THERAPEUTIC ALTERNATIVES FOR MULTIPLE DRUG RESISTANT BACTERIAL ISOLATES

A Dissertation
Submitted to the Central Department of Microbiology
Tribhuvan University

In Partial Fulfillment of the Requirements for the Award of the Degree
of
Master of Science in Microbiology
(Medical Microbiology)

By

Bhuwaneswor Prasad Kandel

Central Department of Microbiology
Tribhuvan University
Kirtipur, Kathmandu, Nepal
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2010

Exam Roll No. : 54/2009
Date of Submission : 6th Oct, 2010

RECOMMENDATION

This is to certify that **Mr. Bhuwaneswor Prasad Kandel** has completed this dissertation work entitled **"Therapeutic Alternatives for Multiple Drug Resistant Bacterial Isolates"** as a partial fulfillment of M.Sc. Degree in Medical Microbiology under our supervision. To our knowledge, this is an original research work of him and has not been submitted for any other degree.

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ABSTRACT

Worldwide emergence of multiple drug resistance among bacterial pathogens is a growing health problem and demands for its proper monitoring and control in different clinical settings. This study aimed to determine the prevalence of multidrug resistance and ESBL production among various clinical bacterial isolates and to search for effective therapeutic alternatives. A total of 2141 various clinical specimens including urine, sputum, throat swabs, eye and ear specimens, pus and body fluids received at microbiology laboratory of National Public Health Laboratory, Nepal, from March, 2009 to February, 2010, were processed and isolates were identified following standard microbiological procedures. Antibiotic susceptibility testing and ESBL detection was done by Disk Diffusion methods according to CLSI guidelines. Of the 2141 various clinical specimens processed, 20.2% showed significant growth, 64.9% isolates were MDR and most of them (78.1%) were urinary isolates. Significant MDR phenotype was seen among major gram negative pathogens such as *P. aeruginosa* (92.3%), *Klebsiella* spp. (82.4%), *E. coli* (75.5%), *Acinetobacter* spp. (71.4%) and *C. freundii* (66.7%), and gram positive *E. faecalis* (80.0%) and *S. aureus* (52.5%). Of the 142 MDR isolates tested for ESBL production, 59.9% isolates produced ESBLs and *E. coli* (77.6%) remained predominant ESBL producer followed by *K. oxytoca* (75.0%). Among conventional antibiotics, nitrofurantoin and chloramphenicol were effective against most MDR isolates. Clindamycin and vancomycin remained the drug of choice for MRSA isolates. Temocillin, meropenem, imipenem, and combination of cefepime, ceftazidime and cefotaxime with clavulanate were effective against most ESBL producers. Resistance rates for fluoroquinolones (75.4-97.6%) and aminoglycosides (44.4-85.2%) were relatively higher. Fosfomycin was found to be the best drug against all MDR isolates with lowest resistance (17.6%) followed by tigecycline (23.2%). ESBL production and increased spectrum of drug resistance was statistically significant ($p<0.05$). To the best of our knowledge, the study reports resistance patterns of MDR clinical bacterial isolates to fosfomycin, temocillin and tigecycline for the first time in Nepal. The higher predominance of ESBL production and MDR phenotypes among common clinical isolates mandates proper control measures and more potent drugs.

Key words: ESBL, MDR, fosfomycin, temocillin, tigecycline, Nepal

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LIST OF ABBREVIATIONS

AAC : Aminoglycoside acetyltransferase
ABC : ATP binding cassette
ABL : AmpC -lactamase
ANT : Aminoglycoside nucleotidyltransferase
APH : Aminoglycoside phosphatase
ASM : American Society for Microbiology
ATCC : American type culture collection
BSAC : British Society for Antimicrobial Chemotherapy
CAT : Chloramphenicol acetyl transferase
CDC : Center for Disease Control and Prevention
CFU : Colony forming units
CLSI : Clinical and Laboratory Standards Institute
DHFR : Dihydrofolate reductase
DMT : Drug/metabolite transporters
EPI : Efflux pump inhibitor
ESBL : Extended spectrum -lactamase
FDA : Food and Drug Administration
FNAC : Fine needle aspiration cytology
GISA : Glycopeptide-intermediate *S. aureus*
HCP : Healthcare professional
HPA : Health Protection Agency
ICU : Intensive care unit
LCR : Ligase chain reaction
LTCF : Long term care facility
MATE : The multidrug and toxic compound extrusion
MBL : Metallo- -lactamase

MDR	:	Multidrug resistant
MFS	:	The major facilitator superfamily
MHA	:	Mueller Hintor agar PABL plasmid abls
MHB	:	Mueller Hinton Broth
MIC	:	Minimum inhibitory concentration
MOP	:	Multidrug/oligosaccharidyl-lipid/polysaccharide flippase
MRSA	:	Methicillin resistant *S. aureus*
MSU	:	Mid stream urine
NBUs	:	Non-replicating *Bacteroides* units
NCCLS	:	National Committee for Clinical Laboratory Standards
NNIS	:	National Nosocomial Infections Surveillance
NPHL	:	National Public Health Laboratory
PABA	:	Para-aminobenzoic acid
PBP	:	Penicillin binding protein
PCR	:	Polymerase chain reaction
PDR	:	Pandrug resistant
RND	:	The resistance nodulation division
SAT	:	Streptogramin Acetyl Transferase
SMART	:	Study for Monitoring Antimicrobial Resistance Trends
SMR	:	The small multidrug resistance
SPSS	:	Statistical package for social science
TUTH	:	Tribhuvan University Teaching Hospital
UTI	:	Urinary tract infections
VRE	:	Vancomycin resistant enterococci
VRSA	:	Vancomycin resistant *S. aureus*
WHO	:	World Health Organization
XDR	:	Extensively drug resistant

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CHAPTER-I

1. INTRODUCTION

The optimism of antimicrobial discovery has been tempered by the emergence of bacterial strains resistant to various antimicrobial agents and the development of new antibiotics at an unprecedented pace in recent years has been paralleled by the appearance of resistance to these antibiotics. Thus, antibiotic resistance has become a growing health problem of global magnitude (Bonomo and Rossolini, 2008; Levy, 1982).

Antimicrobial use and misuse in past decades has led to emergence and evolution of antibiotic resistance among bacterial pathogens in both community and hospital settings. The emergence of multidrug resistant (MDR) strains among common clinical microbes frequently causes treatment failure, prolonged period of hospital stay along with increased health service cost, morbidity and mortality. Therefore, we are in the midst of an emerging crisis of antibiotic resistance throughout the world (Spellberg *et al*., 2008; Stephen *et al*., 2004; Wilcox, 2004).

MDR bacteria arising from animal and environmental sources may pose greater clinical risk to human health. Extensive and indiscriminant use of various antimicrobials in clinical, veterinary and agricultural practices has been creating the major selective force for emergence and global dissemination of resistant strains and resistance genes (Goossens *et al*., 2005; Shea, 2003; Aryal, 2001). Misuse and overuse of antibiotics by clinicians, their unnecessary dispensing by retailers, their use by patients in suboptimal dose and duration, and use of leftover antibiotics by patients, all have contributed to emergence and spread of MDR bacteria (Kardas *et al*., 2007; Wachter *et al*., 1999). Residents of long-term care facilities (LTCF) are among the main reservoirs of most of antibiotic-resistant bacteria. Severe and chronic illness, increased antimicrobial exposure, altered physiological states, use of indwelling devices, surgery, etc. are reported risk factors for infection with MDR organisms (Engel, 2009).

Among the MDR organisms, MRSA (Methicillin resistant *Staphylococcus aureus*), VRSA (Vancomycin resistant *S. aureus*), VRE (vancomycin resistant Enterococci), ESBL (extended spectrum -lactamase)-producing gram-negative bacteria, *Pseudomonas*, *Acinetobacter, Klebsiella, Enterobacter* species, and *Escherichia coli* warrant special attention because of their limited therapeutic options (Reddy *et al*., 2009; Siegel *et al.,* 2007; Hasan *et al*., 2007). Extensively drug resistant (XDR) and pandrug resistant (PDR) *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* are also emerging increasingly and frequently in hospitalized patients for which no adequate therapeutic options exist (Souli *et.al*., 2008; Kuo *et al*., 2004).

Extended-spectrum -lactamases (ESBLs) are plasmid-mediated bacterial enzymes that confer resistance to the penicillins, first-, second-, and third-generation cephalosporins, aztreonam, and related oxyimino-blactams (but not the cephamycins or carbapenems) by hydrolyzing these antibiotics, and are inhibited by -lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Livermore and Woodford, 2006). Most of them also have the ability to hydrolyze fourth-generation cephalosporins e.g. cefepime and cefpirome. Most common types include TEM, SHV, CTX-M and Toho- -lactamase, OXA class D types. PER, VEB, GES and IBC type - lactamases are uncommon and found only in specific geographic regions (Al-Jasser, 2006).

Predominant ESBL producers include *E. coli* and *Klebsiella* species, and they are also found in *Acinetobacter* spp., *Alcaligenes fecalis*, *Burkholderia cepacia*, *P. aeruginosa*, *Salmonella enterica* and *Serratia marcescens* (Bush, 2008; Al-Jasser, 2006; Paterson and Bonomo, 2005; Bradford, 2001). All ESBL producing organisms should be considered resistant to all penicillins (except temocillin), cephalosporins (except cefoxitin and cefotetan) and aztreonam (Livermore and Woodford, 2004). In spite of their significant activity against ESBLs *in vitro*, -lactam- -lactamase inhibitor combinations may not be optimal therapeutic alternatives for serious infections by ESBL producers and their clinical effectiveness is controversial. Moreover, ESBL-producing bacteria are also resistant to fluoroquinolones, aminoglycosides, tetracyclines, chloramphenicol and co-

trimoxazole extremely limiting the antibiotic options in treating infections caused by them (Sharma *et al*., 2010; Giske *et al*., 2008; Paterson and Bonomo, 2005).

The search for more potent agents appears to be otiose presently. Appropriate use of both newer (e.g., tigecycline,), and many older antibiotics (e.g., fosfomycin, cotrimoxazole, aminoglycosides, chloramphenicol, etc.) can be the valuable alternatives for the treatment of difficult-to-treat infections (Flagas *et al*., 2009). Imipenem or meropenem alone or in combination with aminoglycosides can be the good choice for severe infections by ESBL-producing gram-negative bacteria (Rahal, 2008; Wright and Eiland, 2008). Tigecycline can be an alternative in treating many complicated infections by multiresistant pathogens, like *Acinetobacter* spp., ESBL producers, MRSA and enterococci (Garau, 2008; Nathwani, 2005; Livermore, 2005). Fosfomycin can be a safe and effective option in treating infections by MDR bacteria including ESBL-producers (Flagas *et al*., 2009; Cueto *et al*., 2006).

Multiple drug resistant organisms render therapy more precarious and costly, and sometimes unsuccessful. Individuals may succumb to MDR infections because all available drugs have failed, especially in the developing world. The progressive increase of ESBL-producing MDR pathogens has called for a re-evaluation of current antibiotic therapy. Incorporation of feasible and sensitive ESBL detection methods has become mandatory for rationalizing the use of third and fourth generation cephalosporins. Monitoring MDR organisms in different healthcare settings is important to detect newly emerging antimicrobial resistance profiles, to identify vulnerable patient populations, and to assess the need for and effectiveness of interventions including antibiotic stewardship programs. Hence, this study was carried out to determine prevalence and resistance patterns of various clinical isolates, particularly focusing on multidrug resistance and ESBL production with testing for broader antibiotic panels and searching for newer therapeutic alternatives.

CHAPTER-II

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

To determine the prevalence of multiple drug resistance among bacterial isolates from various clinical specimens focusing on ESBL production and their susceptibilities to broader antibiotic panels

2.2 SPECIFIC OBJECTIVES

- To isolate and identify bacterial pathogens from various clinical specimens
- To determine the prevalence of multiple drug resistance among bacterial isolates
- To screen and confirm the MDR isolates for the possible presence of ESBLs
- To determine the susceptibility of MDR isolates to broader antibiotic panels

CHAPTER-III

3. LITERATURE REVIEW

3.1. Antimicrobial Resistance

Antibiotic resistance is a usual and expected phenomenon in the environment when potent and specific antimicrobial agents are used against diverse group of microorganisms. Clinically antibiotic resistance is a relative concept and is indirectly related to the microbiologic techniques often used to detect it, inoculum effect, intrinsic susceptibility and tolerance of microbes to a particular antibiotic. The antimicrobial resistance is recognized and categorized according to the determinations made by different standard-setting bodies such as BSAC, CLSI, etc. (Murray *et al*., 2003).

Antimicrobial resistance is a common problem that complicates the treatment of both community-acquired and nosocomial infections. It is the temporary or permanent ability of an organism and its progeny to remain viable or multiply under environmental conditions by opposing the inhibitory (bacteriostatic) or killing (bactericidal) effects of antibiotics and such resistance usually arises from random mutations in existing genes or from intact genes that already served a similar purpose. Antimicrobial resistance increases morbidity, mortality, length of hospital stay, and health care costs. These adverse outcomes may be the result of ineffectiveness of antibiotics by antimicrobial resistance or a delay in therapy (Cosgrove, 2006; Wilcox, 2004; Stephen *et al*., 2004). Antimicrobial resistance may be cross-resistance (resistance to a whole class of antibiotics), co-resistance (presence of many resistance mechanisms in the same organism) and co-selection (selection of multiple antibiotic resistance genes).

3.1.1. Emergence of antimicrobial resistance

Emergence of antibiotic resistance among bacterial pathogens has been threatening the human health all the times (Spellberg *et al*., 2008). Emergence of antibiotic resistance in

bacteria is linked to the clinical or other use of antimicrobial agent against which the resistance arises. Resistance is the result of selective pressure on the microbe due to its prolonged exposure and interaction with an antibiotic in a sub-inhibitory concentration whether in host, or in the environment. Resistance genes may originate from the antibiotic-producing organism, e.g. aminoglycoside modifying enzymes. Resistant bacteria may appear rapidly after antibiotic use, but disappear slowly, even in the absence of the selecting antibiotic. Moreover, resistance to antibiotics that are not in clinical use is often not tested and it remains unnoticed (Murray *et al*., 2003). Extensive use of antibiotics in agricultural and veterinary fields (Shea, 2003; Aryal, 2001), excessive outpatient antibiotic use (Goosens *et al*., 2005), unnecessary prescriptions of antibiotics by physicians due to promotional gifts (Guldal and Semin, 2000), and suboptimal use of antibiotics by patients (Kardas *et al*., 2007) all have contributed significantly to the emergence of antibiotic resistance. The survival of the resistant strain after its emergence is determined by the level of resistance expressed, antibiotic tolerance, linkage to other resistance genes, site of primary colonization, and others. Severe illness, immunocompromise, use of newer devices and procedures, increased introduction of resistant organisms from community, increased antibiotic prophylaxis, ineffective infection control and isolation practice, increased empirical polymicrobial antibiotic therapy, and higher antibiotic use contribute to increased emergence of antibiotic resistance (ASM, 2009; Denyer *et al*., 2007; Murray *et al*., 2003).

3.1.2. Spread of antimicrobial resistance

Dissemination of antibiotic resistance genes by horizontal transfer has led to the rapid emergence of antibiotic resistance among clinical isolates, and occurs between different bacterial species and genera easily and frequently in nature, even between bacteria that normally reside in different sites. Selected resistance genes and their hosts spread and propagate under continued antimicrobial selection to amplify and extend the problem to other hosts and other geographic locations. Integrons are important horizontal gene transfer systems of resistance genes in clinical isolates and integron-positive isolates were

more likely to be multiresistant than integron-negative isolates (Lin and Biyela, 2005; Carattoli, 2001; Fluit and Schmitz, 1999; Martinez *et al*., 1998). Multiresistant bacteria from environmental water and infected animals may be the potential reservoirs of resistance genes that can spread to other members of the Enterobacteriaceae (Sidjabat *et al*., 2007; Lin and Biyela, 2005). Self-transmissible and mobilizable plasmids, conjugative transposons, non-replicating *Bacteroides* units (NBUs), transposons, gene cassettes and integrons are different elements involved in resistance gene spread (Salyers and Amabile-Cuevas, 1997). Host and clone specificity, plasmid and clone specificity, virulence, interactions with commensal flora, duration of selection pressure, and variable gene expression significantly determine the emergence and spread of resistant strains, e.g. the staphylococcal -lactamase gene is now almost universally present within staphylococci, but not in enterococci (ASM, 2009; Murray *et al*., 2003; CDC, 2002).

Lack of appropriate infection control practices in hospitals and community, improper hygienic practices of patient, their visitors, and the health care professionals (HCP) who are transiently or persistently colonized with resistant bacteria, can transfer resistant bacteria from patient to patient. Exposure of people to day care centers, long term care facilities and nursing homes, and repeated movement to tertiary care centers and back, also transmit resistant microorganisms (Adcock *et al*., 1998; Sherertz *et al*., 1996; Reichler *et al*., 1992). Excessive and nonhuman use of antibiotics and use of counterfeit drugs also contribute to emergence and spread of resistant microorganisms, e.g. emergence of vancomycin resistant enterococci due to the excessive use of a glycopeptide avoparcin as a growth promoter in food animals (Wegener *et al*., 1999). Spread of resistant organisms may be through the food supply or due to population mobility, e.g., salmonellae acquired from meat, or eggs. Population mobility is a main factor in globalization of public health threats and risks, especially distribution of antibiotic resistant microorganisms. These all factors indicate for the establishment of an authority to provide proper guidelines for control and management of infection (MacPherson *et al*., 2009; Siegel *et al*., 2007; CDC, 2006).

3.1.3. General mechanisms of antimicrobial resistance

A. Molecular mechanisms

After exposure to antibiotics, bacteria develop novel mechanisms to overcome their effects. Single or multiple mechanisms together are involved in evolution and exchange of resistant genes among bacterial pathogens. Usually point mutations occur in bacteria, leading to changes in a receptor or binding sites on the antibiotic targets making the antibiotics ineffective, e.g. rifampin resistance by mutation in *rpoB* gene coding RNA polymerase, streptomycin resistance by ribosomal mutation, fluoroquinolone resistance by mutation in topoisomerases, (Murray *et al*., 2003; Cloutier, 1995). Mutations may also activate the expression of silent genes coding for resistant variants of the drug target, result in the production of specific drug inactivating enzymes, or provide an alternative biochemical pathway to avoid drug action, e.g. mutations in a cellular amidase gene (*ampD*) in *Enterobacter* spp. result in buildup of a cell wall breakdown product and increases the expression of *ampC* gene, downregulation of expression of the porin OMP2 in *P. aeruginosa* associated with imipenem resistance, etc. Most, but not all, resistance-determining mutations and accessory elements engender some fitness cost, but those costs are likely to be ameliorated by subsequent evolution (Anderson and Levin, 1999).

Bacteria can acquire the resistance genes in various ways either from antibiotic producers or from other resistant bacteria living in the same ecological niche. Drug resistance, especially multiple-drug resistance, in bacteria is often associated with integrons which link antibiotic-resistant genes together to form large multiple loci of antimicrobial resistance within the genome, e.g. acquired *cfr* gene, when linked with *ermB* gene and coexpressed as *ermB/cfr*, confers resistance to almost all antibiotics whose target is the large ribosomal subunit. Acquired resistance is usually distinguished after several months or even after many years (Toh *et al*., 2007; Bass *et al*., 1999).

Some competitive bacteria can absorb naked DNA molecules from the surroundings under suitable conditions and incorporate them into their chromosomes. The source of

DNA with resistance genes may be the dead and disrupted normal or genetically modified bacteria (Heuer and Kornelia, 2007). Transformation does not account much for clinical cases of resistance transfer. Bacteria may acquire resistance genes via bacteriophages by the process of transduction which may be either specialized or generalized. The high prevalence of -lactamase production and methicillin resistance in staphylococci is probably due to phage mediated transfer of non- conjugative plasmid having -lactamase gene (Stewart and Rosenblum, 1980). The major mechanism of transfer of drug resistance in gram negative bacteria is conjugation and occurs more frequently by the transfer of cojugative R-plasmids among specific but versatile hosts. Transferable drug resistance in Enterobacteriaceae involves all antibiotics in common use. Production of ESBLs, MBLs, carbapenemases and ABLs may also be mediated by conjugative plasmids. Moreover, a single R-plasmid often contains many resistance genes and its conjugative transfer can remarkably contribute to multidrug resistance Conjugation is capable of mediating very broad host range gene transfers than transformation or transduction (Nikaido, 2009; Salyers and Amabile-Cuevas, 1997). The further mutation of acquired genes can lead to development of even broader spectrum of antimicrobial resistance, e.g. over 100 mutational variants of the TEM -lactamases in *K. pneumoniae* (Woodford and Ellington, 2007, Jacoby and Medeiros, 1991).

Antibiotic resistance may also be transferred among bacteria by non-replicative genetic elements, called transposons, e.g. Tn916-mediated tetracycline and minocycline resistance through *tet*(M) resistance gene in *Enterococcus faecalis* and other various hosts, Tn1545-mediated tetracycline, minocycline, erythromycin and kanamycin resistance in *Streptococcus pneumonia*, transposon-mediated vancomycin resistance in *Enterococcus faecium* strains, etc. Transposons may also encode genes for efflux of antibiotics from the cell. Conjugative transposons can also mobilize coresident plasmids or some small integrated elements called NBUs (nonreplicating *Bacteroides* units). Non-conjugative transposons also transfer resistance most commonly by integrating themselves into the transferable plasmids either transiently or permanently, e.g. Tn917

confers erythromycin resistance, Tn1546 confers vancomycin resistance, and Tn4001 confers gentamicin resistance (Salyers *et al*., 1995; Shaw *et al*., 1993).

B. Biochemical mechanisms

Many antibiotic modifying enzymes have been known including the -lactamases, aminoglycoside modifying enzymes (O-phosphotransferases, N-acetyl transfereases, and nucleotidyl transferases), streptogramin acetyl transferases (SATs) and chloramphenicol acetyl transferases (CATs). Most of these enzymes are acquired, and some of them are intrinsic to certain species, though expressed at low levels under normal conditions, e.g. chromosomal -lactamases are intrinsic to almost all gram-negative bacteria (Rice *et al*., 2000; Jacobs *et al*., 1995). Aminoglycoside modifying enzymes are sometimes intrinsic to bacterial species as well, e.g. chromosomal acetyltransferases of *Providencia stuartii* and *Serratia marcescens* Generally, antibiotic modifying enzymes confer high levels of resistance. However, vancomycin does not have such enzymes against it (Murray *et al*., 2003; Rather *et al*., 1993; Shaw *et al*., 1993).

Minor alterations of the highly specific binding sites on target molecule may have pronounced effect on antibiotic binding. Expression of the novel penicillin binding proteins (PBPs) alter the interaction of -lactams with these proteins. Change in PBP2 or PBP2a resulted in the emergence of MRSA. Interaction between erythromycin-ribosomal methylase confers resistance to the macrolide-lincosamide-streptogramin B classes of antibiotics. Most of these alterations are the result of mutations, but some resistance genes are also found on plasmids, e.g. plasmid-mediated vancomycin resistance by the substitution of lactate for D-alanine in peptidoglycan synthesis. Over-expression of the drug target InhA leads to a low-level isoniazid (INH) resistance in *Mycobacterium tuberculosis* (Murray *et al*., 2003). Mutations in the porin genes, resulting in their reduced expression or activity, accounts for much of the observed decrease in membrane permeability. These mutations occur frequently in gram-negative bacteria and resistance to -lactam antibiotics, aminoglycosides, chloramphenicol, and tetracycline may be partially attributed to the decreased uptake (Bellis *et al*., 2000). Examples include -lactam resistance in *E. coli*,

penicillin and tetracycline resistance in *Neisseria gonorrhoeae*, imipenem resistance in *P.aeruginosa*, cefepime resistance in *Enterobacter cloacae* and cefoxitin or ceftazidime resistance in *K. pneumoniae* (Livermore, 1992; Lee *et al*., 1991). Barriers to entry can also exist in the cytoplasmic membrane, e.g. aminoglycosides are inactive against anaerobes as their movement across the cytoplasmic membrane requires oxygen.

Some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA) for folic acid synthesis, but utilize preformed folic acid which in turn is required for bacterial nucleic acid synthesis. Many bacteria are intrinsically resistant to antimetabolites (co-trimoxazole) and some are capable of transferring this genetic capacity to others via plasmids. Moreover, bacteria acquire unaltered wild type genes for drug resistant dihydropteroate synthetase and dihydrofolate reductase from another source expressing both drug sensitive and resistant enzymes. Such type of resistance is usually shown by Staphylococci, Streptococci, *Neisseria* and Enterobacteriaceae (Denyer *et al*., 2007; Cloutier, 1995). Moreover, the prodrug antibiotic itself has no direct activity against the bacteria and requires its activation by a bacterial enzyme, e.g. KatG (catalase-peroxidase) for activation of isoniazide (INH), which produces a range of reactive metabolites including reactive oxygen species and then reactive organic radicals, which then inhibit multiple targets, including mycolic acid synthesis. Metronidazole is activated through RdxA (nitroreductase) forming reactive species that damage the DNA. Thus, mutations in these enzymes cause resistance to these drugs (Wei *et al*., 2005; Land and Johnson, 1999).

Formation of intact biofilms by persister cells confers resistance among *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa* which are genetically similar to susceptible cells (Okajima *et al*., 2006; Keren *et al*., 2004; Drenkard and Ausubel, 2002; Kloos and Bannerman, 1994). Salycylate also mediates non-heretable resistance in some bacteria by binding to MarR to release the suppression of the MarAB operon thus increasing transcription of the efflux pump *acrAB* and membrane channel *tolC* leading to increased efflux of drug. MarA also enhances the transcription of micF (an antisense RNA for *ompF*) which shuts down the expression of ompF leading to reduced drug intake (Price *et al*., 2000).

C. Efflux pumps

Antibiotic efflux was first described in 1980 as a mechanism for tetracycline resistance in enterobacteria. Various efflux pumps have become common ways for bacteria to resist the action of numerous classes of antibiotics nowadays. Most of these pumps are located in the cytoplasmic membrane and use protons as the motive force for efflux. An increased efflux of antibiotic from the bacterium produces a reduction in drug accumulation and an increment in the MIC. The most common antimicrobials expelled by the efflux pumps are macrolides, tetracyclines and quinolones (Barker, 1999). Phylogenetically, bacterial antibiotic efflux pumps belong to five superfamilies that are classified in two mechanistically distinct types. The highly drug specific primary transporters or ATP binding cassette (ABC) transporters extrude drugs from the cell with ATP hydrolysis. They are mostly found in antibiotic-producing organisms and in staphylococci and enteroccocci conferring resistance to macrolides and bacitracin. Secondary transporters are energized by trans-membrane electrochemical gradients of either H^+ or Na^+ ions (Webber and Piddock, 2003).

Secondary transporters include MFS, RND, SMR, MATE, etc. efflux pumps. Efflux pumps of the major facilitator superfamily (MFS) are found in both gram positive and gram negative bacteria; narrow spectrum, e.g. NorA of *S. aureus,* PmrA of *S. pneumonia* and EmeA of *E. faecalis,* EmrB of *E. coli* and various Rv efflux pumps in *M. tuberculosis*. The efflux pumps belonging to resistance nodulation division (RND) superfamily are mainly found in gram-netative bacteria and have broad spectrum of activity. They share genetic homology within and among different bacterial species and function with auxiliary proteins present in the outer membrane (the channel-forming OMF) and the periplasm, e.g. AcrAB-TolC of *E. coli* and MexAB-OprM of *P. aeruginosa*, AdeABC of *A. baumannii*, etc. (Piddock, 2006).

Efflux pumps designated as the small multidrug resistance (SMR) subfamily of drug/metabolite transporters (DMT) superfamily, only found in bacteria, are the smallest drug efflux proteins known and function as homo-or hetero-oligomeric complexes. They

are involved in the efflux of lipophilic cationic drugs, e.g. Smr of *S. aureus* and EmrE of *E. coli.* The MFS, RND and SMR families are proton antiporters. The efflux pumps classified as multidrug and toxic compound extrusion (MATE) subfamily of the multidrug/oligosaccharidyl-lipid/polysaccharide flippase (MOP) superfamily are either Na^+ or H^+ antiporters. They are found in various bacteria like *Vibrio parahaemolyticus* (NorM), *V. cholerae* (VcrM, VcmA), *Haemophilus influenzae* (HmrM), *P. aeruginosa* (PmpM), *Clostridium difficile* (CdeA), and *S. aureus* (MepA) conferring resistance to various antibiotics (Piddock, 2006; Webber and Piddock, 2003; Chung and Saier, 2001).

Efflux pump inhibitors (EPIs) such as valinomycin, dinitrophenol, peptidomimetics, promethazine, nocardamine, antihypertensives (reserpine and verapamil), etc., inhibit various efflux pumps potently, decrease the intrinsic bacterial resistance to antibiotics, reverse acquired resistance due to efflux pump overexpression, reduce the bacterial virulence *in vivo* (RND efflux pumps) and reduce the frequency of the emergence of resistant mutant strains. EPIs target either driving force of MDR pumps or inhibit them competitively or noncompetitively and can be used as adjunct therapy (Zechini and Versace, 2009; Mahamoud *et al*., 2007; Lomovskaya *et al*., 2006, Li and Nikaido, 2004).

3.2. Resistance to Common Antibiotics

Antimicrobial chemotherapy has played a vital role in the treatment of human infectious diseases in the 20th century. However, emergence of resistance against almost all antibiotics available has challenged the clinicians and microbiologists to decide which agents are appropriate for inclusion in routine and specialized susceptibility testing and therapeutic implications according to the current resistance patterns.

