# CHAPTER I

# INTRODUCTION AND OBJECTIVES

## 1.1 Background

Bacterial infections represent a common and most important health problem for patients with renal failure undergoing maintenance hemodialysis. Considerable gains have been made in deciphering the pathogenesis of bacterial infections in this high-risk population. Inspite of these gains the therapeutic goal of preventing bacterial infections in hemodialysis patients remains unfulfilled. Endocarditis, oesteomyelitis, urinary tract infections, bloodstream infections are example of some of the appalling conditions in hemodialysis patients resulting from the infection of microorganisms such as *Staphylococcus aureus, Enterococcus* spp*, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* etc. (Aurora, 2008; D'Agata, 2004).

Acquisition of drug resistance by these pathogenic strains has posed serious challenge for the therapeutic management of clinical cases. Infections that are caused by drug resistant bacteria are associated with up to five times higher mortality rates compared with infections that are caused by susceptible bacteria (Schwaber et. al., 2006). These resistant organisms need to be identified and apposite action be taken before scenario gets worse.

There are increasing reports of Extended Spectrum β-Lactamase (ESBL) and Metallo β-Lactamase (MBL) producing isolates expressing multidrug resistance (MDR), defined as concomitant resistance to at least two different antibiotic classes (Boyd *et. al*., 2004; Morosini *et al.*, 2006). Patients at high risk of developing colonization or infection with ESBL producing microorganisms are often seriously ill patients with prolonged hospital stays, e.g. undergoing hemodialysis and in whom invasive medical devices are present (Paterson and Bonomo, 2005).

Extended-spectrum β-Lactamase and metallo β-Lactamase producing bacteria are emerging concern for health professionals as they are associated with more

severe form of disease and antibiotic resistance. ESBL and MBL have been studied well in Nepalese community (Pokhrel *et al*., 2006; Poudyal, 2010) yet the data remains lacking for those organisms among hemodialysis patients.

Chronic kidney disease is a major public health problem worldwide with enormous cost burdens on health care systems in developing countries. Infection by microorganisms in this vulnerable population not only adds to outlay but may also be fatal. Infection is the second leading cause of death among dialysis patients, accounting for 33 deaths per 1000 patient years in the prevalent US Renal Data System (USRDS) cohort for 2001 through 2003. Septicemia, which is poorly defined, accounts for 79.7% of infectious deaths (USRDS, 2005). The problems which are associated with ESBLs and MBL include multidrug resistance, difficulty in detection and treatment and increased mortality. Awareness and the detection of these enzymes are necessary for optimal patient care. The judicious use of antimicrobial agents and improved infection control methods must become health care priorities**.**

Many laboratories make no effort to detect ESBL production, or are ineffective at doing so inspite of Clinical and Laboratory Standard Institute's (CLSI) recommendations that clinical microbiology laboratories perform specialized tests for detection of ESBL. The findings in case of ESBL and MBL in dialysis patients have important implications, because current measures aimed at preventing the spread of antimicrobial-resistant bacteria have focused on VRE and MRSA (CDC, 2001). To make efforts for safeguarding the efficacy of antibiotic accessible to common people by rationalizing their usage and discouraging their overuse and underuse, steps have to be taken at an earliest. In such scenario, not only efficient detections, early reporting and rationale in treatment are important but aggressive infection control practices have to be employed. Therefore, this research was conducted with the aim of determining the prevalence and antibiotic resistance pattern of Gram negative bacteria on hemodialysis and chronic kidney patients, and to access the prevalence of ESBL and MBL producing strains among them.

## 1.2 OBJECTIVES

### 1.2.1 GENERAL OBJECTIVE

To determine the prevalence of ESBL and MBL producing bacterial pathogens among the MDR Gram negative bacteria isolated from hemodialysis and Chronic Kidney patients**.**

### 1.2.2 SPECIFIC OBJECTIVES

1. To isolate and identify the bacterial pathogens in the urine and blood specimen from chronic kidney patients and patients undergoing hemodialysis at NKC.
2. To perform the antimicrobial susceptibility testing of the isolates.
3. To determine the prevalence of multidrug resistance among the pathogens.
4. To evaluate the status of ESBL and MBL producing strains among the isolated organisms.
5. To analyze the association of different screening agents and their efficacy among the combined disk assay for ESBL and MBL detection among pathogenic strains.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Hemodialysis

In medicine, hemodialysis is a method that is used to achieve the extracorporeal removal of waste products such as creatinine and urea and free water from the blood when the kidneys are in a state of renal failure (Abel *et al.*, 2002). Hemodialysis is one of three renal replacement therapies (the other two being renal transplant and peritoneal dialysis). Dialysis uses a membrane as a filter and a solution called dialysate to regulate the balance of fluid, salts and minerals carried in the bloodstream. A dialyser works on the principle of blood flowing along one side of a semi-permeable membrane made of cellulose or a similar product, with the dialysate flowing along the other side. The dialysate contains a regulated amount of minerals normally present in the blood, but in renal failure they are present in excess. The membrane has tiny holes of different sizes so that the excess fluid and substances in the blood pass through at different rates, small molecules quickly and larger ones more slowly, to be taken away in the dialysate until a correct balance in the blood is achieved. A kidney machine regulates blood flow, pressure and the rate of exchange.

As only a very small amount of blood is in the dialyser at any given time, blood needs to circulate from patient to dialyser to patient for about 4 hours. Treatment is usually 3 times per week. The time and strength of dialysis can be programmed for each patient, depending upon the seriousness of the damage. Blood is carried from the patient to the dialyser and returned through dialysis lines (plastic tubes) which are connected to the patient by either fistulas or catheters.

### 2.1.1 Bacterial infections in hemodialysis patients

Various studies have revealed several types of infection among this very vulnerable population. The most common infectious organisms are Gram positive bacteria, especially *Staphylococcus aureus*, Enterococci, *Clostridium difficile, Stahphylococcus pneumonia*e, followed by Gram negative organisms. Gram negative organisms are the major cause of bacteremia, sepsis, urinary tract infections (Naqvi and Collins, 2006). Besides, Hemodialysis patients are also susceptible to blood borne infections such as HIV, Hepatitis B, Hepatitis C, etc.

Patients with chronic kidney disease have abnormalities in their immune system which impair their ability to fight infection. Although the white blood cell count in the typical laboratory testing profile may be normal, the dialysis patient's white blood cells (which are the primary line of defense against infection) typically do not function normally (Wish, 2002). Since hemodialysis requires access to the circulatory system, patients undergoing hemodialysis may expose their circulatory system to microbes, which can lead to sepsis, an infection affecting the heart valves (endocarditis) or an infection affecting the bones (osteomyelitis). Other common infections in hemodialysis patients include Urinary tract infection, bloodstream infections, pneumonia, etc (D'Agata *et al*., 2000). The risk of infection varies depending on the type of access used (fistula or catheter). Bleeding may also occur; again the risk varies depending on the type of access used. Infections can be minimized by strictly adhering to infection control best practices.

Rates of antibiotic-resistant bacteria are among the highest in patients who require hemodialysis (D'Agata, 2002). Vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) are among the antimicrobial-resistant bacteria that have been intensely investigated in this patient population (D'Agata, 2002; Finelli *et al*., 2005). In the past few years, however, a concerning increase in the prevalence of infections caused by multidrug-resistant Gram-negative bacteria (MDRGN) has been documented in other patient populations (Bradford, 2001; D'Agata, 2004). Infections that are caused by these MDRGN are associated with up to five times higher

mortality rates compared with infections that are caused by susceptible Gram-negative bacteria (Schwaber *et al*., 2006). Among chronic hemodialysis patients, approximately 25% of blood stream infections are caused by Gram-negative bacteria (Marr *et al*., 1997), and this percentage is increasing steadily (NIH, 2000). Patients who are colonized with MDR Gram negative bacteria are at greater risk for subsequently developing an infection with these bacteria.

### 2.2 Antibiotic resistance

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; Davies and Davies, 2010; Forbes *et. al*. 2007).

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 **(**Davies and Davies, 2010**).** According to WHO Antimicrobial resistance (AMR) is resistance of a microorganism to an antimicrobial medicine to which it was originally sensitive. Resistant organisms (they include bacteria, fungi, viruses and some parasites) are able to withstand attack by antimicrobial medicines, such as antibiotics, antifungals, antivirals, and antimalarials, so that standard treatments become ineffective and infections persist increasing risk of spread to others (WHO, 2013). The evolution of resistant strains is a natural phenomenon that happens when microorganisms are exposed to antimicrobial drugs, and resistant traits can be exchanged between certain types of bacteria. The misuse of antimicrobial medicines accelerates this natural phenomenon. Poor infection control practices encourage the spread of AMR.

Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness and greater risk of death. The

death rate for patients with serious infections treated in hospitals is about twice that in patients with infections caused by non-resistant bacteria. AMR reduces the effectiveness of treatment, thus patients remain infectious for a longer time, increasing the risk of spreading resistant microorganisms to others. Many infectious diseases risk becoming untreatable and uncontrollable, which could derail the progress made towards reaching the targets of the health-related United Nations Millennium Development Goals set for 2015. When infections become resistant to first-line medicines, more expensive therapies must be used. The longer duration of illness and treatment, often in hospitals, increases health-care costs and the economic burden to families and societies. The achievements of modern medicine are put at risk by AMR. Without effective antimicrobials for care and prevention of infections, the success of treatments such as organ transplantation, cancer chemotherapy and major surgery would be compromised. The growth of global trade and travel allows resistant microorganisms to be spread rapidly to distant countries and continents through humans and food. The development of AMR is a natural phenomenon. However, certain human actions actually accelerate the emergence and spread of AMR. AMR is a complex problem driven by many interconnected factors so single, isolated interventions have little impact and coordinated actions are required (WHO, 2013).

### 2.2.1 Multidrug resistance

Multidrug resistance has been defined by various researhers and organizations in different ways in different clinical settings. Some of the the most 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).

#### 2.2.1.1 Multidrug resistance: A global perspective

One of the biggest challenges being faced by the health sector of world is the emergence of multidrug resistance. Along with the previous tasks of managing bacterial infections caused by known commensals and pathogenic bacteria, that by acquisition of arsenals of new resistance mechanism have eluded the action of simple antibiotics, the evolution of resistance in responsible pathogens have worsened the scenario making it difficult for prompt treatment with use of optimum use of antibiotics.

Growing resistance among Gram-positive and Gram-negative pathogens that cause infection in the hospital and in the community has been greatly increasing. To emphasize their importance and their ability to "escape" the effects of antibacterial drugs they were termed "ESKAPE" pathogens i.e. *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter* spp. The Centers for Disease Control and Prevention (CDC) has reported rapidly increasing rates of infection due to methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *E. faecium* (VRE), and fluoroquinolone-resistant *P. aeruginosa* (Rice, 2008). Furthermore, pan antibiotic-resistant (PDR) and newly reported cases of extremely drug resistance (XDR) infections due to highly resistant Gram-negative pathogens—namely *Acinetobacter* spp., multidrug-resistant (MDR) *P. aeruginosa* producing metallo β-lactamases (MBL) has been increasingly reported from around the world (Bollero *et al*., 2001; Boucher *et al*., 2008; Falagas *et al*., 2006; Paterson *et al*., 2007).

#### 2.2.1.2 Multidrug resistance in Nepal

Emergence of multidrug resistance in Nepal and its pattern has been studied by many researchers and they all concur use of antibiotics without prescription, without benefit of guidance from a clinician or even a pharmacist, their indiscriminate usage without regard for specific symptoms

and without any information about the organism at the root of the problem has favoured the increasing trend of antibiotic resistance.

In a study of *Salmonella* serovars isolated from urban drinking water supply of Nepal, 35 *Salmonella* isolates were MDR and all the isolates of *S. enteritidis* and four isolates of *S.* typhimurium were resistant to ceftriaxone and indicated presence of one of the ESBL genes $bla_{SHV}$ on PCR amplification (Bhatta *et al.*, 2007). In another study of nosocomial isolates in Kathmandu Medical College (KMC), *Citrobacter* spp. 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). Similarly, another study conducted in Tribhuvan University Teaching Hospital, Kathmandu revealed that 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). Another study on the resistance pattern of fluoroquinolone to *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). However, no significant study have been done till date in the field of the infection among the hemodialysis patients and patients with renal failure.

## 2.3 β-LACTAMASES

β-lactam antibiotics are a broad class of antibiotics, consisting of all antibiotic agents that contains a β-lactam ring in their molecular structures. This includes penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems (Holten *et. al.*, 2000). β-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.

