being most predominant (79.0%) followed by *Citrobacter* species , *Klebsiella pneumoniae*., Proteus species and *Morganella morgani*. This might suggests the lowered immunity with the age. These findings match with the work of Baral, (2008); Bomjan, (2005).

Antimicrobial resistance is one of the main global issues and has a significance implication on health and patient care. It is associated with high mortality and morbidity, high health-care cost and prolonged hospital stay. It is more troublesome to developing countries like Nepal. WHO and Europian Commission (EC) have recognized the importance of studying the emergence and determinants of resistance and the need for strategies for its control (Rosaus *et al.,* 1997).

In this study, out of 15 different antibiotics used, imipenem was the drug of choice with susceptibility of 98.11% but this is used if there are no alternative second line drugs of choice. Similar result was found in the study of Baral, (2008); Poudyal, (2010). This was followed by Amikacin, meropenem, Chloramphenicol, Piperacillin+Tazobactam, Gentamicin, Ciprofloxacin,

Cefotaxime and Ceftazidime with susceptibility of 89.55%, 89.47% , 78.35%, 74.64%, 69.18%, 53.50%, 52.90% and 42.34% respectively. Similar pattern was observed in the studies of Poudyal, (2010); Baral, (2008); Bomjan, (2005) indicating improper use of antibiotics at primary level.

Amikacin, Gentamicin, Ciprofloxacin, Cefotaxime and Ceftazidime were most effective drugs for choice. This finding was also supported by findings of Poudyal, (2010); Baral, (2008); Puri, (2006); Bomjan, (2005); Paneru, (2002); Oteo *et al.,* (2001); Dhakal, (1999).

Similarly, amoxicillin and amoxycillin+clavulanic acid were least effective drugs with susceptibility of 0.00% and 7.55% respectively. This finding was supported by the work of Hadziz et al., (2003); Ghimire *et al*., (1994); Dhakal *et al*., (1999); Poudyal, (2010); Baral, (2008); Bomjan, (2005); Shrestha, (2007); Arosio *et al*., (1978); Obi et al., (1996); Gautam *et al*., (1997); Sahm *et al*., (2001).

Out of total *E. coli* isolated imipenem followed by amikacin, nitrofurantoin and meropenem were the drug of choice with susceptibility of 100%, 93.22%, 94.97% and 87.50% respectively. Similar findings were obtained from the works of Sahmm *et al*., (2001); James *et al*., (2002); Kahlmeter *et al*., (2003); Andrew *et al*., (2005); Zhamel *et al*., (2005); Fadda *et al*., (2005); Biswas *et al*., (2006); Dhakal *et al*., (2000); Baral, (2008); Poudyal, (2010).

With total *E. coli* isolates, amoxicillin was the least effective drug which was similar with the findings of Sahm *et al.,* (2001); Kurupete *et al*., (2005); Dhakal *et al.,* (1999); Dhital *et al.,* (2000); Baral, (2008); Poudyal, (2010). Similarly, Ciprofloxacin and Cotrimoxazole showed the susceptibility of 48.0% and 42.0% which was similar to the studies of Sahm *et al*., (2001); Andrew et al., (2005); Arshlan *et al*., (2005); Kurutepe *et al*., (2005); Biswas *et al*., (2006). However, higher susceptibility were seen in the work of James *et al.,* (2002); Kahlmeter *et al*., (2003); Zhamel *et al*., (2005); Ghimire *et al*., (1994). This suggests that higher susceptibility to these drugs during early of the decades and it gradually decreased due to uncontrolled use of these drugs later on.

Regarding third generation cephalosporins, *E. coli* showed 54.33% and 43.33% susceptibility to cefotaxime and ceftazidime respectively. These data resembles with the studies of Meyer *et al*., (2008); Poudyal, (2010); Baral, (2008). This suggests the frequent use of these drugs during recent times and their poor management.

With total enterobacteria isolated, 178 (59.33%) were multidrug resistant (MDR). Such higher rates were seen in the works of Shrestha *et al*., (2005); Pokhrel *et al*., (2004); Bomjan, (2005); Baral, (2008); Poudyal, (2010).