3.2.1. Resistance to -lactam antibiotics

Penicillins, cephalosporins, monobactams and carbapenems produce bactericidal effects by inhibiting PBPs, transpeptidations, transglycosylations, and carboxypeptidations during peptidoglycan synthesis. PBPs are serine peptidases that, like -lactamases,

interact with -lactams (structural analogues of the peptidyl-D-alaninyl-D-alanine termini of peptidoglycan precursors) by catalytically disrupting the -lactam bond, resulting in a serine ester-linkage of the acylenzyme derivative. Some bacterial species are intrinsically resistant to some -lactams by virtue of decreased PBP affinity, e.g. cephalosporin resistance in enterococci, nafcillin and oxacillin resistance in staphylococci, and ticarcillin and carbenicilin resistance in *Pseudomonas* spp. PBP-mediated resistance may also be due to overexpression of PBPs, e.g. methicillin resistance in staphylococci (overexpressed PBP4), penicillin resistance in enterococci (overexpressed PBP5), etc. Acquisition of foreign PBPs also accounts for -lactam resistance, e.g. methicillin and all -lactam resistance in staphylococci due to acquired low-affinity PBP2a encoded by *mec*A gene. Finally, PBP-mediated -lactam resistance may result from mutations within the pbp genes producing lower affinity PBPs, e.g. mutation in PBP5 of *E. faecium* raises penicillin MIC very much (Murray *et al*., 2003).

Production of -lactamases is the main mechanism of bacterial resistance to the -lactam class of antibiotics. Penicillinase was the first -lactamase to be identified by Abraham and Chain in 1940 from gram-negative *E. coli*. The -lactamases are members of a superfamily of active-site serine proteases and confer resistance to penicillins, cephalosporins, cephamycins and carbapenems. Most -lactamases are composed of -helices and -pleated sheets, have structural similarities, and catalytically hydrolyse the -lactam (amide) bond to release penicilloyl or cephalosporyl moiety with 2-3000 times higher hydolysis rates than PBPs (Knox *et al*., 1995; Ghuysen *et al*., 1991).

The -lactamases can be chromosome, plasmid, or transposon encoded and produced in a constitutive or inducible manner. Chromosomal -lactamases, *e.g.,* AmpC cephalosporinases have some physiological role in peptidoglycan assembly or defend against -lactams produced by environmental bacteria and fungi. Resistance due to these enzymes is non-transferable. The first plasmid-mediated -lactamase TEM-1 was reported in 1965 from an *E. coli* isolate belonging to a patient (Temoniera) in Athens, Greece. The TEM-1 -lactamase has spread worldwide among various bacterial species.

Other commoner plasmid-mediated -lactamase is SHV-1 (sulfhydryl “variable” active site). Plasmid-mediated -lactamases are also called extended-spectrum -lactamases (ESBLs) and are inhibited by clavulanic acid. These can be transferred between various species of Enterobacteriaceae. Most -lactamases are secreted into the periplasmic space in gram-negative bacteria or into the surrounding medium by their gram-positive counterparts (Livermore, 1995).

Novel -lactamases among gram-negative bacteria have evolved and the simultaneous production of multiple types of -lactamases encoded by interchangeable or spreadable chromosomal and plasmid genes has challanged the therapy by -lactams. Synergism between -lactamase action and other resistance mechanisms may lead to emergence of multiple or total drug resistance (Thomson *et al*., 2000; Ahmad *et al*., 1999).

3.2.1.1 Classification of -lactamases

Various classification schemes have been proposed by many researchers but the molecular classification scheme by Ambler in 1980 based on similarities in nucleotide and amino acid sequences, and whether they are plasmid- or chromosome-encoded and functional classification scheme based on correlation of substrate and inhibitory properties with molecular structure by Bush in 1989 which was later modified, are the most frequently used classification schemes. The Bush-Jacoby-Medeiros scheme integrates functional and molecular characteristics and puts 178 -lactamases from naturally occurring bacterial isolates into four groups based on substrate and inhibitor profiles (Bush *et al*., 1995). Classification is summarised in the table in Appendix-IX.

3.2.1.2 Extended spectrum -lactamases (ESBLs)

Microbial resistance through ESBLs was reported first in the early 1980s in Europe and subsequently in the United States, soon after the introduction of third-generation cephalosporins in clinical practice. Probably, the continuous, prolonged and rampant use of third generation cephalosporions contributed to such emergence. There are now over 200 recognized ESBLs

in various gram-negative bacilli conferring resistance to penicillins, cephalosporins, a monobactam, and even carbapenems (Wright and Eiland, 2008).

Extended-spectrum -lactamases (ESBLs) are plasmid-mediated bacterial enzymes that confer resistance to the penicillins, first-, second-, and third-generation cephalosporins, aztreonam, and related oxyimino-blactams (but not the cephamycins or carbapenems) by hydrolyzing these antibiotics, and are inhibited by -lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Livermore and Woodford, 2006). Most of them also have the ability to hydrolyze fourth-generation cephalosporins e.g. cefepime and cefpirome. ESBLs are included in 2be and 2d group of the -lactamases in Bush-Jacoby-Medeiros classification system whereas ESBLs except OXA-type have been grouped in class A in the classification scheme of Ambler (Bush *et al*., 1995). Predominant ESBL producers include *E. coli* and *Klebsiella* species, and they are also found in *Acinetobacter* spp., *Alcaligenes fecalis*, *Burkholderia cepacia*, *P. aeruginosa*, *Salmonella enterica* and *Serratia marcescens* (Bush, 2008; Al-Jasser, 2006; Paterson and Bonomo, 2005; Bradford, 2001).

3.2.1.3 Types of ESBLs

SHV (sulfahydril variable) type of ESBLs may be found in clinical isolates more frequently than any other type of ESBLs. There are relatively few derivatives of SHV-1. More than 50 SHV varieties are described worldwide. Most of them possess the ESBL phenotype and a few are inhibitor-resistant. SHV-type of ESBLs has been detected in a wide range of Enterobacteriaceae, *P. aeruginosa* and *Acinetobacter* spp. The first SHV-type ESBL was reported in 1983 in *Klebsiella ozaenae*. The TEM type ESBls are the mutational derivatives of TEM-1 and TEM-2 -lactamases and are also called complex mutants of TEM (CMT-1 to 4). They hydrolyze third-generation cephalosporins and are resistant to inhibitor. They and are reported in enteric bacteria and in *P. aeruginosa* (Al-Jasser, 2006). Cefotaximases (CTX-M) have become the most prevalent ESBLs worldwide (Livermore *et al*., 2007). They have potent hydrolytic activity against cefotaxime and hydrolyze cephalothin better than benzylpenicillin and cefotaxime over

ceftazidime. CTX-M-type -lactamases also hydrolyze cefipime with high efficiency (Tzouvelekis *et al*., 2000). They are better inhibited by tazobactam than sulbactam or clavulanic acid. These are produced by plasmid-acquired -lactamase genes normally found in chromosome of *Kluyvera* spp. rather than by chromosomal mutation of TEM or SHV genes. More than 40 varients of CTX type ESBLs have been reported and their numbers are rapidly expanding. Most CTX-M-15 producers are resistant to multiple antibiotics. Toho-1 and Toho-2 -lactamases are structurally related to CTX-M types and have similar hydrolytic activity against cefotaxime.

OXA types ESBLs are so named because of their greater hydrolytic activity (>50%) for cloxacillin and oxacillin than that for benzylpenicillin. They are found predominantly in *P. aeruginosa.* Many OXA-type ESBLs have been derived from the original OXA-10 -lactamase (e.g., OXA-11, 14, 6 and 17). Majority of them confer resistance to ceftazidime while OXA-17 confers resistance to cefotaxime and ceftriaxone rather than ceftazidime. A novel OXA-18 is found to be inhibited by clavulanate. PER types ESBLs share 25-27% homolgy with SHV and TEM type ESBLs, efficiently hydrolyse penicillins and cephalosporins, and are inhibited by clavulanate. VEB-1 has greater structural homology to PER-1 and PER-2 and confers high level resistance to ceftazidime, cefotaxime and aztreonam. It is inhibited by clavulanic acid. Other VEB type enzymes have also been detected in various geographic regions. Other rarely found ESBLs are GES, IBC, BES, SFO and TLA. These are either plasmid-mediated or integron-associated (Mavroidi *et al*., 2001; Bonnet *et al*., 2000).

3.2.1.4 Clinical implications of ESBLs

ESBL-producing organisms have been increasingly detected worldwide and their prevalence varies geographically from country to country and from institution to institution. Most ESBL-producing organisms are usually prevalent in tertiary care centers. Higher prevalence of ESBL producers in clinical settings complicates therapy and increases healthcare cost. All ESBL producers should be considered resistant to all penicillins (except temocillin), cephalosporins (except cefamycins) and aztreonam

(Livermore and Woodford, 2004). Optimal therapy in serious infections due to ESBL-producing organisms may not be achieved with -lactam- -lactamase inhibitor combinations and their clinical effectiveness is controversial. ESBL-producing bacteria are also resistant to other class of antibiotics such as fluoroquinolones, aminoglycosides, tetracyclines, chloramphenicol and co-trimoxazole extremely limiting the antibiotic options in treating infections caused by them (Sharma *et al*., 2010; Giske *et al*., 2008; Paterson and Bonomo, 2005; Nathisuwan *et al*., 2001). ESBL producers contribute to the selection and persistence of multidrug-resistant ESBL strains and plasmids in both clinical and community settings (Canton *et al*., 2008; Morosini *et al*., 2006).

3.2.1.5 Screening for ESBLs

Clinical and Laboratory Standards Institute (CLSI) has developed disk diffusion and broth microdilution screening tests for ESBL production with the use of five cephalosporins, viz. cefpodoxime, ceftazidime, ceftriaxone, cefotaxime and aztreonam. (Table 3.2.1.5)

Table 3.2.1.5 ESBL screening breakpoints (BP)

Antibiotic disks and their strengths (µg)	Susceptible BP (mm)	Resistance BP (mm)	ESBL screening BP (mm)	Susceptible MICs (µg/ml)	Resistant MICs (µg/ml)
Cefpodoxime (10)	27	17	17	8	8
Ceftazidime (30)	18	14	22	8	2
Aztreonam (30)	22	15	27	8	2
Cefotaxime (30)	23	14	27	8	2
Ceftriaxone (30)	21	13	25	8	2

Disk diffusion method: The CLSI has proposed disk diffusion methods for screening for ESBL production in *Klebsiella* spp*.*, *E. coli* and *Proteus mirabilis* by noting specific inhibition zone diameters which indicate possible ESBL production (NCCLS, 2005). Cefpodoxime (10µg), ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg), or aztreonam (15µg) can be used for screening. The disks are so arranged that the distance between them is approximately twice the radius of the inhibition zone produced by the

cephalosporins tested on its own. The results are interpreted using the size of zones of inhibition given in table 3.2.1.5. *K. pneumonia* ATCC 700603 (ESBL producer) and *E. coli* ATCC 25922 (ESBL nonproducer) are taken as controls.

Cefpodoxime and ceftazidime have been proposed as indicators of ESBL production as compared to cefotaxime and ceftriaxone. An institution where only cefotaxime and ceftriaxone are used in the routine sensitivity testing panel may have difficulty in detecting ESBLs (Nathisuwan *et al*., 2001). The detection may also be affected by the inoculum size. Not all ESBL producers are universally resistant to any one of extended spectrum cephalosporins. They vary in their substrate specificity and may not phenotypically express resistance to its own substrate. ESBLs can also be induced by certain antibiotics, amino acids or body fluids and organisms possessing genes for inducible -lactamases show false susceptibility if tested in the uninduced state (Revathi and Singh, 1997). For TEM and SHV type ESBLs, ceftazidime is a good detector while for CTX-M type ESBLs, cefotaxime is more useful. All ESBLs show obvious resistance to cefpodoxime. Therefore, use of either cefpodoxime or both cefotaxime and ceftazidime resistance improves the sensitivity of ESBL detection (HPA, 2008).

ChromID ESBL: Bacterial strains are cultured in the chromogenic chromID ESBL agar medium (bioMerieux, France) aerobically at 37ºC for 18 to 48 hours. Colonies of ESBL producers develop species-specific colors (*E. coli* shows pink to burgundy coloration of -glucuronidase-producing colonies; *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., and *Citrobacter* spp. show green and/or blue coloration of -glucosidase producing colonies; and *Proteus* spp., *Providencia* spp., and *Morganella* spp. show orange to brown coloration of deaminase-expressing colonies) on chromID ESBL agar. Non-ESBL producers grow with colorless colonies or will not grow (Farber *et al*., 2008).

Screening by dilution antimicrobial susceptibility tests: The CLSI has proposed this method for *Klebsiella* spp. and *E. coli*. Ceftazidime, cefotaxime, ceftriaxone, or aztreonam can be used at a screening concentration of 1µg/ml. MIC of cephalosporins at a range of 2µg/ml is suspicious of ESBL production.

3.2.1.6 Phenotypic confirmation of ESBLs

ESBLs confer resistance to oxyimino- -lactams (*e.g.* ceftriaxone, cefotaxime, ceftazidime, cefpodoxime and aztreonam) and are inhibited by -lactamase inhibitors, usually clavulanate and others sulbactam and tazobactam. Some phenotypic confirmatory tests for the suspected ESBL producers include:

Cephalosporins/clavulanate combination disks: According to CLSI guidelines, cefotaxime (30µg), cefpodoxime (30µg) and ceftazidime (30µg) disks with and without clavulanate (10µg) or cefpodoxime (10µg) alone and cefpodoxime (10µg) plus clavulanate (1µg) are placed on the already inoculated Mueller Hinton agar with standard suspension of test organism and incubated for 18-24 hours. Regardless of the zone diameters, a 5mm increase in a zone diameter for an antimicrobial agent tested in combination with clavulanic acid versus its zone size when tested alone indicates phenotypic confirmation of ESBL production. These methods, though useful, may not detect those ESBLs that are poorly inhibited by -lactamase inhibitors (Nathisuwan *et al*., 2001). ESBLs are harder to detect in those Enterobacteriaceae with inducible AmpC chromosomal enzymes (e.g. *Enterobacter* spp., *C. freundii, M. morganii, Providencia* spp. and *Serratia* spp.). The AmpC enzymes may be induced by clavulanate and may attack the cephalosporin, masking synergy arising from inhibition of the ESBL. Cefepime or cefpirome is a more reliable detection agent for ESBLs in isolates simultaneously producing AmpC- -lactamase, as this drug is stable to AmpC -lactamases, but labile to ESBLs (Guleri *et al*., 2004; Livermore and Woodford, 2004). ESBL tests are not developed for *Acinetobacter spp., P. aeruginosa* and *S. maltophilia* and should not be used for them.

Broth microdilution: It utilizes ceftazidime (0.25 to128µg/ml), ceftazidime plus clavulanic acid (0.25-128/4 µg/ml), cefotaxime (0.25-64µg/ml), and ceffotaxime plus clavulanic acid (0.25-64/4 µg/ml). Both of these antibiotic should be used. Phenotypic confirmation is considered as 3-twofold-serial-decrease in MIC of either of cephalosporin in the presence of clavulanic acid compared to its MIC when tested alone.

Double disk synergy/disk approximation test: This test incorporates the use of cefotaxime (30µg), ceftazidime (30µg), or cefpodoxime (10µg) disks which are placed on either side of co-amoxiclav (20+10µg) disk on an already inoculated Mueller Hinton Agar (MHA) plate with the bacterial suspension adjusted to 0.5 McFarland turbidity standard, at a center to center distance of 20-30mm. Plates are incubated at 35-37°C for 18-24 hours. Enhancement of zone of inhibition of either cephalosporin is indicative of ESBL production. This method is not advocated for routine use as critical disk spacing for various strains is of utmost imporance (Livermore and Woodford, 2004). Alternatively, cefoxitin (inducer) disk is placed at a distance of 2.5cm from cephalosporin disk. Production of inducible -lactamase is indicated by flattening of the zone of inhibition of the cephalosporin disk towards inducer disk by >1mm.

E-test for ESBLs: Two E-test combination strips, e.g. ceftazidime plus ceftazidime-clavulanate and cefotaxime plus cefotaxime-clavulanate, having a cephalosporin gradient at one end and a cephalosporin plus clavulanate gradient at the other, are employed to perform the phenotypic confirmatory testing. These strips are applied on the inoculated surface of the agar plate and incubated overnight. Any reduction of >3 log 2 (doubling) dilution or ≥8-fold reduction in cephalosporin MICs in the presence of clavulanate, is considered as positive. Strains with substrate specificities other than ceftazidime may not be detected with the ceftazidime/clavulanic acid strip alone hence cefotaxime is also used. The reported sensitivity of this method is 87-100% and specificity is 95-100%.

Disk replacement method: Three amoxycillin/clavulanate disks are applied to a MHA plate inoculated with the test organism. After one hour at room temperature, these antibiotic disks are removed and replaced on the same spot by disks containing ceftazidime, cefotaxime and aztreonam. Control disks of these three antibiotics are simultaneously placed at least 30mm from these locations. A zone increase of 5mm for the disks which have replaced the amoxycillin/clavulanate disks compared to the control disks gives positive test. This method is somewhat unreliable in ESBL detection.

Disk-on-disk test: In this test cefotaxime and ceftazidime disks are tested against test organism both alone and in combination with co-amoxiclavulanic acid disk being placed on top of the cephalosporin disk. The enhancement of inhibition zone by 10mm by placing co-amoxyclav disks than their use alone indicates ESBL production.

The above tests distinguish AmpC -lactamases from ESBLs.

Molecular and other instant methods of ESBL detection: Molecular methods assess genetic variations, *e.g.* presence or absence of plasmids, restriction endonuclease profiles, number and positions of repetitive elements, precise nucleotide sequence, mutations that are associated with pattern variation to measure inter-strain relatedness. These methods characterize ESBLs genotypicallly such as TEM, SHV, OXA, CTX-M, etc. and their epidemiological patterns. Methodologies include DNA probes, various polymerase chain reaction (PCRs), oligotyping, ligase chain reaction (LCR), nucleotide sequencing, etc. The multiplex PCR assay detects ESBLs and PABLs with 100% sensitivity, identifies them efficiently and reduces the time for their classification (Kim *et al.*, 2009). Other faster methods include:

- Vitek ESBL Cards utilize cefotaxime and ceftazidime alone and in combination with clavulanic acid.
- BD Phoenix automated Microbiology System uses growth response to cefpodoxime, ceftazidime, ceftriaxone, and cefotaxime with or without clavulanic acid.
- Agar supplemented with clavulanate: 30µg disks of each ceftazidime, cefotaxime, ceftriaxone and aztreonam are placed on 4µg/ml clavulanate-supplemented and on clavulanate free MHA plates. A difference in -lactam zone width of 10mm on the two media is considered positive for ESBL production.
- MicroScan Walkaway Panels: This is based on hydrolysis of fluorogenic substrates, pH changes following substrate utilization, and rate of production of specific metabolic byproducts after 2.5 hours incubation in the instrument.

3.2.1.7 Treatment options in infections by ESBL producers

Of all the available -lactams, carbapenems such as meropenem, imipenem, doripenem and etrapenem still remain effective (Mehrgan and Rahbar, 2008). But, community outbreaks of ESBL producers will lead to increased carbapenem use and this may lead to a much serious problems in treating infections by carbapenemase producers. However, MBL-producing strains are less prevalent than those with other mechanisms of resistance (Trevino *et al*., 2009). Cephamycins and latamoxef are often effective in the treatment of such infections. Urinary tract infections may be treated safely with -lactam/ -lactamase inhibitor combination due to higher concentration of -lactamase inhibitor in urine to counteract the hydrolytic activity of ESBLs (Nordmann, 1998). Non -lactam antimicrobial agents (aminoglycosides, fluoroquinolones) may be beneficial; however, co-resistance rates against these agents are frequent. Fosfomycin can be a safe and effective alternative in treating infections by multiple drug bacteria including ESBL-producers (Flagas *et al*., 2010; Flagas *et al*., 2009; Cueto *et al*., 2006). Tigecycline can also be of newer and better option against various ESBL producers in treating various types of infections (Livermore, 2005; Garau, 2008).

3.2.1.8 Other -lactamases

AmpC -lactamases (ABLs) cephalosporinases are species-specific chromosomally-encoded -lactamases, common but not ubiquitous in Enterobacteriaceae and Pseudomonaceae, which have also become mobilized onto transmissible plasmids. They mediate resistance to oxyiminocephalosporins (ceftazidime, cefotaxime, ceftriaxone, etc.), cefamycins (cephalothin, cefazolin, cefoxitin), most penicillins, monobactams such as aztreonam, and -lactam/ -lactamase inhibitor combinations. The hydrolysis of benzylpenicillin, cefepime, cefpirome and carbapenems are very low (Jacoby, 2009, Ding *et al*., 2008). Types of ABLs include CMY, MIR, MOX, LAT, FOX, DHA, ACT, etc. Pathgens harbouring plasmids for ABLs often carry resistance genes for other multiple antibiotics and even for ESBLs thus indicating a significant therapeutic challange (Shahid *et al*., 2009). The emergence of metallo- -lactamases (MBLs) with activity

against carbapenems (e.g. the VIM, GIM, NDM, SIM, SPM and IMP families of enzymes) has compromised the clinical utility of this class of antibiotics. Resistance to carbapenems may also be induced due to increased production of either AmpC or ESBL, coupled with a decrease in porin production or increased efflux MBLs can hydrolyse all clinical -lactams, with the exception of aztreonam. These enzymes are frequently reported in *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*. The appearance and rapid spread in the USA, China, Israel and Europe of molecular class A carbapenem-hydrolysing KPC-type -lactamases is the most recent development in the epidemiology of carbapenem resistance (Hawkey and Jones, 2009; Walsh, 2008).

3.2.2 Resistance to aminoglycosides

Most aminoglycosides are derived either from *Micromonospora* spp. (gentamicin, sisomicin, and netilmicin) or from *Streptomyces* spp. (streptomycin, neomycin, kanamycin, tobramycin, and paromomycin). Aminoglycosides contain amino sugars in their structure and exert bactericidal activity against most aerobic bacteria. They inhibit bacterial protein synthesis by binding irreversibly to the bacterial 30S ribosomal subunit, thus blocking the translocation of mRNA during protein synthesis, thereby leading to cell death. Aminoglycosides also cause misreading of the genetic code producing toxic nonsense proteins. Bacterial uptake of these agents is facilitated by -lactams and vancomycin, providing synergistic action. All aminoglycosides exert intrinsic irreversible ototoxic and reversible nephrotoxic effects.

Though resistance to aminoglycosides was reported in *P. aeruginosa* clinical isolates, it is now too common in any clinical specimens and is virtually present in all areas of the world. The aminoglycoside-resistant *P. aeruginosa* isolates carry multiple (i.e., two to five) modifying enzymes and exhibit broad-spectrum aminoglycoside resistance as a result. Aminoglycosides are inactivated by modifications that reduce the net positive charges on these polycationic antibiotics (Wright, 1999; Davies and Wright, 1997). There are three classes of these enzymes: four aminoglycoside acetyltransferases (AACs), seven aminoglycoside phosphatases (APHs) and four aminoglycoside nucleotidyltransferases

(ANTs). Plasmid-encoded enzymes found in many aminoglycoside resistant gram-nagative bacteria are derived form the chromosomal genes of organisms that produce these antibiotics or microorganisms in the environment, especially the soil. Resistance to all aminoglycosides is often associated with reduced aminoglycoside accumulation due to reduced permeability and uptake, panaminoglycoside-resistant due to efflux involving efflux systems of RND family such as MexXY, AmrAB-OprA, MexAB-OprM and AcrAD-TolC, and a multidrug transporter of the SMR family, $EmrE_{P.A}$. Resistance to all aminoglycosides and loss of resistance in absence of drug may occur reversibly both *in vitro* and *in vivo*, and is due to aminoglycoside induced efflux systems and enhanced expression of genes associated with anaerobic respiration. Resistance may also be due to altered ribosomal binding site (Poole, 2005; Karlowsky *et al*., 2003[a]; Li *et al*, 2003).

3.2.3 Resistance to fluoroquinolones

Quinolones are potent antibiotics biochemically related to nalidixic acid, which was developed initially as a urinary antiseptic. Fluoroquinolones are new agents derived from modifications of quinolones and contain a fluorine atom attached to the nucleus at position 6. Norfloxacin, enoxacin, lomefloxacin, ciprofloxacin, ofloxacin, levofloxacin, sparfloxacin, trovafloxacin, gatifloxacin, and moxifloxacin are currently available for clinical use. Quinolones inhibit DNA gyrase (a type II DNA topoisomerase) and fluoroquinolones also inhibit DNA topoisomerase IV leading to termination of chromosomal replication killing the bacteria. The DNA gyrase A subunit in gram-negative bacteria and topoisomerase IV in gram-positive bacteria are the main targets of quinolones. Fluoroquinolones actively trap the topoisomerases as drug-enzyme-DNA complexes in which double-stranded DNA breaks are held together. The enzymes are unable to reseal the DNA so that chromosome becomes fragmented. The activity of various quonolones is reduced by lower pH, urine, and presence of divalent cations like Ca^{+} and Mg^{+} (Wolfson and Hooper, 1989).

Bacterial resistance to quinolones may occur either by mutations in the coding regions of the gyrase subunits (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*),

mutations in regulatory genes governing bacterial outer membrane permeability to the drug, and expression or overexpression of energy-dependent efflux pumps that can actively remove drugs from the bacterial cell (Hooper, 2000). Mutations in *gyrB* and *parE* leading to resistance are uncommon. Acumulation of mutations in several of these genes increases the MIC in a stepwise manner (Drlica and Malik, 2003). There is also a plasmid-encoded target DNA protection mechanism enabled by the *qnr* genes, among some clinical strains of *E. coli* and *K. pneumonia*, with both genes being found on plasmids carrying $bla_{\text{CTX-M}}$ and bla_{CMY} that inactivate third-generation cephalosporins (Cattoir and Nordmann, 2009; Lavilla *et al.*, 2008). In addition, a widely distributed plasmid-coded ciprofloxacin resistance gene encodes for a mutant aminoglycoside acetylase, the AAC (6'')-Ib that acetylates the amino group of ciprofloxacin. Possibly, low level plasmid-encoded fluoroquinolone resistance has provided a selective advantage for bacteria exposed to fluoroquinolones for the easier selection of high-level resistance mutations in *gyrA*, thus explaining the association of high-level quinolone resistance with plasmid-encoded ESBL genes (Robicsek *et al.*, 2006[b]; Wang *et al.*, 2004).

3.2.4 Resistance to tetracyclines

First isolated in 1945, tetracyclines are broad-spetrum bacteriostatic antibiotics with the hydronaphthacene nucleus containing four fused rings, e.g. tetracycline, chlortetracycline, oxytetracycline, doxycycline and minocycline. Tetracyclines enter bacterial cell by an active process and prevent protein synthesis by reversibly binding to the 30S ribosomal subunits thus blocking the access of aminoacyl-tRNA to the RNA-ribosome complex. Resistance to tetracyclines develops relatively slowly, but there is cross resistance. Bacterial resistance to tetracyclines is due to active efflux of the drug from the cell, an altered ribosomal target site, or production of modifying enzymes that inactivate the drug (Spear *et al.*, 1992). The *tet*(A-E), *tet*(G), *tet*(H), *tet*(K), *tet*(L) and *tet*(X) genes have been identified in tetracycline-resistant *E. coli* strains, *tet*(B) gene being the most prevalent gene. The *tet*(X) gene encodes an enzyme which modifies and inactivates tetracyclines instead of efflux (Denyer *et al.*, 2007; Wilkerson *et al.*, 2004).

Tigecycline: A new glycylcycline antibiotic

Tigecycline is a bacteriostatic glycylcycline derived from minocycline and was licensed for use in the United States in 2005 (O'Neill, 2008; Zhanel *et al*., 2004). Tigecycline binds reversibly and 5-fold more strongly to the 30S subunit of the bacterial ribosome in a different orientation than classical tetracyclines and blocks the binding of amino-acyl-tRNA to the acceptor site on the mRNA-ribosome complex. This prevents the incorporation of amino acids to the growing peptide chain, thereby inhibiting protein synthesis (Nathwani, 2005; Bauer *et al*., 2004). Tigecycline evades the Tet(A-E) and Tet(K) efflux pumps, and works on *tet*(M)-protected ribosomes. This enhanced binding, probably, overcomes the ribosomal protection mechanisms mediated *tet*(M) gene. Like tetracyclines, tigecycline also forms chelation complexes with divalent cations such as Ca^{+}, Mg^{+} and Fe^{++} resulting in food-drug and drug-drug interactions, thus influencing its anti-microbial and pharmacokinetic properties (Garrison *et al*., 2005; Fluit *et al*., 2005).

Tigecycline is an FDA (Food and Drug Administration) approved drug and can be used as an empiric monotherapy to treat a variety of both hospital and community acquired serious bacterial infections, including complicated skin/skin-structure infections, complicated intra-abdominal infections, community-acquired bacterial pneumonia including cases with concomitant bacteremia., MDR *A. baumannii* meningitis in combination with meropenem and netilmicin, deep soft tissue infections and infected ulcers (Tutuncu *et al*., 2009; Frampton, 2005). Tigecycline in higher doses can also be used successfully in treating UTI and urospesis caused by ESBL-producing MDR strains of *E. coli*, *K. pneumoniae* and *A. baumanii* (Cunha, 2009; Krueger *et al*., 2007). Tigecycline has proven to be beneficial in the treatment of serious infections in patients with cancer (Chemaly *et al*., 2009). It is currently under review by regulatory agencies worldwide for other indications and due to its proven activity against highly resistant organisms, it should be reserved only for life-threatening situations.

Tigecycline is highly effective against MRSA, glycopeptide-intermediate *S. aureus* (GISA), VRE, and penicillin resistant *S. pneumoniae* (Sorlozano *et al*., 2006;

Felmingham, 2005). Tigecycline shows high potency against gram-negative bacilli such as MDR (including carbapenem-resistant) *Acinetobacter* spp., *S. maltophilia* and *K. pneumonia* and other members of Enterobacteriaceae. Tigecycline is also active against clinically relevant species of Enterobacteriacea*e*, including ESBL and/or MBL producing strains, pan-resistant isolates, *Legionella pneumophilia, Chlamydia*, rapidly growing non-tuberculosis bacteria and various anaerobes (Volles and Branan, 2008; Sorlozano *et al*., 2006; Souli *et al*., 2006; Meagher *et al*., 2005; Ogtrop *et al*., 2000). Tigecycline is not active against certain *Proteus* strains, including *P. mirabilis*, *Pseudomonas*, *Morganella*, or *Providencia* (Greer, 2006). However, the activity of tigecycline is not universally consistent, and may be affected if a more conservative breakpoint is adopted.

