CHAPTER-I

1. INTRODUCTION

Antibiotics today are used either prophylaxis, empiric therapy or pathogen directed therapy which plays a central role in the control and management of infectious diseases. There are a number of antibiotic resistant organisms causing concern at present worldwide. Notable Gram-positive organisms include methicillin resistant *Staphylococcus aureus* (MRSA), methicillin resistant coagulase-negative staphylococci (CONS), glycopeptide intermediate sensitivity *S. aureus* (GISA) and vacomycin-resistant *Enterococcus* (VRE) species. Concerns among the Gram-negative organisms include multidrug resistant *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and members of the Enterobacteriaceae. So, detection of antibiotic resistance is an essential component of the practice of clinical microbiology which allows physicians to make accurate choices with all forms of antimicrobial therapy-prophylaxis, empiric therapy and pathogen directed therapy.

The morbidity and mortality of infectious disease has increased in parallel with the greater acquisition of antibiotic resistance by organisms, especially in regards to strains that are completely resistant to antibiotics (e.g. some enterococci). Antibiotic resistance has also driven the cost of health care up as we continue to search for more and more expensive antimicrobials. Hence, without surveillance for the development of antibiotic resistance, serious infections may lead to death due to inadequate and inappropriate antibiotic therapy and also affect other sectors of antimicrobial therapy (Levi, 2001).

In microorganisms, resistance to antibiotics could be explain on biochemical basis or genetic basis. The major biochemical mechanism of antibiotic resistance is inactivation of the antibiotic in question by enzymes. For instance, β-lactamase splits amide bond of the β-lactam ring in drugs like penicillin and cephalosporin.

Genetic acquisition of antibiotic resistance is mediated by gene transfer mechanism between bacterial species, successful genetic mutation(s) and a combination of mutational and gene transfer events (Forbes *et al*., 2002).

Genetic exchange is likely to arise in soil and the general environment as well as the gut of humans and animals. Due to misuse and overuse of antibiotics, most clinically relevant bacterial pathogens have acquired a selection process to adapt to the pressures of antimicrobial attack, so that certain strains are now no longer susceptible to one or more of these antimicrobial agents (Levi, 2001). Agriculture and veterinary use of antibiotics also makes an important and unhelpful contribution (Denyer *et al*., 2004).

In many countries, antimicrobials can be obtained outside of recognized treatment centers, and taken without medical authorization or supervision. This leads to the inappropriate use of antimicrobials and consumed at sub-optimal dosages and for an insufficient length of time. Often the high cost of an antibiotic results in an incomplete course being purchased, sufficient only to alleviate symptoms. Broad spectrum antibiotics are frequently used prophylactically, e.g. tetracycline. Developing countries are often unable to afford costly second-line antibiotics to treat infections due to resistant organisms. This results in prolonged illness with longer periods of infectivity and the further spread of resistant strains. All these factors contribute to emerge antibiotic resistance worldwide, however conditions is even worst in developing countries (WHO, 2004).

Often antibiotic resistant bacterial isolates are multidrug resistant. In a random sample of 1,080 drug resistant *E. coli* isolates, 90% exhibited a multidrug resistance (MDR) phenotype from stool samples among children (Bartoloni et *al*, 2006). The vast majority of studies have looked at the detection and diagnosis of resistance caused by one class of antimicrobial agents among the certain samples while this research have looked at the detection as well as characterization of resistance to multiple antimicrobial agents

from different types of infections because MDR strains are associated with an emergence of treatment failure.

The defining criterion for Multidrug Resistance (MDR) in this study was resistance to ≥2 of the antimicrobial agents belonging to different structural classes (Bartoloni *et al.*, 2006 and Wright *et al*., 2000).