Among total enterobacteriaceae*, E. coli* accounted for 60.76% (144) MDR which was similar with the works of Kurutepe *et al*., (2005); Manandhar *et al*., (2005); Bomjan, (2005); Baral, (2008); Poudyal, (2010). However much higher rate of MDR *E. coli* was seen in the work of Hawser *et al*., (2009) with >80% *E. coli* beind MDR. Similarly, works of Daniel *et al*., (2008); Selman et al., (2000); Goesch *et al*., (2000); Sahm *et al*., (2001); Tuladhar *et al*., (2000) showed the lower prevalence of MDR *E. coli* with the rate of <10%. This might suggests that the rate of MDR is increasing day by day which might be due to improper management of antibiotics in treatment of disease and transfer mechanisms of resistant genes through plasmids. However there is no significance association between MDR and non-MDR strains between male and female patients. Also no significance association was seen between MDR and culture positivity amog genders.

Of total *Klebsiella pneumoniae* 40% (6/15) were MDR which was similar with the work of Hawser *et al*., (2009); Thapa *et al*., (2009); Bomjan, (2005) and Baral, (2008). However, the work of Poudyal, (2010) showed the higher (83.3%) rate of MDR positivity. Similarly, 69.44% *Citrobacter* species were MDR. Higher rate of MDR *Citrobacter* species was seen in the study of Thapa *et al.,* (2009).

Cent percent MDR were seen with *Proteus mirabilis*, *Enterobacter* species and *Morganella morganii*. Such higher percentage of rate of MDR was seen in the studies of Hadzia *et al.,* (2003); Gautam *et al*., (1997); Bomjan, (2005).

CHAPTER VI
CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

The study was carried to determine the Multidrug resistant bacterial isolates of family enterobacteriaceae isolated from different clinical samples collected at Nobel Medical College Teaching Hospital and Research Centre.

The main findings of this study are that there is significant association between culture positivity and type of patients i.e. higher positivity was seen among in-patients ($p<0.05$). However, statistical analysis failed to show significant difference of MDR strains between genders as well as between culture positivity and gender. The highest susceptibility was shown to imipenem (98.11%) followed by amikacin and chloramphenicol. The overall rate of MDR was found to be 59.33%. Among MDR isolates, there is a high resistace rate to more than five drugs except few. Higher resistance was seen to third generation cephalosporins and fluoroquinolones. This necessitates a reevaluation of first and second line therapies for treatment of infections due to these organisms and regular monitoring of the usage of antimicrobials in order to make reliable information available for optimal empirical therapy.

In conclusion, this study revealed the magnitude of the problems of multiple drug resistance among bacteria of family enterobacteriaceae which are the common isolates isolated in different infections whether community acquired or hospital acquired. Hence routine investigation and monitoring of their antibiogram is necessary for proper management of disease.

6.2 Recommendations

1. Since the study found higher rate of resistivity of the pathogens towards third generation cephalosporins (42.34-52.90%). So, third generation cephalosporins could be started as empirical therapy before the antibiotic sensitivity results are available.
2. Antibiotic sensitivity testing should be done for each and every isolates to confirm their sensitivities.
3. Since higher percentage of growth seen among indoor patients, it should be investigated for nosocomial infections or any other causes.

4. As this study was confined to NMCTHRC, it does not necessarily reveal the picture of the country, therefore systematic prospective surveillance should be carried out throughout the year covering wide geographical region in order to obtain information on seasonal, geographical and ethnic variation of pathogens and their antibiotic susceptibility profile.

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APPENDIX-I

I. Composition and Preparation of Different Culture Media

The culture media used were from Hi-Media Laboratories Pvt. Limited, Bombay, India.

(Final pH of media was tested by pH meter, Sterility testing was performed by incubating prepared plates at 37^0C for 24 hours in an incubator and observed for any growth.)

1. Blood agar (BA)

Blood agar base (infusion agar) + 5% 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. Final pH was measured by pH meter and performance test was checked by inoculating a single plate per lot with *S. aureus* and observed for haemolysis after 24 hours of incubation at 37^0C.

2. MacConkey Agar (MA)

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, cooled and plated in sterile petriplates.