Tigecycline is not yet available in Nepal. It is currently available only for intravenous (IV) use in other countries. The recommended dose of tigecycline is 50mg every 12 hours given over 30-60 minutes after a 100mg loading dose for 5-14 days (Annonymous, 2005). Tigecycline has excellent tissue distribution profile and long half-life (~36 hours) with good post antibiotic effect (Ogtrop *et al*., 2000). The major mode of its excretion is biliary route with <15% unchanged tigecycline through in urine. The pharmacokinetics of tigecycline are unaffected by food, age, gender, renal disease or hepatic disease (Nathwani, 2005). Though it has good safety profiles in normal adults, its use during tooth development may cause permanent discoloration of teeth (Rello, 2005).

The FDA has proposed susceptibility breakpoints of 0.25mg/L for streptococci, 0.5mg/L for staphylococci, 2mg/L for Enterobacteriaceae (inhibition zone diameter 19mm for susceptibility and 14mm for resistance) and 4mg/L for anaerobes. The British Society of Antimicrobial Chemotherapy (BSAC) disk breakpoints are 24mm and 19mm for susceptibility and resistance, respectively (Hope *et al*., 2007). MICs of 2µg/ml and a breakpoint zone diameter of 16/ 12mm to define susceptibility/resistance respectively assessed by CLSI methods (CLSI, 2006) have consistent intermethod accuracy, instead of US FDA breakpoints (19/ 14mm, respectively) for Enterobacteriaceae, giving physicians greater confidence in the

laboratory susceptibility test result (Jones *et al*., 2007). The potential development of resistance to tigecycline during the course of therapy is of concern (Karageorgopoulos *et al*., 2008). Recently tigecycline resistance has been reported in *P. miribalis* and *P. aeruginosa* strains due to its extrusion by chromosomally-encoded multidrug efflux pumps and Tet(X), a tetracycline-degrading mono-oxygenase rarely found in *Bacteroides* spp. (Livermore, 2005; Yang *et al*., 2004). High resistance rates to tigecycline with higher MICs in multiple clones of MDR *A. baumannii* have been reported perturbingly (Capone *et al*., 2008; Navon-Venezia *et al*., 2007).

3.2.5 Resistance to sulfonamides and trimethoprim

Sulfonamides (sulfamethoxazole or SMX, sulfadiazine, sulfisoxozole, and sulfamethizole) are chemical analogs to p-aminobenzoic acid (PABA) which competitively inhibit the bacterial modification of p-aminobenzoic acid into dihydrofolate, whereas trimethoprim (TMP) is a pyrimidine analog that inhibits bacterial dihydrofolate reductase, a synergistic action. This sequential inhibition of folate methabolism ultimately prevents the synthesis of bacterial DNA. Co-trimoxazole (trimethoprim:sulfamethoxazole in 1:5 ratio) has proven to be very effective in the treatment of many infections by many gram-positive cocci, including staphylococci and streptococci, and most gram-negative bacilli, except *P. aeruginosa*. However, 10-50% of *S. pneumoniae* strains are resistant in many parts of the world. Sulfonamides and trimethoprim have irregular *in vitro* activity influenced by inoculums size and composition of test media. The antibacterial activity of co-trimoxazole may be reduced in patients receiving high doses of folinic acid. Resistance to trimethoprim can be caused by overproduction of host dihydrofolate reductase (DHFR) and acquisition of the *dfr* gene encoding resistant forms. DHFR of type I, II, or V of the 15 types is the most common mechanism of trimethoprim resistance among the Enterobacteriaceae. For all species of Enterobacteriaceae, co-trimoxazole resistance was more commonly observed in isolates with a single-drug resistance phenotype (Karlowsky *et al*., 2003[b]).

3.2.6 Resistance to other common antibiotics

Chloramphenicol contains a nitrobenzene ring and is originally derived from *Streptomyces venezuelae*. It is highly effective broad-spectrum antimicrobial agent and inhibits protein synthesis by binding reversibly to the peptidyltransferase component of the 50S ribosomal subunit and prevents the transpeptidation process of peptide chain elongation. Resistance to chloramphenicol is mainly due to its inactivation by plasmid-mediated chloramphenicol acetyltransferases (CATs) in both gram-negative and gram-positive bacteria; however, decreased outer membrane permeability and active efflux have been identified in gram-negative bacteria. Macrolide resistance is due to reduced outer membrane permeability, target modification of 23S rRNA by adenine-N-methyltransferase encoded by *erm* gene and increased efflux mediated by *mef* and *msr* genes. Resistance to nitrofurantoin may be due to alteration in enzyme activity that hydrolyzes the prodrug followed by efflux and reduced uptake. Vancomycin resistance is mediated by five types of genes *vanA* to *vanE*. Phenotypic VanA resistance mediated by a seven gene cluster in transposon Tn1546 is most common and confers high-level vancomycin and teicoplanin resistance (Denyer *et al.*, 2007).

3.3 Fosfomycin: An Old Antibiotic with New Scope

Fosfomycin is a phosphoenoyl pyruvate analogue (1,2-epoxypropylphosphonic acid) originally isolated in 1969 from *Streptomyces fradiae* and other *Streptomyces* species but now produced synthetically. Both oral (fosfomycin calcium and fosfomycin trometamol) and intravenous (fosfomycin disodium) formulations are available. It exerts bactericidal effect by inactivating the enzyme enolpyruvyl transferase, thereby irreversibly blocking the peptidoglycan synthesis in gram-positive and gram-negative bacteria (Forsgen and Walder, 1983). It also reduces bacterial adherence to uroepithelial cells. Fosfomycin can have synergistic effects with -lactams, aminoglycosides and fluoroquinolones (Tessier and Quentin, 1997). Its action requires glucose-6-phosphate induced transport system which enhances drug penetration into bacterial cells. The loss of this transport system

either *in vitro* by the presence of high concentrations of phosphate or glucose in the growth medium or *in vivo* abolishes the action of fosfomycin (Takahata *et al*., 2010).

Fosfomycin has excellent tissue penetration property and it is excreted unchanged in the urine. The drug can be used safely even in pregnancy if needed clearly. Fosfomycin can be used in treating a diversity of infections such as bacteremias, and urinary, respiratory, osteoarticular, and gynecological infections (Gobernado, 2003; Murray *et al*., 2003). Fosfomycin is active against many bacterial strains, both *in vitro* and in clinical infections (Greenwood *et al*., 1992; Barry, 1991). The susceptibility in terms of MIC (NCCLS, M7-A3, 1993) and zone of inhibition in disk diffusion techniques for disk containing fosfomycin 200µg and glucose-6- phosphate 50 µg (NCCLS, M2-A5, 1993) is interpreted as: Susceptible (16mm or MIC 64µg/mL), Intermediate (13-15mm or MIC=128µg/mL) and Resistant (12mm or MIC 256µg/mL).

Fosfomycin has excellent *in vitro* activity against MRSA, and various ESBL-producing and other urinary isolates (Flagas *et al*., 2010; Maraki *et al*., 2009; Cueto *et al*., 2006; Tharavichitkul *et al*., 2005). It has remarkable *in vitro* activity against vancomycin-resistant enterococci (Superti *et al*., 2009). Fosfomycin can be an effective alternative in treating infections by ESBL-producing MDR organisms (Paterson and Bonomo, 2005; Giske *et al*., 2008; Sharma *et al*., 2010). The *in vitro* activity depends on the culture, inoculums, medium and method of testing. Fosfomycin may also be suitable for treatment of soft tissue infection (Frossard *et al*., 2000). Fosfomycin in combination with other antibiotics such as antipseudomonal -lactam, imipenem and aminoglycoside may be a good alternative regimen for the treatment of MDR *P. aeruginosa* infections with a low side effect profile (Flagas *et al*., 2009; Mirakhur *et al*., 2003). Fosfomycin has a protective effect against nephrotoxicity of aminoglycoside by inhibiting aminoglycoside-induced histamine release from mast-cell destruction. It also increases the oxygen levels in the mitochondria and cyclic-AMP in the mast cells (Bedirdjian *et al*., 1978).

There is presumably little opportunity for the resistance due to lack of glucose-6-phosphate induced transport system to develop *in vivo,* since the selection pressure is

minimal, but mutations in the constitutive -glycerophosphate pathway that the drug also uses may give rise to clinical resistance. These types of resistance involve chromosomal genes, but plasmid-mediated resistance associated with enzymic inactivation of fosfomycin has also been described (Arca *et al*., 1988; Llaneza *et al*., 1985). Moreover, urinary *E. coli* isolates can acquire fosfomycin resistance from other fosfomycin resistant and CTX-M-15-producing *E.coli* strains (Oteo *et al*., 2009). In multiple-dose use, rapid chromosome- or plasmid-mediated bacterial resistance to fosfomycin may emerge rapidly. Fosfomycin has no cross resistance with other classes of antibiotics as it differs from other agents its chemical structure and site of action (Murray *et al*., 2003; Reeves, 1994). Novel amino acid substitutions in MurA or the loss of function of transporters confer fosfomycin resistance in clinical isolates of *E. coli* (Takahata *et al*., 2010).

3.4 Multiple Drug Resistance

The definition of multidrug resistance depends on different clinical settings, distribution pattern of pathogens, the drugs used to treat them, and their drug resistance patterns. Most commonly multidrug resistance can be defined as resistance to at least two or more different classes of antibiotics (Shorr, 2009; CDC, 2006; Huys *et al*., 2005; Ortega *et al*., 2004). Multidrug-resistant (MDR) bacteria were detected in the late 1950s and early 1960s. Enteric gram-negative bacilli, such as *Escherichia coli*, S*higella*, and *Salmonella*, were the first MDR bacteria identified (Levy, 2002). Thenafter, other multidrug resistant bacteria have been emerging continuously challenging the antibiotic chemotherapy. Recently, the term extreme drug resistance (XDR) has been suggested to designate gram-negative bacilli that are resistant to all authorized antimicrobial agents except tigecycline and the polymyxins, and pan-drug resistance (PDR) to those bacteria that are also resistant to the latter two drugs (Paterson and Doi, 2007).

In most instances, infections by MDR organisms have clinical manifestations that are similar to infections caused by susceptible pathogens. However, options for treating patients with these infections are often extremely limited, e.g. until recently, only vancomycin provides effective therapy for potentially life-threatening MRSA infections

and during the 1990's there were virtually no antimicrobial agents to treat infections caused by VRE. Moreover, infections due to MDR organisms increase the health cost significantly (Wilcox, 2004; Stephen *et al*., 2004). Although antimicrobials are now available for treatment of MRSA and VRE infections, resistance to each new agent has already emerged in clinical isolates. Resistance to multiple, commonly prescribed antimicrobials among multiple drug resistant gram-negative bacteria raises concerns about available therapeutic options to infections by them (O'Fallon *et al*., 2009).

Therapeutic options are limited for ESBL-producing isolates of gram-negative bacilli, strains of *A. baumannii* resistant to all antimicrobial agents except imipenem and intrinsically resistant *Stenotrophomonas* spp. (Clarke *et al*., 2003; Simor *et al*., 2002). These limitations may influence antibiotic usage patterns in ways that suppress normal flora and create a favorable environment for development of colonization when exposed to potential MDR pathogens, i.e. selective advantage. Increased lengths of hospital stay, costs, and mortality also have been associated with MDR organisms. To reduce the selection pressure for resistance by extensive use of broad spectrum antibiotics, hospital patients can be treated with more narrow-spectrum and target-specific antibiotics after proper antibiotic susceptibility testing of the etiological isolate (Gorgani *et al*., 2009).

Multidrug resistance in bacteria is mainly due to the expulsion of more than one class of drug by multidrug efflux pumps that recognize a broad range of structural and chemically different substrates. However, transfer of the genes coding for these pumps across organisms is uncommon because of the inherent genetic complexity of these pumps. Efflux pumps responsible for multiple drug resistance include highly drug specific ABC transporters and major secondary transporters, viz. MFS, RND, SMR, DMT and MATE transporters that are involved in multiple drug resistance (Poole, 2002).

The chromosomally encoded RND pumps in gram-negative bacteria can be overexpressed easily and some of them can easily pump out most of the antibiotics currently in use. Sometimes combinations of different types of efflux pumps can have synergy between outer membrane impermeability and chromosomally-encoded multidrug

efflux pumps of the RND-MFP-OMF type (Li *et al*., 2000; Germ *et al*., 1999) resulting in higher level of intrinsic multidrug resistance (Nikaido, 2009; Lee *et al*., 2000). Four RND-MFP-OMF type MDR efflux systems have been described in *P. aeruginosa*, including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM. Homologues of these pumps play an equally important role inintrinsic and acquired resistance to antimicrobials in a number of gram-negative organisms (Poole, 2001).

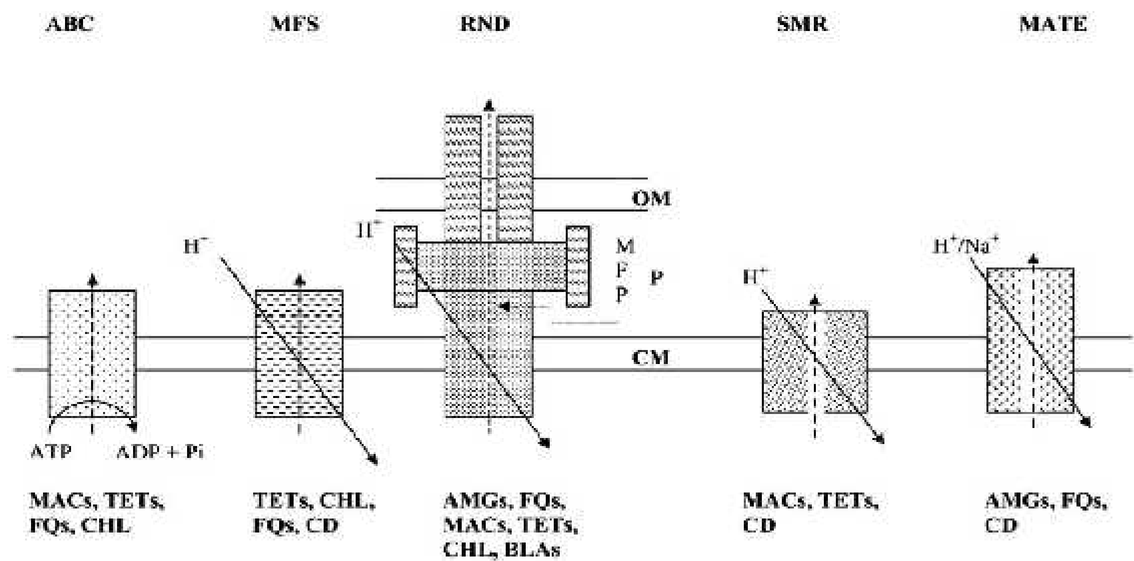


Fig. 1 Bacterial efflux pumps. The figure shows diagrammatically the five structural classes of antibiotic transporters. Abbreviations: OM: outer membrane, P: periplasm, CM: cytoplasmic membrane, MFP: membrane fusion protein, MACs: macrolides, TETs: tetracyclines, FQs: fluorquinolones, CHL: chloramphenicol, CD: cationic drugs, AMGs: aminoglycosides, BLAs: -lactams (Zechini and Versace, 2009).

Limited numbers of bacterial ABC pumps confer MDR phenotype, e.g. LmrA from *Lactococcus lactis* and others described in gram-positive and gram-negative bacteria, such as *E. faecalis* and *V. cholerae*, as well as in *Mycoplasma* pathogens. MacB in *E. coli* confers resistance to macrolides when overexpressed. DrrAB, an ABC efflux pump in *M. tuberculosis*, confers resistance to tetracycline, ethambutol, macrolides, aminoglycosides and chloramphenicol (Piddock, 2006; Chung and Saier, 2001).

The antibiotic susceptibility of bacterial cells is also affected by their physiological states such as occurrence of “persister” cells which is a strategy whereby bacteria naturally generate mixtures of phenotypically different populations, such that one of them can be advantageous to a changing environmental demand limiting the efficacy of antibiotic therapy (Nikaido, 2009). Many bacteria such as *S. aureus* and *P. aeruginosa* become resistant to various antibiotics by biofilm formation (Rao *et al*., 2008).

3.4.1 Multiple drug resistance: A global daunting challange

Multidrug resistance among bacteria is a growing problem in the world. The relentless threat posed by microbial drug resistance has achieved the dimension of a global pandemic, with a relevant impact in terms of morbidity, mortality and health-care associated costs (Cosgrove, 2006). The major resistance challenges are encountered among Enterobacteriaceae, *P.aeruginosa* and *A. baumannii*, which are among the most important causes of nosocomial infections and some Enterobacteriaceae are also important causes of community-acquired infections. Among these gram negative bacteria, clustering of multiple resistance determinants to various classes of antimicrobial agents is a common finding which results in complex MDR phenotypes. MDR and VRE, MRSA, MDR *M. tuberculosis* and MDR gram-negative bacteria are responsible for this emerging crisis (Giske *et al*., 2008; Werner *et al*., 2008; WHO, 2002). Carbapenemase-producing members of Enterobacteriaceae have now been identified in hospitals in at least 20 states in the United States, as well as in other parts of the world, including South America, Israel, China, and, less commonly, Europe (Nordmann *et al*., 2009). The problem is worsened by the dearth of new agents active on multidrug-resistant gram-negatives in the pipeline (Rossolini *et al*., 2007). The emergence of extensively drug resistant (XDR), or even increasingly resistant to vitrually all active antimicrobial agents or pandrug-resistant (PDR), i.e. resistant to all available antibiotics, *A. baumannii*, *P. aeruginosa* and Enterobacteriaceae in Europe has been reported (Souli *et al*., 2008). *S. maltophilia* and *N. gonorrhoeae* also require special attention (Engel, 2009). The growing threat posed by increasing prevalence of ESBLs and carbapenemases among

gram-negative bacteria has resulted in infections that can be extremely difficult to treat, leading to increased morbidity and mortality (Coque *et al*., 2008). Carbapenems may also be losing their effectiveness (Paterson and Bonomo, 2005).

According to a study conducted in Spain, of the 3112 blood isolates of *S. aureus*, 24.5% were oxacillin resistant and among them 68.1% were MDR (Oteo *et al*., 2004). A similar study in India showed methicillin resistance in 34.78% of *S. aureus* isolates, of which 37.5% were found to be resistant to all commonly used antibiotics (Saikia *et al*., 2009). Analysis of data from the National Nosocomial Infections Surveillance System from 1986 to 2003 demonstrated a tenfold increase in resistance among *Klebsiella* spp, twofold increase in resistance among *E. coli*, threefold increase in multidrug resistance among *P. aeruginosa*, and 20% increase in carbapenem-resistant *Acinetobacter* spp. The Study for Monitoring Antimicrobial Resistance Trends (SMART) demonstrated increased detection of ESBL-producing bacteria from 2003 to 2004. In 2004, the percentages of ESBL-producing *E coli*, *Klebsiella* spp, and *Enterobacter* spp. were 10%, 17%, and 22%, respectively (Shorr, 2009; Rossi *et al*., 2006; Gaynes and Edwards, 2005; NNIS, 2004). Increase in ESBL production and carbapenem resistance in *K. pneumoniae* has been reported form Pakistan (Khan *et al*., 2010). All 500 clinical isolates from hospital inpatients and outpatients in Iran were resistant to more than 3 antibiotic classes and a total of 53.8% of them expressed ESBL production. *E. coli* and *K. pneumoniae* were most common in outpatients, and inpatients samples respectively (Mansouri and Abbasi, 2010). Most of urinary isolates isolates in India were resistant to 4 or more number of antibiotics with 42.0% of isolates producing ESBL (Akram *et al*., 2007). A study in Nigeria showed that of the 300 *K. pneumoniae*, 186 (62.0%) isolates produced ESBLs. ESBL producing *K. pneumoniae* were most frequently isolated from blood 76 (40.0%) followed by urine 66 (30.5%) and sputum 44 (23.6%) (Romanus *et al*., 2009). In Singapore, of the 1846 clinical *E.coli* and *Klebsiella* spp. isolates, 28.2% isolates produced ESBL (Chlebicki and Oh, 2004). These studies show the higher prevalence and emergence of multidrug resistance globally among common clinical pathogens.

3.4.2 Multiple drug resistance and ESBL production in Nepal

Nepal is frequently facing a number of different problems and challenges pertaining to antimicrobial resistance. Indiscriminate use of antibiotics by clinicians or pharmacists without the proper clinical and microbiological diagnosis remain the major caouse of emergence of multidrug resistance. No measures are designed to monitor content and quality of antibiotics in various commercial formulations and control the non-medical use of antibiotics also. Large scale researches for antimicrobial resistance surveillance are also deficient. Routine detection of ESBLs is not being practiced.

Besides these causes, the real scenario of emerging antibiotic resistance in the country has been revealed by various studies. In a study of antibiotic resistance pattern of *S. aureus*, carried out in Manipal Teaching Hospital, of the 117 *S. aureus* isolates tested, 15.4% were found to be MRSA, 14 (77.8%) of which were resistant to all agents tested (Subedi and Brahmadathan, 2005). In a study on urinary isolates carried out at National Public Health Laboratory (NPHL), 45.0% of various isolates were MDR among which *E. coli* (51.3%) predominated (Shrestha *et al*., 2007). Of the 121 *S. enterica* serovars Typhi and Paratyphi A, 7 *S*. Typhi isolates were MDR and and all of them had integron-associated multidrug resistance (Tamang *et al*., 2007). A study to assess the load and the antibiotic resistance pattern of bacterial isolates of healthcare liquid waste generated in 10 central hospitals in Nepal showed that more than 50.0% bacteria were multidrug resistant (Sharma, 2004). A study at a tertiary hospital showed that about 56.0% isolates were multi-drug resistant which was most common among *E. faecalis* followed by *P. aeruginosa* (Basnet *et al*., 2009).

ESBL-producing Enterobacteriaceae form a significant percentage (29.6%) of isolates both from the hospital and the community in Nepal. There was increased resistance to other antibiotics in the *E. coli* and *K*. *pneumonia* isolates that had ESBL and/or AmpC activity. Nearly one-third of these infections are becoming untreatable with the available antibiotics in a country that has limited healthcare resources. Fluoroquinolone resistance exceeds 75%, probably reflecting antibiotic selection pressure as some fluoroquinolones

are available over the counter in Nepal (Hammer *et al*., 2007). Many researches have recorded the production of ESBL by various clinical isolates. In a study conducted at Tribhuvan University Teaching Hospital (TUTH), 47.6% of the isolates from the sputum and 60.4% of urinary isolates were MDR strains among which 24.3% and 16.0% of the isolates from sputum and urine respectively were ESBL producers (Pokhrel *et al*., 2006). Another study of 541 blood isolates of *S. enterica* in TUTH showed that 5.0% isolates were MDR strains with 3 isolates of *Salmonella* Paratyphi A demonstrating ESBL activity (Pokhrel *et al*., 2006). ESBLs of SHV type among environmental *Salmonella* Enteritidis isolates has also been detected in Nepal (Bhatta *et al*., 2007). In a similar study at TUTH, 68.3% of the urinary and 71.4% of the sputum isolates showed multidrug resistance among which 12 urinary and 3 sputum isolates produced ESBL (Bomjan, 2005). Similarly, among various clinical isolates in Kathmandu Model Hospital, 41.1% of the clinical isolates were found to be MDR with *E. coli* (46.1%) being the most predominant MDR strain and 100% the MDR *E. coli* demonstrated ESBL production (Baral, 2008). In a study among 203 various clinical isolates at Patan Hospital, ESBLs were detected in 29.6% and AmpC -lactamase in 11.8%; 5.4% isolates had both ESBL and AmpC (Hammer *et al*., 2007).

Controlling antibiotic resistant bacteria and subsequent infections more efficiently necessiates the prudent and responsible use of antibiotics. It is mandatory to prevent the needless use of antibiotics and to improve the timely prescription of appropriate antibiotics to a patient so as to prevent the spread of infection by resistant organisms.

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 Materials, Equipment and Reagents

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

4.2 Methodology

4.2.1 Study site and study period

The study was conducted prospectively at the microbiology laboratory of National Public Health Laboratory (NPHL), Teku, Kathmandu, from March, 2009 to February, 2010.

4.2.2 Study population

The study included patients of all age groups and both sexes visiting NPHL, from whom the samples were sent for routine culture and antibiotic susceptibility testing.

4.2.3 Number and types of specimen

A total of 2141 different specimens including urine, pus, sputum, body fluids, ear and eye specimens, and throat swabs that were sent for routine culture and antibiotic susceptibility testing were processed during the study period. Specimens obtained in a clean, leak proof container without visible contamination and proper label with sufficient patient information were processed. Otherwise, a second proper specimen was requested.

4.2.4 Data collection

The demographic parameters, brief clinical history, prior antibiotic use, if yes duration and type, were recorded using a standard questionnaire as shown in Appendix I.

4.2.5 Specimen collection and transport

4.2.5.1 Urine specimens

The older children, non-menstruating women, and men patients were asked to collect 10-20 ml of clean-catch first morning mid stream urine (MSU) in a sterile, dry, wide necked, leakproof container, instructing them not to stop and restart the urination for a midstream urine collection but preferably move the container into the path of the continuous stream of voiding urine. Catheterized specimens or supra-pubic aspirates were collected with the assistance of a physician from infants and patients who are unable to provide clean-catch specimens because of urologic or neurologic problems including impaired consciousness. The container was then properly labelled and immediately delivered to the laboratory with the request form for further processing. In case of any delay, the specimen was refrigerated at 4-6°C, and when a delay of more than 2 hours in delivery was anticipated, boric acid (1.8% w/v) was added as preservative to the urine.

4.2.5.2 Sputum and throat swab specimens

The sputum sample was collected in a sterile, leakproof, disposable container. The patient was asked to gargle the mouth with sterile water (not with mouthwash) prior to sample collection and to collect specimen (4-6ml sputum) resulting from deep cough, not the saliva or post-nasal discharge, preferably in the morning soon after he wakes up. The patient was also instructed not to soil the outside of the container with the sputum. The container was then properly labelled and immediately delivered to the laboratory. For collecting throat swab, first the patient's head was leaned in a backward position as far as possible and the swab was inserted through the mouth with the aid of tongue depressor. The swab was rubbed over the inflamed tonsillar area and posterior pharynx, selecting inflamed or membranous areas. The swab was rotated over all of the affected areas and withdrawn avoiding the touch to tongue or other areas of the mouth. It was then replaced in Stuart's Transport Medium (STM), labeled and transported to the laboratory promptly. In case of delayed transport, specimen was refrigerated.

4.2.5.3 Eye and ear specimens

For conjunctival swab, first the excess debris from the outside of the eye was cleaned with normal saline and guaze pad, wiping from inner to outer canthus. Then, one or two drops of topical anesthetic were instilled and a moistened swab with sterile physiologic saline was taken and carefully rubbed over lower conjunctiva to collect epithelial cells without touching other surfaces. The swab was held parallelly to the eye rather than pointed directly to it to avoid irritation. One swab per eye was used. For conjunctival/corneal scrapings, specimens were collected by ophthalmologist using a wire culture loop to get epithelial cells. The conjunctival swab or wire culture loop was then transferred into STM, properly labeled and transported to the laboratory. Vitreous taps (0.1-0.3ml) were collected by ophthalmologist by needle aspiration method.

For external ear specimens, the excess debris was cleaned from the patient’s ear by using normal saline and guaze pads. The sterile swab was inserted into the ear canal and rotated gently against the walls of the canal avoiding damage to the eardrum. The swab was drawn out without touching the other surfaces to prevent contamination, transferred into STM, properly labeled and immediately transported to the laboratory.

4.2.5.4 Pus specimens

Pus specimens were usually obtained from wounds or abscesses that were clinically infected or deteriorating or stubborn. In case of closed wounds and aspiration, surface disinfection was done with 2.0% cholorohexidine followed by an iodine solution, whereas for open wound, the wound was first debrided and rinsed thoroughly with sterile normal saline prior to collection of pus specimen. Pus specimen was collected aseptically in such a way that it contained the deepest portion of the lesion or exudate rather than superficial debris. Mostly, aspirates (~ 5ml pus) or biopsy samples were preferred as far as possible, and in other cases swabbing was done with sterile cotton wool swab by gently rolling over the surface of the wound approximately five times, particularly focusing the obviously purulent, inflamed or necrotic tissue. The pus swabs thus

collected were transported to the laboratory in Stuart's Transport Medium, properly labelled with demographic information, type of specimen and anatomic site. For fine needle aspirarion cytology (FNAC) and other aspirated specimens, the syringe was properly capped, labelled and immediately dispathced to the laboratory.

4.2.5.5 Body fluid specimens (pleural, peritoneal, cerebrospinal and synovial fluids)

Body fluid specimens were obtained with the help of trained physicians by aseptic percutaneous aspiration. The puncture site was first cleaned and disinfected with alcohol followed by iodine solution. Then, about 1-5ml of the fluid was drawn with syringe and transported to laboratory, properly labelled, in a sterile tube or vial for further processing.

Cerebrospinal fluid (CSF) specimens were aseptically collected with the help of physicians either by lumbar or ventricular puncture. The puncture site was disinfected with the help of antiseptic solution and alcohol prior to collection then 1-2ml of CSF was allowed to drain slowly into dry, sterile, leakproof tubes. The tubes were properly labelled, and dispatched to laboratory as soon as possible. In case of delay in processing, they were kept at room temperature rather than refrigerated.

4.2.5.6 Semen

The patient was given a sterile, clean, dry, leakproof container and requested to collect the semen at home by masturbation, not by coitus interruptus, following 3-7 days of sexual abstinence. The patient was also instructed to label the specimen properly with collection time, period of abstinence and dispatch to laboratory as soon as possible at around body temperature by wrapping it in plastic bag and carrying it in his clothing.

4.2.6 Gross and microscopic examination

The urine specimens were grossly observed for color, turbidity, presence of blood and crystals, deposits, and reported accordingly. The sputum specimens were observed for presence of only saliva or real sputum. If it was found only to be watery, it was reported

as ‘unsuitable for microbiological examination’ and another proper specimen was requested. Body fluid specimens also were observed for presence of blood, turbidity, quantity, any deposits or suspended particles, visible contamination, and proper labeling. Other specimens such as pus, swabs, scrappings, etc. were observed whether they were sent in proper transporatation media or not. Microscopy of smears of sputum and throat swab, pus, body fluids, various swabs and scrapings was also done following appropriate staining techniques for the presumptive diagnosis of the infection.

