CHAPTER I:

INTRODUCTION AND OBJECTIVES

~~INTRODUCTION~~

1.1 Background

can be defined as a spectrum of disease (UTIs) Urinary tract infections involving the microbial invasion of tissues lining the urinary tract extending UTIs are important complications of .meatus from the renal cortex to urethral and structural and neurologic ,renal transplantation ,renal diseases ,diabetes Forbes) abnormalities that interfere with urine flow*et al*Etiologic .(2007 ,. :agents commonly involved in UTIs include

Gram positive

Staphylococcus aureuss ,*taphylococcus saprophyticus* ,Other coagulase negative staphylococci (CONS) ,*Enterococcus faecalis*

Gram negative

Escherichia coli ,*Proteus* spp., *Pseudomonas aeruginosa*, *Klebsiella* spp., .spp *Acinetobacter* ,.spp *Citrobacter* ,.spp *Enterobacter*

~~Antibiotics have revolutionized medicine in many respects and have saved countless lives. Regrettably, it was accompanied by the rapid appearance of resistant strains.~~ The emergence of ~~development of generations of~~ antibiotic-resistant bacteria~~t microbes~~ and their ~~distribution~~circulation is a global public health problem. Similarly, third generation cephalosporins that are considered effective till date also have been ineffective against many Extended spectrum β-lactamase(ESBL) producing bacterial isolates~~ in microbial populations throughout the biosphere are the results of many years of unremitting selection pressure from human applications of antibiotics, via underuse, overuse and misuse~~. (Brun-Buisson *et al*., 1987; ~~Davies *et al*., 2010~~Knothe *et al*., 1983; ~~).~~ ~~The introduction of the third-generation cephalosporins into clinical practice in the early 1980s was heralded as a major breakthrough in the fight against lactamase-mediated bacterial resistance to antibiotics. These cephalosporins~~

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had been developed in response to the increased prevalence of lactamases in certain organisms (for example, ampicillin hydrolyzing TEM-1 and SHV-1 lactamases in *Escherichia coli* and *Klebsiella pneumoniae*) and the spread of these lactamases into new hosts (for example, *Haemophilus influenza* and *Neisseria gonorrhoeae*) (Paterson and Bonomo *et al.*, 2005). The first report of plasmid encoded lactamases capable of hydrolyzing the extended spectrum cephalosporins was published in 1983 (Knothe *et al.*, 1999). The gene encoding the lactamase showed a mutation of a single nucleotide compared to the gene encoding SHV-1. Other lactamases were soon discovered which were closely related to TEM-1 and TEM-2, but which had the ability to confer resistance to the extended spectrum cephalosporins (Brun-Buisson *et al.*, 1987;; Sirot *et al.*, 1987). Hence, these new lactamases were coined extended spectrum β lactamases (ESBLs).

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Multidrug Resistance (MDR) is defined as resistance to ≥2 of the antimicrobial agents belonging to different structural classes .(2006 ,CDC) Multidrug resistance among common bacterial pathogens has resulted into ,treatment failures and increased economic burden to contain these pathogens .(2009 ,ASM) thus dictating their early and reliable detection

-β lactamases are the major defense of Gram negative bacteria against-β The .lactam antibioticsThe β lactamases are a heterogenous group of proteins with structural similarities, composed of α heix and β pleated sheets and the members of a superfamily of active site serine proteases (Knox, 1995). These enzymes are the major cause of bacterial resistance to β lactam antibiotics and can be either chromosome, plasmid or transposons encoded and produced in a constitutive or inducible manner. They are secreted in the periplasmic space in gram Gram negative bacteria or into the surrounding medium by their gram Gram positive counterparts whereas membrane associated enzymes have been rarely reported (Jacoby *et al.*, 2005; Livermore *et al.*, 1995; Jacoby *et al.*, 2005).

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~~Extended spectrum β-lactamases (~~ESBLs~~)~~ are plasmid-mediated bacterial enzymes that confer resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and are inhibited byβ -lactamase inhibitors such as clavulanic acid. All ESBL producing organisms should be considered resistant to all penicillins (except temocillin), cephalosporins (except cefoxitin and cefotetan) and aztreonam. The first definitively characterized ESBL, TEM-3 (cefotaxime-hydrolyzing enzyme type 1), was discovered in *K.~~lebsiella~~ pneumoniae* isolates recovered from intensive care unit patients in France (~~–~~and Segreti Pfaller~~et al~~2006 ~~,~~,~~). Although ESBLs have been reported most frequently in~~ *E.~~schericia~~ coli* and *Klebsiella* species, they have been found in other bacterial species including *S.~~almonella~~ enterica*, *P.~~seudomonas~~ aeruginosa*, and *Serratia~~erratia~~ marcescens*2008 ,Bush) ~~;~~ ~~–~~and Bonomo Paterson~~et al~~and Pfaller ;2005 ~~,~~,~~ ,Segreti~~et al~~ ;2006~~,~~ ~~and~~ ,and Ambrose Ramphal~~;et al~~ ,Bush ~~and~~ 2006~~,~~ .(~~2008~~

ESBL denotes those β-lactamases of Bush-Jacoby-Medeiros group 2be and those of group 2d which share most of the fundamental properties of group 2be enzymes (Bush *et al*., 1995). The 2be designation shows that these enzymes are derived from group 2b β-lactamases (for example, TEM-1, TEM-2 and SHV-1); the e of 2be denotes that the β-lactamases have an extended spectrum. Group 2b enzymes hydrolyze penicillin and ampicillin, and to a lesser degree carbenicillin or cephalothin~~–~~. They are not able to hydrolyze extended-spectrum cephalosporins or aztreonam to any significant degree. TEM-1 is the most common plasmid-mediated β-lactamase of ampicillin resistant enteric ~~gram~~Gram-negative bacilli (for example, *E.~~schericia~~ coli*), while SHV-1 is produced by the vast majority of *K.~~lebsiella~~ pneumoniae* (Livermore *et al*., 1995). TEM-2 is a less common member of the same group with identical biochemical properties to TEM-1. The ESBLs derived from TEM-1, TEM-2, or SHV-1 differ from their progenitors by ~~as few as one~~few amino acids. This results in a profound change in the enzymatic activity of the

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ESBLs, so that they can now hydrolyze the third-generation cephalosporins or aztreonam (hence the extension of spectrum compared to the parent enzymes). 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. They are able to hydrolyze the penicillins, narrow-spectrum and third-generation cephalosporins, and monobactams. The ESBLs have hydrolysis rates for ceftazidime, cefotaxime, or aztreonam (aminothiazoleoxime β-lactam antibiotics) at least 10% that for benzylpenicillin. They are inhibited by clavulanic acid (Bush *et al*., 1995). This property differentiates the ESBLs from the AmpC-type lactamases (group 1) produced by organisms such as *Enterobacter cloacae* which have third-generation cephalosporins as their substrates but which are not inhibited by clavulanic acid. Selection of stably derepressed mutants which hyperproduce the AmpC-type β-lactamases has been associated with clinical failure when third-generation cephalosporins are used to treat serious infections with *Enterobacter* spp. (Chow *et al*., 2003; Cosgrove *et al*., 2002;~~,~~ Kaye *et al*., 2006~~1~~). In general, the fourth-generation cephalosporin, cefepime, is clinically useful against organisms producing Amp C-type β-lactamases (Sanders, 1993~~6~~), but may be less useful in treating ESBL producing organisms~~-~~.

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The infection due to ESBL- producing organisms can cause the failure of treatment with extended spectrum (third generation) cephalosporins. ~~Besides, ESBL producing bacteria are typically associated with multidrug resistance (MDR) (Araj *et al*., 2003).~~ Antibacterial choice is often complicated by multi-resistance ~~(Rupp *et al*., 2003; Shah *et al*., 2004; Rupp *et al*., 2003)~~. Thus infection due to ESBL-producing bacteria can result unavoidable failure of treatment and increased cost in patients who have received inappropriate antibiotic treatment (Ahm~~a~~ed *et al*., 1999~~2003~~). Colonization and infection with these bacteria have also been associated with indiscriminate use of antibiotics, prolonged hospitalizations, increasing numbers of immunocompromised patients, and medical progress resulting in increased use of invasive procedures and devices ~~(Gold *et al*., 1996)~~. This study will help to

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know the prevalence of strains producing ESBLs and their existing trend of antibiotic resistance.

Various studies have reported circulation of ESBL producing bacterial strains in Nepal. However, this study aims at finding circulation of ESBL producing isolates in a hospital setting (Alka Hospital) at Lalitpur.

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1.2 ~~Chapter II:~~ OBJECTIVES

1.2.1 ~~2.1~~ GENERAL OBJECTIVES

~~To determine the aAntibiotic susceptibility pattern of uUrinary isolates with reference to extended spectrum βBeta lactamase producing bacterial pathogens~~To determine the antibiotic susceptibilty pattern of urinary isolates with reference to extended spectrum β-lactamase producing bacteria.

1.2.2 SPECIFIC OBJECTIVES

1. ~~To find out the status of multidrug resistance among bacterial isolates.~~To study the multidrug resistance development among bacterial isolates.
2. ~~2.~~ To ~~determine the leading organism responsible for ESBL production and the trend of their antimicrobial resistance.~~
2. ~~To~~study antibiotic susceptibility pattern of the urinary isolates.

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~~3.~~ To find out the predominant ESBL producing bacterial isolates.

~~analyze the efficacy of different screening agents and the efficacy among cCombined disk.~~

3.

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~~CHAPTER III:~~

CHAPTER II

LITERATURE REVIEW

~~2Antimicrobial resistance 3.1~~

2.1 Antimicrobial resistance

Antibiotics have been in clinical use for more than 50 years, and a variety of substances with different mechanisms for antibacterial activity have been introduced on the market. Antimicrobials often originate from natural sources secreted as secondary products from bacteria and fungi. Antibiotics are among important clinical tools. Soon after the discovery of penicillin, it was found that bacteria could rapidly develop resistance. During the last decade bacterial resistance has become an increasing problem worldwide. Bacteria, like all living creature adapt to their environment, following the survival of the fittest and developing resistance to antibiotics. ~~Very few drugs are being developed.~~ This places pressure on maintaining the effectiveness of currently available agents as long as possible until newer agents become available. All antibiotics used whether appropriate or inappropriate exerts selective pressure for the emergence of resistant bacteria (Patt~~erson *et al.*,~~on and Bonomo, 2005). Our only means of handling the situation at the moment is through prudent use of antimicrobial agent, improved diagnostics and infection control. ~~(Murray*et al*,).~~ Surveillance of antimicrobial resistance is of great help for selection of empirical therapy, for detecting the emergence and spread of new resistances, and assessing the level of resistance and impact of infection control interventions (Murray *et al*., 2003).~~.~~

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The access to effective antibiotic treatment has created the necessary conditions for advanced modern medicine. Treatment of patients requires antibiotic use during, for example, organ transplantation, implantation of foreign body devices and prevention and immunocompromised periods in malignant diseases. Unfortunately, ~~–~~the use of antibiotics has generated selection of resistance genes. Therapeutic difficulties are now posed by strains of common bacterial species such as pneumococci, staphylococci, enterococci, and enterobacteria, which can acquire resistance to the most useful, and possibly to all agents currently in use. Resistance can result from bacterial modification of the target for the antibiotic, by enzymatic inactivation, efflux or impermeability. Emergence of resistance occurs among 5-10% of treated infections, which leads to clinical failure or longer treatment ~~(Fish, 1995; Milatovic, 1987; Fish, 1995)~~. Some studies have also been able to show that infection by resistant bacteria increases the risk of mortality (Cosgrove *et al.*, 2002~~Kollef, 1999; Crowcroft, 2002; Helms, 2002; Cosgrove, 2003~~). The presence of two resistant pathogens has increased considerably within hospital settings during the 1990s, i.e methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE).

The development of new antibiotics is continuing, assisted by research into microbial genome sequences. However, whether resistance problems can be avoided through the discovery and development of new effective antibiotics is open to question. To sustain the long-term effectiveness of antimicrobial treatments in medicine, we need to develop a better understanding of the processes underlying the development of antibiotic resistance, and use this information to design effective treatment policies. The evolution of antibiotic resistance is directed by several factors such as the level of antibiotic pressure, the influence of the pharmacokinetic and pharmacodynamic properties, the selection in the resident commensal flora, the horizontal transfer of resistance genes and the biological characteristics of bacteria, e.g., mutation rate and biological fitness (Paterson and Bonomo, 2005).

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2Emergence and Spread of Antimicrobial Resistance 3.1.1

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

- Hhost and clone specificity,
- pPlasmid and clone specificity, v
- Virulence,
- iInteractions with other commensal flora, d
- Duration of the selection pressure, and v

Variable gene expression2009 ,ASM) ; and .(2009 ,and ASM 2004 ,WHO

,Soon the new phenotype with resistance appears its spread is favoured by the the ability of the organism to tolerate the ,degree of resistance expressed .site of primary colonization etc ,linkage to other genes ,resistance mechanism lactamase gene was first described shortly after -β taphylococcals the .For eg penicillin was introduced clinically and is now almost universally present *Staphylococci* .taphylococciS within, the gene was first noted in ,owever hH- - EnterococciEnterococcis and has never spread widely in 1980 only in early- Murray ;2002 ,CDC) this genus*et al*.(2002 ,and CDC 2003 ,.

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2Causes of Spread of Antimicrobial Resistance 3.1.2

➢ ailure to fF timicrobial resistance is due tonThe causes of spread of a control techniques both within and outside the adhere to appropriate infection i ,hospital

➢ mproper hygienic practices that cause the transmission of resistant I e ,bacteria

➢ xposure of people to various centers likeEdnursing ,ay care centerD microorganisms are present and homes where probably resistance harbouring ,may get transmitted

➢ presumably by transiently or ,ransmission from patients to patientsTt ,persistently colonized health care workers etc

➢ For .rom nonhuman niches in which antibiotics are used in excessFf A determinant has emerged *van* vancomycin resistant Enterococci with the .eg due to the excessive use ofaas a growth ,a glycopeptide antibiotic ,voparcinA promoter in food animals

(Robberts *et al*., 20091998, Sheretz Sherertz *et al*., 1996; and Wegener *et al*., 1999)

2.1.1 Mechanism of development of Antibiotic Resistance 2 3.1.3 Mechanisms of Antimicrobial Resistance

Resistance in microorganisms can be attributed to various mechanisms either of means and biochemical Genetic .acting singly or in combinations are the primary ways of antimicrobial resistance antimicrobial resistance .acquisition

means of development of drug resistance through genetic One of the prime The various modes are These .means is the mutation of cellular genes

3.1.3.1 antimicrobial resistance Genetic bases of

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~~The various ways in which genetic change from drug sensitivity to resistant :may come are~~

~~1. ,Mutation of cellular genes:~~

~~C~~cellular genes encode for the proteins with important functional responsibilities for cell growth or maintenance. Changing of a single amino acid as a result of the single base change in the gene within the protein, make the antibiotic-protein interaction unfavourable resulting in resistance to that antibiotics. Eg. Rifampin targets cellular RNA polymerase *rpo*B- mutation in *rpo*B gene confers complete resistance (Sharma and Mohan ~~*et al.*~~ , 2006).

~~2. Acquistion of Resistance genes:~~

the acquistion of resistance genes is ,the cellular genes the mutation of ,Beside Resistance genes for most .also an important way of gaining the resistance either in the species that produce the ,antibiotics exists in the microbial world the same ecological niche as the antibiotic or within species that live in Some bacteria are able to absorb naked DNA molecules .antibiotic producers These foreign pieces .from the environment under appropriate circumstances of DNA are then incorporated into bacterial chromosome by recombining Hakenbeck) ross region of sufficient homologyac*et al*.(1998 ,.

The most commonly employed mechanism for genetic exchange is .conjugation and occur more frequently by the transfer of cojugative plasmid a large variety of forms can encode These extrachromosomal replicative DNA ,After entering in a new genus on broad host range plasmids .important genes resistant determinants can readily transfer into more frequently transferable Plasmid may also .plasmids to increase their movement through the new genus ncorporated into the chromosome further stablizing the information they get i ~~-~~and Carias Rice) carry~~*et al*~~ ,In addition .(1998 , ~~,~~R plasmids often contain

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many resistance genes; they are maintained stably in the host strains of bacteria and are transferred very efficiently to neighboring drug-susceptible cells.M ost drug resistance genes are effective when expressed from plasmids andremarkably, many such genes are often present on a single R plasmid, so that multidrug resistance can be transferred to a susceptible bacterium in a single conjugation event(2009 ,Nikaido) .

.e.Transduction is the process of acquisition of genes via a bacterial virus i lactamase gene is related to non conjugative -β taphylococcals Since .Phage the ,*taphylococcis* quently associated withge have been freand pha ,plasmid lactamase production is thought due to phage mediated -β high prevalence of and Alekshun) transfer of these plasmidsLevy ~~*et al*~~The transposons .(2007 ~~,~~ sometimes even code encode their own ability to transfer between replicons and For .for their conjugation allowing them to transfer within bacterial chromosomes encodes resistance toward tetracycline and *faecalis* .*E* described in 916Tn .eg .(2000 ,Rice) minocycline integrate ,however ,The non conjugative transposons .themselves into the transferable plasmids either transiently or permanently Shaw) confers erythromycin resistance 917Tn .For eg*et al*.(1993 ,.