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. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes,cooled and plated in sterile petriplates.

4. **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

1.3 grams of the medium was dissolved in 100 ml distilled water, dispensed about 3-5 ml into clean test tubes and autoclaved at 121^0C for 15 minutes.

5. **Xylose-Lysine Deoxycholate agar (XLD)**

Ingredients	gm/litre
Yeast extract	3.00
L-Lysine hydrochloride	5.00
Lactose	7.50
Xylose	3.75
Sodium chloride	5.00
Sodium deoxycholate	1.00
Sodium thiosulphate	6.80
Ferric ammonium citrate	0.80
Phenol red	0.08
Agar	15.0

Final pH (at 25^0C) 7.4±0.2

5.55 grams of the medium was suspended in 100 ml of distilled water and then transferred to water bath at 50^0C for 10 minutes. It was then plated after cooling to approximately 40^0C.

6. Deoxycholate Citrate Agar (DCA)

Ingredients	gm/litre
Heart Infusion solids	10.00
Proteose peptone	10.00
Lactose	10.00
Sodium deoxycholate	5.00
Neutral red	0.02
Sodium citrate	20.00
Ferric ammonium citrate	2.00
Agar	13.5

Final pH (at 25^0C) 7.5±0.2

7.05 grams of the medium was suspended in 100 ml of distilled water and then boiled to dissolve completely. It was then cooled and plated.

7. Selenite-F broth

Ingredients	gm/litre

Part A	--
Caesin enzymic hydrolysate	5.00
Lactose	4.00
Sodium phosphate	10.00
Part B	--
Sodium hydrogen selenite	4.00

Final pH (at 25^{0}C) 7.0±0.2

0.4 grams of Part B was suspended in 100 ml distilled water and 1.9 grams of Part A was added and mixed well. It was then warmed to dissolve and transferred to sterile test tubes.

8. Cooked meat medium (RCM)

Ingredients	gm/litre
Beef heart solids	98.00
Proteose peptone	20.00
Dextrose	2.00
Sodium chloride	5.00

Final pH (at 25^{0}C) 7.2±0.2

12.5 grams of the medium was suspended in 100 ml of distilled water, mixed thoroughly and allowed to stand for 15 minutes. It was then transferred to test tubes and was sterilized by autoclaving at 121^{0}C (15 lbs pressure) 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 25^0C) 6.9±0.2

1.7 grams of powder was dissolved in 100 ml distilled water. 3 ml of medium was distributed in each test tube and autoclaved at 121^0C 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 25^0C) 6.8±0.2

1.0 gram of the medium was rehydrated in 100 ml cold distilled water and then heated to boiling to dissolve completely and sterilized in the autoclave at 121^0C for 15 minutes at 15 lbs pressure. To sterile medium 10ml of sterile Dextrose was aseptically added 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

3.6 grams of the medium was suspended in 100 ml distilled water and warmed to dissolve completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized by autoclaving.

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

2.42 grams of the medium was dissolved in 100 ml distilled water, warmed to dissolve completely. 3 ml 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^{0}C) 7.4±0.2

6.5 grams of the medium was dissolved in 100 ml of distilled water, warmed to dissolve and about 5 ml medium was distributed into test tubes. It was then sterilized by autoclaving at 15 lbs (121^{0}C) 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^{0}C) 7.4±0.2

2.4 grams of the medium was suspended in 95 ml distilled water and sterilized by autoclaving at 121^{0}C for 15 minutes. After cooling to about 45^{0}C, 5 ml of 40% urea was added and mixed well. Then 5 ml was dispensed in sterile test tubes 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, 2 gm of crystal violet was weighed and transferred to a clean brown bottle. Then, 10 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 1 gm of ammonium oxalate dissolved in 20 ml of D/W was added. Finally the volume was made 100 ml by adding D/W.

(b) Lugol's Iodine

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

Preparation: To 25 ml of D/W, 2 gm of potassium iodide was dissolved. Then 1 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 100 ml by adding D/W.