4.2.7 Culture of Specimens

4.2.7.1 Urine culture

The urine specimens were cultured onto the MacConkey agar (MA) and Blood agar (BA) plates by the semi-quantitative culture technique using a standard calibrated loop that delivered 0.01ml of urine. A loopful of urine was taken by immersing the calibrated loop vertically just below the surface of throughly mixed uncentrifuged/unspun urine specimen and then streaked onto the plate accordingly. The loop was touched to the center of the plate from which the inoculum was spread in a line across the diameter of the plate. Then without flaming or re-entering urine, the loop was drawn across the entire plate, crossing the first inoculum streak numerous times to produce isolated colonies. The plates were then incubated aerobically at 37°C overnight. Colony count was performed so as to calculate the number of colony forming units (CFU) per ml of urine and the bacterial count was reported as:

- 10^4 CFU/ml organisms: **not significant bacteriuria**
- 10^4-10^5 CFU/ml organisms: **doubtful** (suggested for repeated specimen collection)
- 10^5 CFU/mL of urine: **significant bacteriuria**

In case of significant growth of two bacteria, both were identified and tested for antibiotic susceptibility. However, growth of 3 pathogens was reported as mixed bacterial growth and the patient was asked to recollect the specimen and deliver it to laboratory properly.

4.2.7.2 Sputum culture

The culture of sputum specimens was carried out in BA, Chocolate agar (CA) and MA plates. For sputum, a 5µg Optochin disk and a 10U Bacitracin disk were added in CA plate to screen out *S. pneumoniae* and *H. influenzae* respectively, whereas for throat swab, 0.05U bacitracin disk was added to the plate to screen *S. pyogenes*. The CA and BA plates were incubated overnight at 37°C in 5-10% CO_2 environment whereas the MA plate was incubated at 37°C aerobically.

4.2.7.3 Pus, body fluids, CSF, eye and ear specimens, and semen culture

These samples were priomarily inoculated into BA, CA and MA plates for isolation and Mueller Hinton Broth (MHB) tubes for enrichment if the specimen contained very low number of organisms. The BA and CA plates were incubated in a 5-10% CO_2 enriched atmosphere at 37°C whereas MA plates and MHB tubes were incubated aerobically at 37°C overnight. In case of no growth from primary inoculation, the MHB enriched culture was used to re-inoculate the plates.

4.2.8 Bacterial identificaion

The identification of the bacterial isolates was performed by following standard diagnostic procedures. Gram-staining, bacitracin-optochin sensitivity test, bile solubility test, various biochemical tests, etc. were performed to identify gram positive bacteria whereas gram negative bacteria were identified by techniques based on morphological, staining and biochemical properties according to Bergey's Manual of Systematic Bacteriology. Various conventional biochemical tests used are described in Appendix V.

4.2.9 Antimicrobial susceptibility testing

Antimicrobial susceptibility test of the different clinical isolates was performed by following Kirby-Bauer disk diffusion method for the commonly isolated pathogens using Mueller Hinton agar (MHA) whereas for the less frequently isolated or fastidious bacteria

5.0% blood containing MHA or CA were used as described by CLSI. MDR bacteria were screened by testing with routinely used common antibiotics. Isolates showing intermediate susceptibility were interpreted as resistant ones. The MDR isolates were also screened for ESBL production by using all CLSI recommended screening agents and suspected MDR isolates for ESBL production were subsequently subjected to antibiotic susceptibility testing to broader panels of antibiotics and phenotypic confirmation of ESBL production. Any aberrant result obtained during the experiment was confirmed by repeating the test twice and processing accordingly. The detailed account of test procedures is given in Appendix VI.

4.2.10 Preservation of the MDR isolates

The suspected MDR isolates for ESBL production after performing the primary antimicrobial susceptibility testing and ESBL screening were preserved in pure culture in Tryptic Soya Broth containing 20.0% Glycerol and kept at -70°C until subsequent tests for ESBL detection and susceptibility to broader antibiotic panels were performed.

4.2.11 Screening for ESBL production

All MDR isolates were subjected to screening for ESBL production using all CLSI recommended screening agents, viz. ceftriaxone (30µg), ceftazidime (30µg), cefotaxime (30µg), cefpodoxime (10µg), and aztreonam (30µg). The MDR isolates showing reduced susceptibility to one or all of the above with zone of inhibiton diameter for cefpodoxime $\leq$17mm, ceftazidime $\leq$22mm, aztreonam $\leq$27mm, cefotaxime $\leq$27mm, and ceftriaxone $\leq$25mm were considered as the possible ESBL producers.

4.2.12 Confirmation of ESBL production

The suspected ESBL producers were subjected to Combined Disk (CD) test for phenotypic confirmation of ESBL production using MASTDISCS™ ID Extended Spectrum Beta-Lactamase (ES L) Detection Discs (D52C) and MASTDISCS™ ID Cefepime ES L ID Disc Set (D63C). The former kit consisted of ceftazidime (30µg) and

ceftazidime (30µg) plus clavulanic acid (10µg), cefotaxime (30µg) and cefotaxime (30µg) plus clavulanic acid (10µg), and cefpodoxime (30µg) and cefpodoxime (30µg) plus clavulanic acid (10µg). The later consisted of cefepime (30µg) and cefepime (30µg) plus clavulanic acid (10µg). The zone of inhibition for the ceftazidime, cefotaxime, cefpodoxime and cefepime disks was compared with that of disks containing their respective combinations with clavulanic acid and an increase in zone diameter by 5mm in the presence of clavulanic acid for any one or all of the sets was concluded as confirmed ESBL producers. The detailed working protocol is described in appendix VII. *K. pneumonia* ATCC 700603 and *E. coli* ATCC 25922 were used as positive (ESBL producer) and negative (ESBL non-producer) controls, respectively.

4.2.13 Data analysis

All the results obtained were entered in to the worksheet of statistical package for social science (SPSS) software (Version 17.0) and analyzed. Chi-square test was used to determine significant association between dependent variables like resistance to fluoroquinolones, aminoglycosides, etc. to independent variable like ESBL production. Sensitivity and positive predictive value of the screening and confirmatory methods for ESBL detection were also determined.

4.2.14 Quality Control

A. Monitoring and regular evaluation of laboratory euipment, reagents and media

Laboratory equipment like incubator, refrigerator, autoclave and hot air oven were regularly monitored for their efficiency. The temperature of the incubator and refrigerator was monitored twice a day. Reagents and media were regularly monitored for their manufacture and expiry date and proper storage conditions. After preparation, they were properly labelled with prepartion date and self-life. The quality of media prepared was checked by subjecting one plate of each batch for sterility and performance testing.

B. Purity Plate

The purity plate was used to ensure that the inoculation used for the biochemical tests was pure culture and also to check maintenance of aseptic conditions. Thus, while performing biochemical tests, the same inoculum was subcultured in respective medium, incubated and checked for the appearance of pure growth of organisms.

C. Quality control during antimicrobial susceptibility testing

MHA and the antibiotic disks were checked for their lot number, manufacture and expiry date, and proper storage. For the standardization of Kirby-Bauer test and for performance testing of antibiotics and MHA, control strains of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were tested primarily. The thickness of MHA was maintained at 4mm and the pH at 7.2-7.4. Similarly antibiotic disks containing the correct amount of the antibiotic as required by the test were used.

4.2.15 Limitations of the study

Inability to include large number of various clinical specimens from different territories, no determination of MICs and resistance mechanisms of antibiotics tested except ESBL detection, and no genetic analysis of the isolates remained the limitations of the study.

LABORATORY PROCEDURES

A. Organism isolation, identification and antimicrobial susceptibility testing

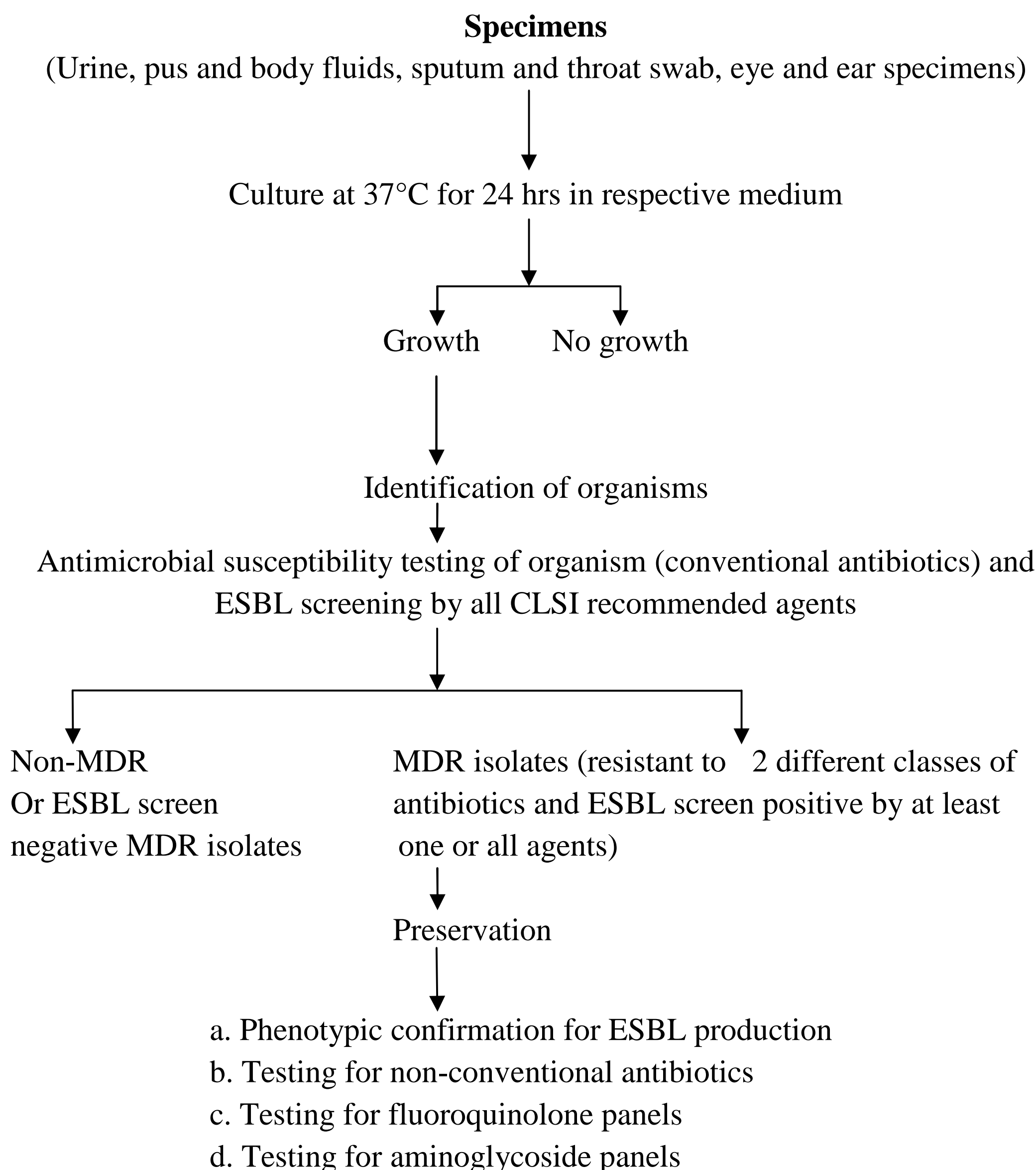
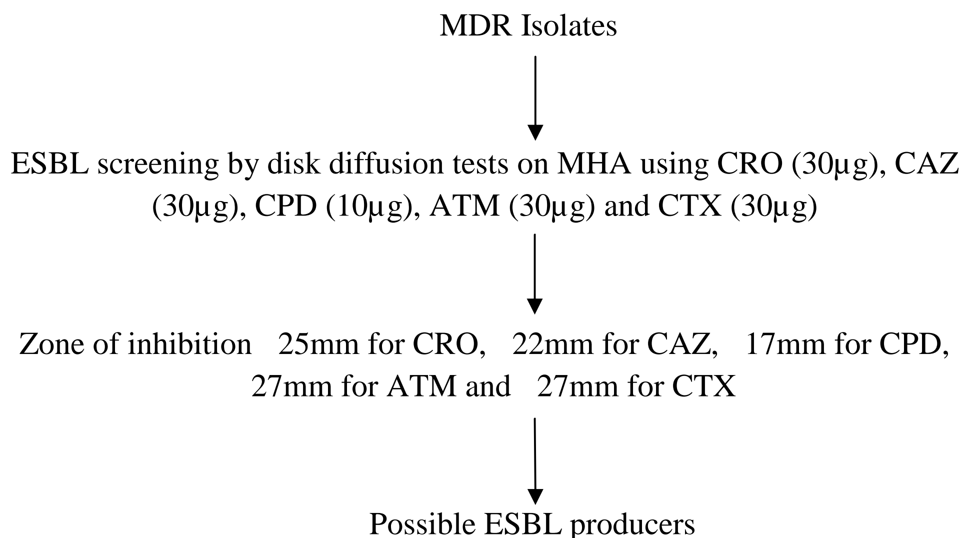


Fig. 2 Flow chart for organism isolation, identification and antibiotic susceptibility testing

B. Detection of ESBL production

1. Screening for ESBL production



2. Phenotypic Confirmation of ESBL Production

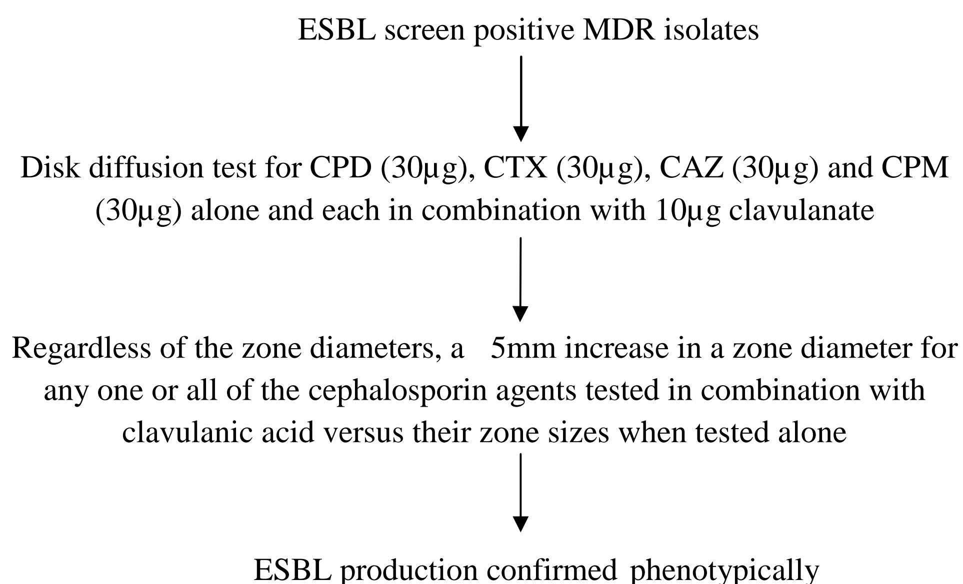


Fig. 3 Flow chart for comparative ESBL screening by different screening agents and phenotypic confirmation of ESBL production by combined disk synergy test (CDST).

CHAPTER-V

5. RESULTS

5.1 Clinical and Microbiological Profiles of Various Clinical Specimens

5.1.1 Clinical profiles of various specimens

Of the total 2141 clinical specimens processed (1710 urinary specimens, 136 pus and body fluids, 170 sputa and throat swabs, and 125 eye and ear specimens of which 53.4% of them were from males and 46.6% were from females), 20.2% specimens grew significantly with maximum isolates (61.2%) from urine. Maximum growth rate was observed in eye and ear specimens (51.2%) followed by pus and body fluids (40.4%), sputum and throat swab (28.8%) and least in urine specimens (15.5%). Among urinary isolates, greater growth rate was observed in females (18.1%) than males (13.1%) and similar was the case among other specimens too. (Table 5.1.1)

Table 5.1.1 Clinical profiles of various specimens

Specimens	Female		Male		Total No. of specimens	Total growth positivity (No. and %)
	No. of specimens	Growth positivity (No. and %)	No. of specimens	Growth positivity (No. and %)		
Urine	815	148 (18.1)	895	117 (13.1)	1710	265 (15.5)
Pus and body fluids	42	22 (52.4)	94	33 (35.1)	136	55 (40.4)
Sputum and throat swabs	75	24 (32.0)	95	25 (26.3)	170	49 (28.8)
Eye and ear specimens	66	37 (56.1)	59	27 (45.8)	125	64 (51.2)
Total	**998**	**231 (23.1)**	**1143**	**202 (17.7)**	**2141**	**433 (20.2)**

5.1.2 Microbiological profiles of various clinical specimens

Of the 433 various bacterial isolates, gram negative bacteria predominated with 292 (67.4%) isolates among which *E. coli* (51.7%) was the most predominant organism followed by *K. pneumoniae* with (14.7%). On the other hand, *S. aureus* and *S. pyogenes* predominated among gram positive bacteria with 41.8% and 28.9% isolates, respectively.

Most of the gram positive bacteria (31.9%) were isolated from eye and ear specimens while most of the gram negative bacteria (83.2%) were isolated from urinary specimens with (56.4%) isolates from females. *S. aureus* was the dominant species among pus and body fluids and eye and ear specimens with 79.4% and 48.9% isolates, respectively. Moreover, *S. pyogenes* was the most frequently isolated gram positive bacteria among sputum and throat swab specimens with 65.0% isolates and *E. faecalis* among urinary specimens with 45.4% isolates. The most predominant isolate among urinary gram negative bacteria was *E. coli* (60.9%) followed by *K. pneumoniae* (11.9%) among which 60.8% *E. coli* and 62.1% *K. pneumoniae* isolates were isolated from females and males, respectively. (Table 5.1.2)

Table 5.1.2 Microbiological profiles of various clinical specimens

Organisms	No. of isolates from different specimens					Total No. of isolates
	Urine		Pus and body fluids	Sputum and throat swabs	Eye and ear specimens	
	Female	Male				
Gram negative bacteria						
*Acinetobacter s*pp.	14	9	3	0	2	28
Alkaligenes spp.	1	0	0	0	0	1
C. freundii	4	3	0	0	2	9
Edwardsiella spp.	1	1	0	0	0	2
Enterobacter spp.	2	1	0	0	0	3
E. coli	90	58	3	0	0	151
H. influenzae	0	0	0	1	2	3
H. parainfluenzae	0	0	0	1	0	1
K. oxytoca	7	5	2	0	0	14
K. pneumoniae	11	18	6	5	3	43
Moraxella spp.	3	0	1	0	8	12
M. morganii	1	1	0	0	0	2
Neisseria spp.	0	0	0	0	1	1
P. mirabilis	2	1	0	0	0	3
P. vulgaris	0	1	0	0	0	1
Providencia spp.	0	3	1	0	0	4
P. aeruginosa	1	5	4	2	1	13
Serratia spp.	0	0	1	0	0	1
Total	**137**	**106**	**21**	**9**	**19**	**292**
Gram positive bacteria						
CONS	0	2	3	0	0	5
E. faecalis	8	2	0	0	0	10
Hemolytic streptococci	0	2	0	0	0	2
S. pneumoniae	0	0	0	4	15	19
S. pyogenes	0	0	2	26	5	33
S. aureus	3	5	27	2	22	59
Viridans streptococci	0	0	2	8	3	13
Total	**11**	**11**	**34**	**40**	**45**	**141**

5.2 Antibiotic Resistance Patterns of Clinical Isolates

5.2.1 Antibiotic resistance among gram negative isolates

Of the 14 primarily tested antibiotics to screen MDR gram negative bacteria from various clinical specimens, amikacin was found to be the drug of choice with only 28.0% resistance. Ceftazidime, nitrofurantoin and chloramphenicol also had relatively lower resistance rates of 28.6%, 29.3% and 30.1%, respectively. Amoxycillin had the highest resistance rate of 89.0% followed by azithromycin with 82.6% and nalidixic acid with 80.5% resistance. Resistance rates to other antibiotics tested were also relatively higher. (Table 5.2.1)

Table 5.2.1 Antibiotic resistance among gram negative isolates

S.N.	Antibiotics used and their potencies	Percentage of resistant isolates	Total No. of isolates tested
1.	Tetracycline (30 µg)	60.6	292
2.	Amoxycillin (30 µg)	89.0	292
3.	Chloramphenicol (30 µg)	30.1	292
4.	Co-trimoxazole (25 µg)	62.2	291
5.	Nitrofurantoin (300 µg)	29.3	246
6.	Nalidixic acid (30 µg)	80.5	256
7.	Ciprofloxacin (5 µg)	66.1	292
8.	Ofloxacin (5 µg)	62.1	288
9.	Norfloxacin (10µg)	62.9	248
10.	Ceftriaxone (30 µg)	43.5	292
11.	Azithromycin (15 µg)	82.6	167
12.	Gentamicin (10 µg)	44.7	282
13.	Amikacin (30 µg)	28.0	282
14.	Ceftazidime (30 µg)	28.6	21

5.2.2 Antibiotic resistance among gram positive isolates

Of the 18 primarily tested antibiotics against various gram positive bacteria, nitrofurantoin and norfloxacin were found to be the best drugs with least resistance of only 9.5% for each. Chloramphenicol, tetracycline, ceftriaxone, nalidixic acid and ceftazidime had lower resistance rates of 10.6%, 12.8%, 14.2%, 14.3% and 14.7%, respectively. Lower resistance patterns were also seen with erythromycin, ofloxacin and penicillin G. Most (47.5%) isolates were resistant to ciprofloxacin followed by gentamicin (44.4%) and co-trimoxazole (41.8%). Fewer (21.3%) isolates were resistant to erythromycin than azithromycin (28.7%). Of the 59 isolates tested, 22.0% isolates were found to be oxacillin resistant of which 76.9% were resistant to methicillin. (Table 5.2.2)

Table 5.2.2 Antibiotic resistance among gram positive isolates

S.N.	Antibiotics used and their potency	Percentage of resistant isolates	Total No. of isolates tested
1.	Tetracycline (30 µg)	12.8	141
2.	Amoxycillin (30 µg)	39.0	141
3.	Chloramphenicol (30 µg)	10.6	141
4.	Co-trimoxazole (25 µg)	41.8	141
5.	Nitrofurantoin (300 µg)	9.5	21
6.	Nalidixic acid (30 µg)	14.3	21
7.	Ciprofloxacin (5 µg)	47.5	141
8.	Ofloxacin (5 µg)	23.4	141
9.	Norfloxacin (10µg)	9.5	21
10.	Ceftriaxone (30 µg)	14.2	141
11.	Azithromycin (15 µg)	28.7	136
12	Gentamicin (10 µg)	44.4	18
13.	Amikacin (30 µg)	35.3	17
14.	Oxacillin (1 µg)	22.0	59
15.	Erythromycin (15 µg)	21.3	141
16.	Penicillin G (10 U)	25.9	135
17.	Methicillin (5 µg)	76.9	13
18.	Ceftazidime (30 µg)	14.7	34

5.3 Distribution and Microbiological Profiles of MDR Isolates among Various Clinical Specimens

5.3.1 Distribution of MDR bacteria among various clinical specimens

Of the total 433 isolates from various clinical specimens, 64.9% of them were MDR isolates. Maximum multiple drug resistance was observed among urinary isolates (78.1%) and least multiple drug resistance was seen among the eye and ear specimens (32.8%). Predominance of MDR isolates among sputum and throat swab (51.0%), and pus and body fluid specimens (50.9%) was almost same. Of the total 281 MDR isolates, most of them (73.7%) were urinary isolates followed by 9.9% pus and body fluid isolates, 8.9% sputum and throat swab isolates and 7.5% eye and ear specimen isolates. Distribution of MDR bacteria among males and females was found statistically insignificant ($p>0.05$). (Table 5.3.1)

Table 5.3.1 Distribution of MDR bacteria among various clinical specimens

Specimens	No. of specimens	Growth positivity (%)	No. of MDR isolates (%) among the genders		
			Males	Females	Total
Urine	1710	15.5	38.1	40.0	78.1
Pus and body fluids	136	40.4	30.9	20.0	50.9
Sputum and throat swabs	170	28.8	20.4	30.6	51.0
Eye and ear specimens	125	51.2	12.5	20.3	32.8
Total	**2141**	**20.2**	**31.4**	**33.5**	**64.9**

5.3.2 Microbiological profiles of MDR bacteria among various clinical specimens

Of the total 281 MDR isolates, 77.9% were gram negative bacteria and most of them (87.7%) were urinary isolates. Least (2.7%) MDR gram negative isolates were isolated from sputa and throat swabs. Among MDR gram positive bacteria, 30.6% were isolated from sputa and throat swabs, 24.2% from urinary specimens and 22.6% from both pus

and body fluids, and eye and ear specimens. Among MDR gram negative bacteria, *E. coli* predominated with 52.0% isolates followed by *K. pneumoniae* (15.5%) and *Acinetobacter spp.* (9.1%) isolates.

Similarly, of the 62 MDR gram positive isolates, *S. aureus* constituted 50.0% of the isolates followed by *S. pyogenes* (17.4%). All the isolates of *Edwardsiella* spp., *M. morganii*, *P. vulgaris* and *Serratia spp*. were MDR. Among gram negative bacteria, higher MDR rate was seen in *K. oxytoca* (92.8%), *P. aeruginosa* (92.3%), *K. pneumoniae* (79.1%), *E. coli* (75.5%), *Providencia* spp. (75.0%) and *Acinetobacter* spp. (71.4%). Multidrug resistance among gram positive bacteria was more frequent among isolates of *E. faecalis* (80.0%) and *S. aureus* (52.5%). Multidrug resistance among viridans streptococci was also considerable (46.1%). (Table 5.3.2)

Table 5.3.2 Microbiological profiles of MDR bacteria among various clinical specimens

Organisms (total No. of isolates)	No. of MDR bacteria among various specimens				Total MDR Isolates (%)
	Urine	Pus and body fluids	Sputum and throat swabs	Eye and ear specimens	
Gram negative bacteria					
Acinetobacter spp. (28)	18	2	0	0	20(71.4)
Alkaligenes spp. (1)	0	0	0	0	0(0.0%)
C. freundii (9)	6	0	0	0	6(66.7%)
Edwardsiella spp. (2)	2	0	0	0	2(100.0)
Enterobacter spp. (3)	2	0	0	0	2(66.7)
E. coli (151)	112	2	0	0	114(75.5)
H. influenza (3)	0	0	0	0	0(0.0)
H. parainfluenzae (1)	0	0	0	0	0(0.0)
K. oxytoca (14)	11	2	0	0	13(92.8)
K. pneumoniae (43)	25	2	5	2	34(79.1)
Moraxella spp. (12)	3	0	0	4	7(58.3)
M. morganii (2)	2	0	0	0	2(100.0)
Neisseria spp. (1)	0	0	0	0	0(0.0)
P. mirabilis (3)	2	0	0	0	2(66.7)
P. vulgaris (1)	1	0	0	0	1(100.0)
Providencia spp. (4)	2	1	0	0	3(75.0)
P. aeruginosa (13)	6	4	1	1	12(92.3)
Serratia spp. (1)	0	1	0	0	1(100.0)
Total (292)	**192**	**14**	**6**	**7**	**219 (75.0)**
Gram positive bacteria					
CONS (5)	1	0	0	0	1(20.0)
E. faecalis (10)	8	0	0	0	8(80.0)
Hemolytic streptococci (2)	1	0	0	0	1(50.0)
S. pneumonia (19)	0	0	2	2	4(21.0)
S. pyogenes (33)	0	0	11	0	11(33.3)
S. aureus (59)	5	13	1	12	31(52.5)
Viridans streptococci (13)	0	1	5	0	6(46.1)
Total (141)	**15**	**14**	**19**	**14**	**62 (44.0)**

5.4 Distribution of MDR Isolates Among Various Clinical Specimens and Their ESBL Production Profiles

5.4.1 Distribution of ESBL producing MDR isolates among clinical specimens

Among 281 MDR isolates from various clinical specimens, 142 isolates suspected of ESBL production were subjected to the ESBL detection test and 59.9% of them were found to produce ESBL. Maximum number of urinary isolates (68.4%) produced ESBLs. ESBL production was also observed among 50.0% of tested isolates from sputum and throat swab and 20.8% isolates from pus and body fluids whereas no isolates from eye and ear specimens were found to produce ESBL possibly in screening test and were not subjected to phenotypic confirmatory test for ESBL. (Table 5.5.1)

Table 5.4.1 Distribution of ESBL producing MDR isolates among clinical specimens

Specimens	No. of MDR strains	No. of isolates tested	No. of ESBL producers (%)	No. of ESBL Non-producers (%)
Urine	207	114	68.4	31.6
Pus and Body Fluids	28	24	20.8	79.2
Sputum and Throat swab	25	4	50.0	50.0
Eye and Ear specimens	21	0	00.0	0.0
TOTAL	**281**	**142**	**59.9**	**40.1**

5.4.2 ESBL production patterns among various MDR bacterial genera

Among 85 ESBL positive isolates, *E. coli* isolates constituted to most of the ESBL producers (77.6%) followed by *K. oxytoca* (7.0%), *K. pneumoniae* (7.0%), *C. freundii* (3.5%), *Acinetobacter* spp. (3.5%) and *P. mirabilis* (1.2%). Moreover, 77.6% *E. coli*, 75.0% K. oxytoca, 66.7% *K. pneumoniae*, 50.0% *C. freundii* and 16.7% *Acinetobacter* spp. tested ESBL positive. One isolate of *P. mirabilis* tested also produced ESBL. No ESBL production was detected in *Providencia* spp., *P. aeruginosa* and *S. aureus*. Most of

the ESBL producers (91.8%) were from urinary specimens among which *E. coli* was most predominant organism with 80.8% isolates. Also, 5.9% ESBL producers were from pus and body fluids and 2.3% were from sputum and throat swabs. (Table 5.4.2)

5.4.2 ESBL production patterns among various MDR bacterial genera

Organisms (No. of MDR isolates tested)	ESBL production	No. of isolates in different clinical specimens			
		Pus and body fluids	Sputum	Urine	Total
Acinetobacter spp. (18)	Positive	1	0	2	3
	Negative	9	0	6	15
C. freundii (6)	Positive	0	0	3	3
	Negative	1	0	2	3
E. coli (85)	Positive	3	0	63	66
	Negative	0	0	19	19
K. oxytoca (8)	Positive	1	0	5	6
	Negative	0	0	2	2
K. pneumoniae (9)	Positive	0	2	4	6
	Negative	0	1	2	3
P. mirabilis (1)	Positive	0	0	1	1
	Negative	0	0	0	0
Providencia spp. (1)	Positive	0	0	0	0
	Negative	0	0	1	1
P. aeruginosa (7)	Positive	0	0	0	0
	Negative	3	0	4	7
S. aureus (7)	Positive	0	0	0	0
	Negative	6	1	0	7
Total (142)	Positive	**5**	**2**	**78**	**85**
	Negative	**19**	**2**	**36**	**57**

5.5 Comparative Efficacy of Different ESBL Screening Agents

Of the 281 MDR bacterial isolates subjected to ESBL screening test by using all five screening agents recommended by CLSI to compare their relative efficacy, altogether 142 isolates were ESBL screen positive by one or all agents. Cefpodoxime and ceftriaxone had the highest sensitivities of 96.5% but cefpodoxime had lowest PPV of 67.2% and ceftriaxone had lower PPV of 68.3%. Aztreonam and cefotaxime both had equal sensitivity of 94.1% and PPV of 67.8%. Though ceftazidime had lowest sensitivity of only 83.5%, it had the highest PPV of 79.8%. The screening results obtained with ceftazidime versus cefotaxime was found statistically insignificant (p>0.05). (Table 5.5)

Table 5.5 Comparative efficacy of different ESBL screening agents

Screening agents for ESBL production	Screening criteria with inhibition zone size (mm)	No. of isolates in screening results		No. of ESBL confirmed isolates	Sensitivity (%)	Positive predictive value (PPV) (%)
Aztreonam (30µg)	27	Positive	118	80	94.1	67.8
		Negative	24	5		
Cefotaxime (30µg)	27	Positive	118	80	94.1	67.8
		Negative	24	5		
Cefpodoxime (10µg)	17	Positive	122	82	96.5	67.2
		Negative	20	3		
Ceftazidime (30µg)	22	Positive	89	71	83.5	79.8
		Negative	53	14		
Ceftriaxone (30µg)	25	Positive	120	82	96.5	68.3
		Negative	22	3		

5.6 Efficacy of Different Combined Disks in ESBL Confirmation

Among 142 MDR isolates tested for confirmation of ESBL production by using four types of combination disks, maximum ESBL producers 97.6% were detected by Cefepime-clavulanate combination disks. Ceftazidime-clavulanate and Cefotaxime-clavulanate combination disks both detected only 95.3% ESBL producers. Ceftazidime and cefotaxime disks each in combination with clavulanate showed 10 and 1 more ESBL producers respectively than in respective screening tests. Cefpodoxime-clavulanate combination disks detected least number of ESBL producers with only 91.8% positive isolates and it detected 4 less ESBL producers than in respective screening test.