Several genetic and other factors contribute to emerge MDR strains today. Integrons, transposons, R-factor are special cases of multidrug resistance.These factors are involved to alter Extended-spectrumof antibiotics by producing mainly β-lactamases, altered binding sites and newer efflux system (Denyer *et al.*, 2004). On these scenario, the plasmid-mediated extended-spectrum beta-lactamases (ESBLs) are of increasing concern. Most are mutants of classic TEM- and SHV-beta-lactamases types. Unlike these parent enzymes, ESBLs hydrolyze oxymino-cephalosporins such as cefuroxime, cefotaxime, ceftriaxone, ceftizoxime, ceftazidime, cefpirome and cefepime, aztreonam, as well as penicillins and other cephalosporins, except for cephamycin (cefoxitin and cefotetan) (Bush *et al*.,1995).

AmpC β-lactamases (ABLs) are another concern of emergenence of MDR strains, demonstrated or presumed to be chromosomally or plasmid mediated, have been described in pathogens e.g. *K. pneumoniae, E. coli, Salmonella* Spp., *P. mirabilis, C. freundii, Acinetobacter* Spp., *Enterobacter* Spp. and *P. aeruginosa* are clavulanate-resistant that confer resistance to a wide variety of beta-lactam drugs as well as other different classes of antibiotics (Black *et al.*,2004).

The advent of carbapenems in the 1980s heralded a new treatment option for serious bacterial infections, carbapenem resistance can now be observed in Enterobacteriaceae and *Acinetobacter* spp. and is becoming commonplace in *P. aeruginosa* is due to production of metallo-ß-lactamases (MBLs) and serine β-lactamse. The emergence of

metallo-beta-lactamase (MBL) producing gram-negative bacteria is a clinical threat for the emergence of MDR strains (Takakura *et al.*, 2002).

However, there are other classes of β-lactamases involved in drug resistance like serine β-lactamase, inhibitor resistant β-lactamase etc but study on these viz ESBL, ABL and MBL have immense clinical significance in the study of MDR strains. This study go through these β-lactamase because there are no guidelines in place for the phenotypic detection of resistance caused by ABLs and MBLs and there is much need for clinical laboratories to address this issue. This study also measures the magnitude of association of multiple drug resistance towards ESBL, ABL and MBL production that have many clinical implications for formulation of antibiotic therapy against MDR pathogens.

Many researchers examined ESBLs are typically plasmid mediated. Until, late 1980s AmpC are typically chromosomally controlled but the plasmid-encoded or 'imported' *ampC* ß-lactamase complicates the scenario in hospital laboratories because plasmid mediated AmpC resistance were found in *Klebsiella* spp., *Escherichia coli*, or *Salmonella* spp. The majority of MBL genes are found on plasmids. So this research focused on ESBL, ABL and MBL mediated multidrug resistance and their dependency onto trasferrable plasmids seems to be pertinent about the reasoning of increasing prevalence of MDR strains and their mechanistic phenomena by going through plasmid DNA analysis.

CHAPTER II

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

To determine the prevalence of multidrug resistant bacterial pathogens and evaluation of extended-spectrum, AmpC, and metalllo β-lactamase producing strains among MDR isolates from different clinical specimens and their likely transfer mechanism by plasmid DNA analysis.

2.2 SPECIFIC OBJECTIVES

- To isolate and identify the bacterial pathogens from different clinical specimens collected from patients visiting KMH.
- To analyze the antibiotic susceptibility pattern of the isolated organisms.
- To find out the prevalence of Multidrug resistant organisms among the total isolates.
- To evaluate the status of Extended-spectrum, AmpC and Metallo ß-Lactamase producing strains from the MDR isolates.
- To anayze the transfer of ESBL, ABL and MBL production phenotypes by conjugation and transformation study.
- To study the transfer of plasmid mediated MDR traits by analyzing plasmid DNA profiles.
- To analyze the level of drug resistance and cause of association of ESBL, AmpC and MBL production.
- To determine the common plasmid types responsible for multidrug resistance.