(c) Acetone-Alcohol Decoloriser

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

Preparation: To 5 ml D/W, 50 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 50 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, 1 gm of safranin was weighed and transferred to a clean bottle. Then 100 ml D/W was added to the bottle and mixed well until safranin dissolved completely.

3. Test Reagents

a. For Catalase test

Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

Preparation: To 48.5 ml of D/W, 1.5 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 0.5 gm of TPD in 50 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-II

List of Equipment, materials and supplies

A. EQUIPMENT

Autoclave

Incubator

Hot air oven

Microscope (Olympus)

Refrigerator 4-8^{0}C

Weighing machine

Water Bath

Gas burners

Glasswares

Inoculating wire and loops

B. MICROBIOLOGICAL MEDIA

Blood Agar
Mac conkey agar
Mueller Hinton Agar
Triple Sugar Iron Agar
Urea Agar Base
BHI broth
Hugh and Leifson Media
Sulphur Indole Motility Media
MRVP Broth
Nutrient broth
Simmon's Citrate agar
RCM medium

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-naphthol in a ratio of 1:3)

Barium Chloride

Conc. H_2SO_4

Gram's reagent

D. ANTIBIOTIC DISKS

The antibiotic disks used for the susceptibility tests that were from Hi-media Laboratories Pvt Ltd. India are as follows

Amikacin (30µg), Amoxycillin (10µg), Cefotaxime (30µg), Ceftazidime (30µg), Cotrimoxazole (25µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Gentamicin (10µg), Imipenem (10µg), Meropenem (10µg), Norfloxacin (10µg), Nitrofurantoin (300µg), Nalidixic acid (30µg), Piperacillin+Tazobactam (100µg+10µg), Amoxycilin+Clavulanic acid (20+10µg) was of HI-Media laboratories Pvt. Ltd. India.

APPENDIX-III

A. 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 air 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 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 60 seconds.
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.

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 100X (Gephart *et al.,* 1981).

APPENDIX IV

1. 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-V

1. **DISC DIFFUSION METHOD FOR THE ANTIMICROBIAL SUSCEPTIBILITY TESTING**

A. Preparation of 0.5 Mc Farland Standard

Add 0.05 ml of 0.048M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) to 9.95 ml of 0.18M H_2SO_4 (1% v/v) with constant stirring.

B. Preparation of inoculum

By touching 2-3 morphologically similar colonies with sterile loop, inoculate into NB and incubate at 37^0C for 2-3 hours until turbidity matches with that of 0.5 Mc Farland Standard.

C. Inoculation of Agar plates

a. The agar plates, canister of discs are brought to room teperature before use. It should be made sure that the agar surface doesn't have any moisture, if so should be dried by keeping it in incubator.

b. Using a sterile swab, a plate of Mueller-Hinton agar is inoculated with the bacterial suspension using carpet culture technique. The plate is left for about 5 minutes to let the agar surface dry.

c. Using sterile forceps, appropriate antimicrobial discs (6 mm diameter) is placed, evenly distributed on the inoculated plates, not more than 6 discs are placed on a 90 mm diameter Petri plate.

d. Within 30 minutes of applying the discs, the plates are incubated at 35^0C for 18-24 hrs.

e. After overnight incubation, the plates are examined to ensure confluent growth. Using a measuring scale, the diameter of each zone of inhibition in mm is measured and results interpreted accordingly.

D. Quality Control

a. QC strains

E. coli ATCC 25922

b. Monitoring Accuracy

a. Running AST for QC strains side by side with pathogenic bacteria

b. Monitoring the expiry date of antibiotic discs and MHA

c. Comparing zone size with CLSI QC tables

APPENDIX-VI

Table: Distinguishing reactions of commoner 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 pneumonia	+	-	++	-	+	+	-	+	+	-	+	+
K oxytoca	+	-	++	+	+	+	-	+	+	-	+	+
E aerogens	+	+	++	-	+	+	-	-	+	-	+	+
E cloacae	+	+	+	-	+	+	-	+/-	-	-	-	+
Hafnia alvei	-	+	+	-	+	-	-	-	+	-	-	+
Serratia marcescens[b]	-	+	+/-	-	+	+	-	-	+	-	+/-	+
P mirabilis	-	+	+	-	+/-	+/-	+	++	-	+	-	-
P vulgaris	-	+	+	+	-	-	+	++	-	+	-	-
M morgani	-	+	+	+	-	-	+	++	-	+/-	-	-
Providencia rettgeri	-	+	-	+	-	+	+	++	-	-	+	-
P stuartii	-	+	-	+	-	+	+	+/-	-	-	+	-
P alcalifaciens	-	+	+	+	-	+	+	-	-	-	-	-
Yersinia enterocolitica[c]	-	-	-	+/-	-	-	-	+/-	-	-	+/-	+