The acquired genes further mutate exhibiting even broad spectrum of isolate resistant to cefotaxime *pneumoniae* .*K* .For eg .antimicrobial resistance lactamase is formed after the mutation of-β by production of TEM*bla* ~~TEM~~~~bla~~ ~~-~~and Medeiros Jacoby) gene~~*et al*~~.(1991 ~~,~~

~~3.1.3.2 Biochemical Bases of Resistance~~

,antimicrobial resistance Beside Genetic means of development of .ecbiochemical processes also leads to the resistance of antimicrobial resistan -β Many antibiotic modifying enzymes have been known including the and chloramphenicol acetyl ,aminoglycoside modifying enzymes ,lactamases in some cases are ,These enzymes in most cases are acquired .stransferase and *Enterobacter* n genera likeI .intrinsic though expressed at low levels with ,these enzymes are under regulatory control *Pseudomonas*

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broad ,high level angements in these regulatory mechanisms resulting inrdear -s ,and Medeiros yJacob) lactam resistance-β spectrum*et al*.(1995-,.

minor ,Since antibiotic interaction with target molecule is quite specific alterations of the target molecule has a pronounced effect on antibiotic axiomatic that an antibiotic must reach its target in order to be It is .binding negative bacteria have an outer membrane that must be -ramgG All .effective Reduction in the .crossed before the cytoplasmic membrane can be reached cumented as an important quantities of presumed porins have been do in cefepime ,*aeruginosa* .*P*.*Ps* contributors to resistance to imipenem in Lee) *pneumoniae* .*K* and cefoxitin or ceftazidime in *Enterobacter cloacaeet al*.(1992 ,Livermore- and ;1991 ,.

more antibiotics from the Efflux pumps are the pumps that remove one or The majority of these pumps are located in the cytoplasmic .bacterial cell ,In some instances.membrane and use proton motive force to drive drug efflux combinations of different types of pumps can result in higher level of - Lee) resistance than achieved by the activity of single pump alone*et al* ,2000 ,. -and Levy nAlekshu*et al* Lee ;2007 , ,.*et al*.(2009 ,Nikaido- and ;2000 ,.

2.2 23.2Emergence of -Multidrug resistance among bacterial isolates

23.2.1 Definition

Multidrug resistance is defined as the resistance to two or more classes of antimicrobial agents (CDC, 2006). Multidrug resistance is the resistance to at least two classes of first line agents including ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, fluroquinolones (ciprofloxacin and oflaxacin), and cephalosporins (cefotaxime, ceftriaxone and ceftazidime) (Koirala *et al.*, 2006).

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~~3.2.2~~ Mechanism of multidrug resistance

~~**a) R-factors**~~

Previously sensitive bacteria may become resistance to multiple antibiotics *via* the acquisition of conjugative plasmid R-factors. For example *E. coli* become multidrug resistance after acquisition of R-factors from *Salmonella* spp. and *Shigella* –spp. A number of R-factors like R1, pSH6 etc. have now be described in the both Gram positive and Gram negative bacteria that confer resistance to many antibiotics (Denyer *et al.*, 2005).

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~~**b) Reduced cellular uptake**~~

Reduced cellular uptake causes the decreased expressions of porins that result in decrease or elimination of flow of antibiotics across the membrane. Resistance to cephamycins and other β-lactam in *E. coli* and ~~*Klebsiella*~~ *Klebsiella.* spp.~~,~~ is mainly due to this mechanism (Anathan and Subha, -2005).

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~~**c) Chromosomal multiple antibiotic resistance (Mar) locus**~~

The multiple-antibiotic resistance (mar) locus was first recognized in *E. coli* and this locus consists of two units, *marC* and *marRAB.* Increased expression of *MarRAB* operon resulting from the mutations in *marO or marR*, or from inactivation or *marR* following exposure to inducible agents leads to development of Mar phenotype which is responsible for resistance to multiple drugs, disinfectants and organic solvents (Denyer *et al.*, 2005).

~~**d) Multidrug Efflux pump**~~

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Majority of Gram negative bacteria harbor the multidrug efflux pump like MexAB-OprM in ~~*Ps.*~~*P. aeruginosa*, AcrAB- TolC in *E. coli* that causes resistance to β-lactams, fluoroquinolones and tetracycline in both. Mutation in the regulatory genes *nalB* causes over expression of MexAB-OprM and consequently confers multidrug resistance. In some instances combination of different type pumps can result in higher level of resistance than achieved by activity of single pump alone (Alekshun and Levy, 2007; Denyer *et al.*, 2005;~~5, Alekshun *et al.*, 2007 and~~ Nikaido, 2009).

2.2.2 ~~3.2.3~~ Multidrug resistance in global scenario

There is great concern among public health authorities around the globe about the threat of increasing antimicrobial resistance which impairs the efficacy of antimicrobial agents and results in substantial increase in mortality, morbidity and health care associated cost (McDonald, 2006). The forces that drive antimicrobial-drug resistance includes failures of hospital hygiene, selective pressures created by overuse of antibiotics, and mobile genetic elements that can encode bacterial resistance mechanisms but despite this extensive knowledge base about resistance and control guidelines, drug resistance has continued to emerge, especially in intensive care units (Weinstein *et al.*,~~,~~ 2006~~1~~).

Hanberger *et al.*, 1999 studied the antibiotic susceptibility of 1196 aerobic Gram negative bacilli in ICU from 5 European countries isolated from respiratory tract, urine, blood, abdomen and skin and soft tissues and found that highest incidence of resistance was in *P~~s~~. aeruginosa* in all countries followed by *Enterobacter* ~~spp,~~spp. *Acinetoacter* ~~spp~~spp. *Sternotrophomonas maltophila* and *Klebsiella* spp. Among the isolates of *Pseudomonas* spp., ~~Thirt~~37%~~y seven percent~~ and 46% of isolates of ~~*Pseudomonas w*~~were resistance to ci~~r~~pfloxacin and gentamicin respectively. (Marquez *et al.*, 2008)

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In one Indian study of MDR nosocomial pathogens in ICU revealed that, *Klebsiella pneumoniae* was the most prevalent isolates from respiratory tract infections followed by *Proteus* spp, *E.~~scherichia~~ coli*, *Staphylococcus* spp. and *Acinetobacter* spp. Resistance to ciprofloxacin and ceftriaxone ranged from 50-100% and 25-83.3% respectively. Staphylococci were 100% resistant to penicillin and tetracycline, 80% to cotrimoxazole, 60% to erythromycin and gentamicin and 40% to amikacin. *Acinetobacter* spp. were highly resistant to most of the antibacterial agents except gentamicin while *Pseudomonas* spp. showed 75% resistance to gentamicin (Singh, 2002).

Similarly, in a study of antimicrobial resistance among Gram negative bacteria from Iran showed that *E. coli* (32.08%) followed by *K. pneumoniae* (31%), *P. aeruginosa* (12.8%) and *Acinetobacter* spp. (9.1%) were the major isolates. Most frequency of resistance to antimicrobial agents was 50% to 100% for ceftriaxone, 50% to 94.1% for ceftazidime, 52.9% to 63.8% for ciprofloxacin, 58.3% to 84.5% for cefepime, 56.9% to 100% for nitrofurantoin, 88.33% to 100% for ampicillin, 58.335 to 87.5% for trimethoprim/sulphamethoxazole, 58.3% to 100% for cefotaxime, 60% for piperacillin-tazobactam and 50% for gentamicin. Further, production of ESBL was found in 46% of *E. coli* and *Klebsiella* spp. (Mohammadi and Feizabadi, -2011).

2.2.3 Emergence of

~~3.2.4~~ Multidrug Resistanet bacteriae in Nepal

Excessive and inappropriate use of antibiotics in hospitals, health care facilities and the community contributes to the development of bacterial resistance and this is especially true for Nepal.~~l. Shankar *et al.*, 2003 reported that 84.5% of the antibiotics were prescribed without bacteriological support in ICU of a teaching hospital in western Nepal.~~

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The intensive care unit (ICU) is a setting where a large number of drugs are administered to patients. Furthermore, widespread use of broad-spectrum antibiotics, crowding of patients into geographically confined areas, presence of invasive medical devices and greater number of critically ill patients may be factors favoring the emergence and spread of resistant organisms and patients with critical illnesses are at risk of acquiring serious nosocomial infections which may lead to escalation in medical expenses, morbidity and mortality. (Manandhar *et al.*, 1996).

Shrestha *et al.*, 20059 studied the AST pattern of nosocomial isolates of S~~taph~~. *aureus* in ~~Tribhuvan University Teaching hospital~~TUTH and reported that among 149 *S.* ~~*taphylococcus*~~ *aureus* isolates, highest resistance was observed against penicillin (91.94%) followed by fluoroquinolone (61.74%), erythromycin (52.94%), gentamicin (46.98%), cotrimoxazole (42.95%), tetracycline (40.94%), whereas susceptibility was observed maximum against chloramphenicol (94.85%) followed by rifampicin (92.61%), tetracycline (59.06%), cotrimoxazole (57.04%), and none of the isolates were resistant to vancomycin and teicoplanin. Of these isolates 44.96 % of the isolates were methicillin resistant S~~taph~~. *aureus* (MRSA). ~~Similarly, Sherchan *et al.*, 2010 found that a prevalence rate of nosocomial infection due to Rotavirus in Nepali childrenschildren under the age of 5 is 30.2%.~~

~~3.~~2.3 ~~.~~ β~~Beta~~-lactams antibiotics, β-lactamases and ESBLs

The mechanism of action of β~~beta~~-lactams is to disrupt bacterial cell wall synthesis by linking covalently to enzymes, i.e. penicillin-binding proteins (PBPs), located in the cell-wall (Richmond ~~MH~~ *et al.*,1973). β~~Beta~~-lactams irreversibly inactivate these peptidoglycan transpeptidases, resulting in cell death. This bactericidal effect, combined with low toxicity, has made the beta-

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lactams currently the most prescribed antibiotic class, constituting over 60% of all clinically used antimicrobial drugs (Lee W *et al.*, 2001)

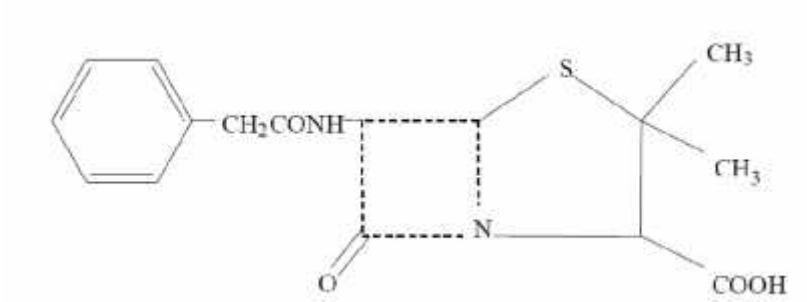

Figure 1. The structure of penicillin G. The beta-lactam ring is represented by dashed lines.

3.4 Beta-lactamases

Beta-lactamases are enzymes located in the periplasm on the outer surface of the inner membrane of the cell envelope. They hydrolyse the beta-lactam ring structure by binding covalently to the amide bond of the beta-lactam ring structure (D.(2005. .SP enyer

Beta-lactamases were present in bacteria long before the introduction of penicillins (Bradford PA *et al.*, 2001), and genes encoding these ancient enzymes were originally located on the bacterial chromosome (Livermore DM *et al.*, 1995). Furthermore, these enzymes are inducible and constitutively expressed in low quantities. In 1965, the first report of a plasmid-encoded beta-lactamase in a Gram-negative bacterium appeared from Greece (Gniadkowski M., –20081). This TEM-1 producing *E. coli* hydrolysed ampicillin, and within a few years after its first isolation, its plasmid-mediated resistance had spread over the world and into many different members of the *Enterobacteriaceae* family, including *P.seudomonas aeruginosa*, *Haemophilus influenzae* and *Neisseria gonorrhoeae*. Additional reports soon followed, describing plasmid-encoded beta-lactamases, which were also able to hydrolyse first generation cephalosporins.

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The spread of plasmid-encoded TEM and SHV enzymes from the mid- 1970s and onwards resulted in therapeutic failures. To counter this, the pharmaceutical agencies introduced several novel classes of beta-lactams with expanded spectra in the late 1970s and the early 1980s. These drugs included the cephamycins, oxyamino-cephalosporins, carbapenems, monobactams, clavulanic acid and penicillanic acid sulfone inhibitors. For reasons of convenience, spectrum, cost and safety, the oxyimino-cephalosporins (principally cefuroxime, cefotaxime, ceftriaxone, ceftazidime and cefepime) became the most-used of these analogues. The cephalosporins are now standard therapies for pneumonia~~s~~, intra-abdominal infections and urinary tract infections worldwide. By the extensive use of these compounds, the selective pressure has increased. Consequently, there has been a dramatic expansion of beta-lactamases (Gniadkowski 2001; Gniadkowski 2008; Medeiros ~~AA.~~ 1997~~, Gniadkowski M. 2001, Gniadkowski M. 2008~~), and new enzymes with wider substrate ranges emerged (Gniadkowski,~~i M.~~ 2008). Today, acquired resistance to beta-lactams is mainly mediated by the ESBLs.

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~~**Bush) Lactamase Classification β 1 Table*et al*(1995 ,.**~~

~~**Bush-Jacoby-Medeiros system**~~	~~**Major subgroup**~~	~~**Ambler System**~~	~~**Main Attributes**~~
~~Group 1 Cephalosporinases~~		~~C (cephalosporinases)~~	~~usually chromosomal, resistance to all β-lactams except carbapenems, not inhibited by clavulanate~~
~~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~~

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	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 3b 3c	B (Metalloenzymes) B B	Zinc dependent carbapenemases
Group 4		Not classified	Miscellaneous enzymes

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(ESBLs) lactamases β Extended Spectrum 3.4.1

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lactamases capable of conferring bacterial resistance to -β ESBLs are such as) and third generation cephalosporins ,-second ,-first ,the penicillins and (ceftriaxone etc ,cefotaxime ,ceftazidime ,cefuroxime ,cefazolin and (Cefotetan eg cefoxitin and) aztreonam but not the cephamycins by hydrolysis of these antibiotics (eg imipenem and meropenem) carbapenems .lactamase inhibitors such as clavulanic acid-β and which are inhibited by be and 2 Medeiros group-Jacoby-lactamases of Bush-β ESBLs include the type have been grouped in -reas ESBLs except OXAd whe2 those of group -and Bonomo Paterson) class A in the classification scheme of Ambler*et al* ,-narrow spectrum and third ,ESBLs are able to hydrolyze penicillins .(2005 hydrolysis rate for generation cephalosporins and monobactams with that for benzyl penicillin %10 or aztreonam at least ,cefotaxime ,ceftazidime -and Bonomo Paterson ;2008 ,Bush)*et al* ;2006 ,and Segreti Pfaller ;2005 ,-and Ambrose Ramphal-;2008 ,Bush*et al* Pfaller ;2006 ,.*et al*.(2006 ,.

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Types of ESBLs 3.4.1.1

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~~1.~~ SHV ~~type: it~~ is the most frequently found ESBL type in clinical isolates than any other type. SHV refers to sulfhydryl variable. It was first reported in 1983 in *Klebsiella ozaenae*.

~~2. TEM type:~~ TEM type ESBLs are the derivative of TEM-1 and TEM-2. Oover 100 TEM type β-lactamases ~~ahve~~ have been reported of which majority are ESBLs.

~~3.~~ CTX-M and Toho β-lactamases ~~: These~~ evolved separately, at least some of them via the escape and mutation of chromosomal β-lactamase of *Kluyvera* spp. The name CTX reflects the potent hydrolytic activity of these β-lactamases toward cefotaximes than ceftazidimes and the number of CTX type ESBLs are rapidly expanding.

Toho β-lactamases are related structurally to CTX-M types and have more potent activity towards cefotaxime than ceftazidimes.

~~4.~~ OXA ~~types: They~~ are so named because of their oxacillin hydrolying activity. These β-lactamases are characterised by hydrolysis of cloxacillin and oxacillin greater than 50% that for benzylpenicillin. They are found predominantly in *P~~seudomonas~~ aeruginosa*.

~~5.~~ PER ~~types: These types of ESBLs~~ efficiently hydrolyse penicillins and cephalosporins, however, shares only 25- 27% homology with SHV and TEM types.