CHAPTER-III

3. LITERATURE REVIEW

3.1 BACTERIAL INFECTIONS AND MULTIDRUG RESISTANCE

3.1.1 Urinary tract infection (UTI)

Urinary tract infection comprises a wide variety of clinical entities which is the result of microbial invasion of tissues lining the urinary tract extending from the renal cortex to the urethral meatus. It also refers to the presence of bacteria undergoing multiplication in urine within the urinary drainage system. Infection of the adjacent structure such as prostrate and epididymis is also included in the definition of urinary tract infection. Infection may be expressed predominantly at a single site, kidney i.e. pelvis and cortex (pyelonephritis), pelvis and ureter (pyelitis), ureter (ureteritis), bladder (cystitis), prostrate (prostitis) and urethra (urethritis) but the entire urinary tract is always at a risk of invasion by bacteria, once any one of its part is infected. As urthra is the common site for urinary tract and genital tract, urethritis is also included in sexually transmitted disease especially if the infection is aused by *Neisseria gonorrhoeae* and *Chlamydia trachomatis*.

UTI is defined as the detection of both bacteriuria (10^5 CFU/ml) and pyuria 10 leucocytes/HPF) (Goya *et al*., 1997). Urinary tract infection can be defined as a spectrum of disease involving microbial invasion of any of the genitourinary tissues extending from the renal cortex to urethral meatus (Singh *et al.*, 1991).

Numerous microorganisms including bacteria, fungi and parasites are involved to cause UTI. The possible bacterial pathogens are follows:

Gram-positive

Staphylococcus aureus, Streptococcus faecalis, Coagulase negative staphylococcus (CONS)

Gram-negative

E. coli, Klebsiella pneumoniae, K. oxytoca, Proteus mirabilis, P. vulgaris, Enterobacter cloacae, E. aerogenes, Providencia rettgeri, P. stuartii, Morganella morganii, Citrobacter freundi , C. diversus, Serratia marcescens, Pseudomonas aeruginosa, Salmonella Typhi, *S.* Partyphi A, *Neisseria gonorrhoeae, Acinetobacter* spp.

UTI is more common in women than in men, at least partially because of the short female urethra and its proximity to the anus. The incidence of infection is highest in women, 20-50% of whom will suffer a clinical episode during their lifetime, however, most of these infections remain undiagnosed and undergo spontaneous remission (Leigh *et al*., 1990). Approximately 95% of infections are urinary re-infections, most occur individuals who are healthy, and few are the cause of serious illnes (Fowler *et al.*, 1990).

There are many factors for higher rate of MDR among uropathogenes. Possible MDR uropathogens are shown in table 2. Some strains of *E.coli* must carry a large plasmid longer than 200 kb that contains genes of multiple drug resistance in addition the genes for cell invasion and cell adhesion. Bacterial biofilms are often associated with long-term persistence of organisms in various environments. Bacteria in biofilm display dramatically increased resistance to antibiotics. The study also showed significant correlation between biofilm production and multi-drug resistance (Snyder et al., 2003 and Suman *et al.*, 2007)

Although urinary tract infection is not usually thought of as a disease associated with community-wide outbreaks, certain multidrug-resistant, uropathogenic lineages of *E. coli* have exhibited epidemic behavior. *E. coli* O15:K52:H1 caused an outbreak of

community-acquired cystitis, pyelonephritis, and septicemia in South London in 1987 and 1988 and is an endemic cause of urinary tract infection in Barcelona, Spain (Manges *et al*., 2001 and Phillips *et al*., 1988)

3.1.2 Skin, Soft tissue and Wound infections

Skin is not only subjected to frequent trauma and thereby is at frequent risk of infection, but also is can reflect internal disease. Skin consists of two major layers: epidermis and dermis which includes glands, hair and nails (Waugh and Grant, 2001). Approximately 15% of all patients who seek medical attention have either some skin disease or skin lesion, many of which are infectious. Many different bacteria, fungi and viruses may be involved (Forbes *et al.*, 2002). Different types of infections of skin may occur. Several of most common are categorized as follows:

a. Infections in or around Hair follicles

Folliculitis, furunculosis and carbuncles are localized abscesses either in or around hair follicles. For the most part, these infections are precipitated by blockage of the hair follicle with skin oils (sebum) or minor trauma. Among the etiologic agent, *S. aureus* is the most common. Members of the family Enterobacteriaceae can also cause folliculitis (Forbes *et al*., 2002). *Pseudomonas aeruginosa* also cause folliculitis and associated with the use of whirlpools, swimming pools and hot tubes (Gustafson *et al.*,1983).

b. Infections in the deeper layers of the epidermis and the dermis

Most infections in the deeper layers of the epidermis and dermis result from the inoculation of microorganisms by traumatic breaks in the skin. Most common infections of this type and etiologies are shown in table 1.