Y pestis	-	-	-	-	-	-	-	-	-	-	-	+/-
Y pseudotuberculosis	-	-	-	-	-	-	-	+	-	-	-	+/-

[a] lac, inos, fermentation of lactose, inositol; mot, motility; gas, gas from glucose; ind, indole production; VP, Voges-Proskauer; cit, Citrate utilization(Simmon's); PDA, phenylalanine deaminase; ure, urease; lys, lysine decarboxylase; H_2S, H_2S production 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 22^0C.

{ Key: +, ≥85% of strains positive; -, ≥85% of strains negative; 16-84% of strains are positive after 24-48 hrs at 36^0C} (Source: Collee *et a*l. 1996)

APPENDIX-VII

LIST OF FIGURES

Table 1: Pattern of type of patients, isolates and MDR strains in different samples

Type of	**Male**	**Female**	**TOTAL**	**MDR**

patients	No. (%)	Isolates (%)	MDR (%)	No. (%)	Isolates (%)	MDR (%)	(Isolate %) (Isolate)	(Isolate %)
OUTDOOR	490 (19.96)	44 (2.92)	30 (68.18)	1017 (41.44)	115 (7.63)	56 (48.69)	1507 (10.55) 159	86 (54.08)
INDOOR	484 (19.72)	66 (6.97)	42 (63.63)	463 (18.86)	75 (7.92)	49 (65.33)	947 (14.89) 141	91 (64.08)
TOTAL	**974 (39.68)**	**110 (4.55)**	**72 (65.45)**	**1480 (60.30)**	**190 (7.74)**	**105 (55.26)**	**2454 (12.22) 300**	**177 (59.0)**

Table2: Age and gender wise distribution of patients requesting for culture and their growth pattern

Age group (in years)	Outdoor patients		Indoor patients		TOTAL (Isolates)	(Isolate%)
	Male (Isolates)	Female (Isolates)	Male (Isolates)	Female (Isolates)		
0-10	123(7)	186(5)	190(14)	133(5)	632(31)	4.90
11-20	79(5)	181(21)	74(8)	45(6)	379(40)	10.55
21-30	135(10)	239(49)	91(15)	71(14)	536(88)	16.42
31-40	51(2)	140(10)	54(11)	74(14)	319(37)	11.60

41-50	23(1)	130(18)	14(5)	76(21)	243(45)	18.52
51-60	31(7)	82(9)	35(7)	33(9)	181(32)	17.67
61-70	22(7)	44(2)	12(4)	16(2)	94(15)	15.95
71-80	18(4)	10(0)	11(2)	11(3)	50(9)	18.0
81-90	8(1)	2(1)	3(0)	4(1)	17(3)	17.65
>90	0(0)	3(0)	0(0)	0(0)	3(0)	0.0
TOTAL	**490(44)**	**1017(115)**	**484(66)**	**463(76)**	**2454(300)**	**12.22**

Table3: Pattern of different clinical samples, status of growth and MDR strains

Specimens	**No. of specimens**	**Growth**		**MDR strains**	
		No.	**%**	**No.**	**%**
Blood	404	14	3.46	7	50.0
Pus	611	108	17.67	72	66.66
Fluid	74	0	0	0	0

Sputum	41	1	2.44	0	0
CSF	27	0	0	0	0
Stool	32	0	0	0	0
Urine	1265	177	14.0	98	55.37
TOTAL	**2454**	**300**	**12.22**	**177**	**59.0**