~~6.~~ VEB-1, BES-1, and other ESBLs ~~: These~~ are either plasmid mediated or integron associated class A enzymes (Bonnet *et al*., 2000~~;~~ Mavroidi *et al*., 2001).

lactam antibiotics such as -β Many ESBL producers are multiresistant to non .ptionsnarrowing treatment o ,aminoglycosides and trimethoprim ,quinolones spreading among patients and ,Some producers achieve outbreak status Paterson ;2005 ,HPA)~~.~~ perhaps owing to particular pathogenecity trait ,locales ,nomoand Bo~~et al.~~(2005~~,~~

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The presence of ESBLs plus the potential for plasmid mediated quinolone and is likely to create significant therapeutic problems in ,carbapenem resistance the future and it is unlikely that newer antibiotics will be available in the next Enhanced infection .resistant infections-years to tackle such multi 10-5 therefore plays an ,programs coupled with antibiotic stewardship ,control .producing organisms-important role in limiting the spread of ESBL

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2.3.1 Screening methods ~~3.4.1.3 Evolution and epidemiology of extended spectrum ßbeta-lactamases~~

~~In 1983, the first report came from Germany by Knothe *et al.* of a mutant of SHV-1 named SHV-2 in a *K. pneumoniae* strain, which was capable of hydrolyzing oxyamino-cephalosporins (Knothe *et al.*, 1983). Two years later, SHV-2 was transferable between bacteria (Kliebe et al., 1985). Just a few years later came reports from French hospitals of problems attributed to *Enterobacteriaceae* carrying mutated TEM-derivates which acted like SHV-2 (Philippon et al.,1 983). The term "extended broad-spectrum beta-lactamases" was coined (Livermore *et al.*, 2008).~~

~~The first-described ESBLs evolved through random point mutations in isolates with broad-spectrum beta-lactamases, i.e. TEM-1, TEM-2, TEM-13, SHV-1 and SVH-11, which were already widespread in clinical settings when expanded-spectrum beta-lactams were introduced (Knothe *et al.*, 1983, Canton et al.,2006, Rossolini *et al.*,2006)). The ESBLs derived from TEM and SHV could differ from their progenitors by only one amino acid. This change was critical and had a profound effect on enzymatic activity, leading to hydrolysis of third-generation cephalosporins and aztreonam. The beta-lactamases had thereby an "extended broadspectrum" activity as compared with the "broad-spectrum" classic TEM and SHV enzymes. The term "broad" was lost and the term became "extendedspectrum beta-lactamase".~~

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~~The continuous pressure exerted by the use of newer expanded spectrum beta-lactams promoted the development of new TEM and SHV derivates. Today, the number of TEM and SHV variants has grown to over 170 and 130, respectively (http://www.lahey.org/Studies, see the web site for updated information).~~

~~ESBLs of the CTX-M type were rare until the end of the 1980s, but Japan, Argentina and Germany reported almost concomitantly findings of this ESBL type (Knothe *et al.*, 1983). The CTX-M family consists of over 80 different CTX-M types (http://www.lahey.org/Studies). The genes encoding CTX-Ms have been mobilised from *Klyuviera* spp. by several genetic events and mechanisms (Canton et al., 2006).~~

~~With the emergence of the CTX-Ms, there has been a marked shift in the epidemiology of ESBLs (Canton et al.,2006, Bonnet et al.,2004,Velasco et al.,2009). Before the mid 1990s, the ESBLs were mainly present in *Klebsiella* spp. in nosocomial settings. The dominating ESBL enzymes were TEM and SHV derivates. Today, the CTXMs are the most prevalent ESBL enzymes, and *E. coli* is the main ESBL producer. CTX-M-15 is derived from CTXM-3 by a single amino acid substitution at position 240 (Asp → Gly). This substitution confers an increased catalytic activity against ceftazidime. The success of the CTX-Ms over the classical ESBL-enzymes SHVs and TEMs is linked to the way by which CTX-M enzymes are spread. Through mobile genetic elements, resistance genes disseminate within the same species and also between bacteria of different species (Velasco et al., 2009), Courvalin et al.,2008). Mobile elements involved in the dissemination of *bla*CTX.M genes have been described in recent reviews (Knothe *et al.*, 1983, Canton et al., 2006, (Canton et al., 2008). Horizontal dissemination of genes encoding ESBLs occurs by conjugative plasmids and transposons (Figure 2).~~

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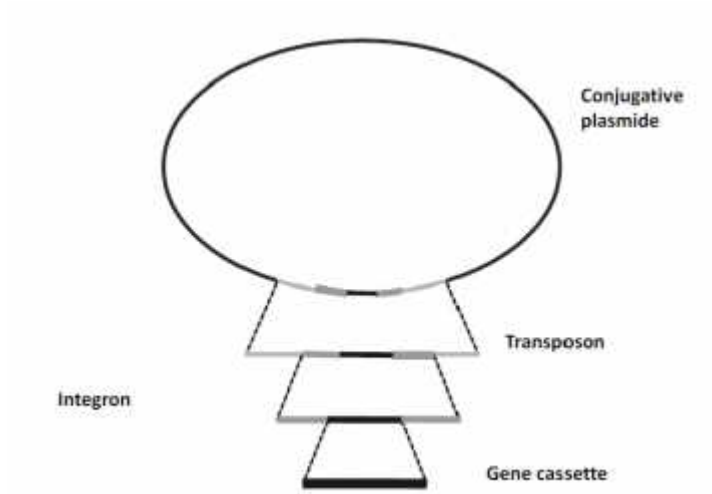

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Figure 2. Anatomy of a resistance plasmid. The plasmid contains mobile transposons and integrons carrying resistance gene cassettes.

The motility and multidrug-resistance of the CTX-Ms is sometimes associated with integrons (Bonnet. 2004) . Integrons are highly efficient recombination and expression systems, which are able of capturing DNA sequences known as gene cassettes by site specific recombination (Courvalin *et al*., 2008). Integrons can move in and out of the genome and in this way remodel it, but integrons are not able to move between bacteria. The gene cassettes, which harbour genes encoding CTX-Ms, usually carry one or several other genes encoding antibiotic resistance. Several different integron classes have been reported according to the homology of their integrase genes . Class 1 integron, followed by class 2 integrons is most commonly found in nosocomial and community settings. Typing of integrons may be a way to surveil the spread of ESBL-producing bacteria.

As a consequence of horizontal gene transfer by transposon-plasmid vectors, most ESBL producing *E. coli* were clonally unrelated until a few years ago (Diaz et al 2010). Recently, CTX-M-15 was identified in an international clone of *E. coli*, which has been detected in both in-patients and out-patients. This clone belongs to the phylogenetic group B2, MLST

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~~type 131 and exhibits a specific lipopolysaccharide type (O25b). This O25b ST131 clone has not only a considerable ability to disseminate, it is also equipped with a high virulence potential, causing significant morbidity and mortality. This is partly explained by its capacity to produce biofilm, which might contribute to their long term persistence in various environments and to their exhibited resistance to antimicrobial agents and disinfectants (Diaz et al 2010). It is also possible that the production of biofilm leads to an increased resistance to host immune defences.~~

~~According to EARSS,(European surveillance system,) resistance to antibiotics among Gram negative bacteria involved in serious infections in humans has now reached 25% or more in many European countries (http://www.rivm.nl/earss/).~~

for ESBL production

~~3.4.1.4 Screening for ESBL Producers~~

has developed disk (CLSI) Clinical and Laboratory Standards Institute diffusiion and broth microdilution screening tests for the possible ESBL .production

Disk Diffusion methoda) : ~~Disk Diffusion method :~~The CLSI has proposed disk diffusion methods for screening for ESBL production by ~~*Klebsiella KKlebsiella.*~~ ~~spp.,~~spp. ~~*Escherichia E.*~~ *coli* and ~~*proteus Proteus.*~~ *mirabilis.* The possible ESBL production can be detected by noting specific zone diameters which indicate a high level of suspicion for ESBL production (CLSI, 2005). Cefpodoxime, ceftazidime, cefotaxime, ceftriaxone, or aztreonam can be used for screening, however, use of more than one of these agents for screening improves the sensitivity of detection.

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~~**b)** The CLSI has **:Screening by dilution antimicrobial susceptibility tests** .spp *Klebsiella* proposed dilution methods for screening ESBL production by or aztreonam can be used at ,ceftriaxone ,cefotaxime ,Ceftazidime .*coli* .*E* and MIC of cephalosporins at a range of .ml/µg1 on ofa screening concentrati ml is suspicious of ESBL production and an indicationfor the organism /µg2≤) to be tested by phenotypic confirmatory testCLSI .(2005 ,~~

The above mentioned organisms should be reported as potential ESBL Ceftazidime ,mm17Cefpodoxime≤ ucers if the screening test results areprod .mm27mm and Aztreonam ≤25Ceftriaxone ≤ ,mm27Cefotaxime ≤ ,mm22≥

~~**as follows:**~~

~~**Disk diffusion** **MIC's**~~

2.3.2 ~~**3.1.4.5 Phenotypic**~~**3.1.4.5** ~~**Phenotypic**~~**confirmatory test for ESBL** ~~**confirmatory test for ESBL production**~~ **producers**

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

a) :clavulanate combination disk/Cephalosporin~~Cephalosporins/Clavulanate Combination Disks:~~ ,(µg30) advocates the use of Cefotaxime lsiThe C– or Cefpodoxime (µg10) disks with or without clavulanate (µg30) Ceftazidime for phenotypic confirmation of the presence of (µg1) plus clavulanate (µg10) mm between the zone 5~~≤~~ greater than or equal to A difference of .ESBLs diameters of either of the cephalosporin disks and their respective tion of clavulanate disk is taken to be phenotypic confirma/cephalosporin .ESBL production

~~**b)** ,(ml/µg128to 0.25) It utilizes ceftazidime **:Broth Microdilution** to 0.25) cefotaxime ,(ml/µg 4/128 to 0.25) ceftazidime plus clavulanic acid~~

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Both of .(ml/µg 4/64 to 0.25) and cefotaxime plus clavulanic acid ,(ml/µg64 -3considered as ≥ Phenotypic confirmation is .these antibiotic should be used decrease in MIC of either of cephalosporin in the presence of -serial-twofold .clavulanic acid compared to its MIC when tested alone

c) This test incorporates the use of **:Double Disk Synergy Test** cefotaxime) 30 µg(and ceftazidime)30 µg(disks whichare placed on either side co-amoxiclav)20+10 µgat a ,on a already inoculated Mueller Hinton Agar plate (-center to center distance of20-30 mm. ESBL production is inferred when the zone of either cephalosporin is expanded by the clavulanate.

3.4 Urinary tract infection and etiological agent

can be defined as a spectrum of disease (UTIs) Urinary tract infections involving the microbial invasion of tissues lining the urinary tract extending ortant complications of UTIs are imp .from the renal cortex to urethral meatus and structural and neurologic ,renal transplantation ,renal diseases ,diabetes Forbes) abnormalities that interfere with urine flow*et al*Etiologic .(2007 ,. :agents commonly involved in UTIs include

Gram positive

Staphylococcus aureuss ,*taphylococcus saprophyticus* ,Other coagulase negative staphylococci (CONS) ,*Enterococcus faecalis*

Gram negative

Escherichia coli ,*Proteus* sp ,.p*Pseudomonas aeruginosa*, *Klebsiella* s ,.pp .spp *Acinetobacter* ,.spp *Citrobacter* ,.spp *Enterobacter*

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~~C~~HAPTER III~~V:~~

~~Materials and methods~~SAND METHOD SMATERIAL

34Materials 1.

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

34Methodology 2.

The study was conductedat Alka Hospital, Jawalakhel, Lalitpur from October to December 2012.years of age of 94 to 1 This study included patients of age ,both sexesadmitted or OPD patientsfrom whom samples were sent for .routine culture and antibiotic susceptibility testing

~~4 Sample size 2.1.~~

~~A total of 1699 urine samples were included in the study.~~

34.2.12 Urine specimen collection, transport and analysis

1699 A total of urine sampleswere included in the study. The patient was given a sterile, dry, wide-necked leak-proof container and requested fora 10-20 ml of~~ first morning~~ mid-stream urine, explaining the need to collect the urine with as little contamination as possible i.e. a clean-catch specimen. The patient was instructed to cleanse the area around the urethral opening with clean water, dry the area and then begin to void and collect the mid-stream

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urine sample. The container labeled with the date, the ~~name and the number of the patient~~hospital number of patient, the time of collection and ~~was~~ delivered to the laboratory along with the request form as soon as possible. When immediate delivery was not possible, the specimen was refrigerated at 4-6^{0}C (Pokhrel *et al.*, 2006). ~~give reference?~~

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~~3.2.24 2.3.M~~3.2.2 Macroscopic examination

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The urine specimen obtained in laboratory was observed for its color, turbidity and odo~~u~~r.

3.2.3 ~~4~~ Microscopic examinations

The urine specimen was examined microscopically as a wet preparation primarily for detecting pus cells. WBCs in excess of 10^4 cells/ml (> 10cells/ml) of urine will indicate significant pyuria. ~~One WBC/LPF corresponds to 3 cells/µL (Cheesbrough, 2000).~~ Other tests in microscopic examination are RBCs, casts, crystals, epithelial cells and bacteria by making smear of deposit after centrifugation at 3500 rpm for 5 -10 minutes under high power microscope~~.~~ (Cheesbrough, 2000).

3.2.4~~4.2.5~~ Culture

The urine sample was cultured onto the Mac Conkey agar and blood agar medium by the semi-quantitative culture technique using a standard loop. After mixing the urine sample in the container thoroughly, a loopful of sample was touched to the center of the plate, from which the inoculum was spread in a line across the diameter of the plate. Without flaming the loop was drawn across the entire plate, crossing the first inoculum streak numerous times to produce isolated colonies. The plates were incubated aerobically at 35-37^{0}C overnight. The approximate number of colonies was counted and the number of bacteria i.e. colony forming unit (cfu) per ml urine estimated in accordance to the volume of urine inoculated previously. For example, 100 colonies on

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inoculating 0.001 ml of urine would correspond to 10^5 cfu/ml (Pokhrel *et al.*, 2006). ~~.~~

Colony count was performed to calculate the number of cfu/ml and bacterial count was reported as following. Less than 10^4/ml organisms: not significant, 10^4-10^5/ml organisms: doubtful significance (suggest repeat specimen). More than 10^5/ml organisms: significant bacteriuria. 10^2 cfu/ml is significant for the catheter collected urine sample .

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

3.2.5~~42..6~~ Identification of the isolates

~~Identificaion of the~~**~~isolates~~**

Identification of significant isolates was done by using microbiological techniques as described in the Bergy's manual which involves morphological appearance of the colonies, staining reactions and biochemical properties (Cheesbrough, 2000; Forbes *et al.*, 2007).

The ~~gram~~ Gram negative isolates were identified by standard diagnostic procedure as: For lactose fermenters, media inoculation for motility, indole production and citrate utilization tests were carried out and incubated overnight. Individual colonies of clinically significant, lactose non-fermenters were inoculated into 2 ml of urea broth and incubated for 4 hours at 37^0 C. Any urease positive culture was then plated to check for purity. The 4 hours suspension would serve as the inoculum for biochemical tests for strains of other genera, and a purity check on Mac Conkey agar.

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Gram positive organisms were identified primarily on the basis of their response to gram's staining, catalase, oxidase and coagulase tests (Cheesbrough, 2000).

3.2.6 ~~42..7~~ Antimicrobial susceptibility testing~~susceptibility Antimicrobial testing~~

Antimicrobial Susceptibility tests of the different clinical isolates towards Bauer disk diffusion method -irbyk various antibiotics were performed by Appendix) (MHA) using Mueller Hinton AgarF.(

~~3.2.7~~ Preservation of the MDR 3.2.7~~42..8Preservation of the MDR isolates~~

isolates

MDR isolates in pure ,After performing the antimicrobial susceptibility testing Glycerol containing Tryptic Soya Broth and %20 culture were preserved in - kept at4 C for the^{0}detections.of ESBL

3.2.8 ~~42..9~~ Screening ~~Screening~~and confirmation for ESBL producers~~for ESBL producers~~

Screening of the suspected ESBL strains was performed according to the guidelines for screening issued by the CLSI in 2005. According to this guidelines or possible ESBL production using MDR isolates were screened f Cefpodoxime ,(µg30) Cefotaxime ,(µg30) Ceftazidime ,(µg30) Ceftriaxone (µg30) and Aztreonam ,(µg10) and isolates showing zone of inhibition to Cefotaxime ≤ 27 mmC ,efpodoxime ≤17mmC ,eftazidime ≤22 mm , Aztreonam ≤27 mmand C ,eftriaxone ≤ 25 mmare the possible ESBL producers. The suspected ESBL producers were tested for confirmatory ESBL production using Lactamase-Extended Spectrum Beta HiMedia™ ~~MASTDISC~~ Detection Discs (LβES). ;The kit consisted of

Clavulanic acid plus (µg30) and Ceftazidime (µg30) Ceftazidime :1 Set plus Clavulanic acid (µg30) and Cefotaxime (µg30) Cefotaxime :2 Set ;(µg10)

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plus Clavulanic (µg10) and Cefpodoxime (µg10) Cefpodoxime :3 Set ;(µg10) .(µg10) plus Clavulanic acid (µg 30) and (µg1) acid

The zone of inhibition for thec ,eftazidimeCc efotaxime andCcefpodoximeC Cefotaxime and Cefpodoxime ,discs was compared to that of the Ceftazidime an increase in zone diameter of ≥ ,plus Clavulanic acid combination discs ic acid from any or all of the sets of the kit mm in the presence of Clavulan5 The detailed working protocol .was concluded as confirmed ESBL producers is described in AppendixG.

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3.3 Quality Control

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3.3.1 Monitoring and regular evaluation of laboratory equipments, reagents and media

~~34.3 Quality Control~~

~~3.3.1 Monitoring and regular evaluation of laboratory euipment, reagents and media~~

autoclave and hot air oven ,refrigerator ,Laboratory equipment like incubator ator The temperature of the incub .were regularly monitored for their efficiency Reagents and media were regularly .and refrigerator was monitored everyday After .monitored for their manufacture and expiry date and proper storage The .~~expiry date ,~~they were properly labelled with prepartion date ,preparation ate of each lot for prepared was checked by incubating one pl quality of media .sterility and using standard control strains for performance testing

~~34 3.2.P~~3.3.2 Purity Plate

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

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3.3.3 Quality control during antimicrobial susceptibility testing

~~34Quality control during antimicrobial susceptibility testing 3.3.~~

Mueller Hinton agar and the antibiotic discs were checked for their lot For the .and proper storage ,manufacture and expiry date ,number Bauer test and for performance testing of antibiotics -standardization of Kirby *S* and (25922ATCC) *coli .E* control strains of ,and MHA~~*taph*~~ ATCC) *aureus* . .were tested primarily (25923Quality of sensitivity tests was maintained by maintaining the thickness of MHA at 4 mm and the pH at 7.2-7.4.

.3.4 Data analysis

~~34.4 Data analysis~~

ocials ackage forp tatisticals All the results were entered in the worksheet of ciencess1 Version) software (SPSS) 6.(0.