Table 5.6 Efficacy of different combination disks in ESBL confirmation

<table>
<tr><th>Combined disks and their potencies</th><th>Criterion for confirmation</th><th>No. of isolates tested</th><th>No. of ESBL confirmed isolates (%)</th><th>No. of ESBL negative isolates</th><th>Total No. of ESBL confirmed isolates (%)</th><th>Total No. of ESBL negative isolates</th></tr>
<tr><td>CPD 10 µg and CPD 10 µg plus CV 1 µg</td><td rowspan="4">Increase in size of inhibition zone by 5 mm for combination disk than the respective disk alone for at least one or all agents</td><td>142</td><td>78 (91.8)</td><td>7</td><td rowspan="4">85 (59.9)</td><td rowspan="4">57</td></tr>
<tr><td>CAZ 30 µg and CAZ 30 µg plus CV 10 µg</td><td>142</td><td>81 (95.3)</td><td>4</td></tr>
<tr><td>CTX 30 µg and CTX 30 µg plus CV 10 µg</td><td>142</td><td>81 (95.3)</td><td>4</td></tr>
<tr><td>CPM 30 µg and CPM 30 µg plus CV 10 µg</td><td>142</td><td>83 (97.6)</td><td>2</td></tr>
</table>

5.7 ESBL Production and Resistance Patterns of MDR Isolates to Various Antibiotics

5.7.1 ESBL production and penicillin resistance

Almost all isolates tested were highly resistant to amoxycillin (97.9%) and ticarcillin (90.1%). Among 85 ESBL positive isolates, maximum resistance (97.6%) was observed against amoxycillin followed by ticarcillin (94.1%). On the flip side, 98.2% ESBL negative isolates were also resistant to amoxycillin and 84.2% were resistant to ticarcillin. All *S. aureus* isolates tested were resistant to amoxycillin, ticarcillin, penicillin G, oxacillin and methicillin.

Altogether, only 27.5% of all MDR isolates were resistant to temocillin. Regarding to ESBL production, 24.7% ESBL positive and 31.6% ESBL negative isolates were resistant to temocillin. Among ESBL positive isolates, 66.7% *Acinetobacter* spp., 50.0% *K. pneumoniae*, and 33.3% of each of *C. freundii* and *K. oxytoca* were resistant to temocillin. Among ESBL negative isolates, all isolates of *C. treundii*, 85.7% of *P. aeruginosa* and 66.7% of *K. pneumoniae* showed resistance to temocillin. No isolate of ESBL negative *K. oxytoca* and fewer (13.3%) ESBL negative *Acinetobacter* spp. were resistant to temocillin. Activity of temocillin against most MDR organisms, particularly against MDR *Acinetobacter spp*. isolates was of note. (Table 5.7.1)

Table 5.7.1 ESBL production and penicillin resistance

Organisms (No. of MDR isolates tested)	ESBL production (No. of organisms)	Antibiotics and No. of resistant isolates					
		A	TC	OX	M	PG	TEM
Acinetobacter spp. (18)	Positive (3)	3	1	-	-	-	2
	Negative (15)	15	15	-	-	-	2
C. freundii (6)	Positive (3)	3	3	-	-	-	1
	Negative (3)	3	2	-	-	-	3
E. coli (85)	Positive (66)	65	64	-	-	-	13
	Negative (19)	18	16	-	-	-	4
K. oxytoca (8)	Positive (6)	6	6	-	-	-	2
	Negative (2)	2	1	-	-	-	0
K. pneumoniae (9)	Positive (6)	5	5	-	-	-	3
	Negative (3)	3	2	-	-	-	2
P. mirabilis (1)	Positive (1)	1	1	-	-	-	0
	Negative (0)	0	0	-	-	-	0
Providencia spp. (1)	Positive (0)	0	0	-	-	-	0
	Negative (1)	1	1	-	-	-	1
P. aeruginosa (7)	Positive (0)	0	0	-	-	-	0
	Negative (7)	7	4	-	-	-	6
S. aureus (7)	Positive (0)	0	0	-	-	-	-
	Negative (7)	7	7	7	7	7	-
Total (142)	Positive (85)	**83**	**80**	**-**	**-**	**-**	**21**
	Negative (57)	**56**	**48**	**7**	**7**	**7**	**18**

5.7.2 ESBL production and resistance to various antibiotics

Nitrofurantoin was the drug of choice for all MDR isolates with least resistance (29.6%) followed by chloramphenicol (39.4%). Highest resistance was seen with azithromycin (93.7%) followed by co-trimoxazole (77.5%). Among ESBL positive isolates, highest resistance (95.3%) was observed with azithromycin followed by co-trimoxazole (82.3%) and lesser resistance was seen with chloramphenicol (40.0%), and least with

nitrofurantoin (21.2%). Altogether, higher nitrofurantion resistance of 61.1% was observed among *Acinetobacter* spp. Among ESBL negative bacteria, highest resistance (91.2%) was seen with azithromycin followed by co-trimoxazole (70.2%). Among *E. coli* and *K. oxytoca* isolates, 84.8% and 84.2% isolates were resistant to co-trimoxazole while 95.4% and 100% isolates were resistant to azithromycin, respectively. Moreover, all 7 ESBL negative MRSA isolates were resistant to co-trimoxazole and 6 isolates to chloramphenicol and azithromycin. All MRSA isolates were susceptible to nitrofurantoin and vancomycin, and only one MRSA isolate was resistant to clindamycin. (Table 5.7.2)

Table 5.7.2 ESBL production and resistance to various antibiotics

Organisms (No. of MDR isolates tested)	ESBL production (No. of organisms)	Antibiotics and No. of resistant isolates					
		C	NF	TS	VA	CD	ATH
Acinetobacter spp. (18)	Positive (3)	2	1	1	-	-	3
	Negative (15)	2	10	2	-	-	15
C. freundii (6)	Positive (3)	2	1	3	-	-	3
	Negative (3)	2	2	2	-	-	3
E. coli (85)	Positive (66)	25	10	56	-	-	63
	Negative (19)	6	4	16	-	-	16
K. oxytoca (8)	Positive (6)	4	2	6	-	-	6
	Negative (2)	0	2	2	-	-	2
K. pneumoniae (9)	Positive (6)	1	3	3	-	-	5
	Negative (3)	1	2	3	-	-	2
P. mirabilis (1)	Positive (1)	0	1	1	-	-	1
	Negative (0)	0	0	0	-	-	0
Providencia spp. (1)	Positive (0)	0	0	0	-	-	0
	Negative (1)	1	1	1	-	-	1
P. aeruginosa (7)	Positive (0)	0	0	0	-	-	0
	Negative (7)	4	3	7	-	-	6
S. aureus (7)	Positive (0)	0	0	0	-	-	0
	Negative (7)	6	0	7	0	1	7
Total (142)	Positive (85)	**34**	**18**	**70**	**-**	**-**	**81**
	Negative (57)	**22**	**24**	**40**	**0**	**1**	**52**

5.7.3 ESBL production and cephalosporin resistance

Altogether, maximum resistance (93.0%) was observed with cephalothin and least resistance (62.7%) was observed with ceftazidime. Among ESBL positive bacteria, lowest resistance (43.5%) was observed with cefoxitin while maximum resistance (98.8%) was seen with aztreonam followed by cefpodoxime (96.5%) and ceftriaxone (96.5%). Fourth generation cephalosporins also showed higher resistance rates of 92.9% for cefpirome and 87.0% for cefepime. Almost all cephalosporins tested showed increased resistance among ESBL producing organisms.

Of the 57 ESBL negative isolates tested against different cephalosporins, ceftazidime showed least resistance (31.6%) while maximum numbers of isolates (89.5%) were resistant to cephalothin. Cefepime and cefpirome also showed higher resistance rates of 57.9% and 59.6%, respectively. All 7 isolates of *P. aeruginosa* and 80.0% of ESBL negative *Acinetobacter* spp. were susceptible to ceftazidime. Moreover, cefepime and cefpirome showed lesser resistance of 21.0% and 26.3%, respectively, among ESBL negative *E. coli* isolates and of 28.7% for both among *P. aeruginosa* isolates. All the MRSA isolates were resistant to all cephalosporins tested. (Table 5.7.3)

Table 5.7.3 ESBL production and cephalosporin resistance

Organisms (No. of MDR isolates tested)	ESBL production (No. of organisms)	Antibiotics and No. of resistant isolates								
		KF	FOX	ATM	CRO	CPD	CAZ	CTX	CPM	CFP
Acinetobacter spp. (18)	Positive (3)	3	2	3	2	2	2	2	2	3
	Negative (15)	15	15	15	15	15	3	15	15	15
C. freundii (6)	Positive (3)	3	3	3	3	3	2	3	3	3
	Negative (3)	3	3	3	3	3	2	3	2	2
E. coli (85)	Positive (66)	63	26	55	64	64	55	63	57	60
	Negative (19)	13	5	5	4	4	3	4	4	5
K. oxytoca (8)	Positive (6)	6	4	6	6	6	6	6	6	6
	Negative (2)	2	0	1	0	0	0	0	0	0
K. pneumoniae (9)	Positive (6)	5	2	6	6	6	5	5	5	6
	Negative (3)	3	3	2	2	3	2	2	2	2
P. mirabilis (1)	Positive (1)	1	0	1	1	1	1	1	1	1
	Negative (0)	0	0	0	0	0	0	0	0	0
Providencia spp. (1)	Positive (0)	0	0	0	0	0	0	0	0	0
	Negative (1)	1	1	1	1	1	1	1	1	1
P. aeruginosa (7)	Positive (0)	0	0	0	0	0	0	0	0	0
	Negative (7)	7	7	4	6	7	0	6	2	2
S. aureus (7)	Positive (0)	0	0	0	0	0	0	0	0	0
	Negative (7)	7	7	7	7	7	7	7	7	7
Total (142)	Positive (85)	**81**	**37**	**78**	**82**	**82**	**71**	**80**	**74**	**79**
	Negative (57)	**51**	**41**	**38**	**38**	**40**	**18**	**38**	**33**	**34**

5.7.4 ESBL production and resistance to carbapenems and combination of various -lactams with clavulanate

Meropenem and imipenem with no and 2.3% resistance, respectively, were found to be the drug of choice among ESBL positive isolates. Imipenem resistance was remarkably high among ESBL negative *Acinetobacter* spp. (86.7%) and MRSA (71.4%), and all MRSA isolates were resistant to meropenem. Carbapenem resistance was also observed among *C. freundii*, *E. coli*, *K. pneumoniae*, *Providencia* spp. and *P. aeruginosa*. Higher degree of carbapenem resistance, 40.3% for imipenem and 19.2% for meropenem, was

seen among ESBL negative bacteria. Also, CPM/CV, CAZ/CV and CTX/CV became the good cephalosporin-clavulanate combinations for ESBL producers with low resistance rates of 2.3%, 7.0% and 10.6%, respectively. However, 87.0% ESBL positive and 82.4% ESBL negative isolates were resistant to augmentin. CAZ/CV was the drug of choice among the ESBL negative isolates with least (28.0%) resistance. (Table 5.7.4)

Table 5.7.4 ESBL production and resistance to carbapenems and combination of various -lactams with clavulanate

Organisms (No. of MDR isolates tested)	ESBL production (No. of organisms)	Antibiotics tested and No. of resistant isolates						
		AUG	CPD/CV	CAZ/CV	CTX/CV	CPM/CV	IMI	MEM
Acinetobacter spp. (18)	Positive (3)	3	3	0	1	0	1	0
	Negative (15)	15	15	2	15	15	13	2
C. freundii (6)	Positive (3)	3	2	1	1	1	0	0
	Negative (3)	2	3	2	2	2	1	1
E. coli (85)	Positive (66)	57	39	3	4	1	1	0
	Negative (19)	14	4	2	3	3	1	0
K. oxytoca (8)	Positive (6)	6	4	1	1	0	0	0
	Negative (2)	1	0	0	0	0	0	0
K. pneumoniae (9)	Positive (6)	5	5	1	2	0	0	0
	Negative (3)	2	3	2	2	2	1	0
P. mirabilis (1)	Positive (1)	0	1	0	0	0	0	0
	Negative (0)	0	0	0	0	0	0	0
Providencia spp. (1)	Positive (0)	0	0	0	0	0	0	0
	Negative (1)	1	1	1	1	1	1	1
P. aeruginosa (7)	Positive (0)	0	0	0	0	0	0	0
	Negative (7)	5	7	0	6	2	1	0
S. aureus (7)	Positive (0)	0	0	0	0	0	0	0
	Negative (7)	7	7	7	7	7	5	7
Total (142)	Positive (85)	**74**	**54**	**6**	**9**	**2**	**2**	**0**
	Negative (57)	**47**	**40**	**16**	**36**	**32**	**23**	**11**

5.7.5 ESBL production and fluoroquinolone resistance

Of the 7 different fluoroquinolones tested, all of them showed very high degree of resistance among both ESBL positive and negative isolates. The maximum resistance of 97.6% was seen with each of nalidixic acid, ciprofloxacin and moxifloxacin among ESBL positive isolates and of 94.0% with naldixic acid and moxifloxacin among ESBL negative isolates. Norfloxacin comparatively showed lower resistance of 85.9% and 75.4% than other quinolones among ESBL positive and negative isolates, respectively. Newer quinolones, viz. gatifloxacin (88.7%) and moxifloxacin (96.5%) showed remarkably higher degree of resistance. ESBL production and fluoroquinolone resistance was statistically significant ($p<0.05$). (Table 5.7.5)

Table 5.7.5 ESBL production and fluoroquinolone resistance

Organisms (No. of MDR isolates tested)	ESBL production (No. of organisms)	Antibiotics tested and No. of resistant isolates						
		NA	CIP	OFX	GAT	MOX	LEV	NX
Acinetobacter spp. (18)	Positive (3)	1	2	1	1	3	1	3
	Negative	15	15	15	15	15	15	13
C. freundii (6)	Positive (3)	3	3	3	3	3	1	1
	Negative (3)	2	2	2	2	2	2	1
E. coli (85)	Positive (66)	66	65	64	64	64	63	58
	Negative	17	13	13	13	16	13	12
K. oxytoca (8)	Positive (6)	6	6	6	6	6	6	6
	Negative (2)	2	2	2	2	2	2	2
K. pneumoniae (9)	Positive (6)	6	6	6	6	6	6	5
	Negative (3)	3	2	2	2	3	2	2
P. mirabilis (1)	Positive (1)	1	1	1	1	1	1	0
	Negative (0)	0	0	0	0	0	0	0
Providencia spp. (1)	Positive (0)	0	0	0	0	0	0	0
	Negative (1)	1	1	1	1	1	1	1
P. aeruginosa (7)	Positive (0)	0	0	0	0	0	0	0
	Negative (7)	7	3	3	3	7	5	5
S. aureus (7)	Positive (0)	0	0	0	0	0	0	0
	Negative (7)	7	7	7	7	7	7	7
Total (142)	Positive (85)	**83**	**83**	**81**	**81**	**83**	**78**	**73**
	Negative	**54**	**45**	**45**	**45**	**54**	**47**	**43**

5.7.6 ESBL production and aminoglycoside resistance

Of the six aminoglycosides tested, fewer MDR isolates (44.4%) were resistant to amikacin while maximum resistance (85.2%) was seen with kanamycin followed by tobramycin (77.5%). Resistance of 35.3% and 57.9% was observed with amikacin among both ESBL positive and negative isolates, respectively. Aminoglycoside resistance was more predominant among ESBL negative *Acinetobacter* spp. Amikacin (14.3%) and tobramycin (42.8%) showed lower resistance against *P. aeruginosa* isolates. All the MRSA isolates were resistant to all aminoglycosides except one isolate was susceptible to amikacin. (Table 5.7.6)

Table 5.7.6 ESBL production and aminoglycoside resistance

Organisms (No. of MDR isolates tested)	ESBL production (No. of organisms)	Antibiotics tested and No. of resistant isolates					
		AK	GM	TN	NET	NE	K
Acinetobacter spp. (18)	Positive (3)	0	1	2	1	1	2
	Negative (15)	15	15	15	14	15	15
C. freundii (6)	Positive (3)	2	3	3	2	2	3
	Negative (3)	2	2	2	2	2	2
E. coli (85)	Positive (66)	26	39	55	39	33	56
	Negative (19)	6	6	10	6	9	14
K. oxytoca (8)	Positive (6)	0	4	4	3	3	5
	Negative (2)	1	0	1	1	1	2
K. pneumoniae (9)	Positive (6)	1	1	4	1	3	5
	Negative (3)	1	2	2	2	2	2
P. mirabilis (1)	Positive (1)	1	1	1	1	1	1
	Negative (0)	0	0	0	0	0	0
Providencia spp. (1)	Positive (0)	0	0	0	0	0	0
	Negative (1)	1	1	1	1	0	1
P. aeruginosa (7)	Positive (0)	0	0	0	0	0	0
	Negative (7)	1	4	3	5	4	6
S. aureus (7)	Positive (0)	0	0	0	0	0	0
	Negative (7)	6	7	7	7	7	7
Total (**142**)	Positive (85)	**30**	**49**	**69**	**47**	**43**	**72**
	Negative (57)	**33**	**37**	**41**	**38**	**40**	**49**

5.7.7 ESBL production and resistance to tetracyclines and fosfomycin

For the 142 MDR isolates tested, fosfomycin was found to be the best drug of choice with lowest resistance of 17.6% followed by tigecycline with lower resistance rate of 23.2%. Highest resistance was observed with tetracycline (85.2%) followed by doxycycline and minocycline with equal resistance rate of 82.4%.

Fosfomycin and tigecycline were the drugs of choice among ESBL producing bacteria with least resistance rates of 4.7% and 14.1%, respectively. However, resistance rates to both of these antibiotics among ESBL non-producing isolates were 4.7% and 36.8%, respectively. Of the 28 isolates, 55.5% of all *Acinetobacter* spp. were resistant to tigecycline and all ESBL negative *Acinetobacter* spp. were resistant to fosfomycin. 71.4% of *P. aeruginosa* were also resistant to tigecycline while only 28.6% of them were resistant to fosfomycin. Moreover, all MRSA isolates were susceptible to fosfomycin while only 14.3% MRSA were resistant to tigecycline. ESBL production and resistance to tigecycline and fosfomycin was statistically insignificant ($p>0.05$). (Table 5.7.7)

Table 5.7.7 ESBL production and resistance to tetracyclines and fosfomycin

Organisms (No. of MDR isolates tested)	ESBL production (No. of organisms)	Antibiotics tested and No. of resistant isolates				
		T	DO	MN	TGC	FOT
Acinetobacter spp. (18)	Positive (3)	3	2	2	0	0
	Negative (15)	15	14	13	10	15
C. freundii (6)	Positive (3)	3	3	3	1	0
	Negative (3)	2	2	1	1	1
E. coli (85)	Positive (66)	54	54	53	8	3
	Negative (19)	17	16	16	2	1
K. oxytoca (8)	Positive (6)	6	6	6	2	0
	Negative (2)	1	1	1	0	0
K. pneumoniae (9)	Positive (6)	5	3	5	0	1
	Negative (3)	1	1	2	2	2
P. mirabilis (1)	Positive (1)	1	1	1	1	0
	Negative (0)	0	0	0	0	0
Providencia spp. (1)	Positive (0)	0	0	0	0	0
	Negative (1)	1	1	1	0	0
P. aeruginosa (7)	Positive (0)	0	0	0	0	0
	Negative (7)	6	7	7	5	2
S. aureus (7)	Positive (0)	0	0	0	0	0
	Negative (7)	6	6	6	1	0
Total (142)	Positive (85)	**72**	**69**	**70**	**12**	**4**
	Negative (57)	**49**	**48**	**47**	**21**	**21**

5.8 ESBL Production and Spectrum of Drug Resistance

Of the 142 MDR isolates tested, 62.7% isolates showed resistance to 21-30 antibiotics. Greater number of the MDR isolates that produced ESBL showed increased resistance towards antibiotics with majority of them (81.2%) resistant to 21-30 drugs and all of them were gram negative bacteria. Among ESBL positives, 2.3% isolates were further resistant to 31-40 drugs tested. Among 57 ESBL negative isolates, 35.1% isolates, which

were all gram negative bacteria, were resistant to 21-30 antibiotics. All 7 ESBL negative MRSA isolates were resistant to greater than 40 antibiotics. ESBL production and increased drug resistance was statistically significant ($p<0.05$). On the other hand, 77.5% MDR isolates were resistant to 5-7 classes of antibiotics of which 61.8% were ESBL positive and all of them were gram negative bacteria. Moreover, 5.9% of ESBL positive and 12.3% of ESBL negative isolates were further resistant to >7 classes of antibiotics and all of them were also gram negative bacteria. All 7 ESBL negative MRSA isolates were also resistant to 5-7 classes of antibiotics. ESBL production and drug resistance to more classes of antibiotics was statistically significant ($p<0.05$).

Table 5.8 Spectrum of drug resistance among MDR isolates and ESBL production

Organism type	ESBL production	Spectrum of drug resistance (No. of bacteria)					Total
		10 Drugs	11 – 20 Drugs	21 – 30 Drugs	31- 40 Drugs	>40 Drugs	
Gram positive	Positive	0	0	0	0	0	0
	Negative	0	0	0	0	7	7
Gram negative	Positive	0	14	69	2	0	85
	Negative	5	18	20	7	0	50
Total		**5**	**32**	**89**	**9**	**7**	**142**

Organism type	ESBL production	Spectrum of resistance to different antibiotic classes (No. of Bacteria)			
		2-4 classes	5-7 classes	>7 classes	Total
Gram positive	Positive	0	0	0	0
	Negative	0	7	0	7
Gram negative	Positive	12	68	5	85
	Negative	8	35	7	50
Total		**20**	**110**	**12**	**142**

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

A total of 2141 clinical specimens (1710 urinary specimens, 136 pus and body fluid specimens, 170 sputa and throat swabs, and 125 eye and ear specimens) were received and processed of which 53.4% were from males. Similar distribution of various specimens was seen among clinical specimens in other studies also (Baral, 2008; Shrestha *et al.*, 2007). Therefore, the overall result of the study may be affected by urine itself. The higher proportion of urinary specimens showed the greater prevalence of the urinary diseases than others. Among the total specimens, 20.2% specimens showed significant growth and maximum percentage (61.2%) of total isolates was contributed by the urinary isolates. A growth rate of 22.3% among different clinical specimens was seen in a similar study (Baral, 2008).

Least growth rate (15.5%) was observed among urine specimens that resembled with low growth rate of 16.3% among urinary specimens observed in India (Ramesh *et al.*, 2008) and with such low growth rates in other similar studies in Nepal by various researchers (Basnet *et al.*, 2009; Baral, 2008; Kattel *et al.*, 2008; Shrestha *et al.*, 2007; Bomjan, 2005; Dhakal, 1999). The low growth rate among urinary specimens may probably be due to request for urine culture in other urogenital problems rather than overt urinary tract infection, non-gonococcal urethritis or other clinical conditions that mimic UTI, higher health consciousness among patients, prior antibiotic consumption and presence of fastidious pathogens. In contrary to the normal patterns, we received more urine specimens (52.3%) from males than from females (47.7%). However, the growth rate was higher among females (18.1%) than males (13.1%). Similar results were obtained in the previous studies (Basnet *et al.*, 2009; Baral, 2008; Shrestha *et al.*, 2007; Bomjan, 2005). It might be attributed to easy access of uropathogens to female urinary tract due to shorter urethra in closer proximity to anus. Moreover, higher growth rate of 43.3% among

sputum specimens was also seen in other studies (Pokhrel *et al.*, 2006). Growth rate among eye and ear specimens (51.2%) and pus and body fluids (48.9%) was higher which may be apparently attributed to poor hygienic practices among persons. The low growth rate (28.8%) of sputum and throat swab may be due to continuing antibiotic therapy, or viral and fungal infection or infection by organisms like *Mycoplasma*, *Chlamydiae*, *Legionella*, etc. which are not routinely cultured (Read and Finch, 1998). Growth patterns among various clinical specimens may differ in different territories depending on the prevalence of the particular disease, hygiene, antibiotic therapy and other disease phenomena.

Of the 433 bacterial isolates, gram negative bacteria predominated with 67.4% isolates and such predominance was also observed in other similar studies (Basnet *et al.*, 2009; Baral, 2008; Bomjan, 2005; Shrestha *et al.*, 2007; Blomberg *et al.*, 2005). Among gram negative isolates, *E. coli* was the most predominant organism with 51.7% isolates followed by *K. pneumoniae* with 14.7% isolates. These results resembled with that of the studies done by various other workers (Baral, 2008; Shrestha, 2007; Pokhrel *et al.*, 2006; Kumari *et al.*, 2005; Sharma, 2004; Tuladhar, 1999). Most *E. coli* isolates were from urinary specimens as they have special virulent properties to cause UTI, thus being the major uropathogens throughout the world. *E. coli* can bind to the glycol-conjugate receptor (Gal 1 4 Gal) of the uroepithelial cells of human urinary tract so it can initiate infection itself (Johnson, 1991). *E. coli* is the most predominant organism to colonize the urethral meatus (Schaeffer and Chmiel, 1983) and perineum (Levy, 2001) before ascending to the bladder.

Maximum numbers of gram positive bacteria were isolated from eye and ear specimens (31.9%). *S. aureus* and *S. pyogenes* predominated among gram positive bacteria with 41.8% and 28.9% isolates. *S. aureus* was the dominant species among pus and body fluids (79.4%) and eye and ear specimens (48.9%). Higher isolation rates of *S. aureus* were also seen in the similar studies done by various other workers, i.e. 25.3% among wound isolates (Banjara, 2002), 70.97% isolates in pus samples (Dhungel, 2001), 49.40% in ear discharges (Aryal, 2001) and 57.66% isolates in pus samples (Tuladhar, 1999). *S.*

aureus is responsible for about 70% of all site specific soft tissue infections in humans (Collier *et al.,* 1998). Because of their rigid body, resistance to dry conditions and high salt concentrations, *S. aureus* is well suited for skin and soft tissue infections. Other major virulent factors like exotoxins, leucocidin, exfoliatins, haemolysins, enzymes (coagulase, phosphatase, dioxyribonuclease, hyaluronidase, staphylokinases, lipases and proteases) are responsible for occurrence of higher prevalence of *S. aureus* mediated skin and soft tissue infections. *S. pyogenes* and *E. faecalis* were the most frequently isolated gram positive bacteria among sputum and throat swab specimens and urinary specimens with 65.0% and 45.4% isolates, respectively. Significant isolation of enterococci from urine specimens has also been reported from India (Ramesh *et al.,* 2008). High frequency of *E. coli* isolation may be due to the predominance of urinary specimens and of *S. aureus* and *S. pyogenes* may be due to their common predominance among pus, swabs and respiratory specimens. Predominance of *E. coli* (60.9%) followed by *K. pneumoniae* (11.9%) among urinary isolates resembled with the findings of other similar studies by various researchers (Basnet *et al*., 2009; Banjara, 2002).