β-lactamases are enzymes (EC 3.5.2.6) produced by some bacteria that provide resistance to β-lactam antibiotics like penicillins, cephamycins, and carbapenems (ertapenem) (Carbapenems are relatively resistant to β-lactamase). β-lactamase provides antibiotic resistance by breaking the

antibiotics' structure. These antibiotics all have a common element in their molecular structure: a four-atom ring known as a β-lactam. Through hydrolysis, the lactamase enzyme breaks the β-lactam ring open, deactivating the molecule's antibacterial properties. β-lactam antibiotics are typically used to treat a broad spectrum of Gram-positive and Gram-negative bacteria. β -lactamases produced by Gram-negative organisms are usually secreted, especially when antibiotics are present in the environment (Neu, 1969).

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

(i) Production of β-lactamase enzymes is the most common and important mechanism of resistance in Gram-negative bacteria.

(ii) Changes in the active site of PBPs through natural transformation and recombination with DNA from other organisms can lower the affinity for β-lactam antibiotics and subsequently increase resistance to these agents. *Neisseria* spp. and *Streptococcus* spp. have acquired highly resistant, low affinity PBPs.

(iii) Decreased expression of outer membrane proteins (OMPs) is another mechanism of resistance. Some *Enterobacteriaceae* (e.g. *K. pneumoniae* and *E. coli*) exhibit resistance to carbapenems based on loss of these OMPs.

Efflux pumps, as part of either an acquired or intrinsic resistance phenotype, are capable of exporting a wide range of substrates from the periplasm to the surrounding environment. These pumps are an important determinant of multidrug resistance in many Gram-negative pathogens, particularly *P. aeruginosa* and *Acinetobacter* spp

### 2.3.1 Classification of β- lactamases

Two major classification schemes exist for categorizing β-lactamase enzymes: Ambler classes A through D, based on amino-acid sequence homology, and Bush-Jacoby-Medeiros groups 1 through 4, based on substrate and inhibitor profile. (Drawz and Bonomo, 2010). A “family portrait” reveals the structural

similarity of class A, C, and D serine β-lactamases. Class B β-lactamases ("a class apart") are metallo- β-lactamases (MBL) (Bush, 1998). MBL possess either a single $Zn^{2+}$ ion or a pair of $Zn^{2+}$ ions coordinated to His/Cys/Asp residues in the active site. (Drawz and Bonomo, 2010).

In general, class A enzymes are susceptible to the commercially available β-lactamase inhibitors (clavulanate, tazobactam, and [less so] sulbactam). The first plasmid-mediated β-lactamase was identified in *E. coli* in 1963 (and reported in 1965), and was named "TEM" after the patient from whom it was isolated. SHV, another common β-lactamase found primarily in *K. pneumoniae,* was named from the term "*s*ulf*h*ydryl reagent *v*ariable." Early studies of SHV-1 showed that *p*-chloromercuribenzoate inhibited the hydrolysis of cephaloridine but not that of benzylpenicillin. TEM and SHV are common β-lactamases detected in clinical isolates of *E. coli* and *K. pneumoniae,* pathogens responsible for urinary tract, hospital-acquired respiratory tract, and bloodstream infections (Buynak 2006; Roy *et al*., 1985).

**Table 1: Classification of β-lactamases (Bush *et. al.*, 1995)**

| **Bush-Jacoby-Medeiros system** | **Major subgroups** | **Ambler System** | **Main Attributes** |
|---|---|---|---|
| Group 1 Cephalosporinases | | C (cephalosporinases) | usually chromosomal, resistance to all β-lactams except carbapenems |
| Group 2 Penicillinases (Clavulanic acid susceptible) | 2a | A (Serine β-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-β-lactamases | 3a<br>3b | B (Metalloenzymes)<br>B | Zinc dependent carbapenemases |
| Group 4 | | Not classified | Miscellaneous enzymes |

### 2.3.2 ESBL

ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by β-lactamase inhibitors such as clavulanic acid (Paterson and Bonomo, 2005). Of all the organisms studied till date, the most potent ESBL producers belong to the family Enterobacteriaceae (*E. coli*, *K. pneumoniae, E. aerogenes*, *Proteus mirabilis*, etc.), (Bradford, 2001; Senekal, 2010). *P. aeruginosa*, a non-enterobacteriaceae Gram negative bacterium, inactivates β-lactamases by ESBL production (approx. 22%) and by MBL production (approx. 8%) (Peshattiwar and Peerapur, 2011).

#### 2.3.2.1 Types of ESBLs

With the exception of OXA-type enzymes (which are class D enzymes), the ESBLs are of molecular class A, in the classification scheme of Ambler. Most ESBLs are derivatives of TEM or SHV enzymes (Jacoby *et al.*, 1991; Paterson and Bonomo, 2005). There are now >90 TEM-type β-lactamases and >25 SHV-type enzymes (Bradford, 2001).

**i. TEM**

TEM-1 is the most commonly encountered β-lactamase in Gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Livermore, 1995). This enzyme is also responsible for the ampicillin and penicillin resistance that is seen in *H. influenzae* and *N. gonorrhoeae* in increasing numbers. TEM-1 is able to hydrolyze penicillins and early cephalosporins such as cephalothin and cephaloridine. TEM-2, the first derivative of TEM-1, had a single amino acid substitution from the original β -lactamase.

**ii. SHV**

The SHV-1 β -lactamase is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this

species (Tzouvelekis and Bonomo, 1999). Unlike the TEM-type β -lactamases, there are relatively few derivatives of SHV-1. The majority of SHV variants possessing an ESBL phenotype are characterized by the substitution of a serine for glycine at position 238. The serine residue at position 238 is critical for the efficient hydrolysis of ceftazidime. The majority of SHV-type ESBLs are found in strains of *K. pneumoniae*. However, these enzymes have also been found in *Citrobacter diversus, E. coli,* and *P. aeruginosa* (Bradford, 2001; Naas *et. al*, 1999).

### iii. CTX-M

In recent years a new family of plasmid-mediated ESBLs, called CTX-M, that preferentially hydrolyze cefotaxime has arisen. They have mainly been found in strains of *Salmonella enterica* serovar Typhimurium and *E. coli*, but have also been described in other species of *Enterobacteriaceae.* These enzymes are not very closely related to TEM or SHV β-lactamases in that they show only approximately 40% identity with these two commonly isolated β -lactamases (Bradford, 2001). Previously, the most closely related enzymes outside this family were thought to be the chromosomally encoded class A cephalosporinases found in *K. oxytoca, C. diversus, Proteus vulgaris*, (Bradford, 2001; Bonnet *et. al*, 1999). Kinetic studies have shown that the CTX-M-type β -lactamases hydrolyze cephalothin or cephaloridine better than benzylpenicillin and they preferentially hydrolyze cefotaxime over ceftazidime (Bradford, 2001). Although there is some hydrolysis of ceftazidime by these enzymes, it is usually not enough to provide clinical resistance to organisms in which they reside. It has been suggested that the serine residue at position 237, which is present in all of the CTX-M enzymes, plays an important role in the extended-spectrum activity of the CTX-M-type β -lactamases (Bradford, 2001).

### iv. OXA

The OXA-type enzymes are another growing family of ESBLs. These β -lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d (Paterson and Bonomo, 2005). The

OXA-type β -lactamases confer resistance to ampicillin and cephalothin and are characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid (Paterson and Bonomo, 2005). While most ESBLs have been found in *E. coli, K. pneumoniae,* and other *Enterobacteriaceae,* the OXA-type ESBLs have been found mainly in *P. aeruginosa*.

**v. Other ESBLS**

Besides the types of ESBLs described above, several other types of enzymes have been reported from around the globe. Some of them include: PER-1 in strains of *P. aeruginosa,* PER-2 in *S. enterica* serovar Typhimurium strains (Bradford, 2001), VEB-1 of *E. coli* and *P. aeruginosa*, TLA-1 in *E. coli,* SFO-1 from *Serratia fonticola*. These enzymes all confer resistance to oxyimino-cephalosporins, especially ceftazidime, and aztreonam. (Bradford, 2001).

The growing number of β-lactamases in *E. coli* and *K. pneumoniae,* as well as the emergence of these enzymes in other pathogens (e.g., *Haemophilus influenzae* and *Neisseria gonorrhoeae*), led to the development of extended-spectrum cephalosporins with an oxyimino side chain, carbapenems, cephamycins, and monobactams (CDC, 1982; Jacoby *et.al*., 2005; Paterson and Bonomo, 2005). ESBLs hydrolyze penicillins, narrow- and extended-spectrum cephalosporins (including the anti-methicillin resistant *S. aureus* [MRSA] cephalosporin ceftobiprole), and the monobactam aztreonam (Paterson and Bonomo, 2005). In contrast, ESBLs cannot efficiently degrade cephamycins, carbapenems, and β-lactamase inhibitors. The majority of ESBLs are from the SHV, TEM, and CTX-M families; less frequently they are derived from BES, GES-1, VEB, and PER enzymes, and sometimes these enzymes do not belong to any defined family (Naas *et al.*, 2008).

### 2.3.3 Methods for ESBL detection

ESBL testing involves two important steps. The first is a screening test with an indicator cephalosporin which looks for resistance or diminished susceptibility, thus identifying isolates likely to be harboring ESBLs. The second one tests for synergy between an oxyimino cephalosporin and

clavulanate, distinguishing isolates with ESBLs from those that are resistant for other reasons.

#### 2.3.3.1 Screening for ESBL producers

**i. Disk-Diffusion methods**

The Clinical and Laboratory Standards Institute (CLSI) has proposed disk-diffusion methods for screening for ESBL production by *Klebsiella pneumoniae, K. oxytoca, E. coli* and *Proteus mirabilis*. Laboratories using disk-diffusion methods for antibiotic susceptibility testing can screen for ESBL production by noting specific zone diameters which indicate a high level of suspicion for ESBL production. Cefpodoxime, ceftazidime, aztreonam, cefotaxime or ceftriaxone disks are used. Since the affinity of ESBLs for different substrates is variable, the use of more than one of these agents for screening improves the sensitivity of detection (CLSI, 2009).However; it is adequate to use cefotaxime, which is consistently susceptible to CTX-M; and ceftazidime, which is a consistently good substrate for TEM and SHV variants. If only one drug can be used, then the single best indicator has been found to be cefpodoxime (Livermore and Paterson, 2006; Steward *et al*., 2001). However, it has been seen that susceptibility testing with cefpodoxime can lead to a high number of false-positive results which can be due to mechanisms other than ESBL production (Livermore and Paterson, 2006).

If isolates show resistance or diminished susceptibility to any of these five agents, it indicates suspicion for ESBL production, and phenotypic confirmatory tests should be used to ascertain the diagnosis.

**ii. Screening by dilution antimicrobial susceptibility tests**

The CLSI has proposed dilution methods for screening for ESBL production by *K. pneumoniae* and *K oxytoca, E. coli* and *Proteus mirabilis*. Ceftazidime, aztreonam, cefotaxime or ceftriaxone can be used at a screening concentration of 1 µg/ml or cefpodoxime at a concentration of 1 µg/ml for *Proteus mirabilis*; or 4 µg/ml, for the others. Growth at or above this screening antibiotic

concentration is suspicious of ESBL production and is an indication for the organism to be tested by a phenotypic confirmatory test (CLSI, 2009).

#### 2.3.3.2 Phenotypic confirmatory tests for ESBL production

**i. Cephalosporin/ clavulanate combination disks**

The CLSI advocates use of cefotaxime (30 µg) or ceftazidime (30 µg) disks with or without clavulanate (10 µg) for phenotypic confirmation of the presence of ESBLs in *Klebsiella* and *E. coli, P. mirabilis* and *Salmonella* spp. The CLSI recommends that the disk tests be performed with confluent growth on Mueller-Hinton agar. A difference of ≥5 mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanate disks is taken to be phenotypic confirmation of ESBL production (CLSI, 2009).

For *Enterobacter* spp. *C. freundii, Morganella, Providencia and Serratia* spp., it is better to use cefepime or cefpirome in the confirmatory tests as they are less prone to attack by the chromosomal AmpC β lactamases, which may be induced by clavulanate in these species (Livermore and Paterson, 2006).

**ii. Broth microdilution**

Phenotypic confirmatory testing can also be performed by broth microdilution assays using ceftazidime (0.25-128 µg/mL), ceftazidime plus clavulanic acid (0.25/4-128/4 µg/mL), cefotaxime (0.25-64 µg/mL), or cefotaxime plus clavulanic acid (0.25/4-64/4 µg/mL) (Queenan *et al*., 2004).Broth microdilution is performed using standard methods. Phenotypic confirmation is considered as ≥3 twofold serial-dilution decreases in minimum inhibitory concentration (MIC) of either cephalosporin in the presence of clavulanic acid compared to its MIC when tested alone.

Steward and colleagues suggested using cefoxitin susceptibility in isolates with positive screening tests but negative confirmatory tests as a means of reducing the mechanism of resistance (Steward *et al*., 2001). ESBL-producing isolates appear susceptible, while those with plasmid AmpC enzymes are

resistant. However, resistance to cefoxitin seems to be increasing in ESBL-producing isolates due to efflux or permeability changes or coexistence of ESBL with AmpC enzymes. The usefulness of this screen test may thus be diminishing.