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CHAPTER IV

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RESULTS

4.1 Clinical profile of urine sample and culture positivity

All together 1699 urine samples were collected during the study period. Out of samples were received from the (%36.8) 626 ,urine samples 1699 the The .samples from female patients (%63.2) 1073 male patient whereas yrs 30-20 maximum number of culture request being received from age group 357 with,years 40-30 followed by age group ,request (%27.3) 463 with (%0.23) 100-90 request and the least being received from age (%21.09) and (1 Table) .(%2.11) 90-80

4.1.1 Number of samples and growth pattern

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~~All together 1699 urine samples were collected during the study period and subjected for bacterial cultures in which significant growth was seen only in 271 cases (16.0%). Total 10^5 cfu/ml was taken as significant growth. In 1428 cases (84%) no growth was observed.~~

Table 12: Growth pattern in urine culture

(%84) 1428 samples and in (%16) 271 ignificant growth was seen in~~The s~~S .samples there was no significant growth.

Bacterial growth	Frequency (n)	Percentage (%)
No growth	1428	84
Significant growth	271	16
Total	1699	100

4.1.2 Age and gender wise distribution of culture positive urine samples

~~4.1.2 Growth status among genders and different age groups~~

~~The significant growth was seen in 271(16%) samples and in 1428 samples there was no significant growth. The maximum number of growth was observed in age group 20-30 yrs (27.3%), followed by age group 30-40 (20.3%). Of the 626 samples from male, 78(12.4%) showed significant growth with maximum no. of growth being observed in age group 60-70 (20.5%), followed by age group 20-40 (16.7%). Similarly, of the 1073 samples from female, 193 (17.9%)showed significant growth with maximum no. of growth being observed in age group 20-30 years (32.6%), followed by 30-40 years (21.8%).~~

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Table 23: Gender wise distribution of significant bacteriuria

Among .cant growthishowed signif (%12.4) 78 ,samples from male 626 Of the .showed the significant growth (%17.9) 193 ,samples from female 1073 (2 Table)

~~urine culture results~~

Gender	Significant bacteriuria		No growth	
	No.~~No.~~	%~~%~~	No.~~No.~~	%~~%~~
Male N=626	78	12.4	548	87.2
Female N=1073	193	17.9	880	81.9
Total=1699	271	16.	1428	84.04

Table 34: Age wise distribution of significant bacteriuria

yrs 30-20 The maximum number of growth was observed in age group of .maximum no ,In males .(%20.3) 40-30 group followed by age ,(%27.3) 70-60 growth being observed in age group(20.5%), followed by age group 20-40 (16.7%) of growth being observed in umbermaximum n ,In females .age group 20-30 years (32.6%), followed by 30-40 years (21.8%).

SN	Age group	Male		Female		Total	
		No	%	No	%	No	%
1	Less than ~~0~~-10	4	5.1	2	1.03	6	2.2
2	10-20	5	6.4	17	8.8	22	8.1
3	20-30	11	14.1	63	32.6	74	27.3
4	30-40	13	16.7	42	21.8	55	20.3

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5	40-50	5	6.4	21	10.8	26	9.6
6	50-60	10	7.8	21	10.8	31	11.4
7	60-70	16	20.5	13	16.7	29	10.7
8	70-80	7	8.9	10	5.2	17	6.3
9	80-90	6	7.7	3	1.55	9	3.3
10	90-100	1	1.3	1	0.5	2	0.7
	Total	78	28.8	193	71.2	271	100.0

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~~**5.2 Correlation of pyuria with culture result**~~

~~Among the total 1699 samples, 1300 (76.5%) showed insignificant pyuria. Among these, 20 (1.5%) of samples gave positive culture results. Similarly, 399 (24.5%) of total samples showed significant pyuria, and among these 251(62.9%) samples gave positive culture results. The results are shown in table 5~~

~~**Table 5: Correlation of pyuria with culture result**~~

~~**Pyuria**~~	~~**Culture positive (%)**~~	~~**Culture negative (%)**~~	~~**Total (%)**~~
~~**Significant (≥5WBC/HPF)**~~	~~251(62.9)~~	~~148~~	~~**399(24.5)**~~
~~**Insignificant (<5WBC/HPF)**~~	~~20 (1.5)~~	~~1280~~	~~**1300(76.5)**~~
~~**Total**~~	~~**271**~~	~~**1428**~~	~~**1699 (100.0)**~~

~~**5.3 Correlation of hematuria with culture result**~~

~~Among the 1699 samples, 1500 (88.3%) of samples showed insignificant haematuria, among these, 200 (13.3%) of samples showed positive culture~~

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~~results. Similarly, 199(11.7%) of total samples showed significant haematuria, and among these 71(35.65) samples showed positive culture results. The results are shown in table 6~~

~~Table 6: Correlation of hematuria with culture result~~

~~Haematuria~~	**~~Culture positive (%)~~**	**~~Culture negative (%)~~**	**~~Total (%)~~**
~~Significant (≥3RBC/HPF)~~	~~71 (35.6)~~	~~228 (60.4)~~	**~~199(11.7)~~**
~~Insignificant (<3RBC/HPF)~~	~~200 (13.3)~~	~~1300 (86.7)~~	**~~1500 (88.3)~~**
~~Total~~	**~~271(16)~~**	**~~1428~~**	**~~1699 (100.0)~~**

~~4.1.3 Correlation of pyuria with culture result~~

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~~Among the total 1699 samples, 1300 (76.5%) showed insignificant pyuria. Among these, 20 (1.5%) of samples gave positive culture results. Similarly, 399 (24.5%) of total samples showed significant pyuria, and among these 251(62.9%) samples gave positive culture results. The results are shown in table 5~~

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~~Table 4: Correlation of pyuria with culture result~~

~~Pyuria~~	~~Culture positive (%)~~	~~Culture negative (%)~~	~~Total (%)~~
~~Significant (≥ 5WBC/HPF)~~	~~251(62.9)~~	~~148~~	~~399(24.5)~~
~~Insignificant (< 5WBC/HPF)~~	~~20 (1.5)~~	~~1280~~	~~1300(76.5)~~
~~Total~~	~~271~~	~~1428~~	~~1699 (100.0)~~

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~~4.1.4 Correlation of hematuria with culture result~~

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~~Among the 1699 samples, 1500 (88.3%) of samples showed insignificant haematuria, among these, 200 (13.3%) of samples showed positive culture results. Similarly, 199(11.7%) of total samples showed significant haematuria, and among these 71(35.65) samples showed positive culture results. The results are shown in table 6~~

~~Table 5: Correlation of hematuria with culture result~~

~~Haematuria~~	~~Culture positive (%)~~	~~Culture negative (%)~~	~~Total (%)~~
~~Significant (≥ 3RBC/HPF)~~	~~71 (35.6)~~	~~228 (60.4)~~	~~199(11.7)~~
~~Insignificant (< 3RBC/HPF)~~	~~200 (13.3)~~	~~1300 (86.7)~~	~~1500 (88.3)~~
~~Total~~	~~271(16)~~	~~1428~~	~~1699 (100.0)~~

~~**M 5.4 4.2icrobiological profile of Urinary isolates**~~

4.2 Microbiological profile of Urinary isolates

Among the 271 isolates, Gram negative bacteria were predominant constituting (%87.8) 238 of the total isolates. Gram positive organisms constituted 33(12.1%). *E. ~~scherichia~~ coli* was the most frequently isolated species with 183(88.2%), followed by *S.~~taphylococcus~~ aureus* with 35 (11.7%)~~.~~, *K. ~~lebsiella~~ pneumoniae* 19 (7 %), ~~,~~ *Acinetobacter* spp.~~.~~, 15 (5.5%). (6 Table)

Table 4 ~~7~~: Microbiological profile of urinary isolates ~~Microbiological isolates profile of Urinary~~

SN	Organism isolated	Total no of isolates	Percentage
	Gram negative organisms		

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1	*Escherichia coli*	183	67.5
2	*Klebsiella pneumoniae*	19	7
3	*Acinetobacter* spp.	15	5.5
4	*Pseudomonas aeruginosa*	11	4.1
5	*Proteus mirabilis*	5	1.8
6	*Proteus vulgaris*	2	7
7	*Citrobacter fruendii*	3	1.1
	Total	238	87.8
	Gram positive organisms		
1	*Staphylococcus aureus*	29	10.7
2	*Enterococcus* spp.	4	1.4
	Total	33	12.1

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~~**5.5 Antibiotic susceptibility pattern of isolates**~~

~~**5.5.1 Antibiotic susceptibility pattern of gram Gram negative isolates**~~

~~Among the common 1st line antibiotics used against all Gram negative bacteria, the most effective antibiotic was found to be nitrofurantoin (84.4%) followed by gentamycin (79.8%), ciprofloxacin (60.9%), norfloxacin(58.8%). Among the 2nd line drugs, the most effective was amikacin(70.70%), ofloxacin (63.45%), ceftazidime (63%). Imipenem, polymixin B, tobramycin were used against resistant strains and were found to bo 100%, 100% and 94.5%~~

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effective respectively. The least susceptible drug was found to be amoxycillin (8.8%) followed by nalidixic acid (36.9%).

Table 8: Antibiotic susceptibility profile of gram negative bacteria

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S.N.	Antibioticics used	Total no. of isolates	Susceptibility Pattern					
			Resistant		Moderate		Susceptible	
			No.	%	No.	%	No.	%
1.	Ciprofloxacin	238	88	36.9	5	2.1	145	60.9
2.	Gentamycin	238	40	16.8	8	3.4	190	**79.8**
3.	Norfloxacin	238	97	40.7	1	0.4	140	58.8
4.	Cotrimoxazole	238	107	44.9	0	0	131	55.0
5.	Amoxycillin	238	214	89.9	3	0	21	8.8
6.	Nalidixic acid	238	150	63.0	1	0.4	88	36.9
7.	Ofloxacin	238	83	34.8	0	0	151	63.4
8.	Nitrofurantoin	238	34	14.3	1	0.4	201	**84.4**
9.	Amikacin	238	32	13.4	0	0	206	86.5
10	Ceftazidime	238	88	36.9	0	0	150	63.0
11.	Imipenem	238	0	0	0	0	238	**100**
12.	Tobramycin	148	8	5.4	0	0	140	**94.5**
13	Polymixin B	231	0	0	0	0	231	**100**

5.5.2 Antibiotic susceptibility pattern of Gram positive bacteria

Among the common 1st line antibiotics used against all Gram positive urine isolates, the most effective antibiotic was found to be nitrofurantoin. (87.9%), followed by gentamycin (75.6%). Among the 2nd line drugs, the most effective was amikacin (78.7%), cefotaxime (75.6%), cotrimoxazole (66.7%), ofloxacin (60%). Vancomycin was 100% sensitive. Ceftazidime was found to have least sensitivity (54.5%).

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Table 9: Antibiotic susceptibility profile of gram-positive bacteria

S.N	Antibiotics used	Total No. of isolates	Susceptibility pattern					
			Resistant		Intermediate		Sensitive	
			No	%	No	%	No	%
1	Ciprofloxacin	33	13	39.4	2	6.0	18	54.5
2	Gentamicin	33	8	24.24	0	0	25	75.6
4	Amikacin	33	7	21.2	0	0	26	78.7
5	Cotrimoxazole	29	11	33.33	0	0	22	66.7
6	Ofloxacin	33	13	39.3	1	3	20	60
7	Nitrofurantoin	33	4	12.12	0	0	29	87.9
8	Vancomycin	33	0	0	0	0	100	100
9	Ceftazidime	33	15	45.5	0	0	18	54.5
10	Cefotaxime	29	8	24.24	0	0	25	75.6

Table 5: Antibiotic susceptibility profile of bacterial isolates

Table (a): Antibiotic susceptibility profile of each isolates of *E. coli*.

Isolates	AMX	AK	CIP	GEN	NIT	CAZ	OF	TOB
EC1	R	S	R	R	R	R	S	S
EC2	R	R	R	S	R	S	R	S
EC3	R	S	R	S	S	R	S	S
EC4	R	S	S	S	S	S	S	S
EC5	R	R	R	R	S	S	R	S
EC6	R	S	IS	R	R	R	S	S
EC7	R	R	S	R	S	S	R	S
EC8	R	S	S	S	S	S	S	S
EC9	S	S	S	S	S	S	S	S

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EC10	R	S	S	S	S	S	S	S
EC11	R	R	S	S	R	R	R	S
EC12	IS	S	S	S	S	S	S	S
EC13	S	S	S	S	R	S	S	S
EC14	R	S	S	S	S	S	S	S
EC15	R	S	S	S	S	S	S	S
EC16	R	R	R	R	S	R	R	S
EC17	R	S	IS	S	R	R	S	S
EC18	S	S	S	S	S	S	S	S
EC19	R	S	R	R	S	R	S	S
EC20	R	S	R	R	S	R	S	S
EC21	R	S	R	R	S	R	S	S
EC22	R	S	R	S	S	R	S	S
EC23	R	S	S	S	S	S	S	S
EC24	S	S	S	S	S	S	S	S
EC25	R	S	S	S	S	R	S	S
EC26	R	R	R	S	R	R	R	S
EC27	R	S	R	R	S	R	S	S
EC28	S	S	S	S	S	S	S	S
EC29	R	S	S	S	S	S	S	S
EC30	R	R	R	S	R	R	R	S
EC31	S	S	S	S	S	S	R	S
EC32	R	S	S	S	S	S	S	S
EC33	R	R	R	R	R	R	R	S
EC34	R	S	R	S	R	R	S	S

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EC35	R	R	R	R	S	S	R	S
EC36	R	R	S	S	S	S	R	S
EC37	R	S	S	S	S	S	S	S
EC38	R	S	R	R	R	R	S	S
EC39	S	R	S	S	S	S	R	S
EC40	R	S	S	S	S	S	S	S
EC41	R	R	R	R	S	S	R	S
EC42	R	S	S	S	S	S	S	S
EC43	IS	S	S	S	S	S	S	S
EC44	R	S	S	S	S	S	S	S
EC45	R	S	R	S	R	R	R	S
EC46	R	S	S	S	S	S	R	S
EC47	S	S	S	S	S	S	S	S
EC48	R	S	R	R	S	R	S	S
EC49	R	S	S	S	S	S	S	S
EC50	R	S	R	S	S	R	S	S
EC51	R	R	R	R	R	S	R	S
EC52	R	S	R	S	S	R	S	S
EC53	R	S	R	S	S	R	S	S
EC54	S	S	S	S	S	S	S	S
EC55	R	R	R	R	S	S	R	S
EC56	R	S	S	S	S	S	S	S
EC57	R	S	R	R	R	R	S	S
EC58	R	S	S	S	S	S	S	S
EC59	R	S	R	S	S	R	S	S

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EC60	R	R	R	S	S	S	R	S
EC61	R	S	R	S	S	R	S	S
EC62	R	S	R	S	R	R	S	S
EC63	R	S	R	S	S	R	S	S
EC64	R	S	S	S	S	S	S	S
EC65	R	S	R	R	S	R	S	S
EC66	R	R	S	S	R	S	R	S
EC67	R	R	R	S	S	R	R	S
EC68	R	S	IS	S	S	S	S	S
EC69	R	S	R	S	S	R	S	S
EC70	R	S	R	S	S	R	S	S
EC71	S	S	S	S	S	S	S	S
EC72	R	S	S	S	S	S	S	S
EC73	R	S	R	S	R	R	S	S
EC74	R	S	R	S	S	R	S	S
EC75	R	S	R	S	S	R	S	S
EC76	R	S	S	S	S	S	S	S
EC77	S	S	S	S	S	S	S	S
EC78	R	S	R	S	S	R	S	S
EC79	R	R	S	S	S	S	R	S
EC80	R	R	R	R	S	S	R	S
EC81	R	S	S	S	S	S	S	S
EC82	R	S	R	S	S	R	S	S
EC83	R	S	S	S	S	S	S	S
EC84	R	S	S	S	S	S	S	S

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EC85	S	S	S	S	S	S	S	S
EC86	R	S	S	S	S	S	S	S
EC87	R	S	S	S	S	S	S	S
EC88	R	S	S	S	S	S	S	S
EC89	R	R	S	S	R	R	R	S
EC90	R	S	S	S	S	S	S	S
EC91	R	R	R	R	R	R	R	S
EC92	R	S	S	S	S	S	S	S
EC93	R	S	S	S	S	S	S	S
EC94	S	S	S	S	S	S	S	S
EC95	R	S	S	S	S	S	S	S
EC96	R	S	R	S	S	R	R	S
EC97	R	S	S	S	S	S	S	S
EC98	R	R	S	R	R	R	IS	S
EC99	R	S	R	R	S	R	IS	S
EC100	R	S	S	S	S	S	S	S
EC101	R	S	S	S	S	S	S	S
EC102	R	R	R	R	S	R	R	S
EC103	R	R	R	R	R	R	R	S
EC104	R	S	S	S	S	S	S	S
EC105	R	S	S	S	S	S	S	S
EC106	R	S	S	S	S	S	S	S
EC107	R	S	S	S	S	S	S	S
EC108	R	R	S	S	R	S	R	S
EC109	R	S	R	R	S	R	S	S

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EC110	R	S	S	S	S	S	R	S
EC111	R	S	R	R	S	R	S	S
EC112	R	R	R	R	R	S	IS	S
EC113	R	R	S	S	R	S	S	S
EC114	R	S	S	S	S	S	R	S
EC115	R	S	S	S	S	S	S	S
EC116	R	S	S	S	S	S	S	S
EC117	S	S	S	S	S	S	S	S
EC118	R	S	R	R	S	R	S	S
EC119	R	R	R	R	R	R	R	S
EC120	R	S	S	S	S	S	S	S
EC121	R	S	S	R	S	R	IS	S
EC122	R	S	S	S	S	S	S	S
EC123	R	S	R	R	S	R	S	S
EC124	R	S	S	S	S	S	S	S
EC125	R	S	S	S	S	S	S	S
EC126	R	R	S	S	S	R	R	S
EC127	R	S	R	R	R	R	S	S
EC128	R	S	S	S	S	S	S	S
EC129	R	S	S	S	S	S	S	S
EC130	R	S	S	S	S	S	S	S
EC131	R	R	R	S	S	S	R	S
EC132	R	S	S	S	S	S	S	S
EC133	R	S	S	S	S	S	S	S
EC134	R	S	S	S	S	S	S	S