Of the 14 primarily tested antibiotics against various gram negative bacteria, amikacin was the best drug with only 28.0% resistance. Ceftazidime, nitrofurantoin and chloramphenicol also had relatively lower resistance rates of 28.6%, 29.3% and 30.1%, respectively. Higher amikacin and ceftazidime susceptibility was also reported among urinary isolates (Das *et al*., 2006). Amoxycillin had the highest resistance rate of 89.0% followed by azithromycin (82.6%) and nalidixic acid (80.5%). This finding resembled the results of other similar works (Puri, 2006; Pokhrel, 2006; Bomjan, 2005; Paneru, 2002; Oteo *et al*., 2001; Dhakal, 1999). Other antibiotics tested also showed relatively higher resistance rates. The lower resistance against nitrofurantoin and chloramphenicol may be due to their rarer use in recent days resulting in reduced selective pressure on bacteria. Higher amoxycillin resistance is due to its extensive and empirical use in minor infections and also in clinical conditions due to viral etiologies. Production of various -lactamases and ESBLs under the extensive selection pressure by excessive -lactam use also has contributed to penicillin and cephalosporin resistance (Goossens *et al*., 2005).

Of the 18 primarily tested antibiotics against various gram positive bacteria, nitrofurantoin and norfloxacin were found to be the drugs with least resistance of only 9.5%. Resistance to most of the other antibiotics among gram positive bacteria was relatively lower as most of the gram positive bacteria except MRSA, VRSA and VRE are still susceptible to most antibiotics worldwide. Maximum resistance was seen with ciprofloxacin (47.5%) followed by gentamicin (44.4%) and co-trimoxazole (41.8%) which were the drugs used most frequently used in the country. Erythromycin showed lower resistance (21.3%) than azithromycin (28.7%). Of the 59 isolates of *S. aureus* tested, 16.9% of them were MRSA. A study in a Malaysian teaching hospital showed 44.1% prevalence of MRSA (Ghaznavi-Rad *et al*., 2010). In Nepal, 15.4-39.6% predominance of MRSA isolates among various clinical *S. aureus* isolates has been recorded (Sanjana *et al*., 2010; Kumari *et al*., 2008; Subedi and Brahmadathan, 2005). Resistance to methicillin is mediated by the chromosomal *mecA* gene which is the part of a mobile genetic element found in many MRSA strains called Staphylococcal Cassette Chromosome (SCCmec). This gene encodes for an altered PBP2a that has lower binding affinity for -lactam antibiotics (Timothy, 2004).

Of the 433 isolates, 64.9% isolates were MDR with maximum multiple drug resistance (78.1%) among urinary isolates with MDR isolates and least multiple drug resistance (32.8%) among the eye and ear specimens. Predominance of MDR isolates among sputum and throat swab, and pus and body fluid specimens was almost same with 51.0% and 50.9% MDR isolates, respectively. Similar results with 47.6-83.0% MDR isolates among urinary isolates and about 60.0% MDR isolates among sputum specimens has been reported nationally and internationally (Ullah *et al*., 2009; Pokhrel *et al*., 2006; Bomjan, 2005). Higher prevalence of MDR isolates from pus specimens has been similarly reported (Banjara, 2002). The multi-drug resistance has been found as a major problem in the management of uropathogens (Akram *et al*., 2007; Hasan *et al*., 2007). Of the total MDR isolates, most of them (73.7%) were urinary isolates. That was because of predominance of urinary specimens among total specimens processed and higher emergence of MDR bacteria among urinary isolates than other specimens due to common

infections and higher antibiotic exposure. Prevalence of MDR organisms varies globally and by different anatomical sites as it depends on the local antibiotic using policies, prevalence of particular type of infection and hence the pathogens too. Moreover, the higher transferable multidrug-resistance may be due to plasmids with several resistance genes which are transferred from one bacterium to another (Ram *et al*., 2000).

Of the total 281 MDR isolates from different clinical specimens, 77.9% were gram negative bacteria and most of them (87.7%) were urinary isolates. Most of the gram positive MDR bacteria (30.6%) were isolated from sputum and throat swab specimens. Of the 219 MDR gram negative bacteria, *E. coli* constituted 52.0% isolates. The higher levels of drug resistance among *E. coli*, *Acinetobacter* spp., *K. pneumoniae* and other bacterial species to -lactams, fluoroquinolones, aminoglycosides, co-trimoxazole and some other antibiotics might be due to production of various -lactamases (ESBL, AmpC and MBL), mutations in *gyrA* and *parC* genes, production of aminoglycoside modifying enzymes and presence of many different multidrug efflux systems (Picao *et al*., 2008; Walsh *et al*., 2005; Sulavik *et al*., 2001; Ozeki *et al*., 1997; Livermore, 1995). As most of the antibiotics are commonly used in the country since several years, increasing selective pressure might have contributed to the emergence of resistance.

In our study, 59.9% ESBL screen positive MDR isolates produced ESBL. Of the 85 ESBL positive isolates, *E. coli* with 77.6% isolates constituted to most of the ESBL producers followed by *K. oxytoca* with 75.0% isolates. Moreover, 66.7% *K. pneumoniae*, 50.0% *C. freundii* and 16.7% *Acinetobacter* spp. also tested ESBL positive. ESBL production was also observed among 50.0% isolates from sputum and throat swab and 20.8% isolates from pus and body fluid. Similar results were obtained among various clinical isolates in other studies in Nepal (Baral, 2008; Bomjan, 2005). Similarly in other study, 8%, 12.5%, 12.5%, 25%, and 5% isolates of *K. pneumoniae*, *E. coli*, *C. freundii*, *A. calcoaceticus* and *P. aeruginosa*, respectively produced ESBLs (Sharma, 2004). In Pakistan, 56.9%% ESBL production among 116 urinary *E. coli* isolates was also observed (Ullah *et al*., 2009). In our study, ESBL production was most predominant

(68.4%) among urinary isolates which resembled the findings of other similar studies (Melzer and Patersen, 2007; Jamal *et al*., 2005). Moreover, in India 100% *K. oxytoca*, 79.0% *E. coli* and 69.4% *K. pneumoniae* produced ESBLs. Higher prevalence of ESBL producing *E. coli* isolates in China (55.0%) and Thailand (50.8%) have been reported (Hawser *et al*., 2009). Prevalence ESBLs varies in different countries as ESBL prevalence of 67%, 42%, and 43% has been reported in *E .coli* from Iran, India and Bangladesh respectively (Mehrgan and Rahbar, 2008; Taneja *et al*., 2008; Rahman *et al*., 2004). Substantial geographical difference in the occurrence of ESBLs among and within countries, in territories, and among hospitals has been observed (Babini and Livermore, 2000). ESBL production is more common in *E. coli* and *Klebsiella* spp. than in other members of Enterobacteriaceae. They are sometimes difficult to detect as clavulanate induces production of chromosomal AmpC enzymes that hydrolyze indicator cephalosporin, thus masking any synergy. Among *C. freundii*, *Enterobacter* spp., *Morganella* spp., *Providencia* spp. and *Serratia* spp., mutational hyperproduction of AmpC enzymes is responsible for most of the cephalosporin resistance. Derepressed mutants of AmpC inducible species copiously produce AmpC enzyme without induction and become resistant to almost all penicillins and cephalosporins (Freeman *et al*., 2009; Livermore *et al*., 2001). However, plasmid-mediated AmpC -lactamases in lower proportions have also been reported among different bacterial strains (Li *et al*., 2008; Moland *et al*., 2006). Variation in ESBL production rates has also been observed among various bacterial genera. These findings bespeak emergence of MDR and ESBL producing organisms worldwide limiting the therapeutic options. This finding suggests for the proper and routine detection of ESBLs before the use of -lactams in therapy as well as evaluation of other old and new potent therapeutic agents.

All 281 MDR isolates were subjected to ESBL screening test by using all five screening agents recommended by CLSI and 142 isolates were found to be screen positive by one or all screening agents. Among them, cefpodoxime and ceftriaxone had the highest sensitivities of 96.5% each but cefpodoxime had lowest PPV (67.2%) and ceftriaxone had lower PPV of 68.3%. Aztreonam and cefotaxime both had equal sensitivity of 94.1% and

PPV of 67.8%. Ceftazidime had lowest sensitivity of only 83.5%, but had the highest PPV of 79.8%. Higher sensitivity of cefotaxime in ESBL screening was reported from Israel with its convenient use and improved negative predictive value for most of the genera in areas where *Klebsiella* spp. are not the dominant ESBL producers (Navon-Venezia *et al*., 2003). In a similar study to investigate the performance of ESBL screening methods for Enterobacteriaceae in South-East England, many isolates found resistant only to cefpodoxime at the source sites proved not to have ESBLs or AmpC and, hence, screening with cefotaxime and ceftazidime allowed better specificity for identification of mechanism-based resistance, as did the automated systems (Hope *et al*., 2007). However, the screening results with ceftazidime versus cefotaxime was found statistically insignificant ($p>0.05$).

Among 142 MDR isolates tested for confirmation of ESBL production by using four types of combination disks, maximum ESBL producers (97.6%) were detected by cefepime-clavulanate combination disks. Higher sensitivity (98.0%) of cefepime-clavulanate in ESBL Etests followed by 83.0% with cefotaxime-clavulanate, and 74.0% with ceftazidime-clavulanate strips among Enterobacteriaceae has also been reported in similar work (Sturenburg *et al*., 2004). According to that study, reliable ESBL detection among *Enterobacter* spp. could only be achieved by the cefepime–clavulanate strip with 100% sensitivity. A limitation of using the cefepime-clavulanate combination strip was less than optimal specificity with K1 phenotypes of *K. oxytoca*: 66.7% cases were false-positive by Etest strips containing cefepime–clavulanate. In a similar study using E-test strips, the cefotaxime ESBL strip detected ESBL activity in 96.5% of the 87 ESBL producing isolates tested while ceftazidime Etest strips detected it only in 54 % of the study isolates (Dashti *et al*., 2006). Moreover, cefotaxime-clavulanate showed 91.0% overall sensitivity and 100% sensitivity among Enterobacteriaceae in detecting ESBLs. Ceftazidime and cefotaxime disks each in combination with clavulanate showed 10 and 1 more ESBL producers respectively than in respective screening tests. Combined use of cefotaxime-clavulanate and ceftazidime-clavulanate showed 100% sensitivity in detecting ESBLs among all various clinical isolates and cefpodoxime-clavulanate did not

add any benefit (Navon-Venezia *et al*., 2003). The use of the combination of both cefotaxime and ceftazidime Etest ESBL strips detected ESBLs in 86 of the 87 test isolates (98.8%). Cefpodoxime-clavulanate combination disks detected least number of ESBL producers with only 91.8% positive isolates. This may be due to presence of some cefpodoxime hydrolyzing -lactamases in some strains that were not sensitive to clavulanic acid, which could have reduced the sensitivity of the test. Moreover, changes in major outer membrane proteins change in porin number and raised production of chromosomal AmpC enzymes might have contributed to the lower sensitivity of this agent (Oliver *et al*., 2002). Higher false-positive ESBL comfirmation may also be due to presence of p*ampC* genes with or without other -lactamase genes and false-negative results may be due to presence of both ESBL and p*ampC* genes (Robberts *et al*., 2009). The differences in sensitivities of various agents in detecting ESBLs might be due to differences in dominant enzymes in various geographical areas. Moreover, the particular enzymes harbored by the tested organisms might have influenced the test performance.

Among 85 ESBL positive isolates, maximum resistance (97.6%) was observed against amoxycillin followed by ticarcillin (94.1%). On the flip side, 98.2% ESBL negative isolates were also resistant to amoxycillin and 84.2% were resistant to ticarcillin. Almost all isolates tested were resistant to amoxycillin and ticarcillin. Temocillin showed remarkably less resistance of 27.5%. Only 24.7% of ESBL positive and 31.6% of ESBL negative isolates were resistant to temocillin. The higher susceptibilities of MDR pathogens, particularly the MDR *Acinetobacter* spp. isolates to temocillin was worthy to note. We adopted the temocillin susceptibility breakpoints according to the BSAC guidelines as diameter of inhibition zone of 20mm for susceptibility and 19mm for resistance for systemic Enterobacteriaceae isolates, and for urinary Enterobacteriaceae isolates, 12mm and 11mm zone diameters were regarded as susceptible and resistant (Andrews *et al*., 2007). All *S. aureus* isolates tested were resistant to amoxycillin, ticarcillin, penicillin G, oxacillin and methicillin. Higher ampicillin resistance among *S. aureus* (81%) and *E. coli* (92.26%) from various clinical specimens was reported in Nepal (Datta, 2004) while around 90.0% amoxycillin resistance was observed among

enteric bacteria in Iran (Mansouri and Abbasi, 2010). Higher amoxycillin resistance is obvious as it remains the most frequently sold antibiotic in Nepal (Kafle *et al*., 2007). Among ESBL positive isolates, all of *Acinetobacter* spp., 83.3% of *K. pneumoniae* and 66.7% of *C. freundii* showed resistance to temocillin. Among ESBL negative isolates, all isolates of *C. freundii*, 85.7% of *P. aeruginosa* and 66.7% of *K. pneumoniae* showed resistance to temocillin. No isolate of ESBL negative *K. oxytoca* and fewer (13.3%) ESBL negative *Acinetobacter* spp. isolates were resistant to temocillin.

Temocillin is -lactamase resistant penicillin. It is stable against hydrolysis by most -lactamases, including extended-spectrum -lactamases (ESBLs) and AmpC-type -lactamases with studies reporting MICs at which 90% of bacteria are inhibited (MIC_{90}s) between 16 and 32 µg/ml (Denyer *et al*., 2007; Glupczynski *et al*., 2007; Livermore *et al*., 2006). In a study on KPC- -lactamase producing *K. pneumoniae* and *E. coli* isolates that were resistant to carbapenem (ertapenem), the MICs ranged between 16 µg/ml and 64 µg/ml (MIC_{90}=32µg/ml) for *K. pneumoniae*. The *E. coli* clinical isolates had MICs between 8 and 16µg/ml. An inoculum effect was not observed at 10^5 CFU, whereas a mild inoculum effect averaging within a twofold MIC difference was seen with *K. pneumoniae* when 10^6 CFU was inoculated (Adams-Haduch *et al*., 2009). The common dosage is 2g intravenously every 12 hours or in severe disease as a single loading dose of 2g is given intravenously followed by a 4g infusion over 24 hours (Jongh *et al*., 2008). Temocillin is thus drawing attention as a potential alternative to carbapenems in treatment of infections caused by the Enterobactericeae producing these broad-spectrum -lactamases. It is not active against gram positive bacteria, anaerobes, *Acinetobacter* species, *Pseudomonas aeruginosa* or bacteria with altered penicillin-binding proteins. Its primary use is against Enterobacteriaceae, and in particular against strains producing extended spectrum -lactamase or AmpC -lactamase (Livermore and Tulkens, 2009; Livermore *et al*., 2006). Temocillin showed comparable efficacy with other IV infusions in treating cystic fibrosis patients (Kent *et al*., 2008).

Altogether, nitrofurantoin was the drug of choice with least resistance of 29.6% followed by chloramphenicol (39.4%). Highest resistance was seen with azithromycin (93.7%) followed by co-trimoxazole (77.5%). Similar high co-trimoxazole resistance has also been reported by other workers (Mansouri and Abbasi, 2010; Ullah *et al*., 2009; Akram *et al*., 2007; Tankhiwale *et al*., 2004). Higher azithromycin resistance in our country can be justified by its excessive empirical use in many enteric and respiratory infections due to its dose convenience. Higher nitrofurantion resistance (61.1%) was observed among *Acinetobacter* spp. reducing its efficacy in UTIs by such bacteria.

All MRSA isolates were ESBL negative and resistant to co-trimoxazole and 85.7% of them were resistant to chloramphenicol and azithromycin. All MRSA isolates were susceptible to nitrofurantoin and vancomycin which was of good remark and only one MRSA isolate was resistant to clindamycin. These findings were in accordance with the results in other similar works (Baral, 2008; Dhungel, 2001; Tuladhar, 1999). Moreover, of the 18 *Acinetobacter* spp. tested, only 3 isolates were resistant to co-trimoxazole and 4 isolates were resistant to chloramphenicol which was also of good note and suggests for the inclusion of these drugs in therapeutic regime for treating *Acinetobacter* infections. However, high resistance of co-trimoxazole among *Acinetobacter* spp. was also observed in a study in Italy (Capone *et al*., 2008). Though older and commoner, these antibiotics can still be used effectively for treating severe infections caused by them. This higher susceptibility may be attributed to the less common infections by such bacteria and thus their lesser previous exposure to these drugs sufficient to develop resistance. However, higher co-trimoxazole resistance was observed among other commoner pathogens the infections by which were empirically treated by this agent in previous years.

Among ESBL positive bacteria, lowest resistance (43.5%) was observed with cefoxitin while maximum resistance (98.8%) among them was seen with aztreonam followed by cefpodoxime and ceftriaxone (96.5%). Lower cefoxitin resistance may imply the lower rate of production of AmpC enzymes (Robberts *et al*., 2009). Fourth generation cephalosporins also showed higher resistance rates of 92.9% for cefpirome and 87.0% for

cefepime. In a study in Indian setting at tertiary care referral hospital by among 516 bacterial isolates, 54.2% (n=177) gram positive isolates, 66% (n=209) Enterobacteriaceae, 70.7% (n=130) *P. aeruginosa* and other nonfermenters were resistant to cefpirome (Chaudhary, 2003). In the 1980s and 1990s, reports from North America, Europe, and Japan have revealed an uniformly higher efficacy of cefpirome as compared to the other -lactams including cephalosporins (Jones *et al.*, 1991). At that time, cefpirome showed better activity than other third generation cephalosporins against both inducible -lactamase producing and not producing Enterobacteriaceae species (Reeves *et al.*, 1993). This scenario may be imperceptibly changing in the light of some reports from Thailand with 13.4% resistance to cefpirome in all gram negative isolates tested and from Japan with 41.7% resistance among 14,216 *P. aeruginosa* isolates but only 15.7% resistance among them to ceftazidime (Nitsuma *et al.*, 2001; Biedenbach *et al.*, 1999). However, no *P. aeruginosa* isolate in our study was resistant to ceftazidime and it remained the good antipseudomonal cephalosporin (Denyer *et al.*, 2007). Resistance to cefotaxime, ceftriaxone and ceftazidime among *Citrobacter* spp. has been reported within range of 70.4-82.2% (Patil and Lakshmi, 2000).

The high frequency of resistance against cefpirome in our study is not surprising as cefpirome belongs to the cephalosporin group and can become a target of potent and constantly evolving -lactamases. Moreover, higher cefepime resistance among clinical isolates of ESBL producing *E. coli* and *K. pneumoniae* has been reported in India (Arya *et al.*, 2007) and in Iran (Feizabadi *et al.*, 2006). Almost all MDR *Acinetobacter* spp. isolates were resistant to cefepime, ceftazidime and aztreonam in Italy (Capone *et al.*, 2008). This higher cephalosporin resistance among these organisms might be due to hyperproduction of AmpC -lactamases rather than ESBLs. The over-the-counter sale of such medicines and use of counterfeit medicines has now become common without any benefits for the patients (Aldhous, 2005). Furthermore, any suboptimal dose and/or duration of therapy would support selection and dissemination of cefepime resistant ESBL-producing *K. pneumoniae* and *E. coli.* There would be some delay in prescribing antibiotics matching the *in-vitro* susceptibility for an ICU isolate.

Almost all cephalosporins tested showed increased resistance among ESBL producing organisms. As cefalosporins are susceptible to various types of ESBLs by definition, higher cephalosporin resistance rates among ESBL producers is obvious. Higher cephalosporin resistance among ESBL producing bacteria have been reported in various studies (Khadri and Alzohairy, 2009). In a similar study carried out in Nigeria on 100 MDR isolates of Enterobacteriaceae, only 54.8% of the *Klebsiella* species isolated were sensitive to ceftazidime, 48.4% to ceftriaxone and 30.7% to cefotaxime (Okesola and Makanjuola, 2009). Higher ceftazidime resistance among enteric bacteria has been reported from Iran (Mansouri and Abbasi, 2010). With *E. coli* however, the susceptibility pattern to the third-generation cephalosporins was better (65.6% were sensitive to ceftazidime, 62.5% to ceftriaxone and 71.9% to cefotaxime). In *Proteus* species, the susceptibility pattern was generally poor to the three classes of antibiotics (50.0% were susceptible to ceftazidime and ceftriaxone, 0.0% to cefotaxime, 33.3% to ciprofloxacin, 50.0% to gentamycin and 0.0% to amoxycillin/clavulanate).

Among 57 ESBL negative isolates, ceftazidime showed least resistance of 31.6% while maximum numbers of isolates (89.5%) were resistant to cephalothin. Cefepime and cefpirome also showed higher resistance rates of 57.9% and 59.6%, respectively. This may be due to production of inhibitor-resistant -lactamases. All (100%) isolates of *P. aeruginosa* and 80% of ESBL negative *Acinetobacter* spp. were susceptible to ceftazidime. Higher ceftazidime susceptibility among *P. aeruginosa* isolates has also been previously mentioned (Denyer *et al*., 2007). Moreover, cefepime and cefpirome showed lesser resistance of 21.0% and 26.3%, respectively, among ESBL negative *E. coli* isolates and of 28.7% for both among *P. aeruginosa* isolates showing their possible therapeutic efficacy against these organisms. All the MRSA isolates were resistant to all cephalosporins tested. Adequate data on fourth-generation cephalosporin use in Nepal was not available as their launch was quite recent, third-generation cephalosporins and penicillins still have a significant market share in Nepal (Kafle *et al*., 2007).

Meropenem and imipenem with no and 2.3% resistance, respectively, were found to be the drug of choice among ESBL positive isolates. Higher degree of carbapenem resistance, 40.3% for imipenem and 19.2% for meropenem, was seen among ESBL negative bacteria. Imipenem was found to be the most effective drug against gram negative isolates in the similar studies (Baral, 2008; Puri, 2006; Oteo *et al*., 2001). Meropenem is more stable than imipenem to the enzyme dehydropeptidase produced by kidney and can be administered without cialistin and hence can be a better therapeutic option (Jones *et al*., 2007). Imipenem resistance was remarkably high among ESBL negative *Acinetobacter* spp. (86.7%) and MRSA (71.4%), and all MRSA isolates were resistant to meropenem. Carbapenem resistance was also observed among *C. freundii*, *E. coli*, *K. pneumoniae*, *Providencia* spp. and *P. aeruginosa*. In India, 16% imipenem resistance among 50 clinical isolates of *P. aeruginosa* has been reported (Hemalatha *et al*., 2005). MBL-mediated imipenem resistance in *P. aeruginosa* is of concern in the therapy of critically ill patients. Resistance to carbapenems is due to reduced permeability due to porin channel downregulation (porin loss), upregulated efflux of the drug and most importantly the production of plasmid borne AmpC like -lactamase, oxacillinases, *Klebsiella pneumoniae* carbapenemases (KPCs 1-4) and metallo- -lactamases. Moreover, co-existance of bla_{AmpC} and $bla_{\text{CTX-M}}$ genes in bacteria with decreased outer membrane permeability may lead to carbapenem resistance (Shahid *et al*., 2009; Wang *et al*., 2009). Higher resistance rates of carbapenem resistance among *Acinetobacter* spp. has also been seen in Israel and Italy (Capone *et al*., 2008; Navon-Venezia *et al*., 2007). The clinical utility of carbapenems is under threat due to emergence of acquired carbapenemases, i.e MBLs among clinically important pathogens, such as *Pseudomonas* spp., *Acinetobacter* spp., and members of Enterobacteriaceae family, which are also capable of integron-mediated transfer. Therefore, early detection of MBL-producing organism is recommended (Picao *et al*., 2008; Franklin *et al*., 2006).

The CPM/CV, CAZ/CV and CTX/CV became the good cephalosporin-clavulanate combinations for ESBL producers with respective low resistance rates of 2.3%, 7.0% and 10.6%. Because there were no published breakpoints for such combinations, we used the

susceptibility criteria for them if the inhibitor combination gave the inhibition zone greater than their ESBL screening criteria. For cefepime, inhibition zone >18mm was interpreted as susceptible. Augmentin showed higher resistance of 87.0% and 82.4% among both ESBL positive and negative isolates. This might be due to excessive use of amoxycillin in past and recent years and also due to production of inhibitor resistant -lactamases by the isolates. CAZ/CV was the drug of choice among the ESBL negative isolates with least (28.0%) resistance. Good stability of drugs against various -lactamases in presence of clavulanate can be attributed to these findings. As relatively higher concentration of -lactamase inhibitor in urine is achieved, safe and successful treatment of UTIs by -lactam/ -lactamase inhibitors has also been described (Nordmann, 1998). In a study on 54 CTX-M -lactamase producing *E. coli* isolates from respiratory, pus and urinary specimens, 96.2% isolates were susceptible to ceftriaxone/sulbactam combination which was equal to imipenem susceptibility; however the susceptibility to ticarcillin/clavulanate and piperacillin/tazobactam combinations was unworthy (Shahid *et al*., 2007). Similarly, in a study among various clinical isolates from ventilator-associated pneumonia in Turkey, cefoperazone-sulbactam showed highest susceptibility (89.0%) against MDR *Acinetobacter* spp. (Erdem *et al*., 2008). These combination agents may be of worthy clinical use in this era of emerging ESBL threat.

All of the 7 different fluoroquinolones tested showed very high degree of resistance among both ESBL positive and negative isolates. The maximum resistance of 97.6% was seen with nalidixic acid, ciprofloxacin and moxifloxacin among ESBL positive isolates and of 94.0% with naldixic acid and moxifloxacin among ESBL negative isolates. Norfloxacin comparatively showed lower resistance of 85.9% and 75.4% than other quinolones among ESBL positive and negative isolates, respectively. Newer quinolones, viz. gatifloxacin and moxifloxacin, showed remarkably higher degree of resistance. Disappointing results of ciprofloxacin resistance among urinary isolates have been reported in eastern part of Nepal (Kumari *et al*., 2005), in Turkey (Erdem *et al*., 2008) and in Iran (Mansouri and Abbasi, 2010). Increasing trend in fluoroquinolone resistance due to their increased use in various clinical cases has also been reported (Neuhauser *et*

al., 2003; Shankar *et al*., 2007). Fluoroquinolones still share a greater market sale percentage in Nepal which has been creating a constant selective pressure on microorganisms (Kafle *et al*., 2007). Greater resistance among ESBL producing *E .coli* isolates to nalidixic acid (63.6%) and ciprofloxacin (54.6%) was seen in a study in Palestine (Astal *et al*., 2004; Astal, 2005). Fluoroquinolone resistance and MDR phenotype is associated with ESBL production. In a study among 34 ESBL producing *E. coli* isolates in Canada, 79.4% isolates were resistant to ciprofloxacin and higher percentage of them were also resistant to gentamicin, doxycycline and co-trimoxazole. CTX-M-14 ESBLs were associated with fluoroquinolone resistance only while CTX-M-15 genotypes were associated with multiple drug resistance (Lagace-Wiens *et al*., 2007). Higher resistance to ciprofloxacin and levofloxacin was also observed in Italy (Capone *et al*., 2008). The higher level of fluoroquinolone resistance observed in the study might be due to involvement of several resistance mechanisms such as mutation of the topoisomerase targets, mutational activation of efflux systems, protection of the topoisomerase target by Qnr proteins and inactivation of the drug by the AAC(6')-Ib-cr variant of the common AAC(6')-Ib aminoglycoside acetyl-transferase (Robicsek *et al*., 2006[b]). Quinolone resistance mostly originates from chromosomal mutations but plasmid-mediated quinolone resistance with the involvement of *qnrA*, *qnrB*, or *qnrS* genes has also been reported in several parts of the world (Oktem *et al*., 2008). These findings question the recent clinical utility of fluoroquinolones and mandates for avoidance of their empirical and monotherapeutic use.

Fewer isolates (44.4%) were resistant to amikacin while maximum resistance (85.2%) was seen with kanamycin followed by tobramycin (77.5%). Lesser resistance of 35.3% and 57.9% was observed with amikacin among both ESBL positive and negative isolates, respectively. Aminoglycoside resistance was more predominant among ESBL negative *Acinetobacter* spp. The presence of integrons containing aminoglycoside-modifying enzymes is associated with resistance to gentamicin and tobramycin but not with resistance to amikacin among *Acinetobacter* isolates (Lin *et al*., 2010). Amikacin (14.3%) and tobramycin (42.8%) showed lower resistance against *P. aeruginosa* isolates. All the

MRSA isolates were resistant to all aminoglycosides tested except that one isolate was susceptible to amikacin. Higher amikacin susceptibility and gentamicin resistance among ESBL producing enteric bacteria has been reported in UAE (Al-Zarouni *et al*., 2008) and higher gentamicin resistance among enteric bacteria in Iran (Mansouri and Abbasi, 2010). Higher amikacin and gentamicin resistance have also been observed in Italy (Capone *et al*., 2008). In similar study, gentamicin and tobramycin typically demonstrated poor *in vitro* activity against ESBL-producing *Klebsiella* species, *E. coli* and *Proteus* species (Spanu *et al.*, 2002). In Pakistan, urinary *E. coli* isolates showed 57% kanamycin resistance and 52% gentamicin resistance (Ullah *et al*., 2009). Such resistant isolates pose serious problems to the physicians as therapeutic options are limited. ESBLs are plasmid-mediated and multidrug resistance is a characteristic feature of strains producing ESBLs. Our study confirms this observation in *Klebsiella* and *Proteus* species, as these isolates were resistant to different classes of antibiotics including aminoglycosides. Amikacin still retains its clinical utility and tobramycin has good efficacy in treating specific *P. aeruginosa* infections. The higher aminoglycoside resistance observed might be due to reduced uptake, decreased permeability, altered ribosomal binding site and most importantly the production of aminoglycoside modifying enzymes, viz. AACs, ANTs and (Mingeot-Leclercq *et al*., 1999; Shaw *et al*., 1993). The much resistance against kanamycin may be due to excessive production of ANT (4’) (4”) I, APH (2”)/AAC (6’), APH (3’) and AAC (3’) III, and to tobramycin may be due to excessive production of ANT(4’)(4”)I, APH(2”)/AAC(6’) (Livermore *et al*., 2001).