Beside these CLSI recommended methods various other rapid and efficient tests are also commercially available for the detection of ESBL in Clinical laboratories.

### 2.3.4 Metallo β-lactamases

Class B enzymes are Zn2+ dependent β -lactamases that demonstrate a hydrolytic mechanism different from that of the serine β -lactamases of classes A, C, and D (Bush *et.al.*, 1995). Organisms producing these enzymes usually exhibit resistance to penicillins, cephalosporins, carbapenems, and the clinically available β -lactamase inhibitors (Walsh *et.al*, 2005). Interestingly, the hydrolytic profile of MBLs does not typically include aztreonam. MBLs likely evolved separately from the other Ambler classes, which have serine at their active site (Drawz and Bonomo, 2010). *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* produce class B enzymes encoded by mobile genetic elements (Drawz and Bonomo, 2010; Hujer *et.al*, 2006). In contrast, *Bacillus* spp., *Chryseobacterium* spp., and *Stenotrophomonas maltophilia* possess chromosomally encoded MBLs, but the majority of these host pathogens are not frequently responsible for serious infections (Walsh *et.al*, 2005). All MBLs hydrolyze imipenem, but their ability to achieve this varies considerably and the rate of hydrolysis may or may not correlate with the bacterium's level of resistance to carbapenems.

At the time, only two transferable types of MBLs had been studied, *Bacteroides fragilis* CcrA and IMP-1 from *P. aeruginosa*, but several other types viz. VIM-, SPM-1, GIM-1, etc. have been detected in several microorganisms (Walsh *et al.*, 2005).

### 2.3.4.1 Types of MBL

In 1989, Bush classified MBLs into a separate group (group 3) according to their functional properties and it yet remains the recommended referencing system for β-lactamases (Bush, 1989). This scheme was primarily based on substrate profiles (in particular imipenem hydrolysis), their sensitivity to EDTA, and their lack of inhibition by serine β -lactamase inhibitors.

At molecular level, MBLs are classified into three classes: Class A, B, and C. (Walsh *et al*., 2005). The rationale of class B1 is that the enzymes possess the key zinc coordinating residues of three histidines and one cysteine and accommodates the transferable MBL IMP, VIM, GIM, and SPM-1. Class B2 includes those that possess asparagines instead of the histidine at the first position of the principal zinc-binding motif and derive from *Aeromonas* spp. and the *Serratia fonticola* enzyme SFH-1. MBL L1 is the sole occupant of the class B3 enzymes, as it is singularly unique among all β -lactamases in being functionally represented as a tetramer.

### 2.3.4.2 Methods for MBL detection

Rather like the accepted ethos for the early detection of extended-spectrum β-lactamases, it is judicious to detect MBLs for precisely the same reasons. Unfortunately, there are no standardized phenotypic methods available and the testing criteria are likely to depend on whether the gene is carried by *P. aeruginosa* or a member of the Enterobacteriaceae, i.e., the evincible level of resistance. It is plausible that for screening Enterobacteriaceae for the presence of MBLs, a plate could contain ceftazidime with and without EDTA, but this would only be effective if the bacterium did not also produce an extended-spectrum β -lactamase, which cannot be assumed.

Given the fact that all MBLs are affected by the removal of zinc from the active site, in principle, their detection should be straightforward, and studies have seized upon this principle and used a variety of inhibitor β -lactam combinations to detect strains possessing these clinically important enzymes. The nonmolecular “gold standard” is well established in research laboratories where bacterial crude cell extracts are examined for their ability to hydrolyze

carbapenems and whether this hydrolysis is EDTA sensitive. For clinical laboratories concerned about implementing a reasonable screening system, Walsh and Colleagues suggest to target key isolates based on ceftazidime and carbapenem MIC data. For example, *P. aeruginosa* isolates with an imipenem MIC of ≥16 µg/ml may be considered appropriate candidates. For *Acinetobacter* spp. isolates, an imipenem MIC of ≥8 µg/ml, whereas for Enterobacteriaceae, an MIC of ≥2 µg/ml may be appropriate (Walsh *et al*., 2005).

# 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 done from March 2013 to August 2013, among the patients undergoing hemodialysis in the National Kidney Centre, Kathmandu using the materials and equipments attached on (Appendix II). The Gram negative bacilli, isolated from the patients, which showed resistance to at least two classes of antibiotic class as per the Clinical Laboratory Standards Institute (CLSI) guidelines, were included in this study**.**

### 3.2.1 Sample population and sample size

Urine and blood samples were taken from patients with renal failure, i.e. patients with chronic kidney disease, undergoing hemodialysis and patients with kidney transplant. Chronic kidney disease is defined as either kidney damage or decreased glomerular filtration rate below 60ml/min/1.73m2 for three months. Kidney damage refers to pathologic abnormalities in kidney including abnormalities in blood (creatinine level >1.4) or urine (loss of protein) (USRDS, 2005).

A total of 517 samples including Urine (496), Blood (21) obtained from the hemodialysis patients and chronic kidney patients visiting National Kidney Centre were included in the study. Samples obtained in a clean, leak proof container with no visible signs of contamination and labeled properly with demographic information of patients were accepted; otherwise a second sample was requested.

### 3.2.2 Collection and transportation of specimen

#### 3.2.2.1 Urine samples

Patients were requested to collect 10-20 ml of clean voided (clean catch) mid stream urine in a sterile, dry, wide necked, leak-proof 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 labeled properly and immediately delivered to the laboratory as soon as possible for further processing. When immediate delivery was not possible, the specimen was refrigerated at 4-6ºC, 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 (Forbes *et al*., 2007).

#### 3.2.2.2 Blood samples

Ten to twelve ml of blood was drawn aseptically from the patient using a sterile needle. The collected blood was transferred to culture media as soon as possible. If delay expected the blood was dispensed in a bottle containing Ethylenediamine tetra-acetic acid (EDTA). The culture media was incubated without delay (Forbes *et al*., 2007).

### 3.2.3 Culture of specimen

#### 3.2.3.1 Urine culture

The urine samples were cultured onto the Mac Conkey agar and Blood agar plates by the semi-quantitative culture technique using a standard calibrated loop.

- A calibrated loop was immersed vertically just below the surface of well-mixed uncentrifuged urine specimen.
- 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.
- The plates were then incubated at $37^0$C overnight.

- Colony count was performed so as to calculate the number of CFU per ml of urine and the bacterial count was reported as:

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

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

#### 3.2.3.2 Blood culture

The blood specimen collected was inoculated aseptically into Brain-Heart Infusion (BHI) broth in a ratio of 1:10, i.e. 10 ml of blood added to 100 ml of broth. The culture media was incubated at 37ºC for up to 1 week. The culture media was observed for any visible signs of growth. If there were signs of bacterial growth the broth was subcultured in Blood agar and MacConkey agar plates. The plates were incubated at 37ºC for overnight and observed for significant bacterial growth by performing blind subculture in addition to daily visual examinations (Forbes *et al*., 2007).

### 3.2.4 Identification of Gram negative isolates

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 (Appendix IV).

### 3.2.5 Antimicrobial susceptibility testing (AST)

Susceptibility tests of the different clinical isolates towards various antibiotics were performed by modified Kirby-Bauer modified disk diffusion method for the commonly isolated pathogens using Mueller Hinton Agar (MHA). The detail procedure of AST is shown in Appendix V.

### 3.2.6 Preservation of the MDR isolates

After performing the antimicrobial susceptibility testing, MDR isolates in pure culture were preserved in 20% glycerol containing tryptic soya broth and kept at -70ºC until subsequent tests for the presence of ESBL and MBL were performed.

### 3.2.7 Screening and confirmation for ESBL producers

The MDR isolates were screened for possible ESBL production using ceftazidime (30µg), cefotaxime (30µg) (CLSI, 2005). According to the guidelines, isolates showing ceftazidime <22 mm, cefotaxime < 27 mm are the possible ESBL producing strains. The procedure of Screening and confirmation of ESBL is shown in Appendix V.

The screen positive isolates i.e. suspected ESBL producers were subjected to Combined Disk (CD) test for confirmation of ESBL production using MASTDISCS$^{TM}$ extended spectrum β-Lactamase (ESBL) detection discs. The kit consisted of:

Set 1: Ceftazidime (30µg) and ceftazidime (30µg) plus clavulanic acid (10µg);

Set 2: Cefotaxime (30µg) and cefotaxime (30µg) plus davulanic acid (10µg).

### 3.2.8 Detection of MBL producers

This study subjected the MDR isolates showing resistance or reduced susceptibility to imipenem and ceftazidime for the detection of possible MBL production using simpler techniques as described. For the detection of production of MBL in *Pseudomonas* spp., the isolates were subjected to EDTA disk synergy test (EDST) (Picao *et al*., 2008). The production of

metallo β-Lactamase was confirmed when an increased growth-inhibitory zone between the ceftazidime and EDTA containing blank disc was seen and in Enterobacteriaceae and others, the isolates were subjected to Combined Disk (CD) assay using imipenem (10µg) and imipenem plus 100mM EDTA (Picao *et al.*, 2008). An increase in the zone diameter of ≥ 5mm in the presence of EDTA than imipenem disk alone confirmed the production of MBL by the test organisms. The detailed of the working protocols are explained in Appendix V.

### 3.2.9 Determination of MIC value of ciprofloxacin

The MIC value of ciprofloxacin towards isolates from blood and urine sample was determined using standard microbiological procedures (EUCAST, 2000). The details of determining MIC value is shown in Appendix VI.

## 3.3 Quality control

### 3.3.1 Monitoring and regular evaluation of laboratory equipment, reagents and media

Laboratory equipment like incubator, refrigerator, autoclave and hot air oven were regularly monitored for their efficiency. The temperature of the incubator and refrigerator was monitored everyday.

Reagents and media were regularly monitored for their manufacture and expiry date and proper storage. After preparation, they were properly labelled with prepartion date, expiry date. The quality of media prepared was checked by incubating one plate of each lot for sterility and using standard control strains for performance testing.

### 3.3.2 Purity plate

The purity plate was used to ensure that the inoculation used for the biochemical tests was pure culture and also to see whether the biochemical tests were performed in an aseptic condition or not. Thus, while performing biochemical tests, the same inoculum was subcultured in respective medium

and incubated. The media were then checked for the appearance of pure growth of organisms.

### 3.3.3 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. For the standardization of Kirby-Bauer test and for performance testing of antibiotics and MHA, control strains of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were tested primarily. 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. 4 Data analysis

All the results were entered in the worksheet of Statistical Package for Social Science (SPSS) software (Version 16.0). Chi-square test was used to determine significant association of dependable variables like susceptibility to ciprofloxacin, carbapenems etc to different independent variables like ESBL, MBL production etc. Sensitivity, specificity, positive predictive value, negative predictive value of the screening methods were also determined. The deatiled of data analysis is shown in Appendix IX.

# CHAPTER IV

# RESULTS

## 4.1. Clinical and microbiological profile of urine samples

### 4.1.1. Clinical profile of the samples and growth status among genders and different age groups

Of the 496 urine samples, 267(53.8%) samples were received from the male patients; the maximum number of culture request being received from age group of more than 65yrs; 101 (20.4%) samples and the least being received from age less than 14 years; 20 (4%) samples. Of the 267 samples from male, 42 (15.73%) showed signifcant growth with maximum number of growth being observed in age group more than 65 yrs; 15/42 (35.71%). Similarly, of the 229 samples from female, 57 (24.89%) showed significant growth with maximum number of growth being observed in age group of more than 65 yrs; 13/57 (22.8%). Of the different isolates of various age groups, isolates from age group less than 14, 25-34 and 55-64 showed 100 % MDR (3/3, 6/6 and 19/19 MDR isolates respectively) while the MDR percentage of age group more than 65 was 87.5% (28/32).

### 4.1.2 Microbiological profile of urinary isolates

Among the processed 496 urine samples 99 Gram negative isolates were obtained, which is 19.95 % of total urine samples. *E. coli* was the most frequently isolated species with 71 (71.71%) isolates, among these, 63 (88.73%) were found to be MDR-strains. Among the 88 MDR strains, highest numbers of isolates, i.e. 49 were isolated from female patients.