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EC135	R	S	S	S	S	S	S	S
EC136	R	S	R	R	S	R	IS	S
EC137	S	S	S	S	S	S	S	S
EC138	R	S	S	S	S	S	S	S
EC139	R	R	R	R	R	R	R	S
EC140	R	S	S	S	S	S	S	S
EC141	IS	S	S	S	S	S	S	S
EC142	R	S	S	S	S	S	S	S
EC143	R	R	S	R	R	R	R	S
EC144	R	S	R	R	S	R	S	S
EC145	R	S	S	S	S	S	S	S
EC146	R	S	S	S	S	S	S	S
EC147	R	S	S	S	S	S	S	S
EC148	R	R	S	R	R	S	R	S
EC149	R	S	S	S	S	S	S	S
EC150	R	S	R	R	R	R	IS	S
EC151	R	R	S	R	S	R	R	S
EC152	R	R	S	R	S	R	R	S
EC153	R	S	S	S	S	S	S	S
EC154	R	R	S	R	R	R	R	S
EC155	R	S	S	S	S	S	S	S
EC156	R	S	S	S	S	S	S	S
EC157	R	S	S	R	S	R	S	S
EC158	R	S	IS	R	S	S	S	S
EC159	R	R	S	R	S	R	R	S

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EC160	R	S	S	S	S	S	S	S
EC161	R	S	S	S	S	S	S	S
EC162	R	R	S	R	S	S	S	S
EC163	R	S	R	S	S	R	S	S
EC164	R	S	S	S	S	S	S	S
EC165	S	S	R	S	S	S	R	S
EC166	R	S	S	S	S	S	S	S
EC167	R	S	S	S	S	S	S	S
EC168	R	S	S	S	S	S	S	S
EC169	R	R	S	R	S	R	S	S
EC170	R	R	S	R	S	R	R	S
EC171	R	S	S	S	S	S	S	S
EC172	R	R	S	R	S	R	R	S
EC173	R	R	S	R	R	S	R	S
EC174	R	S	S	S	S	S	S	S
EC175	R	S	R	S	S	R	S	S
EC176	R	S	S	S	S	R	S	S
EC177	S	S	S	S	S	S	S	S
EC178	R	S	S	S	S	S	S	S
EC179	R	R	S	R	R	R	S	S
EC180	IS	S	S	S	S	S	S	S
EC181	R	S	S	S	S	S	S	S
EC182	R	S	S	S	S	R	S	S
EC183	R	R	S	R	S	R	S	S

Table (b): Antibiotic susceptibility profile of each isolate of *K. pneumoniae*

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Isolates	AMX	AK	CIP	GEN	NIT	CAZ	OF	TOB
KP1	R	S	S	S	S	S	S	S
KP2	R	S	S	S	S	S	S	S
KP3	R	R	R	S	R	R	R	S
KP4	S	S	S	S	S	S	S	S
KP5	R	S	S	R	S	S	S	S
KP6	R	S	R	S	S	S	S	S
KP7	R	R	R	R	S	R	R	S
KP8	R	S	S	S	S	S	S	S
KP9	R	S	S	I	S	R	S	S
KP10	R	S	S	S	S	S	S	S
KP11	R	S	S	S	S	R	R	S
KP12	R	S	S	S	S	S	S	S
KP13	S	S	S	S	S	S	S	S
KP14	R	R	R	S	S	R	S	S
KP15	R	S	S	S	R	S	S	S
KP16	R	S	S	S	S	R	S	S
KP17	R	R	R	R	S	R	R	S
KP18	R	S	S	S	S	S	S	S
KP19	R	S	R	R	R	R	R	S

Table (c): Antibiotic susceptibility profile of each isolates of *Proteus* spp.

Isolates	AMX	AK	CIP	GEN	NIT	CAZ	OF	TOB
PV1	R	S	R	R	R	S	R	S
PV2	R	IS	S	S	R	S	S	S
PM1	R	S	S	R	R	R	S	S

PM2	R	R	R	R	R	S	R	S
PM3	R	S	S	S	R	R	S	S
PM4	R	S	R	S	R	R	S	S
PM5	R	S	S	S	R	S	S	S

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Table (d): Antibiotic susceptibility profile of each isolates of *Citrobacter freundii*

Isolates	AMX	AK	CIP	GEN	NIT	CAZ	OF	TOB
CF1	R	S	R	S	R	R	R	S
CF2	R	S	S	S	S	S	S	S
CF3	R	S	S	S	S	S	S	S

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Table (e): Antibiotic susceptibility profile of each isolates of *P. aeruginosa*

Isolates	AMX	AK	CIP	GEN	NIT	CAZ	OF	TOB	PB
PA1	R	S	S	S	S	S	S	S	S
PA2	R	R	R	R	R	R	R	S	S
PA3	R	S	S	S	R	S	S	S	S
PA4	R	R	R	R	R	R	R	S	S
PA5	R	S	S	S	R	S	S	S	S
PA6	R	R	S	S	R	S	S	S	S
PA7	R	S	R	R	R	R	S	S	S
PA8	R	R	S	S	R	S	S	S	S
PA9	R	R	R	R	R	R	R	S	S
PA10	R	S	S	S	R	R	R	S	S
PA11	R	S	S	S	R	S	S	S	S

Table (f): Antibiotic susceptibility profile of each isolates of *Acinetobacter* spp.

Isolates	AMX	AK	CIP	GEN	NIT	CAZ	OF	TOB
AC1	R	S	R	R	R	R	R	S
AC2	R	S	S	S	S	S	S	S
AC3	R	S	R	S	R	R	S	S
AC4	R	R	R	R	R	R	R	S
AC5	R	S	R	S	S	R	S	S
AC6	R	R	R	R	R	R	R	S
AC7	R	R	R	R	R	R	R	S
AC8	R	S	R	S	R	R	S	S
AC9	R	S	R	S	R	R	S	S

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AC10	R	S	R	S	R	R	S	S
AC11	R	S	R	S	S	R	S	S
AC12	R	S	R	S	R	R	S	S
AC13	R	S	R	S	R	R	S	S
AC14	R	S	R	S	R	R	R	S
AC15	R	S	R	S	R	R	S	S

Table (g): Antibiotic susceptibility profile of each isolates of *S. aureus*

Isolates	AMX	AK	CIP	GEN	NIT	CAZ	OF	CX	VAN
SA1	R	S	S	S	S	S	S	S	S
SA2	R	R	R	S	R	R	S	R	S
SA3	S	S	S	S	S	S	S	S	S
SA4	R	S	S	S	S	IS	S	S	S
SA5	R	R	R	R	R	R	R	R	S
SA6	S	S	S	S	S	S	S	S	S
SA7	R	S	S	S	S	R	S	R	S
SA8	R	S	S	S	S	R	S	~~R~~S	S
SA9	R	R	R	R	R	R	R	R	S
SA10	R	S	S	S	S	S	S	S	S
SA11	R	S	S	S	S	S	S	R	S
SA12	R	R	R	R	R	R	R	R	S
SA13	R	S	S	S	S	R	S	~~R~~S	S
SA14	R	S	R	R	R	R	R	~~R~~S	S
SA15	S	S	S	S	S	S	S	S	S
SA16	R	S	S	S	S	R	S	IR	S
SA17	R	S	R	S	S	IS	S	~~R~~S	S
SA18	S	S	S	S	S	S	S	S	S
SA19	R	S	R	R	R	R	R	~~R~~S	S

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SA20	R	S	S	S	S	S	S	RS	S
SA21	R	S	R	R	S	R	R	RS	S
SA22	R	S	R	S	S	R	S	RS	S
SA23	S	S	S	S	S	S	S	S	S
SA24	R	S	S	S	S	R	S	RS	S
SA25	R	S	S	S	S	S	S	S	S
SA26	R	S	R	R	S	R	R	RS	S
SA27	S	S	S	S	S	S	S	S	S
SA28	R	S	S	S	S	R	S	IR	S
SA29	S	S	S	S	S	S	S	S	S

Table (i): Antibiotic susceptibility profile of each isolates of *Enterococcus* spp.

Isolates	AMX	AK	CIP	GEN	NIT	CAZ	OF	CX	VAN
ES1	R	S	S	S	S	R	R	S	S
ES2	S	S	S	S	S	R	S	S	S
ES3	R	S	S	S	S	R	S	S	S
ES4	R	S	S	S	S	R	S	S	S

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~~5.5.3 Antibiotic~~5.5.3 Antibiotic susceptibility ~~profile~~ of ~~*E. Escherichia coli*~~

In all 183 *E. coli* isolates 148 (80.87%) isolates were sensitive to Nitrofuran toin, 130 ~~was found to be most effective drug in Urinary tract infection with (80.87%) followed by gentamycin~~ (71.15%) were sensitive to gentamycin ~~susceptibility followed by Amikacin , 142(73.8%) were sensitive to amikacin~~. Among the 2nd line drugs, amikacin was (77.6%), ofloxacin was 135 (73.8%) and ceftazidime was 120~~15~~(65.5~~2.8~~%) sensitive. *E. coli* was least sensitive to ~~amoxicillin~~amoxycillin 17 (9.25%) and 100% sensitive to tobramycin. (Table 7, 8)

Table 8 ~~10~~: Antibiotic sensitivity profile of E.~~scherichia~~ coli

Antibiotics used	Total isolates	Resistant		Intermediate		Sensitive	
		No	%	No	%	No	%
Amoxycillin	183	162	88.5	4	2.1	17	9.2
Amikacin	183	41	22.4	0	0	142	77.6
Ciprofloxacin	183	59	32.24	4	2.1	120	65.6
Gentamycin	183	53	28.9	0	0	130	71.1
Nitrofurantoin	183	35	19.1	0	0	148	80.87
Ceftazidime	183	63~~8~~	37.1	0	0	120~~15~~	65.5~~2.8~~
Ofloxacin	183	43	23.5	5	2.7	135	73.8

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Tobramycin	183	0	0	0	0	100	100

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~~5.5.4~~ Antibiotic susceptibility profile of *K.~~lebsiella~~ pneumoniae*

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In all 19 isolates of *K.~~lebsiella~~ ~~-~~pneumoniae* 16 isolates (84.2%) were ~~found~~ to ~~be~~ sensitive to nitrofurantoin ~~(84.2%)~~, amikacin was sensitive to 15 (78.9%) isolates, g~~G~~entamycin was sensitive to 14 (73.6%) isolates. The isolates are resistant to ~~amoxicillin~~amoxycillin (10.6%) and ceftazidime (57.9%). Table)7, (9

Table 9 ~~11~~: Antibiotic sensitivity profile of *K. ~~lebsiella~~ pneumoniae*

Antibiotics used	Total isolates	Resistant		Intermediate		Sensitive	
		No	%	No	%	No	%
Amoxycillin	19	17	89.5	0	0	2	10.6
Amikacin	19	4	21.0	0	0	15	78.9
Ciprofloxacin	19	6	31.5	0	0	13	68.4
Gentamycin	19	4	21.0	1	5.2	14	73.6
Nitrofurantoin	19	3	15.8	0	0	16	84.2
Ceftazidime	19	8	42.1	0	0	11	57.9
Ofloxacin	19	5	26.3	0	0	14	73.6
Tobramycin	19	0	0	0	0	100	100

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~~5.5.5~~ Antibiotic susceptibility profile of *P. ~~roteus~~ vulgaris*

~~*Proteus vulgaris* isolates~~Tobramycin was ~~were~~ found to be 100% sensitive to *P. vulgaris* isolates ~~tobramycin~~ and amikacin, ciprofloxacin, gentamycin and ofloxacin were 50% sensitive ~~to amikacin, ciprofloxacin, gentamycin and ofloxacin~~. Amoxycillin, nitrofurantoin and ceftazidime ~~The ispolates arew~~were 100% sensitive to *P. vulgaris* isolates ~~amoxicillin, nitrofurantoin and ceftazidime~~. Table)7, (10

Table 1~~0~~2: Antibiotic sensitivity profile of *P.~~roteus~~ vulgaris*

Antibiotics used	Total isolates	Resistant		Intermediate		Sensitive	
		No	%	No	%	No	%
Amoxycillin	2	2	100	0	0	0	0
Amikacin	2	0	0	1	50	1	50
Ciprofloxacin	2	1	50	0	0	1	50
Gentamycin	2	1	50	0	0	1	50
Nitrofurantoin	2	2	100	0	0	0	0
Ceftazidime	2	0~~2~~	0~~100~~	0	0	2~~0~~	100~~0~~
Ofloxacin	2	1	50	0	0	1	50
Tobramycin	2	0	0	0	0	2	100

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~~5.5.6~~ Antibiotic susceptibility profile of *P. ~~roteus~~ mirabilis*

Amikacin and ofloxacin was the effective drug with 80% susceptibility. Tobramycin was 100% sensitive. Table)7, (11

Table 11~~3~~: Antibiotic sensitivity profile of *P. ~~roteus~~ mirabilis*

Antibiotics used	Total isolates	Resistant		Intermediate		Sensitive	
		No	%	No	%	No	%
Amoxycillin	5	5	100	0	0	0	0
Amikacin	5	1	10	0	0	4	80
Ciprofloxacin	5	2	40	0	0	3	60
Gentamycin	5	2	40	0	0	3	60
Nitrofurantoin	5	5	100	0	0	0	0
Ceftazidime	5	1~~5~~	10~~100~~	0	0	4~~0~~	80~~0~~
Ofloxacin	5	1	10	0	0	4	80
Tobramycin	5	0	0	0	0	5	100

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~~5.5.7~~ Antibiotic susceptibility profile of *C. ~~itrobacter~~ freundii*

Tobramycin and amikacin was the most effective drug with 100% susceptibility. The isolates are 66.67% susceptible to ciprofloxacin, nitrofurantoin, ofloxacin. Table)7, (12

Table 124: Antibiotic sensitivity profile of *C. ~~itrobacter~~ freundii*

Antibiotics used	Total isolates	Resistant		Intermediate		Sensitive	
		No	%	No	%	No	%
Amoxycillin	3	3	100	0	0	0	0
Amikacin	3	0	0	0	0	3	100
Ciprofloxacin	3	1	33.33	0	0	2	66.7
Gentamycin	3	0	0	0	0	3	100
Nitrofurantoin	3	1	33.33	0	0	2	66.7
Ceftazidime	3	12	33.366.7	0	0	21	66.6733,33
Ofloxacin	3	1	33.33	0	0	2	66.7
Tobramycin	3	0	0	0	0	3	100

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~~5.5.8~~ Antibiotic susceptibility profile of *P.~~seudomonas aeuroginosa~~aeruginosa*~~.~~

Tobramycin and polymixin ~~–~~B were 100% susceptible to *P. aeruginosa* ~~*Acinetobacter*~~ isolates. Table)7, (13~~Among 1st line drugs , the effective drugs are ciprofloxin , gentamycin, ofloxacin with each (63.6%) susebtibility.~~

Table 1~~3~~5: Antibiotic sensitivity profile of *P.~~seudomonas aeuroginosa~~aeruginosa*

Antibiotics used	Total isolates	Resistant		Intermediate		Sensitive	
		No	%	No	%	No	%
Amoxycillin	11	11	100	0	0	0	0
Amikacin	11	5~~11~~	45.5~~100~~	0	0	6~~0~~	54.5~~0~~
Ciprofloxacin	11	4	36.3	0	0	7	63.6
Gentamycin	11	4	36.3	0	0	7	63.6
Nitrofurantoin	11	1~~0~~1	100	0	0	1~~0~~	9.7~~0~~
Ceftazidime	11	5	45.45	0	0	6	54.5
Ofloxacin	11	4	36.3	0	0	7	63.6
Tobramycin	11	0	0	0	0	11	100
Polymixin ~~–~~B	11	0	0	0	0	11	100

~~5.5.9~~ Antibiotic susceptibility profile of *Acinetobacter* spp.~~.~~

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~~The most effective drug found was a~~Amikacin were 12 (80%) to *Acinetobacter* spp.~~,~~ followed by gentamycin (73.3%) ~~,~~and ofloxacin (66.67%~~5~~). Tobramycin was ~~(86.7%)~~ sensitive among 86.7% isolates. Amoxycillin was least sensitive drug with (100%) resistance followed by nitrofurantoin (80%). Table)7, (14

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Table 1~~4~~6: Antibiotic sensitivity profile of *Acinetobacter* spp.~~,~~

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Antibiotics used	Total isolates	Resistant		Intermediate		Sensitive	
		No	%	No	%	No	%
Amoxycillin	15	15	100	~~0~~0	~~0~~0	0	0
Amikacin	15	3	20	~~0~~0	~~0~~0	12	80
Ciprofloxacin	15	1~~4~~5	93.3~~100~~	~~0~~0	~~0~~0	~~1~~0	~~6.7~~0
Gentamycin	15	4	26.7	~~0~~0	~~0~~0	11	73.3
Nitrofurantoin	15	12	80	~~0~~0	~~0~~0	3	20
Ceftazidime	15	1~~4~~5	93.3~~100~~	~~0~~0	~~0~~0	~~1~~0	~~6.7~~0
Ofloxacin	15	5	33.3	~~0~~0	~~0~~0	10	66.7
Tobramycin	15	2	13.3	~~0~~0	~~0~~0	13	86.67

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~~5.5.10~~ Antibiotic susceptibility profile of *S.~~taphylococcus~~ aureus*

Amikacin was sensitive to 25 ~~found to be most effective drug with~~ (86.2%) of the isolates ~~susceptibility,~~ followed by nitrofurantoin (79.3%). Vancomycin showed (100%) susceptibility. Table)7, (15

Table 1~~7~~: Antibiotic sensitivity profile of *S.~~taphylococcus~~ aureus*

Antibiotics used	Total isolates	Resistant		Intermediate		Sensitive	
		No	%	No	%	No	%
Amoxycillin	29	22	75.9	0	0	7	24.13
Amikacin	29	4	13.8	0	0	25	86.2
Ciprofloxacin	29	10	34.4	3	10.34	18	62.0
Gentamycin	29	7	24.13	0	0	22	75.9
Nitrofurantoin	29	6	20.6	0	0	23	79.3
Ceftazidime	29	15~~4~~	51.737~~.9~~	2	6.9	12~~16~~	41.455~~.17~~
Ofloxacin	29	7	24.13	0	0	22	75.9
Cloxacillin	29	~~1~~86	~~62.06~~20.70	~~0~~3	~~0~~10.30	~~11~~26	~~37~~68.90
Vancomycin	29	0	0	0	0	29	100

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~~5.5.11~~ **Antibiotic susceptibility profile of *Enterococcus* spp.~~.~~**

-Vancomycin, amikacin, ciprofloxacin, gentamycin, nitrofurantoin were found effective with (100%) susceptibility. Ceftazidime showed 75% susceptibility and ~~amoxicillin~~*amoxycillin* showed (50%) susceptibility. Table)7, (16

Table 1~~6~~8: Antibiotic sensitivity profile of *Enterococcus* spp.