Among tetracyclines, the highest resistance rate was observed with tetracycline (85.2%) followed by doxycycline and minocycline with equal resistance rate of 82.4%. Higher tetracycline resistance among enteric bacteria has also been reported from Iran (Mansouri and Abbasi, 2010). In contrary to our finding, higher susceptibility (92%) of doxycycline among 25 *A. baumannii* strains but higher MICs for 35 *P. aeruginosa* was seen in other study (Timurkaynak *et al*., 2006). This higher resistance observed in our study might be due to consequence of excessive and imprudent use of these antibiotics in the past years and the involvement of various resistance mechanisms such as various efflux pumps,

alteration of ribosomal target site that prevents binding of the drug, or production of modifying enzymes that inactivate the drug (Spear *et al*., 1992). The *tet*(A-E), *tet*(G), *tet*(H), *tet*(K), *tet*(L) and *tet*(X) genes have been identified in tetracycline-resistant organisms. The *tet*(X) gene encodes an enzyme which modifies and inactivates tetracyclines instead of efflux (Denyer *et al*., 2007; Wilkerson *et al*., 2004). Cross resistance has also been reported.

For 142 MDR isolates tested, fosfomycin was the best drug with lowest resistance (17.6%). Similar higher susceptibility rate to fosfomycin among both gram positive and gram negative bacteria was reported in Japan (Wachino *et al*., 2010) and Greece (Maraki *et al*., 2009). Though we observed such slight resistance, evidence for the *in vivo* development of fosfomycin resistance is lacking. Fosfomycin has been extensively used for more than 20 years in Japan and Europe for treatment of UTI, but resistance rate as low as <2% among urinary *E. coli* isolates has been reported (Shimizu *et al*., 2000). Fosfomycin was also found considerably active against both ESBL positive and negative isolates of *E. coli* and *K. pneumoniae* and similar results have also been reported by different workers (Flagas *et al*., 2010; Maraki *et al*., 2009; Cueto *et al*., 2006; Tharavichitkul *et al*., 2005). As ESBL-producing bacteria are also resistant to other various antibiotics, fosfomycin can be an effective alternative in treating infections by such MDR organisms (Paterson and Bonomo, 2005; Giske *et al*., 2008; Sharma *et al*., 2010). Fosfomycin can also be a good option for treatment of soft tissue infection (Frossard *et al*., 2000). However, all ESBL negative *Acinetobacter* spp. isolates of our study were resistant to fosfomycin. Similar higher fosfomycin resistance of 96.5% (82/85) among *Acinetobacter baumannii* has been reported in other studies (Flagas *et al*., 2009; Maraki *et al*., 2009). Though, the drug was never used previously in Nepal and this is the test for the first time, observance of resistance among these isolates was of note and makes question on its use in treating infections by such organisms. The observed resistance rate could be confirmed by MIC tests, genetic analyses and large scale studies.

Only 28.6% of the *P. aeruginosa* isolates were resistant to fosfomycin. Similarly, 10% and 10-50% resistance rates of *P. aeruginosa* among 7/19 and 4/19 relevant studies, respectively have been identified (Flagas *et al.*, 2009). Fosfomycin in combination with other antibiotics such as antipseudomonal -lactam, imipenem and aminoglycoside has shown a very good efficacy in the treatment of MDR *P. aeruginosa* infections with a low side effect profile and can be a good alternative regimen for such infections (Mirakhur *et al.*, 2003; Flagas *et al.*, 2009). Moreover, all seven MRSA isolates in our study were susceptible to fosfomycin and similar good *in vitro* activity of fosfomycin against MRSA and other staphylococci alone or in combination with other antibiotics was observed in the similar studies (Flagas *et al.*, 2009; Maraki *et al.*, 2009; Flagas *et al.*, 2010). Fosfomycin also showed remarkable *in vitro* activity against more than 98% of vancomycin-resistant enterococci (Superti *et al.*, 2009). Therefore, fosfomycin is a very good therapeutic alternative in various clinical conditions by a vast array of MDR bacteria. It has good pharmacological properties and better safety profiles in both oral and parenteral administration (Woodruff *et al.*, 1977). There is presumably little chance of *in vivo* fosfomycin resistance due to lack of glu-6-phosphate induced transport system since the selection pressure is minimal.

Plasmid genes encoding thiol transferase enzymes FosA and FosB confer low level fosfomycin resistance. The resistance may be due to mutation of chromosomally encoded -glycerolphosphate transport (GlpT) transport system, involvement of transferable plasmid-mediated genes *fosA*, *fosB* and *fosC* which efficaciously inactivate fosfomycin through glutathione *S*-transferase activity which add glutathione to fosfomycin and a nontransferable ATP dependent novel mechanism comparable to mechanisms by *fosC*, and *fomA* and *fomB* of fosfomycin producing *Pseudomonas syringae* and *Streptomyces wedmorensis* (Wachino *et al.*, 2010; Shimizu *et al.*, 2000; Arca *et al.*, 1997).

The next drug of choice was found to be the tigecycline with resistance rate of (23.2%). Except *Acinetobacter* spp. and *P. aeruginosa*, which contributed this higher resistance rate, all the tigecycline susceptibility rates for other organisms tested were in accordance

with the studies by some other workers (Brown and Traczewski, 2007; Hope *et al.*, 2006). In clinical studies to evaluate the clinical efficacy of tigecycline on MDR Enterobacteriaceae, 69.7% of the 33 reported patients treated with tigecycline achieved resolution of an infection caused by a carbapenem-resistant or ESBL-producing or MDR Enterobacteriaceae (Kelesidis *et al.*, 2008). Tigecycline showed excellent activity against a collection of difficult-to-treat and various -lactamase producing pathogens (Sorlozano *et al.*, 2006; Souli *et al.*, 2006; Bouchillon *et al.*, 2005). Tigecycline showed higher efficacy over classical tetracyclines because it binds more strongly to the 30S subunit of the bacterial ribosome in a different orientation than classical tetracyclines to halt protein synthesis (Nathwani, 2005; Bauer *et al.*, 2004). Tigecycline also evades the Tet(A-E) and Tet(K) efflux pumps, and works on *tet*(M)-protected ribosomes (Garrison *et al.*, 2005; Fluit *et al.*, 2005). This enhanced binding, probably, overcomes the ribosomal protection mechanisms mediated *tet*(M) gene.

Of the 28 isolates, 55.5% of all *Acinetobacter* spp. were resistant to tigecycline. Similar high level of tigecycline resistance (66%) among 82 clinical isolates and 62% resistance among 58 clinical isolates of MDR *Acinetobacter* spp. was observed in Israel and India (Navon-Venezia *et al.*, 2007; Behera *et al.*, 2009). Recent data on the activity of tetracyclines against *A. baumannii* are conflicting. In large surveys from the UK, the USA, Germany and Italy, the frequency of *A. baumannii* isolates resistant to tigecycline, minocycline and doxycycline varied in the ranges 4% to 6%, 0% to 18% and 6% to 44%, respectively, in association with high overall rates of resistance to tetracycline (Mezzatesta *et al.*, 2008; Seifert *et al.*, 2006; Henwood *et al.*, 2002). Moreover, 3.8%, 16.3% and 86.3% of the the 80 presumed MDR *A. baumannii* isolates have been reported resistant to minocycline, doxycycline and tetracycline, respectively (Capone *et al.*, 2008). Significant tetracycline and minocycline resistance with the involvement of *tet*(M), *tet*(K) and *tet*(A) genes and high tigecycline susceptibility among MRSA, MDR *E. coli*, *K. pneumoniae*, *Acinetobacter* spp., *Enterobacter* spp. and *P. aeruginosa* has been reported from Italy (Patersen *et al.*, 2010). In our study, of the 15 ESBL negative *Acinetobacter* spp. isolates, 13 were imipenem resistant isolates, and similarly 10 (66.7%) such isolates

were also resistant to tigecycline which coincided with the results of study in Israel (Navon-Venezia *et al.*, 2007). However, this study reported only 37% minocycline resistance and our results substantially differed with those findings for minocycline as we observed higher minocycline resistance of 83.3% among *Acinetobacter* spp. and 82.4% among all isolates tested. This indicated its least usefulness. Minocycline has limited clinical implications due to the unavailability of its intravenous formulation.

Though resistance mechanisms for tetracyclines were not determined in this study and data regarding to the minocycline susceptibility was not available in Nepal, we assumed the involvement of common tetracycline resistance mechanisms, making the drug clinically ineffective. The high resistance to tigecycline observed in our study differs substantially from previous reports in which *Acinetobacter* spp. isolates were almost uniformly susceptible to tigecycline, even in studies that tested MDR *Acinetobacte*r isolates with high imipenem resistance rates (Souli *et al.*, 2006; Pachon-Ibanez *et al.*, 2004). We used the FDA breakpoints for susceptibility criteria in disk diffusion techniques and the different susceptibility rates in other works may be referring to the less strict breakpoints. Recently, non-susceptibility to tigecycline has also been reported in a single clone of MDR *A. baumannii* in the Chicago area (Lolans *et al.*, 2006). The higher degree of tigecycline resistance among *Acinetobacter* spp. might be, at least partly, due to the involvement of increased levels an efflux pump AcrAB (Peleg *et al.*, 2007). Resistance to tigecycline have also been reported in *Enterobacter cloacae* due to treatment with ciprofloxacin, a substrate of AcrAB in *E. cloacae*, which possibly selected for cross-resistance to tigecycline as a result of RamA-mediated AcrAB upregulation (Hornsey *et al.*, 2010)[a]. Moreover, adoption of FDA breakpoints for disk diffusion tests might have reported the overresistance among these isolates as advocated by the study of (Jones *et al.*, 2007) who have suggested the correct disk diffusion breakpoints of 16mm for susceptible and 12mm for resistant. Furthermore, susceptibility breakpoint of 13mm for *Acinetobacter* spp. with 99.0% sensitivity and 100% specificity has been suggested (Thamlikitkul *et al.*, 2007). This report is the first description of a high *in vitro* tigecycline resistance among multiple clones of MDR *A. baumannii* in Nepal.

Tigecycline resistance, particularly among MDR *Acinetobacter* spp. isolates that had not previously been exposed to the drug is worrysome and limits the antibiotic options available to clinicians to combat these bacteria. When treating patients with severe infections caused by MDR *Acinetobacter* spp., *in vitro* susceptibility to tigecycline should be evaluated. Also 71.4% of *P. aeruginosa* and one isolate of *P. mirabilis* were resistant to tigecycline. Nons-susceptibility of tigecycline among *P. aeruginosa* and *P. mirabilis* has also been described in other studies (Greer, 2006). The higher tigecycline resistance among *P. aeruginosa* might be due to their intrinsic resistance to tigecycline mediated by various efflux pumps such as MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM and tigecycline also induces transcription of mexZ, mexX and mexY genes. Overexpression of MexAB-OprM efflux pump is also responsible for higher tigecycline and tetracycline resistance among *P. aeruginosa* (Dean *et al*., 2003). Therefore, a combination of tigecycline and the efflux pump inhibitor to augment its efficacy may present an attractive therapeutic option (Rajendrana *et al*., 2010).

Up-regulation of endogenous SdeXY-HasF-mediated efflux is associated with tigecycline resistance in *S. marcescens* along with MIC rises for tetracycline, ciprofloxacin and cefpirome (Hornsey *et al*., 2010)[b]. In addition to various other severe infections by MDR bacteria, off-level use of tigecycline can also treat UTIs as a single agent that covers a polymicrobial infection due to VRE and MDR *Acinetobacter* spp. and avoids nephrotoxic antimicrobials such as colistin and amikacin, and 22.0% of tigecycline is excreted unchanged in the urine (Bantar, 2007; Anonymous, 2007). Only 1 of 7 MRSA isolates in our study was resistant to tigecycline. Higher susceptibilities of >98.0% to tigecycline among MRSA has also been reported (Verkade *et al*., 2010; Souli *et al*., 2006).

Fosfomycin and tigecycline were the drug of choice among ESBL producing bacteria with least resistance rate of 4.7% and 14.1%, however, resistance rate to both of these antibiotics among ESBL non-producing isolates was same and somewhat higher (36.8%), respectively. The association between such resistance was statistically insignificant ($p>0.05$). Despite the resistance among certain MDR isolates, fosfomycin and tigecycline were found the best drugs to treat infections by highly resistant MDR bacteria.

Increasing spectrum of multidrug resistance was seen with ESBL production, particularly among gram negative isolates, which was statistically significant ($p<0.05$). Most of the ESBL producers (81.2%) were found to be resistant to 21-30 different drugs tested and some of them (2.3%) were further resistant to 31-40 drugs tested further increasing the spectrum of antibiotic resistance. In a similar study carried out in Iran, 92.8% ESBL producing isolates were resistant to nine antibiotics tested (Mansouri and Abbasi, 2010). More than one-third (35.1%) of ESBL negative isolates were also resistant to 21-30 antibiotics. Considerable resistance rate observed among ESBL negative isolates indicates involvement of other mechanisms of drug resistance or masking of ESBL detection. Moreover, comparison among smaller sample size might have given the larger resistance percentage. All MRSA isolates were ESBL negative and resistant to more than 40 antibiotics. On the other hand, 77.5% MDR isolates were resistant to 5-7 classes of antibiotics of which 61.8% were ESBL positive and all of them were gram negative bacteria. ESBL production and drug resistance to more classes of antibiotics was found statistically significant ($p<0.05$). All the MRSA isolates were also resistant to 5-7 classes of antibiotics. Similar significant multidrug resistance against commonly used antibiotics such as fluoroquinolones, aminoglycosides, tetracyclines, chloramphenicol and co-trimoxazole has also been observed among ESBL-producing bacteria (Sharma *et al*., 2010; Tsering *et al*., 2009; Giske *et al*., 2008; Paterson and Bonomo, 2005). Multidrug resistance among MRSA is usual. Though data on resistance to such larger number of antibiotics is scanty to the best of our knowledge, higher resistance against commonly used antibiotics among MRSA has been reported (Sanjana *et al*., 2010; Kumari *et al*., 2008). This is probably due to the indiscriminate and empirical use of the drugs due to relatively cheaper price and easy availability over-the-counter in Nepal. This higher proportion of resistance among bacterial pathogens extremely limits the therapeutic options in treating infections caused by them, directs toward the prudent use of antibiotics and sophisticated testing methods, and urges for finding more potent options.

6.2 Conclusion

Emergence of multiple drug resistance among common bacterial patohgens limits the therapeutic options in the management of several bacterial infections. Higher percentage of multiple drug resistance (64.9%) prevailed among the common clinical isolates in this study. *E. coli*, *S. aureus*, *Klebsiella* spp., *Acinetobacter* spp. and *P. aeruginosa* were the most frequently isolated bacterial etiologies among various infectious clinical conditions with high level of multiple drug resistance. Predominance of multiple drug resistance was higher among urinary isolates than others. Higher rate of ESBL production (59.9%) and increased multiple drug resistance among the tested MDR bacterial isolates was statistically significant ($p<0.05$). Use of both cefotaxime and ceftazidime in screening ESBL producers and use of cefepime and cefepime plus clavulanate in phenotypic ESBL confirmation was found to be most effective. Temocillin showed greater efficacy against most MDR isolates including ESBL producers than other penicillins. Chloramphenicol and nitrofurantoin still remained useful against various bacterial pathogens. Other conventional first line drugs, newer fluroquinolones, aminoglycosides and fourth generation cephalosporins showed high level of resistance. Vancomycin and clindamycin had best activity against MRSA isolates. Carbapenems, fosfomycin, tigecycline and combination of third and fourth generation cephalosporins with clavulanate remained highly active against most MDR isolates. The higher predominance of ESBL production and MDR phenotypes among common clinical isolates mandates for immediate control measures and search for more potent drugs.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

1. Of the 2141 specimens (1710 urine, 136 pus and body fluids, 170 sputum and throat swab, and 125 eye and ear specimens) processed, 20.2% specimens showed significant growth.
2. Slightly higher rate of infection was observed among females (23.1%) than males (17.7%). However, the association between growth rate and gender was found statistically insignificant ($p>0.05$). Among urinary isolates, statistically significant greater growth rate was observed in females (18.1%) than in males (13.1%).
3. Among 433 various clinical isolates, gram negative bacteria predominated with 67.4% isolates and most of them (83.2%) were from urine. Altogether 25 different bacterial species were isolated (18 gram negative and 7 gram positive) from 4 different types of clinical specimens. *E. coli* (51.7%) predominated among them followed by *K. pneumoniae* (14.7%) and *Acinetobacter* spp. (9.6%). *S. aureus* (41.8%) predominated among gram positive bacteria and most of them were from pus and body fluids.
4. Amikacin was found as the drug of choice among gram negative isolates with low (28.0%) resistance rate followed by ceftazidime, nitrofurantoin and chloramphenicol with relatively lower resistance rates of 28.6%, 29.3% and 30.1%, respectively.
5. Nitrofurantoin and norfloxacin were found to be the best drugs among gram positive isolates with least resistance of only 9.5% followed by chloramphenicol, tetracycline, ceftriaxone, nalidixic acid and ceftazidime with lower resistance rates of 10.6%, 12.8%, 14.2%, 14.3% and 14.7%, respectively. Erythromycin, ofloxacin and penicillin G also showed relatively lower resistance patterns.
6. Of the 59 *S. aureus* isolates, 16.9% of them were MRSA.

7. Of the 433 various clinical isolates, 281 (64.9%) isolates were MDR. Maximum multiple drug resistance was observed among urinary isolates with 78.1% MDR isolates followed by about 51.0% among sputum and throat swab, and pus and body fluid specimens, and 32.8% among eye and ear specimens.
8. Of the total 281 MDR isolates from different clinical specimens, 77.9% were gram negative bacteria and most of them (87.7%) were urinary isolates. Multidrug resistance was more predominant among gram negative bacteria (75.0%) than gram positive bacteria (44.0%). Most of the gram positive MDR bacteria (30.6%) were isolated from sputum and throat swab specimens. *E. coli* (52.0%) and *S. aureus* isolates were the most predominant MDR isolates.
9. Significant multidrug resistance was seen among major gram negative pathogens such as *E. coli* (75.5%), *Acinetobacter* spp. (71.4%), *C. freundii* (66.7%), *Klebsiella* spp. (82.4%) and *P. aeruginosa* (92.3%). Multidrug resistance was also more pronounced among *E. faecalis* (80.0%) and *S. aureus* (52.5%).
10. Of the 281 MDR isolates, 142 isolates were ESBL screen positive of which 59.9% produced ESBLs.
11. Of the 85 ESBL positive isolates, *E. coli* with 77.6% isolates remained predominant ESBL producers followed by *K. oxytoca* (75.0%). Other enteric bacteria also produced ESBLs in varying degrees. No ESBL production was detected among isolates of *Providencia* spp., *P. aeruginosa* and *S. aureus*.
12. Most of the ESBL producers (91.8%) were from urinary specimens among which *E. coli* was most predominant organism. Also, 5.9% ESBL producers were from pus and body fluids and 2.3% were from sputum and throat swabs.
13. In comparative ESBL screening, cefpodoxime and ceftriaxone had the highest sensitivities of 96.5% but with lower respective PPV of 67.2% and 68.3%. Aztreonam and cefotaxime both had equal sensitivity of 94.1% and PPV of 67.8%. Ceftazidime had lowest sensitivity (83.5%) and highest PPV (79.8%). The screening result obtained with ceftazidime versus cefotaxime was found statistically insignificant ($p>0.05$).

14. Four types of combination disks were utilized to confirm ESBL production. Of 85 ESBL positive isolates, maximum ESBL producers (97.6%) were detected by Cefepime-clavulanate combination disks while least number of ESBL producers (91.8%) was detected by cefpodoxime-clavulanate combination.

15. All penicillins tested showed high level of resistance except temocillin which showed 72.5% susceptibility among all isolates. Good activity of temocillin against MDR *Acinetobacter* spp. was of note.

16. Altogether, nitrofurantoin was the drug of choice with least resistance of 29.6% followed by chloramphenicol (39.4%) against MDR isolates tested. Other commoner drugs showed higher level of resistance. Clindamycin and vancomycin remained the drug of choice for MRSA isolates.

17. Cefoxitin showed least *in vitro* resistance (43.5%) among ESBL producers while ceftazidime showed least *in vitro* resistance (31.6%) among ESBL non-producers. Other third and fourth-generation cephalosporins showed high level of *in vitro* resistance among ESBL producers. All isolates of *P. aeruginosa* and 80% ESBL negative *Acinetobacter* spp. isolates were susceptible to ceftazidime. All the MRSA isolates were resistant to all cephalosporins tested.

18. Meropenem and imipenem with no and 2.3% resistance, respectively, were found to be the drug of choice among ESBL positive isolates. Higher degree of carbapenem resistance, 40.3% for imipenem and 19.2% for meropenem, was seen among ESBL negative bacteria. Imipenem resistance was remarkably high among ESBL negative *Acinetobacter* spp. (86.7%) and MRSA (71.4%), and all MRSA isolates were resistant to meropenem.

19. Carbapenem resistance was also observed among *C. freundii*, *E. coli*, *K. pneumoniae*, *Providencia* spp. and *P. aeruginosa*. Also, CPM/CV, CAZ/CV and CTX/CV became the good cephalosporin-clavulanate combinations for ESBL producers with low resistance rates of 2.3%, 7.0% and 10.6%, respectively. CAZ/CV was the drug of choice among the ESBL negative isolates with least (28.0%) resistance.

20. Resistance rates for all newer and fluoroquinolones ranged from 75.4-97.6%. Fewer MDR isolates (44.4%) were resistant to amikacin. Aminoglycoside resistance was more predominant among *Acinetobacter* spp. Almost all MRSA isolates were resistant to all aminoglycosides tested. ESBL production and fluoroquonolone resistance was statistically significant ($p<0.05$).
21. Fosfomycin was found to be the best drug with lowest resistance (17.6%) followed by tigecycline with lower resistance rate of (23.2%). However, 55.5% of all *Acinetobacter* spp. and all ESBL negative *Acinetobacter* spp. were resistant to tigecycline and fosfomycin, respectively. Moreover, 71.4% of *P. aeruginosa* were also resistant to tigecycline while only 28.6% of them were resistant to fosfomycin. Moreover, all MRSA isolates were susceptible to fosfomycin while only 14.3% MRSA were resistant to tigecycline.
22. Greater number of the MDR isolates, i.e. 81.2% that produced ESBL showed increased resistance towards antibiotics with majority of them resistant to 21-30 drugs. Also, 2.35% ESBL positive ones were further resistant to 31- 40 drugs tested. Moreover, 35.1% ESBL negative isolates were resistant to 21-30 antibiotics. All MRSA isolates were resistant to 40 antibiotics. Moreover, 77.5% MDR isolates were resistant to 5-7 classes of antibiotics. ESBL production and increased spectrum of drug resistance was statistically significant ($p<0.05$).

7.2 Recommendations

1. Prevalence of MDR organisms among various clinical specimens should be determined in large scale using most frequently used antibiotics on regular basis to know the exact scenario of drug resistance.
2. Organism specific use of antibiotics after proper *in vitro* susceptibility test should be encouraged for treatment rather than the empirical use of broad spectrum antibiotics.
3. Use of two screening agents, viz. cefotaxime and ceftazidime should be used in routine ESBL screening to detect most ESBLs rather than using single agent.
4. Inclusion of routine ESBL detection tests in all clinical laboratories should be encouraged.
5. ESBL producers should be reported resistant to all penicillis (except temocillin), cephalosporins (except cephamycins) and aztreonam regardless of their *in vitro* susceptibilities determined by disk diffusion and MIC determination methods.
6. Molecular characterization of ESBLs should be done in large scale to determine the exact prevalence of dominant type of ESBLs in different territories, their source and transfer phenomena.
7. Isolates that are ESBL non-producers but still resistant to cephalosporins and carbapenems should be subjected to tests for detection of ABLs and MBLs.
8. Efficacy of commonly used, non-conventional, older and newer antibiotics in different clinical settings should be regularly evaluated.
9. Dissemination of such types of findings to health care professionals and educating patients to avoid self-medication, overuse and underuse of antibiotics should be emphasized. Any problems regarding antimicrobial chemotherapy should be immediately addressed.
10. Establishment of authority to devise guidelines for antibiotic policy with antibiotic stewardship programme is mandatory.
11. Fosfomycin, tigecycline and temocillin should be included in therapeutic regime in treating difficult-to-treat infections by MDR organisms.

CHAPTER-VIII

8. REFERENCES

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APPENDIX-I

PATIENT'S REQUEST FORM

Name: … … … … … … … … … … … … … … … Lab No: … … …
Age / Gender: … … … … … … … …… … … … … Date: … … … …
Address: … … … … … … … … … … … … … …
Brief Clinical History:
Culture requested for: … … … … …
Patient under antibiotic treatment: a). Yes … … b). No … …
If Yes, Antibiotic(s) taken: 1) … … … … … … … … 2) … … … …… … … …

Duration of treatment: … … … …… … … …… … … …

MICROBIOLOGICAL PROCEDURES

Day 1

Date: … … … … …
Specimen type: … … … … … … Time of specimen collection: … … … … … …
Method of specimen collection: … … … … … … … … …
Time of specimen receipt at the laboratory: … … … …
Condition of specimen: … … … … … … … … …
Culture on: 1) … … … … 2) … … … … … 3) … … … … … …
Incubation: 1) Aerobic 2) Anaerobic 3) Microaerophilic
Incubation temperature: … … … … … … … …Incubation time: … … … … … … … …

Day 2

Date: … … … … …

Observation for colony characteristics, Gram-staining reaction preliminary tests on primary culture plates:

Specimen ID	Isolate ID	Colony characteristics									Gram's reaction
		Medium	Shape	Size	Color	Texture	Margin	Elevation	Opacity	Consistency	

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

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

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

Inoculation on different biochemical test media 1) … … … … … 2) … … … … … 3) … … … … … 4) … … … … …

Day 3

Date: … … … … …

A. Observation of Biochemical Tests

Sample ID	Isolate ID	SIM	MR	VP	TSI	Citrate	Lysine	Urease	Ornithine	Others	Identified organism

Note: SIM, Sulfide Indole Motility test; MR, Methyl Red; VP, Voges Proskauer; TSI, Triple Sugar Iron agar test; Lysine and Ornithine, decarboxylase tests.

B. Antibiotic susceptibility Test: Kirby-Bauer Method

Date: … … … … …

Disks Manufacturer: … … … … … Manufacture Date: ... … … ... Expiry Date: … … …

Medium used/Manufacturer: … … … … … … Medium Manufacture Date: … … … … Medium Expiry Date: … … … … … … Medium preparation Date: … … … …

Isolate ID	Organisms	Antibiotic disks used and Disk contents											Remarks (MDR, R to 2 antibiotic classes)
		A 10 µg	T 30 µg	TS 25 µg	C 30 µg	NF 300 µg	NA 30 µg	CIP 5 µg	GM 10 µg	…	…	…	

Note: S = Susceptible, I = Intermediate, R = Resistant

Day 4

Date: … … … …

C. Screening of the Multi-drug resistant bacteria for ESBL Production

Isolate ID	Organisms	Diameter of Zone of Inhibition (mm) of antibiotic disks used for screening and their disk contents					Interpretation
		CTX 30µg	CRO 30µg	CAZ 30µg	CPD 10µg	ATM 30µg	

Day 5

Date: … … … … …

D. ESBL Confirmatory Test

Isolate ID	Organisms	Diameter of zone of inhibition (mm) of antibiotic disks alone and in combination with clavulanate and their disk contents used for ESBL phenotypic confirmation								Interpretation
		CTX30 µg	CTX30 µg + CV10 µg	CAZ30 µg	CAZ30 µg + CV10µg	CPD30 µg	CPD 30 µg + CV10 µg	CPM30 µg	CPM30 µg + CV10 µg	

Day 6

Date: … … … … …

Disks Manufacturer: … … … … … Manufacture Date: ... … … ... Expiry Date: … … …

Medium used/Manufacturer: … … … … … … Medium Manufacture Date: … … … … Medium Expiry Date: … … … … … … Medium Preparation Date: … … … …

E. Testing for susceptibility of ESBL-Positive and ESBL-Negative Isolates towards wide spectrum of conventional and newer antibiotics

Isolate ID	Organisms	Antibiotic disks used and Disk contents											Remarks
		TGC 15 µg	DO 30 µg	MN 30 µg	FOT 200µg	TEM 30µg	CFP30 µg	GAT 5 µg	TN 10 µg	…	…	…	

APPENDIX-II

I. Composition and Preparation of Different Culture Media

The culture media used were manufactured by following companies, viz. Hi-Media Laboratories Pvt. Limited, Bombay, India, Oxoid Unipath Ltd., Basingstoke, Hampshire, England, and Mast Diagnostics, Mast house, Derby Road, Bootle.

1. Blood agar (Hi-Media, M 073)

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

Ingredients	gm/liter
Beef heart infusion	500.00
Tryptose	10.00
Sodium Chloride	5.00
Agar	15.00

Final pH (at 25°C) 7.3±0.2

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

Chocolate agar (CA)

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

2. MacConkey Agar (Hi-Media, M 008)

(Without sodium taurocholate, salt and crystal violet)

Ingredients	gm/liter
Peptone	20.00
Lactose	10.00
Sodium taurocholate	5.00
Sodium chloride	5.00
Neutral Red	0.04
Agar	20.00

Final pH (at 25°C) 7.4±0.2

Preparation: As directed by the manufacturer, 55 grams of the medium was suspended in 1000ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes.

3. Mueller Hinton Agar (Mast, DM 170D)

Ingredients	gm/liter
Beef, Infusion form	300.00
Casein Acid Hyrolysate	17.50
Starch	1.50
Agar	17.00

Final pH (at 25°C) 7.4±0.2

Preparation: As directed by the manufacturer, 38 grams of the medium was suspended in 1000ml distilled water and the medium was warmed to dissolve. Then the medium was sterilized by autoclaving at 121°C

(15 lbs pressure) for 15 minutes. The sterilized medium was then poured into sterile Petri plates and allowed to cool.

4. Nutrient Agar (Hi-Media, M 001)

Ingredients	gm/litre
Peptone	10.00
Sodium Chloride	5.00
Beef Extract	10.00
Yeast Extract	1.50
Agar	12.00

Final pH (at 25°C) 7.4±0.2

Preparation: As directed by the manufacturer, 37 grams of the medium was suspended in 1000ml of distilled water and then boiled to dissolve completely. The medium was sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes, and poured into sterile Petri plates.