**Table 2. Microbiological profile of urinary isolates and their genderwise distribution**

| Organisms | Male | | Female | | Total MDR (%) | Total no. of isolates |
|---|---|---|---|---|---|---|
| | No. of isolates | MDR (%) | No. of isolates | MDR (%) | | |
| *E. coli* | 28 | 26(92.85) | 43 | 37(86.04) | 63(88.73) | 71 |
| *K. pneumoniae* | 9 | 9(100) | 12 | 10(83.33) | 19(90.47) | 21 |
| *P. aeruginosa* | 3 | 2(66.66) | 0 | 0 | 2(66.66) | 3 |
| *Proteus vulgaris* | 2 | 2(100) | 2 | 2(100) | 4(100) | 4 |
| Total | 42 | 39(92.85) | 57 | 49(85.96) | 88(88.88) | 99 |

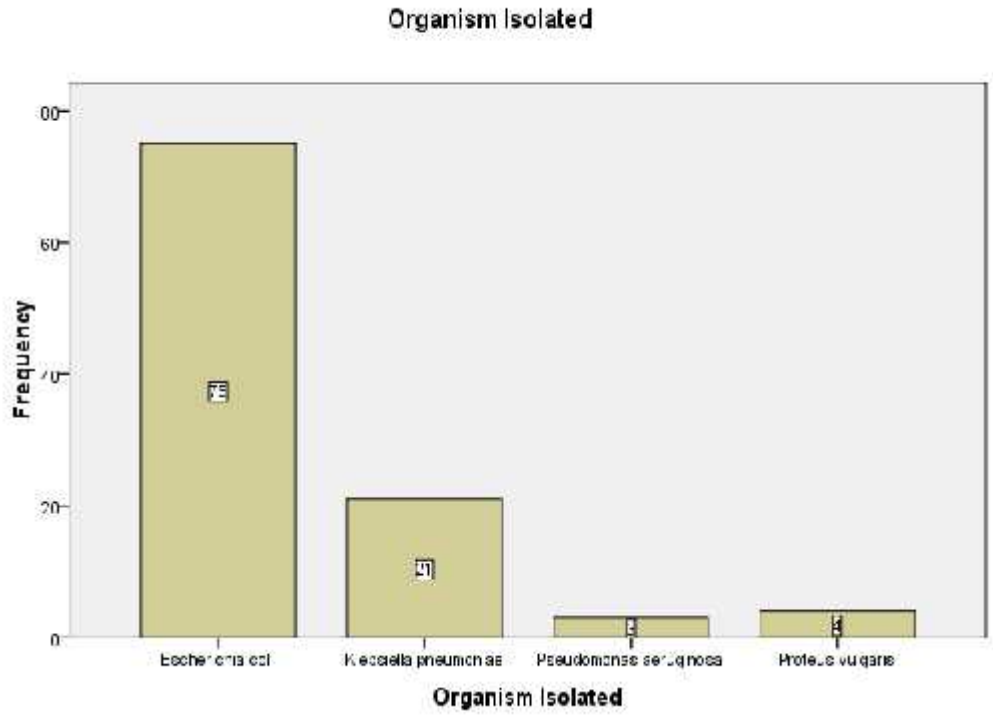


**Fig 1: Number and types of different bacterial pathogens isolated**

## 4.2 Clinical and microbiological profile of blood samples

Of the 21 blood samples only 4 showed growth on culture (19.04%). Two organisms each were isolated out of 10 male and 11 female patients respectively. Maximum number of cultures were requested from patients of age group 45-54; 6 (28.57%). All of the four isolates were *Escherichia coli*, and none of them were MDR.

## 4.3 Clinical and microbiological profile of isolates according to morbidity

### 4.3.1 Pattern of sample according to morbidity

Out of 517 samples most of the samples 384 (74.27%) were obtained from chronic kidney patients visiting the OPD with high protein leakage in urine and high creatinine in their blood. All the samples cultured from chronic kidney patients were urine. Likewise, all together 129 samples were taken from hemodialysis patients; of which 108 (83.7%) was urine and 21 (16.3%) was blood sample. From renal transplant patients four urine samples were obtained.

### 4.3.2 Pattern of isolates and MDR according to morbidity

Of the total 103 isolates, 81 were obtained from chronic kidney patients; 21 from hemodialysis patients and single isolate was obtained from renal transplant patient. 51 out of 57 *E. coli* isolates from chronic kidney patients were MDR whereas the number was 64.70% (11/17) in hemodialysis patients. All of the *P. aeruginosa* isolated were from chronic kidney patients and 66.66% (2/3) of them were MDR. Altogether 88.88 % (16/18) of the *K. pneuomniae* isolated from kidney patients were MDR whereas 100 % (3/3) of them were MDR when isolated from hemodialysis patients. However, the pattern of isolates was found to be statistically insignificant to the morbidity on performing chi-square test.

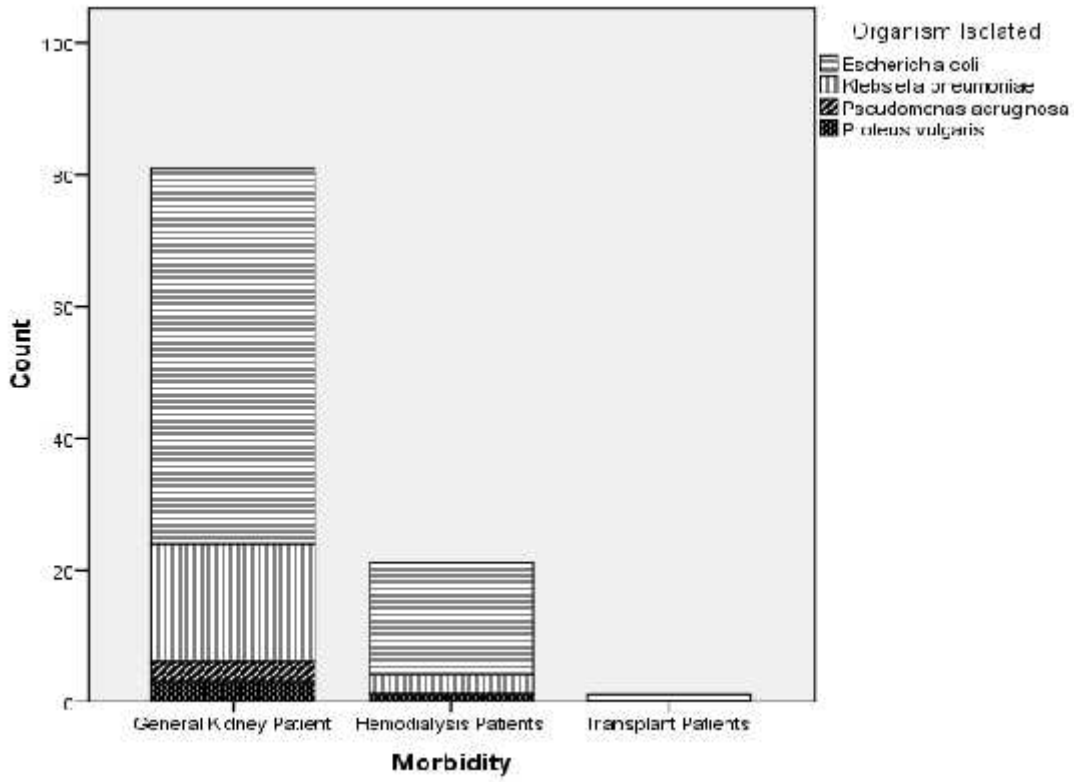


**Fig 2: Organisms isolated with respect to morbidity**

**Table 3. Microbiological profile MDR isolates and their distribution in different types of patients**

| Organisms | Chronic Kidney Patients | | Hemodialysis | | Transplant | | Total MDR (%) | Total no. of isolates |
|---|---|---|---|---|---|---|---|---|
| | No. of isolates | MDR (%) | No of isolates | MDR (%) | No. of isolates | MDR (%) | | |
| *E. coli* | 57 | 51(89.47) | 17 | 11(64.70) | 1 | 1(100) | 63(84) | 75 |
| *K. pneumoniae* | 18 | 16(88.88) | 3 | 3(100) | 0 | 0 | 19 (90.47) | 21 |
| *Ps. aeruginosa* | 3 | 2(66.66) | 0 | 0 | 0 | 0 | 2 (66.66) | 3 |
| *P. vulgaris* | 3 | 3(100) | 1 | 1(100) | 0 | 0 | 4 (100) | 4 |
| *Total* | 81 | 72(88.88) | 21 | 15(71.42) | 1 | 1(100) | 88(85.43) | 103 |

### 4.3.3 Morbidity and age group

Out of the 382 samples cultured from kidney patients the highest number of samples was obtained from age group of more than 65 years; 82 (21.4%) and least number of samples were obtained from the age group less than 14; 20 (5.4%). Among the 129 samples from hemodialysis patients 29 (22.5%) samples were of patients of age group 25-34, and a single sample was obtained from age group less than 14. Out of the 4 samples from transplant patients 2 (50%) were from age group 55-64 and a sample each was cultured from patients of age group 25-34, 35-44. The association between age group and morbidity was statistically insignificant.

**Table 4. Age group-wise distribution of different types of patients**

| | | Age Group | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | less than 14 | 15-24 | 25-34 | 35-44 | 45-54 | 55-64 | More than 65 | Total |
| Morbidity | General Kidney Patient | 20 | 30 | 59 | 65 | 64 | 64 | 82 | 384 |
| | Hemodialysis Patients | 1 | 13 | 29 | 23 | 23 | 20 | 20 | 129 |
| | Transplant Patients | 0 | 0 | 1 | 1 | 0 | 2 | 0 | 4 |
| Total | | 21 | 43 | 89 | 89 | 87 | 86 | 102 | 517 |

### 4.4 Antibiotic susceptibility pattern of the isolates

#### 4.4.1 Antibiotic susceptibility pattern of the Gram negative isolates

Of the 12 different antibiotics used against Gram negative isolates, imipenem was found to be the drug of choice with a susceptibility of 75.7% (78/103) followed by amikacin with 63.1% susceptibility. Among others doxicycline hydrochloride, cotrimoxazole and nitrofurantoin were found to be more effective with 44%, 42.7% and 38.8% susceptibility respectively. Whereas, cephalexin, nalidixic acid and ceftriaxone were found to be the least effective drugs with susceptibility of 0.97%, 3.9%, 14.16% respectively. (Table 5)

**Table 5. Antibiotic sensitivity pattern of Gram negative isolates from different samples**

| Antibiotic used | Sensitive | | Intermediate | | Resistant | | Total |
|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | |
| Imipenem | 78 | 75.7 | 12 | 11.7 | 13 | 12.6 | 103 |
| Doxicycline | 34 | 44 | 22 | 21.4 | 47 | 45.6 | 103 |
| Norfloxacin | 27 | 26.2 | 11 | 10.7 | 65 | 63.1 | 103 |
| Cephalexin | 1 | 0.97 | 4 | 3.88 | 98 | 95.15 | 103 |
| Nitrofurantoin | 40 | 38.8 | 21 | 20.4 | 42 | 40.8 | 103 |
| Ciprofolxacin | 25 | 24.3 | 10 | 9.7 | 68 | 66 | 103 |
| Cotrimoxazole | 44 | 42.7 | 3 | 2.9 | 56 | 54.4 | 103 |
| Amikacin | 65 | 63.1 | 21 | 20.4 | 16 | 15.5 | 103 |
| Ofloxacin | 32 | 31.1 | 4 | 3.9 | 67 | 65 | 103 |
| Nalidixic Acid | 4 | 3.9 | 16 | 15.5 | 83 | 80.6 | 103 |
| Ceftazidime | 20 | 19.4 | 7 | 6.8 | 76 | 73.8 | 103 |
| Ceftriaxone | 15 | 14.6 | 4 | 3.9 | 84 | 81.6 | 103 |

### 4.5. Multidrug resistant bacterial isolates and their β lactamase production profile

Of 99 bacterial isolates from urine, 88 (88.88%) were multi-drug resistance. However, none of the four isolates from blood samples were MDR. The MDR isolates were suspected as ESBL producers on the basis of reduced susceptiblity to ceftazidime and cefotaxime and possible MBL producer if it showed reduced susceptibility to impienem. On this ground 59 MDR isolates suspected of being the possible producers of β lactamases were subjected to the detection of ESBL production and 11 isolates to MBL production. Of the 59 isolates, 35 isolates were found to be ESBL producers (35 from urine, none from blood) whereas a single isolate was MBL producer. Urinary isolates were among the predominant β-lactamase producers with 35/59 ESBL producers, 1/11 MBL producers. (Table 6)

**Table 6. Distribution of MDR isolates among different samples and their β-lactamase production profile**

| Specimen | No. of bacterial isolates | MDR strains | | No. of ESBL producers | No. of MBL producers |
|---|---|---|---|---|---|
| | | No. | % | | |
| Urine | 99 | 88 | 88.88 | 32 | 1 |
| Blood | 4 | 0 | - | - | - |
| Total | 103 | 88 | 85.43 | 32 | 1 |

### 4.6. ESBL production profile among various bacterial genera

Of 59 MDR isolates tested for ESBL production, 35 (59.32%) bacterial isolates tested positive for ESBL production. The majority consisted of *E. coli* i.e. 29/35(82.85%) followed by *K. pneumoniae* 4/35 (11.42%) and *P. aeruginosa* 2/35(5.71%). ESBL production was not detected in *P. vulgaris*. (Table 7)

**Table 7. Profile of ESBL producing bacterial strains from different sample**

| Organisms | Total isolates | No. of MDR strains | No.of suspected ESBL producers | No. of cases confirmed (%) | Negative cases on confirmation |
|---|---|---|---|---|---|
| *E. coli* | 75 | 63 | 45 | 29 (64.44) | 16 |
| *K. pneumoniae* | 21 | 19 | 10 | 4 (40) | 6 |
| *Ps. aeruginosa* | 3 | 2 | 2 | 2 (100) | 0 |
| *P. vulgaris* | 4 | 4 | 2 | 0 (0) | 2 |
| Total | 103 | 88 | 59 | 35 (59.32) | 24 (27.27) |

### 4.7. Screening for ESBL production using different screening agents

Of the total 88 MDR isolates, 59 MDR isolates that were suspected of being producers of various β lactamases were screened for ESBL production using ceftazidime and ceftriaxone as the CLSI recommended screening agents. Of the agents used, ceftazidime had the highest value for both sensitivity and PPV (94.2% and 76.7% respectively). Ceftriaxone was found to have low sensitivity and PPV of 91.42% and 61.53% respectively. However, the screening results obtained from ceftazidime and ceftriaxone were found statistically significant with the confirmatory results of ESBL production ($p < 0.05$).