To a large degree, the organisms isolated from subcutaneous abscesses depend on the site of infection e.g. anaerobes are commonly isolated from abscesses of the perineal, inguinal and buttock area, whereas nonperineal infections are caused by mixed facultative anerobic organisms.

Table 1: Common infections of epidermis and dermis and their aetiologies

Infection	Etiologies
Erysipelas	*S. pyogenes*, sometimes groups B,C or G streptococci
Erythrasma	*Corynebacterium minutissimum*
Erysipeloid	*Erysipelothrix rhusiopathiae*
Impetigo	Nonbullous-*S. pyogenes* ,Bullous- *S. aureus*
Cellulitis	Group A streptococci, *S.aureus* , Less common -*Aeromonas* spp., *Vibrio* spp. and *Haemophilus influenzae*
Hidradenitis	*S. aureus, S. milleri* group, anaerobic streptococciand *Bacteroides* spp.
Infected pilonidal tuft cyst	*B. fragilis* group, *Prevotella, Fusobacterium* , anaerobic GP cocci and *Clostridium* spp.
Cutaneous ulcers	*C. diphtheriae, Bacillus anthracis, Nocardia* spp. and *M.marinum*

Meleney's ulcer is a slowly progressive infection of the subcutaneous tissue with associated ulceration of overlying skin. The organism involved is a microaerobic *streptococcus*, but anaerobic streptococci may be involved (Forbes *et al*., 2002).

Necrotizing fasciitis is a serious infection of soft tissue involving overlying muscle groups. The organisms involved are Group A streptococci, *S. aureus, Bacteroides* and *Clostridium* spp. Progressive bacterial synergistic gangrene is usually a chronic gangrenous condition of the skin most often encountered as a postoperative complication. Organisms involved for this gangrene are microaerobic streptococci, *S. aureus, Proteus* spp. and other facultative bacteria. Myositis involves various infectious agents. (George *et al*.,1989 and Goldstein 1992).

c. Postoperative infections

Sources of surgical wound infections can include the patient's own normal flora or organisms present in the hospital environment that are introduced to the patient by

medical procedures and/or a specific underlying disease or trauma that my interrupt a mucosal or skin surface. The nature of infecting flora depends on the underlying problem and the location of the process. Bacterial pathogens encountered are follows: *S. aureus*, CONS, *S. pyogenes, Streptococcus milleri* group streptococci, microaerobic streptococci, *Enterococci*, *Proteus* spp., *Morganella* spp., *E. coli, Pseudomonas* spp., Other Enterobacteriaceae, *Bacteroides* spp., *Prevetella* spp., *Fusobacterium* spp., *Clostridum* spp., *Peptostreptococcus* spp. and other non-spore former anaerobes (Forbes *et al*., 2002).

d. Bites and burns

Human bites and clenched-fist injuries consist of α-hemolyic streptococci, *S. aureus*, group A streptococci and *Eikenella corrodens.* Dog bites introduce organisms *Pasteurella* spp., CDC group EF-4, *Weeksella* spp., *S. intermedius*. Cattle and snakes bites cause infection of *Pseudomonas* spp., Clostridial and various Gram-negative bacilli.

The possible MDR pathogens encountered with skin, soft tissues and wound infections include *S. aureus,* Enterobacteriaceae, *Enterococci,* CONS, *P. aeruginosa* and *Acinetobacter* spp (Gupta *et al.,* 2008). These MDR pathogens with their resistance traits are shown in table2.

Highest drug resistance was seen amongst the gram-negative bacilli to ciprofloxacin (68.42%), gentamicin (60.41%), tobramycin (66.67%) and beta-lactam group of antibiotics (50%). This could be due to increasing production of extended spectrum beta lactamase (ESBL), MDR efflux pump or co-resistance (Gupta *et al.,* 2008).