Table4: Antibiotic susceptibility pattern of the pathogens

ORGANISMS		**Am**	**AMC**	**CTX**	**CAZ**	**PT**	**G**	**Ak**	**NA**	**Nx**	**CIP**	**IMP**	**MP**	**C**	**Co**	**Nf**	**Te**
Escherichia	S	0	4	113	70	81	176	220	63	66	106	38	167	56	21	151	-
Coli	I	0	0	5	5	9	9	5	0	0	9	0	0	1	0	4	-
	R	130	132	90	86	17	54	11	169	90	106	0	20	11	29	4	-
Citrobacter spp.	S	0	0	8	6	9	9	18	1	2	21	5	1	10	0	1	7
	I	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1
	R	5	10	17	15	7	17	7	2	1	5	1	0	4	1	1	3
Citrobacter	S	-	0	3	2	4	4	5	1	2	4	2	-	4	-	1	5
koseri	I	-	0	0	0	0	0	0	0	0	0	0	-	0	-	1	0
	R	-	2	4	1	0	2	2	1	0	2	0	-	0	-	0	0
Citrobacter	S	-	-	1	1	2	0	2	-	-	2	-	-	0	-	-	0
freundii	I	-	-	0	0	0	0	0	-	-	0	-	-	0	-	-	0
	R	-	-	1	1	0	2	0	-	-	0	-	-	1	-	-	2
Klebsiella	S	0	0	11	3	5	11	10	6	6	11	6	2	6	2	5	1
pneumoniae	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0

	R	3	2	4	4	1	5	4	1	1	4	0	0	3	3	1	1
Enterobacter spp.	S	-	0	0	-	1	0	0	-	-	0	1	-	0	-	-	1
	I	-	0	0	-	0	0	0	-	-	1	0	-	0	-	-	0
	R	-	1	1	-	0	1	1	-	-	0	0	-	1	-	-	0
Proteus mirabilis	S	-	-	-	0	-	1	1	-	-	-	-	-	0	0	-	0
	I	-	-	-	0	-	0	0	-	-	-	-	-	0	0	-	0
	R	-	-	-	1	-	0	0	-	-	-	-	-	1	1	-	1
Morganella morganii	S	-	-	1	1	1	1	1	-	-	1	-	-	-	-	-	0
	I	-	-	0	0	0	0	0	-	-	0	-	-	-	-	-	0
	R	-	-	0	0	0	0	0	-	-	0	-	-	-	-	-	1

Abbreviations: Am-Amoxycillin; CTX-cefotaxime; CAZ-ceftazidime; PIT-piperacillin+tazobactam; G-gentamicin; AK-amikacin; NA-nalidixic acid; NX-norfloxacin; CIP-ciprofloxacin; OF-ofloxacin; IMP- imipenem; MP-meropenem; C-chloramphenicol; Co-co-trimoxazole; NF-nitrofurantoin and TE-tetracycline

Table5: Antibiotic susceptibility pattern of ***E. coli*** isolated from different samples

Antibiotic used	**Sensitive**		**Moderately sensitive**		**Resistant**		**Total**
	No. of isolates	**%**	**No. of isolates**	**%**	**No of isolates**	**%**	

Amoxycillin	0	0.00	0	0.00	130	100	**130**
Amoxycillin+Clavulanic acid	4	10.53	0	0.00	132	89.47	**136**
Cefotaxime	113	54.33	5	2.40	90	43.27	**208**
Ceftazidime	70	43.48	5	3.10	86	53.42	**161**
Piperacillin+Tazobactam	81	75.70	9	8.41	17	15.88	**107**
Gentamicin	176	73.64	9	3.76	54	22.60	**239**
Amikacin	220	93.22	5	2.12	11	4.66	**236**
Nalidixic acid	63	27.15	0	0.00	169	72.84	**232**
Norfloxacin	66	42.30	0	0.00	90	57.70	**156**
Ciprofloxacin	106	48.00	9	4.07	106	48.00	**221**
Imipenem	38	100	0	0.00	0	0.00	**38**
Meropenem	167	87.50	0	0.00	20	12.50	**187**
Chloramphenicol	56	82.35	1	1.47	11	16.17	**68**
Co-trimoxazole	21	42.00	0	0.00	29	58.00	**50**
Nitrofurantoin	151	94.97	4	2.51	4	2.51	**159**
Tetracycline	41	37.50	0	0.00	68	62.50	109

APPENDIX-VIII

1. **Association between Culture positivity among genders**

		Growth		Total
		Positive	Negative	
Gender	Male	110	864	974
	Female	190	1290	1480
Total		300	2154	2454

Test statistics is χ^2

H_O: There is no significant association between culture positivity among genders.