**Table 8. Screening for ESBL production using different screening agents**

| Screening Agents | Screening Criteria | ESBL Screening | | No. of confirmed ESBL producers | Sensitivity (%) | positive predictive value (PPV)(%) |
|---|---|---|---|---|---|---|
| Ceftazidme (30µg) | ≤22mm | Screen positives | 43 | 33 | 94.2 | 76.7 |
| | | Screen negatives | 16 | 2 | | |
| Cefotaxime (30µg) | ≤27mm | Screen positives | 52 | 32 | 91.42 | 61.53 |
| | | Screen negatives | 7 | 3 | | |

## 4.8 Confirmation of ESBL production using different combination disc assay

Of the 59 MDR isolates subjected for ESBL confirmation test using two different combination disks, regardless of their screening results, the ceftazidime-clavulanate and combined disk detected all the 35 (100%) ESBL positive isolates. The cefotaxime-clavulanate missed two isolates; only 33/35 (94.28%) were correctly identified (Table 9).

**Table 9. Patterns of ESBL production according to the use of various combination disks**

| S.N | Combination disks (CD) Assay | Criteria for confirmation | No. of suspected ESBL producers | No. of confirmed cases | Total confirmed cases | Negative cases after confirmation |
|---|---|---|---|---|---|---|
| 1 | CAZ (30µg)<br>CAZ (30µg) plus CV (10µg) | Increase in zone size of ≥5 mm with ≥1 of the combination disks | 59 | 35 | 35 | 24 |
| 2 | CTX (30µg)<br>CTX (30µg) plus CV (10µg) | | | 33 | | |

## 4.9 Pattern of quinolone (ciprofloxacin) and aminoglycoside (amikacin) resistance MDR isolates and their association with ESBL production

Of the 88 MDR strains, only 25 (24.3%) isolates showed sensitivity towards ciprofloxacin. However, 4 of the 25 (16%) sensitive isolates were ESBL positive. Of the remaining 39 resistant isolates, 29 (74.34%) produced ESBL whereas; of the 10 intermediate isolates, 2 (20%) produced ESBL and all others were ESBL negative. The association between the production of ESBL and resistance shown towards ciprofloxacin was found statistically significant ($p<0.05$). Similarly, of the 88 MDR isolates, 16 (18.18%) were resistant to amikacin, of which 8 (50%) showed ESBL production. Of the total 65 amikacin susceptible isolates, 20 (30.76%) showed ESBL production and 7 out of 21 (33.33%) amikacin intermediate isolates showed ESBL production. The association between the production of ESBL and resistant shown towards amikacin was found statistically insignificant ($p>0.05$). (Appendix IX)

### 4.10. Statistical pattern of results

Statistical differences in etiologies of various dependent variables were determined by $\chi^2$ test. The highest percentage of growth seen in our result among the female patients of different bacterial infections was found to be statistically significant ($p < 0.05$). However, the higher number of culture isolates from urine samples were statistically insignificant ($p > 0.05$). Likewise, higher pattern of growth seen among chronic kidney patients suffering from different bacterial isolates was also not significant statistically ($p > 0.05$).

The null hypothesis that there is no association between MDR isolates and their ESBL production is rejected statistically ($p > 0.05$). Among the different classes of antibiotics used the statistical pattern of resistance of ciprofloxacin, norfloxacin, ceftazidime, ceftriaxone and ESBL production each was statistically significant ($p < 0.05$). However none of the resistance among other drugs showed statistically significant results with ESBL production. The results are shown in Table 10, Table 11, Table 12 and Appendix IX.

**Table 10. Statistical pattern of gender and morbidity-wise association with bacterial infections**

| N=103 | Bacterial infections | | P value |
|---|---|---|---|
| Independent Variables | Positive(%) | Negative(%) | |
| Gender (Female) | 24.59 | 75.41 | **0.035** |
| Morbidity (Kidney patients) | 21.31 | 78.69 | 0.911 |

**Table 11. Comparison of antimicrobial resistance patterns of ESBL producing (n=35) and non-producing (n=68) isolates**

| Antimicrobials | ESBL producer (N=35) | ESBL non Producer (N=68) | P value |
|---|---|---|---|
| Imipenem | 6 | 7 | 0.465 |
| Doxicycline | 17 | 30 | 0.490 |
| Norfloxacin | 28 | 37 | **0.029** |
| Cephalexin | 35 | 63 | 0.259 |
| Nitrofurantoin | 17 | 25 | 0.462 |
| Ciprofolxacin | 29 | 39 | **0.034** |
| Cotrimoxazole | 22 | 34 | 0.453 |
| Amikacin | 8 | 9 | 0.450 |
| Ofloxacin | 26 | 41 | 0.370 |
| Nalidixic Acid | 31 | 52 | 0.330 |
| Ceftazidime | 33 | 43 | **0.003** |
| Ceftriaxone | 32 | 53 | **0.048** |

**Table 12. Statistical pattern of gender and morbidity and MDR pattern wise association with ESBL production**

| N=35 | ESBL production | | P value |
|---|---|---|---|
| Independent Variables | Positive (%) | Negative (%) | |
| Gender (Male) | 43.18 | 56.81 | 0.089 |
| Morbidity (Hemodialysis patients) | 47.61 | 52.38 | 0.113 |
| MDR | 36.36 | 63.64 | 0.216 |

## 4.11 Minimum inhibitory concentration of ciprofloxacin among MDR isolates

The MDR isolates showed high degree of resistance towards Ciprofloxacin and their association was also statistically significant. Therefore, MIC value of isolates for ciprofloxacin was calculated. Most strains of the isolates had the MIC value of >4 µg/ml and 8 µg/ml for ciprofloxacin. Similarly one sample showed highest MIC value of 128 µg/ml. The results are shown in Appendix VIII.

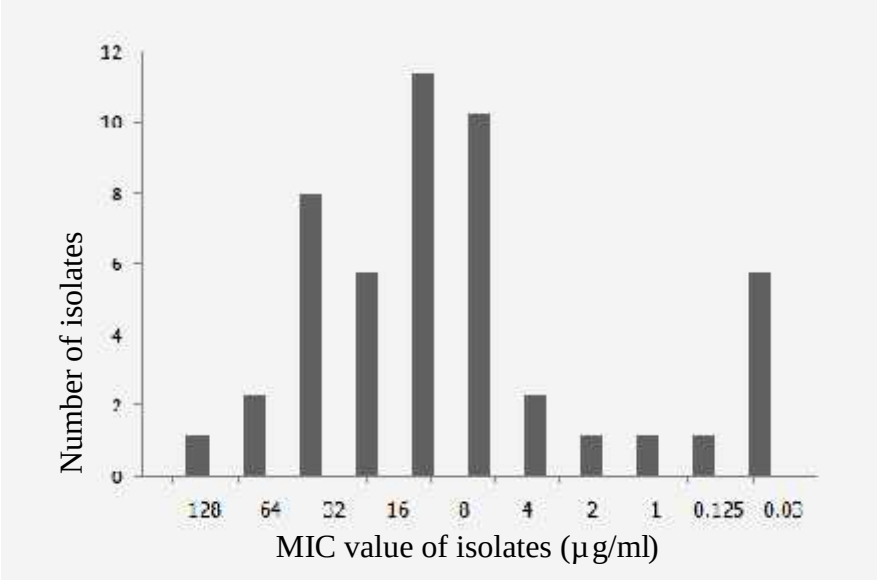


Fig 3: MIC value of ciprofloxacin among the various isolates

# CHAPTER V

# DISCUSSION

## 5.1 DISCUSSION

Of the 517 samples consisting of various clinical specimens (urine, blood), only 103 (19.92%) showed significant growth. Of the total isolates 88 (85.83%) were found to be multidrug resistance. A similar study by Baral (2008) showed the low culture positivity of 22.35% among various samples and a high prevalence of multidrug resistance i.e. 41.07% among various clinical isolates. Another study conducted by Poudyal (2010) in National Public Health Laboratory also showed 19.61% growth and 61.27% of MDR among the isolates. On another study conducted in Germany 176 of the 15,034 *E. faecalis* were vancomycin-resistant (VRE), and 677 of the 15 516 *E. coli* and 438 of the 6139 *K. pneumoniae* isolates were resistant to third-generation cephalosporins (3GCR) (Meyer *et al*., 2008). In a study by Bomjan (2005), a high proportion (60%) of multidrug resistance pattern among the urinary and sputum isolates was reported. Pokhrel ***et al.*** (2005) in a study of urinary and sputum isolates in TUTH showed 47.57% and 60.40% of the sputum and urinary isolates respectively were multidrug resistance.

Among the 496 urine samples, 99 (19.95%) showed significant growth. Similar results were obtained by Poudyal (2010) where 16.88% urine samples showed significant growth**.** The low number of growth positivity among urine samples was observed in similar studies carried out by Baral (2008), Bomjan (2005), Chettri *et. al***.** (2001) and Dhakal (1999). The referral of all the patients seeking intervention regarding problems of urinary tract to urine culture, the prior use of the antibiotics, or the possible presence of the fastidious bacteria are some of the reasons behind the poor growth positivity seen in urine culture. Contrary to the earlier studies in which large female population seeking urine culture were reported, this study saw an increasing number of male patients requesting urine culture. However, high culture positivity was seen among female than males and so was the case with the distribution of MDR strains among gender with 55.68% MDR isolates obtained from female.

Similar results were seen in the earlier studies by Bomjan (2005) and Baral (2008). A study by Falhal *et. al.* (2012) in hemodialysis patients of Baghdad showed 15% growth, which is similar to our findings.

Only the Gram negative isolates were further processed for AST and other microbiological testings. This is because most of the predominant pathogens isolated from hemodialysis patients are of Gram negative class (Paterson and Bonomo, 2005). Of the 103 Gram negative isolates, *E. coli* was the predominant pathogen adding up to 72.81% of the total isolates. High numbers of Gram negative isolates were reported by the investigations conducted by Baral (2008), Bomjan (2005), Falhal *et. al.* (2012), Manandhar (1996), Mathai *et al*. (2001), Marquez *et al*. (2008), Poudyal (2010) and Puri (2006). Among the 99 isolates from urine sample, 71 (71.71%) were *E. coli*, followed by *K. pneumoniae* (21.42%). Similarly low number, 3 and 4 isolates each of *P. aeruginosa* and *P. vulgaris* were obtained. These results were in harmony with the results obtained in smilar studies by Baral (2008), Dhakal (1999), Farrell *et al*. (2003), Gales *et al*. (2002)**,** Kahlmeter (2000), Mathai *et al***.** (2001) and Poudyal (2010).

A higher rate of infection was found among female patients (57.28%) than male patients (42.72%). Association between the culture positivity among gender was found statistically significant ($p<0.05$). This result is in harmony with the results obtained by various researchers who also found more number of infections in female as compared to the male patients. (Baral**,** 2008; Bomjan**,** 2005**;** Dhakal, 1999; Falhal *et. al.*, 2012**;** Manandhar,1996**;** Pokhrel *et al*., 2005**;** Poudyal**,** 2010; and Puri, 2006)

Of the 75 *E. coli* isolates 63 (84%) were MDR, *K. pneumoniae* isolates showed 90.47% MDR whereas all the four isolates (100%) of *P. vulgaris* were MDR. These results resembled the outcomes of previous studies by Baral (2008), Bomjan (2005)**,** Koljalg *et. al*. (2009), Marquez *et. al*. (2008), Pokhrel *et. al*. (2005) and Poudyal (2010).