Antibiotics used	Total isolates	Resistant		Intermediate		Sensitive	
		No	%	No	%	No	%
Amoxycillin	4	3~~2~~	75~~50~~	0	0	1~~2~~	25~~50~~
Amikacin	4	0	0	0	0	4	100
Ciprofloxacin	4	0	0	0	0	3~~4~~	100
Gentamycin	4	0	0	0	0	4	100
Nitrofurantoin	4	0	0	0	0	4	100
Ceftazidime	4	4~~1~~	100~~25~~	0	0	0~~3~~	0~~75~~
Ofloxacin	4	1	25	0	0	3	75
Cloxacillin	4	0	0	0	0	4	100
Vancomycin	4	0	0	0	0	4	100

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4.3 Multidrug resistance among bacterial isolates~~MUltidrug 5.6 isolates resistance among different~~

Out of total 271 bacterial isolates, 190 (70.11%) isolates showed multidrug resistance. The high multi drug resistance in the study was showed by ~~*Acinitobacter*~~*Acinetobacter* spp.~~*p.*~~, –(–93.3%), followed by *P.*~~*seudomonas aeuroginosa*~~*aeruginosa* -(90.9%). Table)7, (17

Table 17: Distribution of MDR isolates among urine samples and their β-lactamase production profile

~~Table 19 Distribution of MDR isolates among different samples and their β lactamase production profile~~

Organisms	Total isolates	MDR strains	
		No	%
~~*Escherichia* *E.*~~ *coli*	183	123	67.2
Klebsiella pneumoniae	19	11	57.9
Acinetobacter ~~*spp.*~~,spp.	15	14	93.3
Pseudomonas ~~*aeuroginosa*~~*aeruginosa*	11	10	90.9
Proteus mirabilis	5	3	60
Proteus vulgaris	2	1	50
Citrobacter freundii	3	1	33.33
Staphylococcus aureus	29	24	82.7
Enterococcus ~~*spp.*~~,spp.	4	3	75
Total	271	190	70.11

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4.45.7 ESBL production profile among isolated strains

Out of 271 bacterial isolates, 111 (35.8%) isolates were screened positive for ESBL, and 77 (28.4%) were confirmed positive for ESBL .production ESBL production was found highest in *E. coli* (30.6%) followed *by K. pneumoniae* (26.3%). (31.6%)isolates gave positive results *coli* .*E* of the and (31.5%) of *K. pneumoniae* isolates.gave positive results ESBL production was not detected in*P.Proteus mirabilis, P. roteus vulgaris, C. itrobacter freundii* and *Enterococcus spp* strains. (1820 Table)

Table 18: ESBL screening using different agents

Screening Agents	Screening Criteria	ESBL Screening		No. of confirmed ESBL producers
Ceftazidme (30µg)	≤22mm	positive	111	77
		negative	39	34
Cefotaxime (30µg)	≤27mm	positive	130	75
		Negative	20	55

Table 19: ESBL confirmation using CD assay

S.N	Combination disks(CD) Assay	Criteria for confirmation	No. of suspected ESBL producers	Total confirmed cases	Negative cases after confirmation
1	CAZ (30µg) CAZ (30µg) plus CV (10µg)	Increase in zone size of ≥5 mm with ≥1 of the combination disks	111	77	34

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Table 20: Profile of ESBL producing bacterial strains

~~**Profile of ESBL producing bacterial strains 20 Table**~~

S.N	Organisms	Total isolates	No. of MDR strains~~,~~ (%)	No. of suspected ESBL producers	No. of cases confirmed (%)~~confirmed (%)~~
1	*Escherichia coli*	183	123(67.2)	6359	58(30.61.6)
2	*Klebsiella pneumoniae*	19	11(57.9)	812	56(26.331.5)
3	*Acinetobacter* spp.~~.~~	15	14(93.3)	142	35(206.7)
4	*Pseudomonas ~~aeuroginosa~~aeruginosa*	11	10(90.9)	510	23(18.827.3)
5	*Proteus mirabilis*	5	3(60)	1	0(0)
6	*Proteus vulgaris*	2	1(50)	0	0(0)
7	*Citrobacter freundii*	3	1(33.33)	1	0(0)
8	~~*Staphyllococcus*~~*Staphylococcus aureus*	29	24(82.7)	15	7(24.1)
9	*Enterococcus* spp.~~.~~	4	3(75)	41	40
	Total	271	190(70.1)	111	77(28.4)

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CHAPTER V~~hapter V: Discussion~~

DISCUSSION ~~& Conclusion~~

5.1 Discussion

Urinary tract infection (UTI) is one of the most important cause of morbidity in the general population and the 2nd most common cause of hospital visits (Das *et~~.~~ al.*, 2006). Nepal is one of the developing countries of South East Asia having comparatively very poor health status due to illiteracy, lack of hygienic and sanitary knowledge, malnutrition, economic status and lack of proper techniques in using medical procedures. So, people are usually victimized by many infectious diseases. UTIs are common type of bacterial infection accounting for reasonably high health care expenditures in people of all ages with more than 35 million medically treated infections each year (Mindbranch Inc, 2004) . Urinary tract infections account for about 8.3 million doctor visits each year. ~~(CDC, 2004).~~ There are an estimated 150 million urinary tract infections per annum worldwide (Stamm and Norrby, 2001). According to annual report published by Department of Health Services, Kathmandu (1996/1997) the morbidity of UTI is 0.42% of the total population and urine sample appears as the second commonest sample (98%) to be submitted to the laboratory after blood (48%)~~ for examination~~. The geographical distribution of UTI amongst the Nepalese population is 0.57% in the mountains, 0.45 % is estimated to be in planes. ~~(DoHS, 2000).~~

~~The present study was conducted at Alka Hospital from October to December 2012 with the objectives to isolate bacteria producing extended spectrum beta-lactamase and shows resistance toward third generation cephalosporins and to determine their~~

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~~antimicrobial resistance trend. During this period, sixteen hundred ninety nine mid-stream urine samples were collected and investigated by conventional semi-quantitative culture technique, microscopy and antibiotic susceptibility test.~~

Al~~l~~ together 1699 urine samples were collected during the study period and subjected for bacterial cultures in which significant growth was seen only in 27~~1 cases~~ (16.0%) cases~~)~~. A similar study carried out by Poudyal *et al.*, ~~(~~2011~~)~~ showed growth rate of (16.88%). This low growth result might~~ay~~ be due to inclusion of every patient ~~requesting for urine culture~~ for routine check up~~regardless of illness and symptom~~, the use~~prior use~~ of antibiotics before the culture request or possible presence of fastidious bacteria (Manandhar *et al.*, 1996)~~.~~. However ~~A similar study carried out by Chhetri *et al* (2001) showed low~~showed low growth positivity of 21.8%.~~the high growth rate) %58.8 Karki, 2010) %64.68 very high growth rate of and (Deep *et al* 2004)~~ has also been reported.~~The low number of growth positivity among urine samples was and ,(2005) Bomjan ,(2008) observed in similar studies carried out by Baral .(1999) Dhakal~~

yrs 30-20 The maximum number of growth was observed in age group ~~culture~~ growth high The .(%20.3) 40-30 followed by age group ,(%27.3) Similar .(%12.4) than in males (%17.9) was seen among female~~ positivity~~ .(2008) and Baral (2005) results were seen in the earlier studies by Bomjan This higher growth positivity seen in females was found to be statistically significant ($p<0.05$) and is attributed to their anatomical structure (short urethra and proximity to anal orifice) leading to easy access for coliform bacilli. Females are more susceptible to UTI than males~~.~~. ~~Women are more prone to UTIs than men because, in females, the urethra is much shorter and closer to the anus. As a woman's estrogen levels decrease with menopause, her risk of urinary tract infections increases due to the loss of protective vaginal flora.(Diclubanza, EJ; Schaeffer, AJ (2011 Jan). The term "honeymoon cystitis" has been applied to this phenomenon of frequent UTIs during early marriage in.~~ In female the maximum number of growth being observed in age group 20-30 years (32.6%), followed by 30-40 years (21.8%). These results are in consistent ~~Similar results~~with previous findings of ~~was found~~were observed ~~in the study by~~ Jha and Bapat (2005), Manandhar *et al.*,

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(1996), Rajbhandari and Shrestha (2002), Regmi *et al.*, (2003), Shrestha *et al.*, (2005) and Steenberg *et al.*, (1969)~~, Manandhar *et al.*, (1996), Rajbhandari and Shrestha (2002), Regmi *et al.*, (2003), Shrestha *et al.*, (2005) and Jha and Bapat (2005)~~. The females of this age group are sexually active and are of child bearing age. A number of studies suggest that sexual activity is an important factor in the pathogenesis of UTI in women. In young sexually active women, sexual activity is the cause of 75–90% of bladder infections, with the risk of infection related to the frequency of sex ~~(T Nicolle LE .2008).~~ . In male maximum number of growth was found in age group 60-70 years (20.5%) followed by the age group 20-30 years (16.7%).

Gram negative isolates were predominant constituting (%87.8) 238 of the total isolates. Gram positive isolates constituted 33(12.1%). In a study done by Karki *et al.*, (201004), 91.1% of the isolates were Gram negative bacilli and 8.8% of them were Gram positive cocci. The higher incidence of UTI by Gram negative bacilli was also accounted in a study conducted by Baral ,(2008) Marquez*et al.*, ,(2006) Puri ,(2008) Shrestha *et al.*, (2005), Thakur *et al.*, (2011). ~~Similar results have been obtained in the study conducted by~~

~~Marquez ,(1996) Manandhar ,(2005) Bomjan ,(2008) Baral*et al.*, ,(2008) Mathai*et al.*, Mathai ,(2008) Baral ,(2006) Puri and (2001) *et al.*, ,(2001) and Marquez ,(2005) Bomjan*et al.*,.(2008)~~ *E.~~sherichia~~ coli* 183(88.2%) was the most frequently isolated species ~~with 183(88.2%),~~ followed by *S.~~taphylococcus~~ aureus* with 35 (11.7%), *K~~lebsiella~~ pneumoniae* 19 (7 %), *Acinet~~t~~obacter* spp., 15 (5.5%). The high prevalence of *E. coli* seen in this study also resembled the study carried ~~out~~by Das *et al.*, (2006), Sharma *et al.*, (2006), ~~and Thakur *et al.* (2011), Poudyl *et al.* (2011),~~ Baral *et al.*, (2012) ~~,~~ ~~Rajan *et al.* (2012)~~ in Nepal. These results were in harmony with the results Gales ,(2008) Baral obtained in smilar studies by*et al.*, Mathai ,(2002) *et al.*, ~~Farrell ,~~(200~~57~~) Shrestha ~~,(2008) Baral ,~~and (2001)~~*et al.*, and Gales ,(2003) *et al.*, (2002)~~

E. coli can bind to the glycoconjugate receptor (Gal α 1-4 Gal) of the uroepithelial cells of human urinary tract so it can initiate infection itself. *E. coli* is isolated in 90.0% of infections and strains are characterized by unique

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virulence determinant, the p pilus (Gal-Gal receptor) ~~(Johnson, 1991)~~. *E. coli* is the most predominant organism to colonize the urethral meatus (Schaeffer and Chmiel, 1983) and perineum (Leigh, 1990) before ascending to the bladder. Strains of *E. coli* appear well adapted to invade urinary tract (serogroups 02, 04, 06, 07, 08, 09) which forms the majority of isolates of UTI ~~(Chakraborty, 2001)~~. This ability of *E. coli* may be the reason to be the most frequent organism to cause UTI in both sexes all over the world. In the study performed in ~~by Ana *et al.* (1998) in~~ USA *E. coli* was the most common urinary pathogen and found that most of the *E. coli* strains possessed urovirulence determinants which include mannose-resistant hemagglutination, F fimbriae, P fimbriae, hemolysins and aerobactin. It reflects that uropathogenic *E.coli* possess a number of potential specific virulence factors that are responsible to overcome the natural defense mechanisms and able to colonization in urinary tract. *S.~~taphylococcus~~ aureus* is the most predominant species colonizing the urethra and the perineum in both sexes. Furthermore, it is an opportunistic pathogen and can cause infection when the immune system is impaired. Presence of this organism in urine often indicates pyelonephritis acquired via hematogenous spread, so a pure culture of *S. aureus* is considered to be significant regardless of the number of CFUs (Forbes *et al.*, 2007~~2~~).

Antibiotic susceptibility testing has become a very essential step for the proper treatment of infectious diseases. *E. ~~.~~coli* was found more suseptible to n~~N~~itrofurantoin, (80.87%) , gentamycin (71.15%). Among the 2nd line drugs, tobramycin has (100%), amikacin has (77.6%), ofloxacin has (73.8%) and ceftazidime has (62.8%) suseptibility. The least sensitive drug was ~~amoxicillin~~amoxycillin (9.25%).

Antibiotic resistance is now accepted as major public health issue and has significant implication on health and patient care. Resistance to antimicrobial drugs is associated with high morbidity and mortality, high health-care cost and prolonged hospitalization. The problem antimicrobial resistance is more troublesome ~~to~~for developing countries. WHO and the European Commission

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(EC) have recognized the importance of studying the emergence and determinants of resistance and the need for strategies for its control.

~~Microscopic observation of the urine was done by wet mount preparation. The purpose of microscopy by wet mount preparation was to determine the number of white cells and red cells. Finding of ≥5WBCs/HPF is of great importance, while erythrocytes and epithelial cells are of poor significance for UTI diagnosis (Merila *et al.*, 1987).~~

~~Eisinger *et al*, (1997) has suggested that the finding of >10WBC/HPF in urine sediments predicts a positive urine culture and hence indicates urinary tract infection. But other many workers (Abyad *et al.*, 1991; Chakraborty, 1995; Wargotz *et al.*, 1987 and Ziloski and Smucker, 1989) concluded that pyuria is significant if >5 leucocytes are seen per HPF.~~

~~In this study, significant pyuria was observed in 399(24.5%) of requests. In this study, out of 1300(76.55%) cases of insignificant pyuria, only 20(1.5%) showed culture positive while remaining 1280 showed culture negative results. Based on this result, the sensitivity and specificity of pyuria as a screening test for UTI were calculated as 96.3% and 91.9%. Positive predictive value of WBC count of ≥5/HPF for growth positive culture was found out to be 77.8%. The study shows that as the number of pus cells increased, the significant pathogenic growth also increased. As mentioned in the earlier text, bacteriuria without significant pyuria often occur in cases of asymptomatic patients, patients with diabetes, enteric fever or bacterial endocarditis whereas significant pyuria with sterile bacterial cultures occur in patients with prior antibiotic use, pregnancy, renal tuberculosis (abacterial pyuria) corticosteroid administration, analgesic nephropathy, renal calculi or in the presence of bacteria that are not able to grow in the media used.~~

~~Similarly, out of 199 cases of significant haematuria (≥3 RBCs/ HPF), 71(35.65%) showed culture positive while remaining 128 showed culture negative; while out of 1500 cases of insignificant haematuria, 200(13.3%) cases were culture positive and 1300 were culture negative. Based on this, the sensitivity and specificity of haematuria were calculated as 23.8% and 89.3%~~

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~~respectively. The predictive value of positive test (PPV) was found to be 39.6% and the predictive value of negative test (NPV) was found to be 79.9%.~~

~~study this In the most effective antibiotic against Gram negative bacteria was was found to be nitrofurantoin (84.4%) followed by gentamycin (79.8%), cipr ofloxacin (60.9%), norfloxacin (58.8%). Among the 2nd line drugs, the most effective was amikacin (70.70%), ofloxacin (63.45%), ceftazidime (63%). Imipenem, polymixin B, tobramycin were used against resistant strains and were found to bo 100%, 100% and 94.5% effective respectively. The least susceptible drug was found to be amoxycillin (8.8%) followed by nalidixic acid (36.9%). Nitrofurantoin was found to be most effective drug against *E. coli* and *K.lebsiella pneumoniae* with (80.87%) and (84.2%) suseptibility. 71.15% of *E. coli* and 73.6% of *K.lebsialla pneumoniae* were susceptible to gentamycin. Nitrofurantoin was found least effective against *Pproteus* spp. with 0% susceptibility. *Ps.eudomonas aeruginosa* was found (63.6%) sensitive both ciprofloxacin and gentamycin. Tobramycin and polymixin B were found most effective with 100% susceptibility against *Ps.eudomonas aeruginosa.*~~

~~against Gram positive ,Vancomycin was found to be most effective drugs A .susceptibility %100 isolate s withmong the common 1st line antibiotics used against all Gram positive urine isolates, the most effective antibiotic was found to be nitrofurantoin. (87.9%), followed by gentamycin (75.6%). Among the 2nd line drugs, the most effective was amikacin (78.7%), cefotaxime (75.6%), cotrimoxazole (66.7%), ofloxacin (60%). Ceftazidime was found to have least sensitivity (54.5%). *S.taphyloccus aureus* was found (86.2%) sensitive to amikacin and (79.3%) sensitive to nitrofurantoin.~~

Out of ~~-~~total 271 bacterial isolates, 190 (70.11%) isolates showed multidrug resistance. The high multi~~-~~drug resistance in the study was showed by *Acin~~e~~itobacter* spp.~~s~~ (93.3%), followed by *P~~ss~~.~~seudomonas~~ ~~aeuroginosa~~aerug inosa* (90.9%), *S.~~taphylococcus~~ ~~—~~aureus* (82.7%), *E. coli* ~~,—~~(%67.2) *K. pneumonia* ~~.—~~(%57.9) eIn a study carried out ~~by Tuladhar *et al.* (2003)~~ at TUTH, MDR bacterial strains were detected in 35.2% cases in which the most predominant was *E. coli* (22.2%) followed by *Klebsiella* spp. (6.1%) and

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S.taphylococcus aureus (2.2%).These results resembled the outcomes of Koljalg ,(2005) Bomjan ,(2008) Baral previous studies by*et al* ,(2009) . Marquez*et al* Pokhrel ,(2008) Baral ,and (2008) .*et al* and Bomjan (2005) . has strains producing multidrug resistance is variation indicatesTh .(2005) .in Nepal been steadily increasing over the past few years The emergence of MDR is clearly related to the quantity of antibiotics and how they are being used (Levy, 1997). Outcome of prevalence of MDR depends on various factors, MDR criterion being the chief one followed by the types of antibiotics used in antibiogram and study population.