5. Nutrient Broth (Hi-Media, M 002)

Ingredients	gm/litre
Peptone	5.00
Sodium Chloride	5.00
Beef Extract	1.50
Yeast Extract	1.50

Final pH (at 25°C) 7.4±0.2

Preparation: As directed by the manufacturer, 13 grams of the medium was dissolved in 1000ml distilled water Then the medium was dispensed in test tube in amount of 3ml in each and and autoclaved at 121°C (15 lbs pressure) for 15 minutes. The sterilized medium was then cooled to room temperature.

6. Mueller Hinton Broth (Oxoid, CM 0405)

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

Final pH (at 25°C) 7.3±0.1

Preparation: As directed by the manufacturer, 21 gram of the media was added to 1000ml of distilled water, mixed well to dissolve, dispensed in screw-capped bottles in amount of 3ml in each and sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes. the sterilized medium was then cooled to room temperature.

7. Mannitol Salt Agar (Hi-Media, M 118)

Ingredients	gm/litre
Proteose peptone	10.00
Beef etract	1.00
NaCl	75.00
D-Mannitol	10.00
Phenol Red	0.025
Agar	15.00

Final pH (at 25°C) 7.4±0.2

Preparation: As directed by the manufacturer, 111.02 grams of the medium was dissolved in 1000ml distilled water, boiled to dissolve completely and sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes. The sterile medium was then poured into sterile petriplates and allowed to cool.

8. Tryptone Soy broth+ 20% Glycerol

Ingredients	gm/litre
Pancreatic Digest of Casein	15.00
Enzymatic Digest of Soybean Meal	5.00
Sodium Chloride	5.00
Glycerol	200ml

Preparation: As directed by the manufacturer, 40 gram of the medium was suspended in 1000ml of distilled water containing 200ml glycerol and mixed thoroughly. It was boiled completely and autoclaved at 121°C for 15 minutes.

II. Biochemical Test Media

1. MR-VP Medium (Hi-Media, M 070I)

Ingredients	gm/litre
Buffered Peptone	7.00
Dextrose	5.00
Dipotassium Phosphate	5.00

Final pH (at 25°C) 6.9±0.2

Preparation: As directed by the manufacturer, 17 grams of medium was dissolved in 1000ml distilled water, heated to dissolve completely, dispensed in the amount of 3 ml in each test tube and autoclaved at 121°C (15 lbs pressure) for 15 minutes. The tubes were then allowed to cool.

2. Hugh and Leifson's Medium (Hi-Media, M 826)

Ingredients	gm/litre
Tryptone	2.00
Sodium Chloride	5.00
Dipotassium Phosphate	0.30
Bromothymol Blue	0.08
Agar	2.00

Final pH (at 25°C) 6.8±0.2

Preparation: As directed by the manufacturer, 19.40 grams of the medium was dissolved in 1000ml distilled water and then heated to boiling to dissolve completely. The medium was then sterilized by autoclaving for 15 minutes at 15 lbs pressure (121°C). To 1000ml sterile medium 100ml of sterile Dextrose was added aseptically, mixed thoroughly and dispensed in 5ml quantities into sterile culture tubes.

3. Sulphide Indole Motility (SIM) medium (Hi-Media, M 181)

Ingredients	gm/litre
Beef Extract	3.00
Peptone	30.00
Peptonized Iron	0.20
Sodium Thiosulphate	0.025
Agar	3.00

Final pH (at 25°C) 7.3±0.2

Preparation: As directed by the manufacturer, 36.23 grams of the medium was suspended in 1000ml distilled water and dissolved completely. It was dispensed into test tubes to a depth of about 3 inches and autoclaved for 15 minutes at 15 lbs pressure (121°C).

4. Simmon's Citrate Agar (Hi-Media, M 099)

Ingredients	gm/litre
Magnesium Sulfate	0.20
Mono-ammonium Phosphate	1.00
Dipotassium Phosphate	1.00
Sodium Citrate	2.00
Sodium Chloride	5.00
Agar	15.00
Bromothymol Blue	0.08

Final pH (at 25°C) 6.8±0.2

Preparation: As directed by the manufacturer, 24.2 grams of the medium was dissolved in 1000ml distilled water. Then 3ml of the medium was dispensed in each test tubes and sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes. After autoclaving tubes containing medium were tilted to form slants during cooling.

5. Triple Sugar Iron (TSI) Agar (Hi-Media, M 021)

Ingredients	gm/litre
Peptic digest of animal tissue	10.00
Casein enzymatic hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Ferrous Sulphate	0.20
Sodium Chloride	5.00
Sodium Thiosulphate	0.30
Phenol Red	0.024
Agar	12.00

Final pH (at 25°C) 7.4±0.2

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

6. Christensen Urea Broth (Hi-Media, M 112)

Ingredients	gm/litre
Peptic digest of animal tissue	1.00
Dextrose	1.00
Sodium Chloride	5.00
Dipotassium Phosphate	1.20
Mono-potassium Phosphate	0.80
Phenol Red	0.012

Final pH (at 25°C) 7.4±0.2

Preparation: As directed by the manufacturer, 24 grams of the medium was dissolved in 950ml distilled water and sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes. After cooling to about 45°C, 50ml of sterile 40.0% urea was added aseptically and mixed well. Then 5ml in each test tube was dispensed and cooled to room temperature.

7. Decarboxylase test medium (Hi-Media, M 912)

Ingredients	gm/litre
Peptic digest of animal tissue	5.00
Yeast extract	3.00
Dextrose	1.00
Bromo-cresol purple	0.02
Final pH (at 25°C) 6.8±0.2	

Preparation: As directed by the manufacturer, 9 grams of the medium was dissolved in 1000ml of the distilled water. The medium was then divided into four equal parts. One part was dispensed in test tubes without adding amino acid, and to the remaining three parts three amino acids (L-Lysine, L-Arginine and L-Ornithine) were added separately to a final concentration of 0.5% and dispensed in test tubes. The tubes were then sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes.

III. Staining and Test Reagents

1. Preparation of gram staining reagents

(a) Hucker's crystal violet stain

In a clean piece of paper, 40gm of crystal violet (90.0-95.0% dye content) was weighed, dissolved in 400ml of ethanol (95.0%), filtered and stored a clean brown bottle at room temperature. For the preparation of working solution of crystal violet, 40ml of stock solution was added to 160ml of filtered ammonium oxalate solution (1.0%).

(b) Lugol's iodine

To prepare stock solution of Lugol's iodine, 25gm of iodine crystals and 50gm of potassium iodide crystals were dissolved in 500ml of distilled water in a brown glass bottle. For the preparation of working solution, 60 ml of Lugol's iodine stock solution was mixed with 220ml of distilled water and 60 ml of 5.0% sodium bicarbonate solution.

(c) Acetone-alcohol decolorizer (1:1)

To 50ml of ethanol (95.0%), 50ml of acetone was mixed well and transferred into a clean bottle. The preparation was labeled with date and stored at room temperature.

(d) Safranin (Counterstain)

The stock solution was prepared by dissolving 5gm of safranine in 200ml of 95.0% ethanol and the working solution was prepared by mixing 20ml of stock solution with 180ml of distilled water.

3. Normal saline

Preparation: In 100ml of distilled water, 0.85gm of sodium chloride was dissolved well in a leak-proof bottle. The bottle was then labeled and stored at room temperature.

4. Test reagents

A. For catalase test

To make 100ml Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3.00ml
Distilled Water	97.00ml

Preparation: To 97ml of distilled water, 3ml of hydrogen peroxide was added and mixed well.

B. For oxidase test

To make 100ml Oxidase Reagent (impregnated in Whatman's No. 1 filter paper)

Tetramethyl *p*-phenylene diamine dihydrochloride (TPDD)	1.00gm

Distilled Water 100.00ml

Preparation: In 100ml of distilled water, 1gm of TPDD was dissolved. To that solution strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then those strips were freeze-dried and stored in a dark bottle tightly sealed with a screw cap.

C. Kovac's reagent for indole test

To prepare 40ml reagent
Isoamyl alcohol 30.00 ml
4-dimethyl aminobenzaldehyde 2.00gm
Conc. hydrochloric acid 10.00ml

Preparation: In 30ml of isoamylalcohol, 2gm of 4-dimethyl aminobenzaldehyde was dissolved in a clean brown bottle. Then, 10ml of conc. HCl was added to that and mixed well.

D. Methyl red solution for methyl red test

To make 50ml solution
Methyl red 0.05gm
Ethyl alcohol (absolute) 28.00ml
Distilled Water 22.00ml

Preparation: To 28ml ethanol, 0.05gm of methyl red was dissolved in a clean brown bottle. Then 22ml distilled water was added to that bottle and mixed well.

E. Barritt's reagent for Voges-Proskauer test
Voges-Proskauer reagent A
To make 100ml
-Napthol 5.00gm
Ethyl alcohol (absolute) 100.00ml

Preparation: To 25ml distilled water, 5gm of -napthol was dissolved in a clean brown bottle. Then the final volume was made 100ml by adding distilled water.
Voges-Proskauer reagent B
To make 100ml
Potassium hydroxide 40.00gm
Distilled Water 100.00ml

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

The three parts of reagent A (5.0% -napthol) was mixed with one part of reagent B (40.0% KOH) to get the working solution of the Barritt's reagent.

APPENDIX-III

I. List of Equipment, Materials and Supplies

A. General microbiology laboratory equipment

(a). Autoclave (Stermite, Japan) **(b).** Incubator (Sakura, Japan) **(c).** Hot air oven (Memmert, Germany; and GallenKamp) **(d).** Microscope (Olympus, Japan) **(e).** Refrigerator, 4-8°C (Sanyo, Japan); -20°C (Videocon, India); -75°C (Sanyo, Japan) **(f).** Weighing machine (Ohaus Corporation, USA) **(g).** Water Bath (Boekel Scientific, USA) **(h).** Gas burner **(i).** Glasswares **(j).** Inoculating loops and wires (k). Sterile cotton swabs **(l).** Sterile Petri plates **(m).** McFarland 0.5 turbidity standards **(n).** Vortex mixer **(o).** Ruler **(p).** Forceps

B. Microbiological media

(a). Blood Agar **(b).** Chocolate Agar **(c).** MacConkey Agar **(d).** Mueller Hinton Agar **(e).** Mannitol Salt Agar **(f).** Mueller Hinton Broth **(g).** Hugh and Leifson Medium **(h).** Sulfide Indole Motility medium **(i).** MRVP broth **(j).** Triple Sugar Iron Agar **(k).** Urea broth **(l).** Simmon's Citrate Agar **(m).** Nutrient Agar **(n).** Tryptone Soy Broth

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% -naphthol in a ratio of 1:3), Barium Chloride, Conc. H_2SO_4, , Glycerol, Gram's reagent, etc.

D. Antibiotic disks

The following antibiotic disks were used for the antibiotic susceptibility tests which were from Mast Group Ltd., Mast House, Derby Road, Bootle, Merseyside, L201EA, UK.

Amikacin (AK/30µg), Amoxycillin (A/20µg), Augmentin (AUG/30µg), Azithromycin (ATH/15µg), Erythromycin (E/15µg), Aztreonam (ATM/30µg), Bacitracin (BA/10U), Bacitracin (BA/0.04IU), Cefotaxime (CTX/30µg), Ceftazidime (CAZ/30µg), Cefpodoxime (CPD/10µg/30µg), Ceftriaxone (CRO/30µg), Cotrimoxazole (TS/25µg), Cephalothin (KF/30µg), Cefoxitin (FOX/30µg), Nalidixic acid (NA/30µg), Ciprofloxacin (CIP/5µg), Ofloxacin (OFX/5µg), Levofloxacin (LEV/5µg), Moxifloxacin (MFX/1µg), Gatifloxacin (GAT/5µg), Tobramycin (TN/10µg), Neomycin (NE/30µg), Gentamicin (GM/10µg), Netilmicin (NET/30µg), Kanamycin (K/30µg), Chloramphenicol (C/30µg), Cefepime (CPM/30µg), Cefpirome (CFP/30µg), Clindamycin (CD/2µg), Imipenem (IMI/10µg), Meropenem (MEM/10µg), Norfloxacin (NOR/10µg), Nitrofurantoin (NI/300µg), Oxacillin (OX/1µg), Penicillin G (PG/10U), Tetracycline (T/30µg), Doxycycline (DXT/30µg), Minocycline (MN/30µg), Tigecycline (TGC/15µg), Ticarcillin (TC/75µg), Fosfomycin (FOT/200µg), and Vancomycin (VA/30µg).

Methicillin (M/5µg) disks used were from Hi-Media Laboratories Pvt. Ltd., India.

E. Disks for ESBL detection

(a). MASTDISCS™ ID Extended Spectrum Beta-Lactamase (ES L) Detection Discs (D52C): Cefotaxime (CTX/30µg), cefpodoxime (CPD/30µg) and ceftazidime (CAZ/30µg) alone and each in combination with clavulanic acid (CV/10µg)

(b). MASTDISCS™ ID Cefepime ES L ID Disc Set (D63C): Cefepime (CPM/30µg) alone and its combination with clavulanic acid (CV/10µg)

APPENDIX-IV

A. Gram-Staining

The method was first devised by Sir Hans Christian Gram during the late 19th century.This method can be used effectively to divide all bacterial species into two major groups: Gram-positive (those that take up and retain the basic dye crystal violet even on washing) and Gram-negative (those that allow the crystal violet dye to wash out easily with the decolorizer of alcohol or acetone). The following steps were performed for Gram-staining:

1. A thin oval smear of the material to be examined was prepared on a clean grease free glass slide and air dried. The material on the slide was then heat fixed and allowed to cool before staining.
2. The slide was flooded with crystal violet stain and allowed to remain without drying for 30-45 seconds. Then the slide was rinsed with tap water to remove the excess stain.
3. Then the slide was flooded with Gram's 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. Again the slide was rinsed with tap water to remove excess iodine.
4. The slide was flooded with acetone/alcohol decolorizer for 10 seconds and rinsed immediately with sufficient tap water until no further color flowed from the slide with the decolorizer. Thicker smear required more aggressive decolorizing.
5. The slide was flooded with counter stain (safranin) for 30 seconds and washed off with tap water.
6. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 1000X.

Quality control

The laboratory staining procedure and reagents were tested prior to use of new lots of each staining and decolorizing agents and at least weekly thereafter, using *Escherichia coli* ATCC 25922 (Gram negative) and *Staphylococcus aureus* ATCC 25923 (Gram positive).

B. Biochemical Tests for Bacterial Identification

Catalase test

Microorganisms produce hydrogen peroxide (H_2O_2) during aerobic respiration, which is lethally toxic 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, with major exception being *Streptococcus* species. About 2-3 drops of 3.0% H_2O_2 was put on the surface of a clean glass slide and a small amount of a culture from Nutrient Agar plate was applied with sterile wooden or glass stick. Presence of effervescence indicated the positive test. A false positive reaction was prevented by avoiding the use of the culture medium with catalase (e.g., Blood Agar) or an iron loop.

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 (TPDD), the cytochrome oxidase oxidizes it into a deep purple colored end product, Indophenol, which is detected in this test. A small aliquot of colony of the test organism was rubbed on the filter paper soaked in oxidase reagent with the help of sterile glass rod. The positive test was 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 products. 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 medically important bacteria are facultative anaerobes. The test organism was stabbed into the bottom of two sets of tubes with Hugh and Leifson's media, containing bromothymol blue as the pH indicator. The inoculated medium in one of the tubes was covered with a 10mm deep layer of sterile paraffin oil. The tubes were then incubated at 37°C for 24 hours. After incubation the tubes were examined for carbohydrate utilization as shown by acid production. Fermentative organisms utilize the carbohydrate in both the open and sealed tubes as shown by a change in colour of the medium from green to yellow. However, oxidative organisms 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. Indole, if present, combines with the aldehyde present in the reagent to produce red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole. A smooth bacterial colony was stabbed on Sulfide Indole Motility (SIM) medium by a sterile inoculating wire and the inoculated media was incubated at 37°C for 24 hours. After 24 hours incubation, 0.5ml of Kovac's reagent was added. Appearance of red color on the top of media indicated the indole positive reaction.

Motility test

Motile organisms migrate from the stabline and diffuse into the medium causing turbidity whereas non-motile bacteria show the growth only along the stabline, and the surrounding medium remains colorless and clear. This test was macroscopically observed in semisolid Sulfide Indole Motility (SIM) medium.

Methyl red test

This test is performed to test the ability of an organism to produce sufficient acid from the fermentation of glucose. The degree of acidity is denoted by intensity of color change of methyl red indicator over a pH range of 4.4-6.0. A pure colony of the test organism was inoculated into 2ml 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 detects 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 2ml 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 detects whether an organism can utilize citrate as a sole source of carbon for metabolism with resulting alkalinity. Organisms capable of utilizing citrate as sole carbon source also utilize the ammonium salts present in the medium as sole nitrogen source. Production of ammonia imparts alkalinity, thus changing the color of indicator Bromothymol blue from green (pH 6.0-7.6) to blue (pH   7.6). 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. Growth of organism and change of color of media from green to blue indicated a positive test.

Triple sugar iron (TSI) agar test

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 stabbed on the butt and streaked on the surface of TSI slant 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 indicated sucrose or lactose fermentation. Phenol red is the pH indicator which gives yellow colour at acidic pH, and red colour at alkaline condition.

Urease test

This test demonstrates the ability of bacteria to produce urease which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced imparts alkalinity and changes the color of phenol red indicator incorporated in the medium. The test organism was inoculated in a medium containing urea and the indicator phenol red and incubated at 37°C overnight. Change in color of the medium to deep pink red indicated positive reaction, i.e. urease production.

Amino acid decarboxylase test

This test identifies the ability of some bacteria to decarboxylate an amino acid to the corresponding amine with the liberation of carbon dioxide. Lower pH induces the production of these decarboxylases and pH rises to the neutrality or above as a result of their action. Lysine, ornithine and arginine are the three principal amino acids used in this test which are converted to cadaverine, putrescine and citrulline respectively by the specific enzymes. A pure colony of test organism was inoculated lightly through the paraffin layer of each broth medium containing lysine, ornithine and arginine separately with a straight wire. The tubes were incubated at 37°C and read daily for 4 days. The development of violet color in the medium indicated decarboxylation.

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 for DNase production by flooding the plate with a weak hydrochloric acid solution as acid precipitates unhydrolysed DNA. Clear areas developed around the DNase producing colonies due to DNA hydrolysis.

Coagulase tests

Coagulase tests differentiate species within the genus *Staphylococcus*: *S.aureus* (usually positive) from *S. epidermidis* (negative). A positive coagulase test is usually the final diagnostic criterion for the identification of *S. aureus.* Free coagulase and bound coagulase are the two types of coagulase possessed by this organism most strains of *S.aureus* possess both free and bound coagulase.

- **Slide coagulase test**

  Slide test detects bound coagulase (Clumping Factor) which is bound to the bacterial cell wall and reacts directly with fibrinogen resulting in alteration of fibrinogen so that it precipitates on the staphylococcal cell, causing cell clumping 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 as a control.

- **Tube coagulase test**

  This test detects free coagulase. Plasma contains coagulase reacting factor (CRF) which activates free coagulase. The activated coagulase converts prothrombin to thrombin which in turn converts fibrinogen into fibrin which is detected as a firm gel (clot) in the tube test. Tube test is performed in case of negative or doubtful results in slide test. The plasma was first diluted 1 in 10 in physiological saline. Four small tubes were taken, one for test organism, other for positive control, another for negative control, and next to observe self clotting of plasma. Then 0.5ml of the diluted plasma was pipetted into each tube and 0.5ml of test organism, 0.5ml of positive control (*Staphylococcus aureus* culture), and 0.5ml negative control (*S. epidermidis* culture) was added to three respective tubes. To the fourth tube, 0.5ml sterile broth was added. After mixing gently, all tubes were incubated at 37°C on

a waterbath for 6 hours and observed for gel formation in every 30 minutes. Any clot formation indicated the positive coagulase test.

Optochin susceptibility test

This test is used to differentiate pneumococci from other streptococci. Optochin (ethyl hydrocupriene hydrochloride) 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 5µg. The pneumococci susceptible to optochin show a zone of inhibition of ≥14mm, while other alpha hemolytic streptococci grow to the edge of the disk. A paper disk containing 5µg 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°C in candle jar for overnight. After incubation the plate was observed for zone of inhibition around the disk.

Bacitracin susceptibility test

This test is used to screen *H. influenzae* from the primary agar plate. *H. influenzae* is resistant to the 10 Unit Bacitracin disc but the viridans streptococci, *Neisseria* spp., diptheroid bacilli and staphylococci are susceptible and get supressed. A paper disk containing 10U 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°C in candle jar for overnight. After incubation, the plate was observed for the colonies of *H. influenzae* growing near the bacitracin disk.

Bile solubility test

This test differentiates pneumococci and viridans streptococci. Bile lyses the pneumococcal cells by attacking the peptidoglycan, whereas the viridans 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 5ml nutrient broth from the blood or chocolate agar and was incubated at 37°C for overnight. Then 0.5ml of bile salt solution was added in one tube while another was taken as control. Incubation was continued for 15-30 minutes. In the positive test, turbidity of broth was cleared as compared to the control tube.

Tests for X and V factor requirements

This test is carried out to differentiate *H. influenzae* from *H. parainfluenzae. H.influenzae* requires both X-factor (haemin or various iron containing compounds such as protoporphyrin IX) and the V-factor (NAD or NADP) as the accessory growth factors. *H. parainfluenzae* requires only the V-factor for growth. Disks impregnated with X factor alone, V factor alone and X and V factor together were placed on Mueller-Hinton agar which had been inoculated with a light suspension of the organism. After overnight incubation at 37°C in candle jar, the plate was examined for growth around each disk. The growth only around the XV disc was indicative of the species being *H. influenzae*.

APPENDIX-VI

1. Antimicrobial Susceptibility Testing by Disk Diffusion Method

A. Principle

A standardized inoculum of bacteria is swabbed onto the surface of MHA plate. Filter paper disks impregnated with antimicrobial agents are placed flat on the agar surface. After overnight incubation, the diameter of the zone of inhibition is measure around each disk. By referring to the tables in the CLSI disk diffusion standard, a qualitative report of susceptible, intermediate or resistant is obtained.

B. Procedures

1. Preparation of 0.5 McFarland Standard turbidity solution: To 0.5ml of 0.048M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$), 99.5ml of 0.18M H_2SO_4 (1%v/v) was added with constant stirring. The solution was then distributed into test tubes, well screw-capped, wrapped with almunium foil and stored in dark till use.

2. The agar plates and antibiotic disk cartridges were brought to room temperature before use. Agar plates were made to contain no excess moisture on their surface by placing them in the 35°C ambient air incubator with lids ajar for evaporation.

3. Inoculum preparation: By touching 2-3 morphologically similar colonies not more than 24 hours old with sterile loop on nonselective agar plate, MHB or NB was inoculated and incubated at 37°C until turbidity matched with that of the 0.5 McFarland Standard turbidity solution.

4. Inoculation of agar plates: Within 15 minutes of adjusting turbidity, a sterile cotton swab was dipped into the inoculums and rotated against the wall of the tube above the liquid to remove excess inoculum. The entire surface of the Mueller Hinton agar plate was swabbed by rotating plates approximately 60° between successive swab strokes to ensure even distribution. Any touching to sides of the plates and aerosol formation was avoided. Finally, the swab was run around the edge of the agar to remove any excess moisture. The plates were left for about 10-15 minutes to let the agar surface dry before the application of the disks.

5. Appropriate antibiotic disks were placed evenly (not closer than 24mm from center to center) on the inoculated agar surface either with dispenser or manually with sterile forceps and a gentle pressure was applied on each disk with sterile forceps to ensure complete contact of disk with agar. Not more than six disks were placed on a 90mm Petri plate. Within 30 minutes of disk application, the plates were incubated in inverted position at 35°C in an ambient air incubator for 16-18 hours.

6. Reading, interpretation and reporting: Only the plates with lawn of confluent or nearly confluent growth were read. The diameter of zones of inhibition was measured in nearest whole millimeter with a measuring scale by observing the plate against nonreflecting surface. In case of sulfonamides, trimethoprim and co-trimoxazole, light growth (<20.0% of lawn growth) was disregarded and zone of inhibition was measured by using the edge of the more obvious margin of the zone. In case of development of discrete colonies within the zone of inhibition (which may represent mixed culture or resistant variants), each morphologically different single colonies were subcultured, re-identified and retested for antibiotic susceptibility. If they were still present, the colony-free inner zone was measured. Any aberrant results were confirmed by repeating the test twice and processing accordingly. Criteria specified by the CLSI (for most antibiotics), BSAC (for temocillin and moxifloxacin) and FDA (for tigecycline) were used unambiguously to interpret the zone of inhibition and the categorical results were reported as either susceptible (S), intermediate (I), or resistant (R).

Quality control

a. QC strains: **i).** *Escherichia coli* ATCC 25922 **ii).** *Staphylococcus aureus* ATCC 25923

b. Monitoring Accuracy: QC strains were tested daily and weekly by following routine procedures and results were recorded. Also the lot number and expiry date of the antibiotic disks and MHA were noted.

The obtained results were compared to expected results (CLSI QC tables) and in case of any aberrant result, the procedure was continued with corrective action.

APPENDIX-VII

A. Inhibitor Potentiated Disk Diffusion (IPDD) Test/Combined Disk Assay for phenotypic confirmation of ESBL production

ESBL production was confirmed among the suspected bacterial strains according to the CLSI (Clinical and Laboratory Standard Institute) guidelines for phenotypic confirmatory testing. According to these guidelines, when confirming ESBL production among the suspects using Combined Disk assay, an increase in zone size of 5 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 onto Mueller Hinton broth and incubated at 35°C until the turbidity matched with 0.5 McFarland standard turbidity solution.

ii). The test organism was carpet-cultured on a MHA plate using a sterile cotton swab.

iii). Using sterile forceps, the ESBL detection disks were applied onto the inoculated medium ensuring that they are evenly spaced and in proper contact with the agar surface.

iv). The plates were incubated at 35-37°C for 18-24 hours and the results were interpreted accordingly.

Interpretation

Zones of inhibition for cefotaxime, cefpodoxime, ceftazidime and cefepime were compared to that of their respective combinations with clavulanic acid. An increase in zone diameter by 5mm in the presence of clavulanic acid for any or all of the disks indicated the presence of ESBL in the test organism.

Quality control

Quality control was maintained by performing the test with at least one organism to demonstrate positive reaction and at least one other organism to demonstrate negative reaction. For positive controls, *Escherichia coli* NCTC 13351 and *Klebsiella pneumonia* ATCC 700603 were tested while for negative control, *Escherichia coli* ATCC 25922 was tested.

Other QC measures related to antibiotic susceptibility test were also strictly followed.

APPENDIX-VIII

Distinguishing biochemical reactions of common and pathogenic Enterobacteriaceae

Organisms	Tests/ Substrates[a]											
	lac	mot	gas	ind	VP	cit	PDA	ure	lys	H_2S	inos	ONPG
Escherichia coli	+	+	+	+	-	-	-	-	+	-	-	+
Shigella groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-
Sh. sonnei	-	-	-	-	-	-	-	-	-	-	-	+
Salmonella (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-
Salmonella typhi	-	+	-	-	-	-	-	-	+	+	-	-
Salmonella paratyphi A	-	+	+	-	-	-	-	-	-	-	-	-
Citrobacter freundii	±	+	+	-	-	+	-	±	-	±	-	+
C. koseri	±	+	+	+	-	+	-	±	-	-	-	+
Klebsiella pneumoniae	+	-	++	-	+	+	-	+	+	-	+	+
K. oxytoca	+	-	++	+	+	+	-	+	+	-	+	+
Enterobacter aerogens	+	+	++	-	+	+	-	-	+	-	+	+
Ent. cloacae	+	+	+	-	+	+	-	±	-	-	-	+
Hafnia alvei	-	+	+	-	+	-	-	-	+	-	-	+
Serratia marcescens[b]	-	+	±	-	+	+	-	-	+	-	±	+
Proteus mirabilis	-	+	+	-	±	±	+	++	-	+	-	-
P. vulgaris	-	+	+	+	-	-	+	++	-	+	-	-
Morganella morganii	-	+	+	+	-	-	+	++	-	±	-	-
Providencia rettgeri	-	+	-	+	-	+	+	++	-	-	+	-
Prov. stuartii	-	+	-	+	-	+	+	±	-	-	+	-
Prov. alcalifaciens	-	+	+	+	-	+	+	-	-	-	-	-
Yersinia enterocolitica[c]	-	-	-	±	-	-	-	±	-	-	±	+
Y. pestis	-	-	-	-	-	-	-	-	-	-	-	±
Y. pseudotuberculosis	-	-	-	-	-	-	-	+	-	-	-	±

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

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

[c] *Yersinia* species are motile at 22°C.

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

(Source: Collee *et al.,* 1996)

APPENDIX-IX

Table of -lactamase classification (Bush *et al.*, 1995).

Bush-Jacoby-Medeiros system	**Major subgroups**	**Ambler System**	**Main Attributes**
Group 1 Cephalosporinases		C (cephalosporinases), also called AmpC enzymes	Usually chromosomal; resistance to all -lactams except carbapenems; not inhibited by claculanate
Group 2 Penicillinases (Clavulanic acid susceptible)	2a	A (Serine -lactamases)	Staphylococcal penicillinases
	2b	A	Broad spectrum: TEM-1, TEM-2, SHV-1
	2be	A	Extended spectrum: TEM-3-??, SHV-2; inactivate 3rd generation cephalosporins and monobactams
	2br	A	Inhibitor resistant Tem(IRT); diminished inhibition by clavulanic acid and sulbactam; still susceptible to tazobactam
	2c	A	Hydrolyse carbenicillin and cloxacillin
	2e	A	Cephalosporinases; hydrolyse monobactams; inhibited by clavulanate
	2f	A	Serine-based carbapenemases; inhibited by clavulanate
	2d	D (Oxacillin, cloxacillin, dicloxacillin hydrolysing)	Cloxacillin hydrolyzing (OXA); poorly inhibited by clavulanate; some are ESBLs
Group 3 Metallo- -lactamases	3a, 3b and 3c	B (Metalloenzymes)	Zinc dependent carbapenemases; not inhibited by clavulanate; hydrolyze penicillins, cephalosporins and carbapenems but not monobactams
Group 4		Penicillinase; no molecular class	Miscellaneous enzymes most not yet sequenced; not inhibited by clavulanate