The high level of drug resistance seen among *E. coli* is due mediated by β-lactamases, which hydrolyze the β-lactam ring inactivating the antibiotic. The

classical TEM-1, TEM-2, and SHV-1 enzymes are the predominant plasmid-mediated β-lactamases of Gram-negative rods (Livermore, 1995). Mutations at the target site i.e. *gyr*A, which is a gyrase subunit gene, and *par*C, which encodes a topoisomerase subunit, confer resistance to fluoroquinolones (Ozeki *et. al., 1*997). In addition to this mechanism, there are more than seven efflux systems in *E. coli* that can export structurally unrelated antibiotics; these multidrug resistance efflux pump (MDR pump) systems contribute to intrinsic resistance for toxic compounds such as antibiotics, antiseptics, detergents, and dyes (Sulavik *et al*., 2001).

Similarly higher level of drug resistance seen among *K. pneumonia*e is mediated by the production of different kind of β-lactamases primarily ESBL, AmpC and Metallo β-lactamases. The fact that the carriage of resistance trait for quinolones and aminoglycoside in the plasmid along with the gene for β-lactamases have had a great impact on the drug resistance character shown by these pathogenic bacteria (Lee *et. al*., 2003; Picao *et. al*., 2008; Thomson *et. al*., 2000 and Walsh *et. al*., 2005). The *acr*R and *ram*A genes are involved in expression of the MDR phenotype in strains of *K. pneumoniae* (Denyer *et. al*., 2004). Moreover, various clinical isolates show alteration of nonspecific porins associated with the presence of active drug efflux in these bacteria; both processes maintain a very low intracellular concentration of drugs and contribute to a high resistance level for structurally unrelated molecules including β-lactam antibiotics, quinolones, tetracyclines, and chloramphenicol (Martinez-Martinez *et. al*., 2002).

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

enzymes is the most common mechanism of resistance in Gram negative bacteria causing clinical significant infection (Bush *et.al.,* 1995).

Imipenem with susceptibility of 75.7% was found to be the most effective drug against Gram negative isolates. However, it is the alternative therapeutic agent in absence of other first line drugs. Among others doxicycline hydrochloride, cotrimoxazole and nitrofurantoin were found to be more effective with 44%, 42.7% and 38.8% susceptibility respectively. Cephalexin, nalidixic acid, with the sensitivity of 0.97%, 3.9%, respectively were the least effective drugs. As these both antibiotics are the first line drugs which are easily hydrolysed by the bacterial enzymes and offer less in the treatment of Gram-negative bacterial infections. In concurrence to our findings, imipenem was found to be the most effective drug against Gram negative isolates in the study of Baral (2008), Oteo *et. al*., (2001) and Puri (2006). However, Poudyal (2010) found that meropenem was more effective than imipenem. Meropenem is more stable than imipenem to kidney enzyme dehydropeptidase and can be administered without cialistin hence, may be better therapeutic option than imipenem (Jones *et. al*., 2008).

Of the 129 culture (108 urine and 21 blood) performed from hemodialysis patients, 21 (16.27%) showed growth of pathogens. Although the growth was low, the pattern of MDR was high (71.42%) among the dialysis patients. All of the dialysis patients were suffering from hypertension, which indicates HTN is one of the leading causes of Kidney disease. 93% of the patients in the study by Falhal *et. al.* (2012) were found to be suffering from HTN. In one study conducted by D' Agata *et. al.*, (2000) chronic hemodialysis patients were more prone to infections of various types. Most of them suffered from urinary tract infections, and blood stream infections. Similar results were obtained by Falagas *et al.,* (2005), Linton *et al.,* (1970), Naqvi and Collins (2006) and Pop-vicas *et al*., (2008). The prevalence and acquisition of multidrug-resistant Gram-negative bacteria surpassed that of vancomycin resistant enterococci and methicillin-resistant *Staphylococcus aureus*. Endogenous acquisition, as opposed to patient-to-patient spread, was the predominant mechanism of acquisition. Residence in a long-term care facility

and antibiotic exposure may be important factors promoting the spread of multidrug-resistant gram-negative bacteria among this patient population (Pop-vicas *et al*., 2008).

Most of the *E. coli* (11/17) isolated from dialysis patients were MDR, whereas 100% *K. pneumoniae* and *P. vulgaris* were MDR (3/3 and 1/1 respectively). Similar results were obtained by Aurora *et. al*., (2008) Falagas *et. al.*, (2006), Naqvi and Collins (2006) and Linton *et al.,* (1970).

Although this study did not address infections that are caused by Multidrug resistant Gram negative bacteria (MDRGN), colonization is a necessary prerequisite for subsequent infection. Translocation of MDRGN across the intestinal wall into the blood stream and fecal contamination of vascular devices lead to infections (Donskey, 2004; Tancrede *et. al*., 1985). Thus, patients who are colonized with MDR Gram negative bacteria are at greater risk for subsequently developing an infection with these bacteria. In one study, 15% of hospitalized patients who were colonized with MDR Gram negative bacteria developed a bacteremia caused by the same colonizing strain of MDR Gram negative bacteria (Ben-Ami *et al*., 2006). The co-resistance to multiple antimicrobials among MDR Gram negative bacteria severely limits the therapeutic options that are available to physicians for treating infections that are caused by MDR Gram negative bacteria.

Most of the samples acquired for culture were of age group more than 65 years, which is in accordance with the studies conducted by Aurora *et. al*. (2008), D' Agata *et. al.* (2000), Falagas *et. al.* (2006) and Naqvi and Collins (2006). However, the association between age group and culture positivity was statistically insignificant.

A total of 59 multi-drug resistance bacteria were screened for ESBL production using two of the CLSI recommended screening agents viz. ceftazidime and cefotaxime. The lowest sensitivity (91.42%) was observed with cefotaxime, when the screen positive isolates were subjected to ESBL confirmation using inhibitor potentiated disk diffusion (IPDD) test. Ceftazidime was comparatively more sensitive towards ESBL screening

(94.2%). Poudyal (2010) found that cefotaxime is more effective in screening ESBL than ceftazidime, however the percentage of sensitivity is in harmony with our result. Similar results were also obtained by Baral (2008). In a study to determine the performance of screening methods for ESBL detection in South-East England, Hope *et al*. (2007) found isolates submitted solely on cefpodoxime resistance 256/372 (69%) proved cephalosporin-susceptible or had only borderline resistance with no clear mechanism demonstrable; the proportion decreased to 28/160 (18%) for those submitted on the basis of resistance to ceftazidime, 18/122 (15%) for those resistant to cefotaxime and 26/496 (5%) for those resistant to both cefotaxime and ceftazidime. A relatively high sensitivity of 94.2% and a positive predictive value of 76.7% was observed with ceftazidime. Katz *et. al*. (2004) subjected 115 isolates of *E. coli* and 157 isolates of *Klebsiella* spp. for screening using cefotaxime, ceftazidime, and cefpodoxime disks. The sensitivity of screening criteria ranged between 98.6% for cefotaxime and 92.8% for ceftazidime, and the specificity ranged between 100% for cefotaxime and cefpodoxime and 99.0% for ceftazidime. Similar results were obtained in the study of Ho *et. al*. (2000) and Jain *et. al*. (2007).

In our study, of the 25 isolates sensitive to ceftazidime (Screen negatives) in disk diffusion test, 2 isolates were found to be ESBL producers when tested with other IPDDT suggesting the possible presence of CTX-M type ESBL, however, due to lack of genetic charecterization of the enzyme, it could not be confirmed. CTX-M-type enzymes were reported in Germany and Argentina in 1989, and so far, more than 67 CTX-M-type β-lactamases have been identified, mostly in *E. coli*, *K.pneumoniae*, and *S. enterica* serovar Typhimurium isolates (Gonullu *et. al*., 2008). Diagnostic laboratories may fail to identify CTX-M–positive isolates as ESBL producers if ceftazidime resistance is used as the sole screening criterion since CTX-M producing isolates have typical propensity towards cefotaxime, however, are susceptible to ceftazidime in vitro. CTX-M extended spectrum β-lactamases (ESBLs) differ from those derived from TEM and SHV enzymes by their preferential hydrolysis of cefotaxime and ceftriaxone compared with ceftazidime (Lewis *et. al*., 2007). They also differ from an evolutionary standpoint and are more

closely related to the chromosomal enzymes of *Kluyvera* spp and these enzymes are increasingly described worldwide, particularly in South America, Europe, and East Asia (Decousser *et. al*., 2001; Koh *et. al*., 2004).

In our study, 2 different combination disks were used for the confirmation of ESBL production. The inhibitor potentiated disk diffusion test (IPDDT) identified 35 suspected isolates as confirmed ESBL producers. The ceftazidime-clavulanate combination disk correctly identified only 35 isolates as confirmed ESBL producers. None of the ceftazidime susceptible isolates that were screen negative tested positive with ceftazidime-clavulanate combination disk. However, cefotaxime-clavulanate combination disk correctly identified only 33 of the ESBL producing isolates. This pattern of result is in contrast to the results that were obtained in the study carried out by Baral (2008), Hope *et. al*. (2007), Ho *et. al*. (2000), Jain *et. al*. (2007), Srisangkaew *et. al*. (2004) and Poudyal (2010). However this result is in accordance with the findings by Jonathan (2005) who conducted a study in UK among the urinary tract infection patients. More than 100 different sequence variants of SHV and TEM genes with various levels of activity against ceftazidime, cefotaxime, and ceftriaxone have so far been demonstrated. However the most commonly encountered ones are TEM-3, which confers broad resistance to ceftazidime, cefotaxime, and ceftriaxone, and TEM-10, which confers high-level resistance to ceftazidime and appears to be sensitive in vitro to cefotaxime and ceftriaxone. Most ESBLs are derived from the TEM and SHV genes. These usually confer resistance to cefotaxime but spare ceftazidime. These genes are thought to have evolved by mutation of the chromosomal β lactamase of *Kluyvera* spp.; the mutated forms have now escaped to plasmids and are being distributed among other enterobacteria (Jonathan, 2005).

Patients with kidney failure are more prone to developing urinary tract infections for a number of reasons. First of all, the mere act of passing urine tends to flush out the urinary tract of infectious agents so they cannot gain a foothold and cause problems. Once the kidneys fail and the production of urine is decreased, this normal flushing action is gone. UTIs usually are

caused by bacteria which normally inhabit the bowel and spread to the urinary tract by local extension. Patients with chronic kidney disease have abnormalities in their immune system which impair their ability to fight infection. Although the white blood cell count in the typical laboratory testing profile may be normal, the dialysis patient's white blood cells (which are the primary line of defense against infection) typically do not function normally (Wish, 2002).

The spread of infectious agents from the dialysis machine or procedure to the kidney patient is unusual in the setting of current infection control policies but does rarely occur. Even if the dialysate fluid is contaminated with an infectious agent, the dialyzer membrane material is an effective barrier to the spread of that agent from the machine to the patient's blood. Infections related to the dialysis treatment, although unusual, generally occur during the put-on or take-off process when infectious agents can be introduced into the patient's dialysis catheter or permanent vascular access because of improper sterile technique. Therefore, it is important for patients to become familiar with and insist that sterile technique be used during the beginning and ending of dialysis treatment. The antimicrobial susceptibility tests in this study revealed that amikacin, ceftazidime, and imipenem act well on isolated bacteria. Although aminoglycosides are effective against many Gram negative bacteria, they may be ototoxic and nephrotoxic, especially in patients with diminished renal function (Falahs et. al., 2002).

Of the 103 bacterial isolates consisting of 4 genera 59 isolates were tested for ESBL production based on their reduced susceptibility towards ceftazidime and cefotaxime. ESBL production was seen among 35 (33.98%) isolates. The majority consisted of *E. coli* i.e. 29/35 (82.85%) followed by *K. pneumoniae* 4/35 (11.42%). Two isolates of *P. aeruginosa* showed ESBL production. Similar pattern of results were seen in the study carried out by Baral (2008) who showed the presence of 28.12% ESBL producers out of 96 MDR isolates, Bomjan (2005) who found the presence of 28.3% ESBL producers among various clinical isolates and Sharma (2004) who found 8% *K. pneumoniae*, 12.5% *E. coli*, 12.5% *Citrobacter freundii*, 25% *A. calcoaceticus* and 5% *P.*

*aeruginosa* as ESBL-producing strains. Poudyal (2010) reported 62.72% of ESBL producers of which 86.96% were *E. coli*. From the data of SMART program in the Asia-Pacific region, of 3,004 gram-negative bacilli collected from intra-abdominal infections during 2007, 42.2% and 35.8% of *E. coli* and *Klebsiella* spp. respectively were ESBL positive. Moreover, ESBL rates in India for *E. coli*, *K. pneumoniae*, and *K. oxytoca* were 79.0%, 69.4%, and 100%, respectively. ESBL-positive *E. coli* rates were also relatively high in China (55.0%) and Thailand (50.8%) (Hawser *et. al*., 2009).