H_1: There is significant association between culture positivity among genders.

From $\chi^2 = \sum \frac{(O-E)^2}{E}$ we find $\chi^2 = 1.32$

Thus χ^2_{cal} (1.32) $< \chi^2_{tab}$ at =0.05 and d.f = 1 i.e.3.841

Hence, H_O is accepted i.e. there is no significant association between culture positivity among male and female patients i.e higher proportion of culture positivity seen among female patient is statistically insignificant.

2. **Association between Culture positivity among out-patients and in-patients.**

	Culture positive	**Culture negative**	**Total**
Out-Patients	159	1348	1507
In -Patients	141	806	947
Total	300	2154	2454

Test statistics is χ^2

H_O: There is no significant association of culture positive and negative among out-patient and in- patients.

H_1: There is significant association of culture positive and negative among out-patient and in- patients.

.

From $\chi^2 = \sum \frac{(O-E)^2}{E}$ we find $\chi^2 = 10.18$

Thus χ^2_{cal} (10.18) $>\chi^2_{tab}$ at =0.05 and d.f = 1 i.e.3.841

Hence, H_O is rejected i.e. there is significant association of culture positivity among types of patients i.e. higher proportion of bacterial infections found among in-patients was statistically significant.

3. **Association of multi drug resistance among out-patients and in-patients among different pathogens.**

	MDR- strains	Non-MDR strains	Total
Out-Patients	86	73	159
In -Patients	91	50	141
Total	177	123	300

Test statistics is χ^2

H_O: There is no significant association of multi-drug resistance among out-patients and in-patients among different pathogens.

H_1: There is significant association of multi-drug resistance among out-patients and in-patients among different pathogens.

From $\chi^2 = \sum \frac{(O-E)^2}{E}$ we find $\chi^2 = 3.36$

Thus χ^2_{cal} (3.36) $<\chi^2_{tab}$ at =0.05 and d.f = 1 i.e.3.841

Hence, H_O is accepted i.e. there is no significant association of multi-drug resistance among types of patients suffering bacterial infections i.e. higher percentage of MDR strains found in in-patients was stastically insignificant.

4. Association of multi drug resistance among male and female patients

	MDR- strains	**Non-MDR strains**	**Total**
Male	72	38	110
Female	105	85	190
Total	177	123	300

Test statistics is χ^2

H_O: There is no significant association of MDR and non-MDR strains among male and female patients.

H_1: There is significant association of MDR and non-MDR strains among male and female patients.

From $\chi^2 = \sum \frac{(O-E)^2}{E}$ we find $\chi^2 = 2.97$

Thus χ^2_{cal} (2.97) $<\chi^2_{tab}$ at =0.05 and d.f = 1 i.e.3.841

Hence, H_O is accepted i.e. there is no significant association of MDR and non-MDR strains among male and female patients i.e higher proportion of multi-drug resistance seen among female patients was not found stastically significant.

5. Association of culture isolates and MDR strains among male and female patients

	No. of isolates	**MDR strains**	**Total**
Male	110	72	182
Female	190	105	295

Total	300	177	477

Test statistics is χ^2

H_O: There is no significant association of culture isolates and MDR strains among gender.

H_1: There is significant association of culture isolates and MDR strains among gender.

From $\chi^2 = \sum \frac{(O-E)^2}{E}$ we find $\chi^2 = 0.74$

Thus χ^2_{cal} (0.74) $<\chi^2_{tab}$ at =0.05 and d.f = 1 i.e.3.841

Hence, H_O is accepted i.e. there is no significant association of culture isolates and MDR strains among gender i.e higher proportion of MDR strains seen among female patients is not found stastically significant.