P.Pseudomonas aeruginosa shows a remarkable capacity to resist antibiotics, either intrinsically (because of constitutive expression of β-lactamases and efflux pumps, combined with low permeability of the outer-membrane) or following acquisition of resistance genes (e.g., genes for β-lactamases, or enzymes inactivating aminoglycosides or modifying their target), over-expression of efflux pumps, decreased expression of porins, or mutations in quinolone targets and these mechanisms are often present simultaneously, thereby conferring multiresistant phenotypes (Lambart, 2002; and Misaros *et al.*, 2007).

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. *gyrA*, which is a gyrase subunit gene, and *parC*, which encodes a topoisomerase subunit, confer resistance to fluoroquinolones (Ozeki *et al.*, 19197). In addition to this mechanism, there are more than seven efflux systems in *E.scherichia 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).

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Similarly, higher level of drug resistance seen among *K. pneumoniae* and *Acinetobacter* spp. is mediated by the production of different kind of β-lactamases primarily ESBL, AmpC and Metallo β-lactamases. The fact that the carriage of resistance trait for quinolones and aminoglycoside in the plasmid along with the gene for β-lactamases have had a great impact on the drug resistance character shown by these pathogenic bacteria (Lee *et al*., 2003; Picao *et al*., 2008 and Thomson *et al*., 2000~~; Lee *et al*., 2003; Picao *et al*., 2008 and Walsh *et al*., 2005~~).

Resistance to β-lactau~~u~~m in *Acinetobacter* spp. is associated with the production of β-lactamases like TEM-1, TEM-2, CARB-S, VEB, SHV, PER, CTX-M and ARI-1 located in the plasmid in concert with permeability reduction and altered penicillin binding protein~~, (Berezin and Towner 1996)~~. ARI-1 is a novel β-lactamases that confer resistance to imipenea~~a~~m which was first isolated from a blood culture at Royal Infirmary Edinburgh in 1985 ~~(Paton *et al*., 1993)~~. Resistance to aminoglycosides is due to enzyme that modifies the substrate by adenylation, phosporyltion and acetylation and genes for this are located in plasmid and transposo~~s~~ns~~ (Devaud *et al*., 1982)~~. Similarly, resistance to quinolones is due to changes in structure of DNA gyrase subunit by *gyr*A and *par*C gene mutation, multiple eff~~u~~lux pump and decreased uptake~~ (Seward and Towner 1998 and Hamouda *et al*., 2004)~~. Resistance to tetracycline and their derivatives can be mediated by tetracycline-specific efflux pumps or ribosomal protection and multidrug efflux systems~~ (Magnet *et al*., 2001 and Fluit *et al*., 2005)~~. Carbapenem resistance may be caused in part by impaired permeability, resulting from decreased expression of porins or by modification of penicillin binding protein and carbapenem hydrolyzing β-lactumase especially OXA-type play a major role (Fluit *et al*., 2001)~~ (Ozen *et al*., 2009)~~.

In *S.~~Staph~~ aureus*, resistance to penicillins occurs mainly by 2 mechanisms: the production of the β-lactamase enzyme and the presence of the *mec*A gene. β-lactamases enzyme destroys β- lactam antibiotics such as penicillins and

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cephalosporins and damage bacteria by inactivating penicillin binding proteins (PBPs), enzymes that are essential in the assembly of the bacterial cell wall. Methicillin resistance in *Staph. aureus* is primarily mediated by the *mec*A gene, which codes for the modified penicillin-binding protein (PBP2a). PBP2a has a very low affinity for β-lactam antibiotics. The *mec*A gene is found on a large mobile genetic element called the Sstaphylococcal chromosomal cassette mec (SCC*mec*) and at least 8 SCC*mec* types (SCC*mec* I through SCC*mec* VIII) have been identified (Otter *et al.*, 2010, Pinho *et al.*, 2001 and , Weese *et al.*, 2005 and Otter *et al.*, 2010).

Among various antibiotics like cefpodoxime, ceftazidime, ceftriaxone, cefotaxime and aztreonam recommended by CLSI to screen the presence of ESBL, we used only cephotaxime ceftazidime because of its highest sensitivity and specificity compared with other screening agents. Poudel in 2010 used different screening agents for ESBL detection and found that sensitivity of cefotaxime was 98.6% compared with that of ceftazidime with only 89.9%. Similar results were also obtained by Srisangkaew and Vorachit 2003 and Hope *et al.*, 2007. ESBLs remain to be increasingly complex and diverse in the future and will create increasing challenges for those setting guidelines for detection in the clinical microbiological laboratory thus always dictating the need for continuous monitoring of those criteria (Paterson and Bonomo, *et al.*, 2005) and detection of ESBL producers for the above mentioned reasons. The incidence of ESBL producing strains among clinical isolates has been steadily increasing over the past few years (since their description in the early 1980s) & has risen to prominence among *Eenterobacteriaceae* isolates all over the world.

Out of 271 bacterial isolates, 111 (35.8%) isolates were screened positive for ESBL, and 77 (28.4%) were confirmed positive for ESBL production. Positive ESBL screening result may be due to AmpC β-lactamases more often than to ESBL. Though ABL can interfere with some ESBL screening test

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~~(Munier *et al.*, 2010)~~ by hydrolyzing oxyiminocephalsoporins such as ceftazidime, cefotaxime and ceftriaxone~~,~~ CLSI confirmatory test using clavalunate is still good to detect ESBL as clavalunate is not affected by ABL (Jacoby 2009).

The higher ESBL production was found ~~high~~ in *E.coli* (30.6%) followed by *K. pneumoniae* ~~.~~ (26.3~~31.6~~%), *S. aureus* (24.1%), *Acinetobacter* spp. (20%), ~~*Ps.*~~*P. ~~aeuroginosa~~aeruginosa* (18.8%). ~~of the *E. coli* isolates gave positive results and (31.5%) of *K. pneumoniae* isolates gave positive results.~~ Paterson and Bonomo in 2005 addressed that predilection of ESBL for *Klebsiella* spp. may be due to increased frequency of SHV type ESBL in *Klebsiella* spp. than in other species. Resistance to β-lact~~a~~am in *Acinetobacter* spp. is associated with the production of β-lactamases like TEM-1, TEM-2, CARB-S, VEB, SHV, PER, CTX-M and ARI-1 located in the plasmid in concert with permeability reduction and altered penicillin binding protein~~, (Berezin and Towner 1996).~~ . ARI-1 is a novel β-lactamases that confer resistance to imipen~~e~~am which was first isolated from a blood culture at Royal Infirmary Edinburgh in 1985 (Mavroidi *et al.*, 2001) ~~(Paton *et al.*, 1993)~~.

Similar study conducted at Asian countries from Iran, India, Bangladesh and Pakistan reported the prevalence of ESBL in ~~Ecoli~~*E. coli* 44.5%, 58%, 39.5% and 43% respectively (Rahman *et al.*, 2004; Feizabadi *et al.*, 2006; Jain and Mondal~~, *et al.*~~, 2008). The other limited studies in Nepal showed prevalence of 53.7% in *E.coli* and 14.8% in *Klebsiella* spp.

Seventy seven (40.5%) isolates of total tested MDR were ESBL positive. The increasing pattern of the multidrug resistance seen among ESBL producers was found statistically significant ($p<0.05$). In a similar type of study conducted by Baral *et al.*, 2010, ESBL producers were significantly resistant

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to commonly used antibiotics than non-ESBL producers. Of all the ESBL producers all were resistant to five or more than five of the most commonly used antibiotics. In a similar type of study conducted by Tseringg ~~*et al.*~~ *et al.*, (2009), ESBL producers were significantly resistant to commonly used antibiotics than non-ESBL producers and multidrug resistance was significantly higher (69.14%) in ESBL positive isolates than non-ESBL isolates (21.66%).

The high resistance of uropathogenic bacteria to antimicrobial agents in developing countries (Lester *et al*, 1990) is often due to self-medication, the suboptimal quality of antimicrobial drugs, and poor community and patient hygiene (Walson *et al*, 2001). Second, inappropriate use of antimicrobial agents is widespread as many people can easily buy ~~antibiotics from some pharmacy stores and patent medicine stores, with or~~ without prescriptions. This widespread and inappropriate use of antibiotics is recognized as a significant contributing factor to the spread of bacterial resistance and the development of resistance to antimicrobial agents (Mincey and Parkulo, 2001). Third, there is evidence that for most bacteria, increased usage of a particular antimicrobial agent correlates with increased levels of bacterial resistance to that agent ~~(Granizo *et al*.,2000).~~.

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CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

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65.12 Conclusion

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~~Multidrug resistance among bacterial pathogens is a major health problem in Nepal that thwarts the management of several infectious diseseases and compromises therapy. Thus, controlling antibotic resistant bacteria and subsequent infections more efficiently necessitates the prudent and responsible use of antibiotics.~~

The ~~total of 271 bacteria were isolated.~~ culture positivity was low i.e. 16% (n=271). Gram negative isolates were predominant. ~~constituting 238 (87.8%) of the total isolates.~~ ~~*E.scherichia.scherichia* *coli*~~ was the most frequently isolated species. Among the common 1st line antibiotics used against all Gram negative bacteria the ~~bacteria, the most~~ effective antibiotic was found to be nitrofurantoin ~~(84.4%)~~ followed ~~by~~ gentamycin~~d by gentamycin (79.8%)~~. ~~Imipenem, polymixin B, tobramycin were found to be 100%, 100% and 94.5% effective respectively.~~ Among the common 1st line antibiotics used against all Gram positive urine isolates, the most effective antibiotic was found to be nitrofurantoin~~. (87.9%,),~~ followed by gentamycin. ~~(75.6%).~~ Vancomycin was 100% sensitive against all Gram positive urine isolates.

MDR isolates were ~~One hundred and ninenty~~190190 (70.11%) ~~of the isolates were MDR and~~and 77(28.45%) of isolates were confirmed positive for ESBL production. The high multi drug resistance was showed~~n~~ by ~~*Acinitobacter*~~*Acinetobacter* spp.~~ (93.3%)~~ followed by *P.~~Pseudomonas~~ aeruginosa* ~~(90.9%)~~, *S.~~taphylococcus~~ aureus* ~~(82.7%)~~, *E. coli* , ~~(%67.2)~~ *K. pneumonia*~~(%57.9)~~ ~~e~~). ESBL production was found highest in *E.coli* ~~(31.6%)~~ *followed by K. pneumoniae* ~~(31.5%)~~, *P.~~seudomonas~~ aeruginosa* ~~(27.3%)~~ and ~~,~~ *Acinen~~i~~tobacter* spp ~~(26.7%)~~.

A high prevalence of ESBL producers (40.5%) among the tested MDR isolates was observed with statistically significant level of multidrug resistance in ESBL. ~~producers.~~

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Chapter VI: Summary and recommendation

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6.1 Summary

1. Total 1699 mid stream urine sample were received. The significant growth was seen only in 271 (16.0%) cases .
2. The maximum number of growth was observed in age group 20-30 yrs (27.3%), followed by age group 30-40 (20.3%). The high culture .(%12.4) than in males (%17.9) positivity was seen among female This higher growth positivity seen in females was found to be statistically significant ($p<0.05$).
3. Altogether 9 different bacteria were isolated. Gram negative isolates were predominant constituting 238 (87.8%) of the total isolates.
4. *Escherichia coli* was the most frequently isolated species with 183 (88.2%), followed by *Staphylococcus aureus* with 35 (11.7%),

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Klebsiella pneumoniae 19 (7 %), *Acinetobacter spp*., 15 (5.5%), *Pseudomonas aeruginosa* 11(4.05%).

5. Microscopy of pyuria showed the sensitivity of 96.3% and the specificity of 91.9%. The positive and negative predictive values were found to be 77.8% and 98.8% respectively.
6. Microscopy of haematuria showed the sensitivity of 23.8% and the specificity of 89.3%. The positive and negative predictive values were found to be 39.6% and 79.9% respectively.
7. The most effective antibiotic against Gram negative bacteria was found to be nitrofurantoin (84.4%) followed by gentamycin (79.8%). Among the line second drugs, the most effective was amikacin (70.70%). Imipenem, polymixin B, tobramycin were found to be 100%, 100% and 94.5% effective respectively. The least susceptible drug was found to be amoxycillin (8.8%).
8. Among the common 1st line antibiotics used against all Gram positive urine isolates, the most effective antibiotic was found to be nitrofurantoin. (87.9%), followed by gentamycin (75.6%). Vancomycin was 100% sensitive.
9. MDR was observed in 190 (70.11%) isolates. The high multi drug resistance was showed by *Acinetobacterspp*., (93.3%), followed by *Pseudomonas aeuroginosa* (90.9%), *Staphylococcus aureus* (82.7%), *E. coli* , (%67.2) *K. pneumonia*. (%57.9) e
10. 77 (28.4%) were confirmed positive for ESBL production. ESBL production was found higher in *E.coli* (31.6%) *followed by K. pneumoniae* (31.5%), *Pseudomonas aeruginosa* (27.3%).
11. The increasing spectrum of drug resistance observed among ESBL producers was found statistically sinificant ($p<0.05$),

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6.2 Recommendations

1. Routine ESBL testing in clinical laboratory is recommended.
2. Further study on etiology especially the organisms that cannot grow on the media used and the provided cultural conditions should be carried out as an extension of this study.
3. The large number of multi drug resistant strains were isolated. To limit the spread of resistance, stringent guidelines for antibiotic policy, over prescription, incorrect prescription, non compliance by patients should be addressed scrupulously both within and outside the hospital.
4. Genotypic characterization of MDR strains and ESBL strains should be done in order to ascertain the location of drug resistance genes and to characterize the mechanism of drug resistance.

~~1.~~

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

~~Stringent guidelines for antibiotic policy, over prescription, incorrect prescription, non compliance by patients should be addressed scrupulously both within and outside the hospital.~~

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APPENDIX A

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CLINICAL AND MICROBIOLOGICAL PROFILE OF PATIENT

Clinical Profile:

Name: … … … … … … … … … … … … … … Lab No: … … …

Age / Sex: … … … … … … … …… … … … … Date: … … … …

Address: … … … … … … … … … … … … … …

Brief Clinical History:

Probable Diagnosis: ....

Types of sample: ....

Current empirical antibiotic therapy prescribed by treating physician: ....

Duration of hospital stay (For inpatients).........................

Microbiological Profile:

Day 1 (… … / … … / … … … …)

Specimen: … … … … … … … … …

Time of sample collection: … … … … … … … …

Mode of Collection: … … … … … … … … Receiving time at the laboratory: … … …

Direct Microscopic observation (if necessary):

1) … … … … … … … … …

Incubation: 1) Aerobic 2) Anaerobic 3) Microaerophilic

Incubation temperature: … … … … … … … … Incubation time: … … … … … … … …

Culture on: 1) … … … … 2) … … … … … 3) … … … … …

Day 2 (… … / … … / … … … …)

Reading of Culture Plates:

Media used	*Feature*	*Shape*	*Size*	*Color*	*Texture*	*Opacity*	*Consistency*

Gram staining results: … … … … … … … … … … … … … … … … …

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

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

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

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

Day 3 (… … / … … / … … … …)

BIOCHEMICAL TESTS

TSI: … … … … … … … … … SIM: … … … … … … … … …

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

Others: … … … … … … … … …

Organism Identified as: … … … … … … … … … … … … … … … … … …

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Antibiotic sensitivity test method: **Kirby-Bauer Method**

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

Day 4 (... .../ ... .../ ... ...)