In our study of the 11 isolates with reduced susceptibility to imipenem subjected to MBL detection only a single isolate (9.09%) of *P. aeruginosa* showed positive result. The same isolate was also found to co-produce ESBL. In a study conducted in France in 241 clinical strains of IPM-nonsusceptible *P. aeruginosa* isolated from 2002 to 2004, 110/241 (46%) were MBL positive using phenotypic methods while 107/241 (45%) were PCR positive for MBL genes: 103/241 (43%) for $bla_{VIM}$ and 4/241 (2%) for $bla_{IMP}$ (Pitout *et. al*., 2005). In a similar study conducted by Baral (2008), of the 117 MDR isolates, 33 (28.2%) isolates were MBL producers among them 25 (75.75%) *E. coli* isolates and 3 (9.1%) *Citrobacter freundii* isolates. Zavascki *et al*. (2005) has reported the presence of 77.1% MBL producers in Southern Brazil upon testing 35 isolates of Carbapenem resistant *P. aeruginosa* (CRPA), among which 27 were MBL positive.

In a nationwide study conducted for a 4 month period in Italy, of 14,812 consecutive non replicate clinical isolates (12,245 *Enterobacteriaceae* isolates and 2,567 gram-negative nonfermenters) screened for reduced carbapenem susceptibility, 30 isolates (28 *P. aeruginosa* isolates, 1 *Pseudomonas putida* isolate, and 1 *E. cloacae* isolate) carried acquired MBL determinants (Rossolini *et. al.,* 2008). MBL producers were detected in 10 of 12 cities, with a predominance of VIM-type enzymes over IMP-type enzymes (4:1). Since there are no standardized phenotypic methods available for the detection of MBL, several tests have been employed to detect the MBL production depending upon whether the gene is carried by *P. aeruginosa* or a memeber of *Enterobacteriaceae* and taking advantage of chelating agents, EDTA and thiol

based compounds, to inhibit MBL activity (Walsh *et al*., 2005). Franklin *et al*. (2006), using an Imipenem(10 µg) and Imipenem-EDTA (292µg) combined disk test for MBL detection found a sensitivity and specificity of 100% and 98% respectively. Whereas, in the same bacterial isolates the DDST yielded a sensitivity and specificity of 79% and 98% respectively.

Patients with Chronic Kidney Diseases and undergoing dialysis treatment have an increased risk for getting a healthcare-associated infection (HAI). Hemodialysis patients are at a high risk for infection because the process of hemodialysis requires frequent use of catheters or insertion of needles to access the bloodstream. Also, Kidney patients have weakened immune systems, which increase their risk for infection, and they require frequent hospitalizations and surgery where they might acquire an infection. Multidrug resistance among bacterial pathogens is a major health problem in Nepal that thwarts the management of several infectious diseases and compromises therapy. Thus, controlling antibotic resistant bacteria and subsequent infections more efficiently necessitates the prudent and responsible use of antibiotics.

# CHAPTER VI

# CONCLUSION AND RECOMMENDATIONS

## 6.1 Conclusion

The culture positivity among the samples was found to be low (19.92%) but the rate of multidrug resistance among the isolates was very high (85.83%). Among the various organisms isolated *E. coli* was most frequently isolated (72.81%) followed by *K. pneumoniae*. Higher rate of growth was seen in female which was found statistically significant. Hypertension was found to be the leading cause of Kidney disease. The higher number of isolates obtained from chronic kidney patients (78.64%) was found statistically insignificant. Imipenem was found to be the most effective drug against the Gram negative isolates with sensitivity of 75.7% whereas cephalexin was found to be most ineffective drug with resistance rate of almost 100%. Ceftazidime was found to be better in both screening and confirmation of ESBL production than cefotaxime. Among the MDR isolates suspected of ESBL production by screening 59.60% were confirmed as ESBL producers and roughly 10% isolates were found to produce MBL. The association between high level of resistance towards ciprofloxacin and MDR was found statistically significant and hence its MIC value was determined. Most of the isolates showed MIC value of 8µg/ml towards ciprofloxacin.

## 6.2 Recommendations

1. Overuse of drug should be discouraged as it leads to increase in antibiotic resistance.

2. The practice of using ceftriaxone alone for screening ESBL producers should be discouraged since this may lead to incorrect characterization of ESBL producers as ESBL non-producers.

3. Further charecterization of ESBL should be done and the presence false positive isolates due to hyperproduction of SHV-1 ESBL should be distinguished.

4. Isolates with reduced susceptibility to ceftazidime should also be included for the detection of MBL production in addition to carbapenem resistant isolates.

5. Effective network system to maintain laboratory practices should be established and prudent use of antibiotics should be assured.

## REFERENCES


Abel JJ, Rountree LG and Turner BB (2002). The removal of diffusible substances from circulating blood by dialysis. *Tn Assoc Am Phys*. **28(51**): 1913.

Acharya D (2008). Fluoroquinolone susceptibility pattern of the *Salmonella* isolates from enteric fever patients visiting to National Public Health Laboratory, Nepal. M.Sc. Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

APIC (2010). Guide to the elimination of infections in hemodialysis. Washington, USA.

Aurora PV, Storm J, Stanley K and D'Agata EMC (2008). Multidrug-resistant Gram-negative bacteria among patients who require hemodialysis. *Clin J Am Soc Nephrol*. **3**: 752-758.

Babic M, Hujer AM and Bonomo RA (2006). What's new in antibiotic resistance? Focus on β-lactamases. *Drug Resist Updat.* **9**:142–156.

Baral P (2008). Multidrug resistance among various clinical bacterial isolates and production of different types of β-lactamases with subsequent transfer mechanism by plasmid DNA analysis. M.Sc. Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal.

Ben-Ami R, Schwaber MJ, Navon-Venezia S, Schwartz D, Giladi M, Chimelnistky I, Leavitt A and Carmeli Y (2006). Influx of extended-spectrum β-lactamase-producing Enterobacteriaceae into the hospital. *Clin Infect Dis* **42**: 925–934.

Bhatta DR, Bangtrakulnonth A, Saroj SD, Bandekar JR, Hendriksen JR and Kapadnis BP (2007). Serotyping, PCR, phage-typing and antibiotic sensitivity testing of *Salmonella* serovars isolated from urban drinking water supply systems of Nepal. *Letters in Applied Microbiology* **44**:588-594.

Bollero D, Cortellini M, Stella M, Carnino R, Magliacani G and Iorio M (2001). Pan antibiotic resistance and nosocomial infection in burn patients: therapeutic choices and medicolegal problems in Italy. *Papers Presented in Italian Burn Society.*

Bomjan R (2005) Prevalence of multidrug resistant strains with reference to Extended spectrum β-lactamase producing strains among the bacterial pathogens isolated from different clinical samples at Tribhuvan University Teaching Hospital. M.Sc. Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal.

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

Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B and Bartlett J (2009). Bad bugs, no drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. *Clinical infectious Disease* **48**:1-12.

Boyd DA, Tyler S and Christianson S (2004). Complete nucleotide sequence of a 92 kilobase plasmid harboring the CTX-M-15 extended-spectrum β-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada. *Antimicrob Agents Chemother*. **48**:3758-64.

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

Bush K (1989). Classification of β- lactamases group-2c, group-2d, group-2e, group-3 and group-4. *Antimicrob Agents Chemother*. **33**: 271-276.

Bush K (1998). Metallo-β -lactamases: a class apart. *Clin Infect Dis.* **27(Suppl. 1)**: 48–S53.

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

Buynak JD (2006). Understanding the longevity of the β-lactam antibiotics and of antibiotic β-lactamase inhibitor combinations. *Biochem Pharmacol*. **71**:930–940.

CDC (2006). Management of multidrug resistant organisms in healthcare settings. CDC, Atlanta USA.

Centers for Disease Control and Prevention (2001). Recommendations for preventing transmission of infections among chronic hemodialysis patients. *MMWR Recomm. Rep.***50**: 1–43.

Centre for Disease Control (1982). Global distribution of penicillinase producing *Neisseria gonorrheae* (PPNG). *Morb. Mort Wkly Rep*. **31**:1-3.

Cheesbrough M (2000). District laboratory practice in tropical countries Part 2. Cambridge university press. Pp. 125-137.

Chhetri PK, Rai SK, Pathak UN, Thapa JB, Devkota KC, Shrestha BO and Shrestha RR (2001). Retrospective study of urinary tract infection at Nepal Medical College Teaching Hospital, Kathmandu. *Nep Med Col J* **3:**83-85.

Clinical and Laboratory Standards Institute. Wayne PA: Clinical and Laboratory Standards Institute; 2009. CLSI. Performance Standards for Antimicrobial Susceptibility Testing: Nineteenth Informational Supplement. CLSI document M100-S19.

D'Agata EMC (2002). Antimicrobial-resistant Gram-positive bacteria in the chronic hemodialysis population. *Clin Infect Dis*. **35**: 1212–1218.

D'Agata EMC (2004). Rapidly rising prevalence of nosocomial multidrug-resistant Gram-negative bacilli: A 9-year surveillance study. *Infect. Control Hosp Epidemiol*. **25**: 842–846.

D'Agata EMC, Mount DB, Thayer V and Schaffner W (2000). Hospital-acquired infections among hemodialysis patients. *American journal of kidney diseases* **35(6)**: 1083-1088.

Dahal BK (1999). A prospective study of urinary tract infection based on culture and direct microscopy of urine along with the antibiotic sensitivity of urinary pathogens. M.Sc. Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

Daniel FS, Thornsberry C, Mayfield DC, Jones ME and Karlowsky JA (2001). Multidrug resistant urinary tract isolates of *Escherichia coli*: prevalence and patient demographics in the United States in 2000. *J of Antimicrob Chemother* **45 (5):**1402-1406.

Decousser JW, Poirel L and Nordmann P (2001). Characterization of a chromosomally encoded extended-spectrum class A β-lactamase from *Kluyvera cryocrescens*. *Antimicrobial Agents and Chemotherapy* **45**:3595–3598.

Davies J and Davies D (2010). Origin and evolutions of antimicrobial resistance. *Microbiol Mol Biol Rev*. **74(3)**: 417-433.

Donskey CJ (2004). The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens. *Clin Infect Dis* **39**: 219–226.

Drawz SM and Bonomo RA (2010). Three decades of β-lactamase inhibitors. *Clin. Microb. Rev.* **23(1)**: 160-201.

EUCAST (2000). Determination of minimum inhibitory concentration (MIC) of antibiotics by agar dilution. EUCAST definitive document, E. Def 3.1.

Falagas ME, Koletsi PK and Bliziotis IA (2006). The diversity of definitions of multidrug-resistant (MDR) and pan drug resistant (PDR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *J of Medical Microbiol*. **55**:1619–1629.

Falah SM, Mohammed AA and Ali KA (2012). Urinary tract infections in hemodialysis patients with renal failure. *J Fac Med Baghdad.* **54(1)**: 24-29.

Farrel DJ, Morrisey I, Rubies D, Robbins M and Felmingham D (2003). A UK multicenter study of the antimicrobial susceptibility of bacterial pathogens causing UTI. *J of Infect.* **46:**94-100.

Finelli L, Miller JT, Tokars JI, Alter MJ and Arduino MJ (2005). National surveillance of dialysis-associated diseases in the United States, 2002. *Semin Dial*. **18**: 52–61.

Forbes BA, Sahm DF and Weissfeld AS (2007). Bailey and Scott's diagnostic microbiology, twelfth edition. Elesvier USA. Pp. 514-618.

Franklin C, Liolios L and Peleg AY (2006). Phenotypic detection of carbapenem-susceptible Metallo β-lactamase-Producing Gram negative bacilli in Clinical laboratory. *J of Clin Microbiol.* **44(9)**: 3139-3144.

Gales AC, Sader HS and Jones RN (2002). Urinary tract infection trends in Latin American hospitals: report from the SENTRY antimicrobial surveillance program (1997–2000). *Diag Microbiol and Infect Dis.* **44**: 289–299.

Gonullu N, Aktas Z, Kayacan CB, Salcioglu M, Carattoli A, Yong DE and Walsh TR (2008). Dissemination of CTX-M-15 β-Lactamase genes carried on *inc* FI and FII Plasmids among clinical isolates of *Escherichia coli* in a university hospital in Istanbul, Turkey. *J of Clin Microbiol.* **46(3)**:1110-1112.

Gutmann L (1985). Cross-resistance to nalidixic acid, trimethoprim and chloramphenicol associated with alteration in the outer membrane of *Klebseilla, Enterobacter* and *Serratia*. *J Infec Dis.* **154**; 501-507.

Hawser SP, Bouchillon SK, Hoban DJ, Badal RE, Hsueh P and Paterson DL (2009). Emergence of high levels of extended spectrum β-lactamase-producing Gram negative bacilli in the Asia-Pacific region: Data from the study for monitoring antimicrobial resistance trends (SMART) program, 2007. *Antimicrob agents and chemother*. **53(8)**:3280-3284.