Screening of the Multi-drug resistant isolates for ESBL Production

Screening agents used	***Zone of inhibition (mm)***	***Interpretation***
Ceftriaxone		
Cefotaxime		
Ceftazidime		
Cefpodoxime		
Aztreonam		

Day 5 (... .../ ... .../ ... ...)

ESBL Confirmatory Test

Combination Disks used	***Increase in size of zone of inhibition (mm)***	***Interpretation***
Cefepime-clavulanate		
Cefotaxime-clavulanate		
Ceftazidime-clavulanate		
Cefpodoxime-clavulanate		

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APPENDIX B

I. Composition and Preparation of Different Culture Media

The culture media used were from two companies

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

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

1. Blood agar (BA)

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

Ingredients	gm/liter
Beef heart infusion	500.0
Tryptose	10.0
Sodium Chloride	5.0
Agar	15.0
Final pH (at 25^{0}C) 7.3±0.2	

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

2. Chocolate agar (CA)

The sterilized blood agar was poured in Petri plates and was allowed to solidify and was heated at 75^{0}C in an oven for 30 minutes. By this time, the color changes to chocolate brown.

3. MacConkey Agar (MA)

(Without sodium taurocholate, without salt and crystal violet)

Ingredients	gm/liter
Peptone	20.0
Lactose	10.0
Sodium taurocholate	5.0
Sodium chloride	5.0

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Neutral Red	0.04
Agar	20.0
Final pH (at 25^{0}C)	7.4±0.2

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

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4. Mueller Hinton Agar (MHA)

Ingredients	gm/liter
Beef, Infusion form	300.0
Casein Acid Hyrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25^0C)	7.4±0.2

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

5. Nutrient Agar (NA)

Ingredients	gm/litre
Peptone	10.0
Sodium Chloride	5
Beef Extract	10.0
Yeast Extract	1.5
Agar	12.0
Final pH (at 25^0C)	7.4±0.2

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

6. Nutrient Broth (NB)

Ingredients	gm/litre
Peptone	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Final pH (at 25^0C)	7.4±0.2

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

7. Mueller Hinton Broth

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

Final pH (at 25^{0}C) 7.3±0.1

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

8. Mannitol Salt Agar

Ingredients	gm/litre
Protease peptone	10
Beef etract	1
NaCl	75
D-Mannitol	10
Phenol Red	0.025
Agar	15

Final pH (at 25^{0}C) 7.4±0.1

111 gram of the medium was dissolved in 1000ml distilled water and boiled completely and sterilized by autoclaving at 121^{0}C for 15 minutes.

9. Tryptic Soy broth+ 20% Glycerol

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

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

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II. Biochemical Test Media

1. MR-VP Medium

Ingredients	gm/litre
Buffered Peptone	7.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 25^{0}C)	6.9±0.2

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

2. Hugh and Leifson's Medium

Ingredients	gm/litre
Tryptone	2.0
Sodium Chloride	5.0
Dipotassium Phosphate	0.3
Bromothymol Blue	0.08
Agar	2.0
Final pH (at 25^{0}C)	6.8±0.2

9.4 grams of the medium was rehydrated in 1000 ml cold distilled water and then heated to boiling to dissolve completely. The medium was distributed in 100 ml amounts and sterilized in the autoclave for 15 minutes at 15 lbs pressure (121^{0}C). To 100 ml sterile medium aseptically added 10ml of sterile Dextrose and mixed thoroughly and dispensed in 5 ml quantities into sterile culture tubes.

3. Sulphide Indole Motility (SIM) medium

Ingredients	gm/litre
Beef Extract	3.0

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Peptone	30.0
Peptonized Iron	0.2
Sodium Thiosulphate	0.025
Agar	3.0
Final pH (at 25^{0}C) 7.3±0.2	

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

4. Simmon Citrate Agar

Ingredients	gm/litre
Magnesium Sulfate	0.2
Mono-ammonium Phosphate	1.0
Dipotassium Phosphate	1.0
Sodium Citrate	2.0
Sodium Chloride	5.0
Agar	15.0
Bromothymol Blue	0.08
Final pH (at 25^{0}C) 6.8±0.2	

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

5. Triple Sugar Iron (TSI) Agar

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

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Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0
Final pH (at 25^{0}C)	7.4±0.2

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

6. Christensen Urea Agar

Ingredients	gm/litre
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Dipotassium Phosphate	1.2
Mono-potassium Phosphate	0.8
Phenol Red	0.012
Agar	15.0
Final pH (at 25^{0}C)	7.4±0.2

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

III. Staining and Test Reagents

1. For Gram's Stain

(a) Crystal Violet solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml
Distilled Water (D/W) to make	1 litre

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

(b) Lugol's Iodine

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

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

(c) Acetone-Alcohol Decoloriser

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

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

(d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

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

3. Normal saline

Sodium Cholride	0.85 g
Distilled Water	100 ml

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

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4. Test Reagents

a. For Catalase test

Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

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

b. For Oxidase Test

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

Tetramethyl *p*-phenylene diamine dihydrochloride (TPD)	1 gm
Distilled Water	100 ml

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

c. For Indole Test

Kovac's Indole Reagent

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

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

d. For Methyl Red Test

Methyl Red Solution

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

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

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

Solution A

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

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

Solution B

Potassium hydroxide	40.0 g
Distilled Water	1000 ml

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

f. 100mM EDTA solution for MBL detection

1.86 gm of EDTA (Disodium salt, MW 372.24) was dissolved properly in 50 ml of sterilized distilled water and the reagent was kept aseptically.

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APPENDIX C

List of Equipment, materials and supplies

A. **EQUIPMENT**

Autoclave (Stermite, Japan)

Incubator (Sakura, Japan)

Hot air oven (Memmert, Germany; and GallenKamp)

Microscope (Olympus)

Refrigerator 4-8^{0}C (sanyo, Japan); -20^{0}c (Videocon) and -75^{0}C (Sanyo, Japan)

Weighing machine (Ohaus, USA)

Water Bath (Boekel)

Gas burners

Glasswares

Inoculating wire and loops

B. **MICROBIOLOGICAL MEDIA**

Blood Agar	**Hugh and Leifson Media**
Choocolate Agar	**Sulphur Indole Motility Media**
Mac conkey agar	**MRVP Broth**
Mueller Hinton Agar	**Triple Sugar Iron Agar**
Mannitol Salt Agar	**Urea AgarBase**
Mueller Hinton broth	**Simmon's Citrate agar**

C. CHEMICALS AND REAGENTS

Catalase reagent (3% H_2O_2)

Oxidase reagent (1% Tetramethyl *p*-phenylene diamine dihydrochloride)

Kovac's reagent

Barritt's reagent (40% KOH, 5% α-naphthol in a ratio of 1:3)

Barium Chloride

Conc. H_2SO_4

Ethylene Diamine Tetra Acetate (EDTA)

Glycerol

Gram's reagent

D. Antibiotic Disks

The antibiotic disks used for the susceptibility tests that were from Mast Diagnostics, Mast House, Derby Road are as follows

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Amikacin(30µg(, Aztreonam(30µg(, Bacitracin(10U ,(Bacitracin)0.04IU) Cefotaxime(30µg(, Ceftazidime(30µg(, Ceftriaxone(30µg(, Cotrimoxazole (25µg(, Ciprofloxacin(5µg(, Chloramphenicol(30µg(, Gentamicin(10µg(, Imipenem(10µg(, Nitrofurantoin(300µg(, Nalidixic acid(30µg(, Ofloxacin (5µg(, lin GPenicil(10Units(, Vancomycin(30µg(

E. DISKS FOR ESBL DETECTION

ID ™MASTDISCS

Extended Spectrum beta-lactamase(ESβL) Detection Discs(D52C)

F. DISKS FOR ABL DETECTION

HiMedia Discs

AmpC and Extended Spectrum beta-lactamase(ESβL) Detection Discs (D68C)

APPENDIX D

A. Gram-staining Procedure

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

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

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APPENDIX E

1. BIOCHEMICAL TESTS FOR IDENTIFICATION OF BACTERIA

Catalase test

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

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

Oxidase test

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

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

Oxidation-Fermentation test

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

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

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

Indole Production test

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

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

Methyl Red test

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

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

Voges Proskauer (VP) test

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

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

Citrate Utilization test

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

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

Motility test

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

Triple Sugar Iron (TSI) Agar

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

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

Urea Hydrolysis test

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

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

Coagulase test

This test is used specifically to differentiate species within the genus *Staphylococcus*: *S aureus* (usually positive) from *S epidermidis* (negative). A positive coagulase test is usually

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the final diagnostic criterion for the identification of *Staphylococcus aureus.* Free coagulase and bound coagulase are the two types of coagulase possessed by this organism; most strains possess both free and bound coagulase.

- **Slide Coagulase Test**

Bound coagulase (Clumping Factor) is detected by slide test. The bound coagulase is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in alteration of fibrinogen so that it precipitates on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma.

For slide coagulase test, a drop of physiological saline was placed on three places of a slide, and then a colony of the test organism was emulsified in two of the drops to make thick suspensions. Later a drop of plasma was added to one of the suspensions and mixed gently. Then a clumping was observed within 10 seconds for the positive coagulase test. No plasma was added in second suspension. This was used for the differentiation of any granular appearance of the organism from true coagulase clumping. The third drop of saline was used for a known strain of coagulase positive staphylococci.

- **Tube Coagulase Test**

This test is carried out to detect production of free coagulase. Plasma contains coagulase reacting factor (CRF) which activates free coagulase. The activated coagulase acts upon prothrombin thus converting it to thrombin. Thrombin converts fibrinogen into fibrin which is detected as a firm gel (clot) in the tube test. Tube test is performed when negative or doubtful results are obtained in slide coagulase test.

In the tube coagulase test, plasma was diluted 1 in 10 in physiological saline. Four small tubes were taken, one for test organism, one for positive control, one for negative control, and one to observe self clotting of plasma. Then 0.5 ml of the diluted plasma was pipetted into each tube and 0.5 ml of test organism, 0.5 ml of positive control (*Staphylococcus aureus* culture), and 0.5 ml negative control (*Staphylococcus epidermidis* culture) was added to three tubes, to the fourth tube, 0.5 ml sterile broth was added. After mixing gently, all tubes were incubated at 37^0C on a waterbath for 6 hours and observed for gel formation in every 30 minutes.

DNase (Deoxyribonuclease) test

This test is used to identify *Staphylococcus aureus* which produces deoxyribonuclease (DNase) enzyme. The deoxyribonuclease enzyme hydrolyses the DNA. The test organism was cultured on a medium containing DNA. After overnight incubation, the colonies were tested

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for DNase production by flooding the plate with a weak hydrochloric acid solution. The acid precipitates unhydrolysed DNA. DNase producing colonies are therefore seen as clear areas surrounding colonies due to DNA hydrolysis.

Amino acid Decarboxylase test

This test is based on the ability of some bacteria to decarboxylate an amino acid to the corresponding amine with the liberation of carbon dioxide. The production of these decarboxylases is induced by a low pH and as a result of their action; the pH rises to the neutrality or above. Lysine, ornithine and arginine are the three principal amino acids. Specific enzymes convert these into cadaverine, putrescine and citrulline respectively.

A pure colony of test organism was inoculated lightly through the paraffin layer of each broth medium containing lysine, ornithine and arginine with a straight wire. The tubes were incubated at 37^0C and read daily for 4 days. The occurrence of decarboxylation in the medium was shown by violet color of the medium.

2. Optochin Sensitivity test

This test is used to determine the effect of optochin (ethyl hydrocupriene hydrochloride) on an organism. Optochin lyses pneumococci (positive test); whereas the viridans group of streptococci are resistant (negative test). It can be performed conveniently with a paper disc containing optochin 5 µg. The pneumococci susceptible to optochin show a zone of inhibition of > 14 mm, while other alpha hemolytic streptococci grow to the edge of the disk.

A paper disk containing 5 µg of optochin was placed in the primary inoculum of a chocolate agar plate streaked with the pure culture of suspected pneumococci and the plate was incubated at 37^0C in candle jar for overnight. After incubation the plate was observed for zone of inhibition surrounding the disk.

3. Bacitracin Sensitivity test

This test assists in the screening of *Haemophilus influenzae* from the primary agar plate. A 10 Unit Bacitracin disc suppresses the viridans streptococci, *Neisseria*, diptheroid bacilli and staphylococci. But *Haemophilus influenzae* is resistant to the 10 Unit Bacitracin disc.

A paper disk containing 10 U of bacitracin was placed in the primary inoculum of a chocolate agar plate streaked with the material from the specimen and the plate was incubated at 37^0C in candle jar for overnight. After incubation, the plate was observed for the colonies of *Haemophilus influenzae* growing near the bacitracin disk.

4. Bile Solubility test

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This test is also used to differentiate between pneumococci and viridans streptococci. Bile lyses the cells of pneumococci by attacking the peptidoglycan, whereas the viridans groups of streptococci are unaffected.

Solution of 10% sodium deoxycholate prepared in distilled water was autoclaved and stored at room temperature. Colonies of suspected pneumococci were inoculated in two test tubes containing 5 ml nutrient broth from the blood or chocolate agar and was incubated at 37^0C for overnight. Then 0.5 ml of bile salt solution was added in one tube while another was taken as control. Incubation was continued for 15 to 30 minutes. In the positive test, turbidity of broth was cleared as compared to the control tube.

5. Disc test of X and V requirements

This test is carried out to differentiate *Haemophilus influenzae* from *Haemophilus parainfluenzae.* The former requires both the accessory growth factors, i.e. the X-factor which is the haemin or various iron containing compounds such as protoporphyrin IX, and the V-factor which is a heat labile vitamin like substance: Nicotinamide adenine dinucleotide (NAD) or NAD phosphate (NADP); where as *Haemophilus parainfluenzae* requires only the V-factor for growth.

To establish X and V factor requirements, disks impregnated with X factor alone, V factor alone and X plus V factor were placed on Mueller-Hinton agar an unsupplemented media, which had been inoculated with a light suspension of the organism. After overnight incubation at 37^0C in candle jar, the plate was examined for growth around each disk. If growth was seen only around the XV disc, this was indicative of the species being *Haemophilus influenzae*.

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APPENDIX F

1. DISC DIFFUSION METHOD FOR THE ANTIMICROBIAL SUSCEPTIBILITY TESTING

A. Preparation of 0.5 Mc Farland Standard

Add 0.5 ml of 0.048M $BaCl_2$ (1.17% w/vBaCl2.2H2O) to 99.5 ml of 0.18M H_2SO_4 (1% v/v) with constant stirring.

B. Preparation of inoculum

By touching 2-3 morphologically similar colonies with sterile loop, inoculate into MHB or NB and incubate at 37^0C until turbidity matches with that of 0.5 Mc Farland Standard. Direct colony suspension method can also be used.

C. Inoculation of Agar plates

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

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

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

d. Within 30 minutes of applying the discs, the plates are incubated at 35^0C for 16-18 hrs.

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

D. Quality Control

a. QC strains

Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923

b. Monitoring Accuracy

a. Running AST for QC strains side by side with pathogenic bacteria
b. Monitoring the expiry date of antibiotic discs and MHA
c. Comparing zone size with CLSI QC tables

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APPENDIX G

1. Inhibitor Potentiated Disk Diffusion(IPDD) Test/ Combined Disk Assay for ESBL ID Extended Spectrum beta ™confirmation using MASTDISCS-lactamase(ESβL) Detection Discs(D67C)

according to ESBL production was confirmed among the suspected bacterial strain the gudelines of CLSI(Clinical and Laboratory Standard Institute) for phenotypic confirmatory testing. According to these guidelines, when confirming ESBL production among the suspects using Combined Disk(CD) assay, of ≥ an increase in zone size5 mm from either of the combination disk i.e. clavulanate containing disk indicates the presence of ESBL in the test organism.

i. **The suspected organism was inoculated into Mueller hinton broth and incubated at37 C until the turbidity matched^{0}0.5 and Mc Farl atandard.Using a sterile cotton swab the test orgaism was carpet cultured on a MHA plate.**

ii. **With the help of sterile forcep, the ESBL detection discs were placed onto the inoculated medium ensuring that they are evenly spaced.**

iii. **35 The plate was incubated at- C for37^{0}18-24 hours and the results interpreted.**

Interpretation of results

Compare the zone of inhibition for the ceftazidime, cefotaxime and cefpodoxime discs to that of the ceftazidime, cefotaxime and cefpodoxime plus clavulanic acid cscombination dis. An increase in zone diameter of ≥5 mm in the presence of clavulanic acid from any or all of the discs indicates the presence of ESBL in the test organism.

Quality control

Check for sign of deterioration. Quality control mst be performed with at least one organism to demonstrate positive reaction and at least one to demonstrate negative reaction.

Positive Control: *Escherichia coli* NCTC 13351, *Klebsiella pneumoniae* ATCC 700603

Negative Control: *Escherichia coli* ATCC 25922

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APPENDIX H

1. Association of ESBL production and multidrug resistance

		Multidrug resistance		Total
		Yes	No	
ESBL production	Positive	36	4	40
	Negative	94	37	131
Total		130	41	171

Test statistics is χ^2

H_0: There is no significant association of ESBL production and level of multidrug resistance among MDR ESBL producing isolates

H_1: There is significant association of ESBL production and level of multidrug resistance among MDR ESBL producing isolates

From $\chi^2 = \Sigma \frac{(O-E)^2}{E}$ Calculated $\chi^2 = 5.594$

Since $\chi^2_{cal}(5.594) > \chi^2_{tab}(3.84)$ and $\alpha = 0.05$ and d.f. = 1, H_0 is rejected.

Result: There is significant association of ESBL production and level of multidrug resistance among MDR ESBL producing isolates or increasing level of multidrug resistance seen among ESBL positive MDR isolates were found significant.

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