Ho PL, Tsang DNC, Que TL, Ho M and Yuen KY (2000). Comparison of screening methods for detection of extended-spectrum β-lactamases and their prevalence among *Escherichia coli* and *Klebsiella* species in Hong Kong. *APMIS* **108:** 237–40.

Holten KB and Onusko EM (2000). Appropriate prescribing of oral β-lactam antibiotics. *Am Fam Physician*.**62(3)**:611-20

Hope R, Potz NAC, Warner M, Fagan EJ, Arnold E and Livermore DM (2007). Efficacy of practised screening methods for detection of cephalosporin-resistant Enterobacteriaceae. *J of Antimicrob Chemother*. **59**:110-113.

Hujer KM, Hujer AM, Hulten EA, Bajaksouzian S, Adams JM, Donskey CJ, Ecker DJ, Massire C, Eshoo MW, Sampath R, Thomson JM, Rather PN, Craft DW, Fishbain JT, Ewell AJ, Jacobs MR, Paterson DL and Bonomo RA(2006). Analysis of antibiotic resistance genes in multidrug-resistant *Acinetobacter* spp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. *Antimicrob Agents Chemother*. **50:**4114–4123.

Huys G, Cnockaert M, Vaneechoutte M, Woodford N, Nemec A, Dijkshoorn L and Swings J (2005). Distribution of tetracycline resistance genes in genotypically related and unrelated multiresistant *Acinetobacter baumannii* strains from different European hospitals. *Resistance and Microbiol.* **156**:348-355.

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

Jacoby GA and Munoz- price LS (2005). The new β- lactamases. *New Engl J Med*. **352**: 380-391.

Jain A and Mondal R (2008). Detection of extended spectrum β-lactamase production in clinical isolates of *Klebsiella* spp. *Indian Journal of Medicine and Resistance* **127**:344-346.

Jonathan N (2005). Screening for extended spectrum β-lactamase producing Enterobacteria in district general hospitals. *J Clin Microbiol*. **43(3)**: 1488-1490.

Jones RN, Kirby JT and Rhomberg PR (2008). Comparative activity of meropenem in US medical centers (2007): initiating the 2nd decade of MYSTIC program surveillance. *Diag Microbiol and Infec Dis*. **61**:203–213.

Kahlmeter G (2000). The ECO-SENS project: a prspective, multinational, multicenter epidemiological survey of the prevalence and antimicrobial susceptibility of urinary tract pathogens-interim report. *J of Antimicrob Chemother*. **46** (Suppl.S1):15-22.

Koh TH, Sng LH, Babini GS, Woodford N, Livermore DM and Hall LM (2001). Carbapenem-resistant *Klebsiella pnuemoniae* in Singapore producing IMP-1 β-lactamase and lacking an outer membrane protein. *Antimicrob Agents and Chemother*. **45**:1939–1940.

Koljalg SK, Truusalu K, vainuma I, Stsepetova J and Mikelsaar M (2009). Persistence of *Escherichia coli* clones and phenotypic and genotypic antibiotic resistance in recurrent urinary tract infections in childhood. *Journal of Clinical Microbiology* **47:** 99-105.

Krieg NR (1984). Bergey's manual of Systemic bacteriology. Williams and Wilkins Inc. Vol 1.

Lee K, Lim YS, Yong D, Yum JH, and Chong Y (2003). Evaluation of the Hodge test and the imipinem- EDTA double-disk synergy test for differentiating metallo β-lactamase-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J of Clin Microbiol.* **41**: 4623- 4629.

Lewis JS, Herrera M, Wickes B, Patterson JE and Jorgensen JH (2007). First report of the emergence of CTX-M-type extended spectrum β lactamases (ESBLs) as the predominant ESBL isolated in a U.S. health care system. *Antimicrob Agents and Chemother.* **51(11**):4015-4021.

Linton AL, Lawson DH, Macvarish I and Eakin JS (1970). Antibiotic therapy on patients on regular dialysis treatment. *Brit Med Journal. Editorial.* Pp. 2-5.

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

Livermore DM and Paterson DL (2006). Pocket guide to extended spectrum β-lactamases in resistance. New Delhi: Springer (India) Private Limited. Pp. 10-50.

Manandhar S (1996). Microbiology of the urinary tract infection: a hospital based study. M.Sc. Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal.

Marquez C, Labbate M, Raymondo C, Fernandez J, Gestal AM, Holley M, Borthagaray G and Stokes HW (2008). Urinary tract infections in a South American population: dynamic spread of class 1 integrons and multidrug resistance by homologous and site-specific recombination. *J of Clin Microbiol.* **46(10):**3417-3425.

Marr KA, Sexton DJ, Conlon PJ, Corey GR, Schwab SJ and Kirkland KB (1997). Catheter-related bacteremia and outcome of attempted catheters salvage in patients undergoing hemodialysis. *Ann Intern Med*. **137**: 275–280.

Martinez-Martinez L, Pascual A, Mdel CC, Garcia I, Joyanes P, Domenech-Sanchez A and Benedi VJ (2002). Energy-dependent accumulation of norfloxacin and porin expression in clinical isolates of *Klebsiella pneumoniae* and relationship to extended-spectrum β-lactamase production. *Antimicrob Agents and Chemother***. 46**:3926–3932.

Mathai D, Jones RN and Pfaller MA (2001). Epidemiology and frequency of resistance among pathogens causing urinary tract infections in 1,510 hospitalized patients: A report from the SENTRY Antimicrobial Surveillance Program (North America). *Diag Microbiol and Infectious Dis*. **40**:129–136.

Meyer E, Gastmeier P and Schwab F (2008). The burden of multiresistant bacteria in German intensive care units. *J of Antimicrob Chemother, Research lett*: 1474-1476.

Morosini MI, Garcia CM and Coque TM ( 2006). Antibiotic coresistance in extended spectrum-β-lactamase-producing Enterobacteriaceae and *in vitro* activity of tigecycline. *Antimicrob Agents Chemother*. **50**:2695-99.

Naas T, Philippon L, Poirel L, Ronco E and Nordmann P (1999). An SHV-derived β- lactamase in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. **43**: 1281-1284.

Naas T, Poirel L and Nordmann P (2008). Minor extended spectrum β-lactamases. *Clin Microbiol Infect.* **14(Suppl. 1)**: 42- 52.

Naqvi SB and Collins AJ (2006). Infectious diseases in chronic kidney diseases. *Advances in Chronic Kidney Diseases* **13(3)**: 199-204

National Committee for Clinical Laboratory Standards (2005). Performance standards for antimicrobial susceptibility testing; 15th informational supplement (M100–S15). National Committee for Clinical Laboratory Standards, Wayne, Pa.

National Institutes of Health (2000). 2000 Annual data report: US renal data system, Bethesda, US department of health and human services, National Institutes of Health, National institute of diabetes and digestive and kidney diseases.

Neu HC (1969). Effect of β-lactamase location in *Escherichia coli* on penicillin synergy. *Appl Microbiol*. **17 (6)**:783–6

Oteo J, Campos J, Baquero F and Spanish members of the European Antimicrobial Surveillance System (2001). *Escherichia coli* in 27 Spanish hospitals participating in the European Antimicrobial Surveillance System. *J of AntimicrobChemother.* **42**:16-18.

Ozeki S, Deguchi T, Yasuda M, Nakano M, Kawamura T, Nishino Y and Kawada Y (1997). Development of a rapid assay for detecting gyrA mutations in *Escherichia coli* and determination of incidence of gyrA mutations in clinical strains isolated from patients with complicated urinary tract infections. *J of Clin Microbiol.* **35:**2315-2319.

Paterson DL and Bonomo RA (2005). Extended-spectrum β-lactamases: a clinical update. *Clin Microb Rev.* **18(4)**: 657-686.

Paterson DL and Doi Y (2007). A step closer to extreme drug resistance (XDR) in Gram negative bacilli. *Clin Infec Dis.* **45**:1179–81.

Peshattiwar PD and Peerapur BV (2011). ESBL and MBL mediated resistance in *Pseudomonas aeruginosa*. *J of Clin and Diag Res*. **5(8)**: 1552-1554.

Picao RC, Andrade SS, Nicoletti AG, Campana EH, Moraes GC, Mendes RE and Gales AC (2008). Metallo β-lactamase detection: comparative evaluation of double disk synergy versus combined disk tests for IMP-, GIM-, SIM-, SPM-, or VIM- producing isolates. *J of Clin Microbiol*. **46 (6)**: 2028- 2037.

Pokharel BM, Koirala J, Mishra SK, Dahal RK, Khadga P and Tuladhar NR (2006). MDR and ESBL producing strains causing lower respiratory tract and urinary tract infections. *J of Inst of Med*. **28(3)**:19-27.

Pokhrel BM (2004). A Handbook of Clinical Microbiology. 1st edition. Gorhaknath Desktop and Printing Supports, Kathmandu, Nepal. Pp. 10-15.

Poudyal S (2010). Prevalence of β-lactamase producing multidrug resistant bacterial pathogens isolated from different clinical samples at National Public Health Laboratory. M.Sc. Dissertation submitted to Central Department of Microbiology, Tribhuvan University. pp. 5-74.

Puri N (2006). Study on the incidence of urinary tract infection among the diabetic patients and the prevalence of multidrug resistant strains among the bacterial pathogenic isolates. M.Sc. Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal.

Queenan AM, Foleno B, Gownley C, Wira E and Bush K ( 2004). Effects of inoculum and β-lactamase activity in AmpC- and extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates tested by using National Committee for Clinical Laboratory Standards ESBL methodology. *J Clin Microbiol*. **42**: 269–275.

Rossolini GM, Luzzaro F, Migliavacca R, Spalla M, Amicosante G, Toniolo A and Pagani L (2008). First countrywide survey of acquired metallo β-lactamases in Gram negative pathogens in Italy. *Antimicrob Agents and Chemother*. **52:**4023-4029.

Roy C, Segura CM, Tirado R, Reig M, Hermida D and Foz A (1985). Frequency of plasmid-determined β-lactamases in 680 consecutively isolated strains of Enterobacteriaceae. *Eur J Clin Microbiol.* **4**:146–147.

Schwaber MJ, Navon-Venezia S, Kaye KS, Ben-Ami R, Schwartz D and Carmeli Y (2006). Clinical and economic impact of bacteremia with extended spectrum β lactamase producing Enterobacter. *Antimicrob Agents Chemother*. **50**: 1257–1262.

Senekal MH (2010). Current resistance issues in antimicrobial therapy. *CME.* **28(2)**: 54-57.

Srisangkaew S and Vorachit M (2003). The optimum agent for screening and confirmatory tests for extended spectrum β-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* in Ramathibodi Hospital, Thailand. *J of Infect Dis and Antimicrob Agents.* **21**:1-5

Steward CD, Rasheed JK, Hubert SK, Biddle JW, Raney PM and Anderson GJ (2001). Characterization of clinical isolates of *Klebsiella pneumoniae* from 19 laboratories using the National Committee for Clinical Laboratory Standards extended-spectrum β-lactamase detection methods. *J Clin Microbiol*. **39**:2864–72.

Sulavik MC, Houseweart C, Cramer C, Jiwani N, Murgolo N and Greene J (2001). Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. *Antimicrob Agents and Chemother*. **45**: 1126-1136.

Tancrede CH and Andremont AO (1985). Bacterial translocation and Gram-negative bacteremia in patients with hematological malignancies. *J Infect Dis* **152**: 99–103.

Thapa B, Adhikari P, Mahat K, Chhetri MR and Joshi LN (2009) . Multidrug-resistant nosocomial *Citrobacter* in a Hospital in Kathmandu. *Nep Med col J* **11(3)**: 195-199.

Thomson KS and Moland ES (2000). Version 2000: the new β-lactamases of Gram-negative bacteria at the dawn of new millenium. *Microbes and Infection* **2**: 1225-1235.

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

US Renal Data System (2005). USRDS 2005 annual data report: atlas of end-stage renal disease in the United States, Bethesda, National Institutes of Health, national institute of diabetes and digestive and kidney diseases.

Walsh TR, Toleman MA, Poirel L, and Nordmann P (2005). Metallo β-lactamases: the quiet before the storm? *Clin Microbiol Rev*. **18**:306–325.

WHO (2013). Antimicrobial Resistance.

Available at http://www.who.int/mediacentre/factsheets/fs194/en/ accessed 23rd August 2013.

Wish J (2002). What kinds of infections can occur in hemodialysis patients? *aakpRENALIFE*. **18(3)**:24-27.

Zavascki AP, Gaspareto PB, Martins AF, Goncalves AL and Barth L (2005). Outbreak of carbapenem-resistant *Pseudomonas aeruginosa* producing SPM-1 Metallo-β-lactamase in a teaching hospital in Southern Brazil. *J of Antimicrob Chemother*. **56**: 1148-1151.