Various nonfermenters (*P. aeruginosa* and *Acinetobacter* spp.) also showed high level of resistance which varied from 90% resistance to ciprofloxacin, 83.33% to augmentin**,** 78.37% to gentamicin and 56.09% to cefepime. This high rate of could be due to the

fact that ours is a tertiary care hospital with widespread usage of broadspectrum antibiotics, leading to selective survival advantage of pathogens (Gupta *et al.,* 2008)

.

3.1.3 Upper Respiratory Tract Infection (URTI)

The respiratoy tract is arbitrarily divided at the level of the lower border of the cricoid cartilage into upper and lower parts. The nasal cavity, pharynx,epiglottis,larynx constitutes the upper respiratory tract (Forbes *et al*., 2002).

In the upper respiratory tract, microorganisms live primarily in areas bathed with the secretions of the mucous membranes. The pathogenic organisms adhere to the walls of nasopharynx and oropharynx, colonize locally and elaborate a toxin that is disseminated systemically, adhering preferentially to the CONS cells and muscle cells of the heart (Madigan *et al*., 1997).

The common form of infections caused by bacterial pathogens in URT are common cold, purulent rhinitis, pharyngitis/tonsilitis, less common are diphtheria, whooping cough, laryngitis and epiglottitis. The most common Gram-negative bacterial pathogens of URTI are *E. coli*, *Haemophilus* spp., *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Bordetella pertusis* and *Neisseria meningitidis*. Gram-negative bacteria occasionally encountered are *Aeromonas* spp., *Pasterurella* spp., *Corynebacterium diphtheriae*, *Nocardia* spp., *Listeria* spp. and *Francisella tularensis.*

Gram-positive bacterial pathogens frequently encountered are coagulase positive *Staphylococcus aureus*, *Streptococcus pneumoniae* and beta hemolytic streptococci (Shrestha *et al*., 2002).

There are several risk factors for both colonization and infection of MDR pathogens of URTI like severity of illness , previous exposure to antimicrobial agents, underlying diseases or conditions, invasive procedures (such as tracheostomy), repeated contact

with the healthcare system and previous colonization of by a multidrug-resistant organisms (CDC, 2000) .Possible MDR pathogens of URTI are shown in table 2.

3.1.4 Lower Respiratory Tract Infection (LRTI)

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

Various viruses, bacteria, fungi and actinomycetes are encountered in LRTI. A wide variety of bacteria are encountered in LRTI either as primary causes of illness or as secondary invader or when the defences are impaired. The possible pathogens are –

Gram-positive	Gram-negative
Streptococcus pneumoniae	*Haemophilus influenzae*
Staphylococcus aureus	*Klebsiella pneumoniae*
S. pyogenes	*Pseudomonas aeruginosa*
Mycobacterum tuberculosis	*Proteus* spp.
M.avium -intracellulare	Other Enterobacteriaceae
M. kansasii	*Mycoplasma pneumoniae*
	Legionella pneumoniae
	Yersinia pestis
	Bordetella pertusis
	Moraxella catarrhalis
	Prevotella spp.

(Forbes *et al*., 2002)

Diseases of the LRT are bronchitis (acute and chronic), pneumonia (Community acquired and hospital acquired) and chronic infection. The etiology of acute pneumonias is strongly dependent on age such as > than 80% of pneumonias in infants

and children are caused by viruses, whereas > than 10% to 20% of pneumonias in adults are viral.Chronic infection of LRT is most commonly caused by *M. tuberculosis* but other factors like genetic disorder, underlying disease is also involved (Collier *et al*., 1998 amd Forbes *et al*., 2002).

MDR bacterial pathogens associated with LRTI are shown in table 2 Multidrug resistance (MDR) in Gram-negative bacteria associated with LRTI is on the rise, but its effect on patient outcomes is not well established. Forty-one patients (31.8%) were infected with MDR bacteria among 129 adult surgical intensive care unit (SICU) patients treated for Gram-negative pneumonia (Kwa *et al*., 2007).

3.1.5 Bloodstream Infection

Microbial invasion of the bloodstream can have serious immediate consequences, including shock, multiple organ failure, disseminated intravascular coagulation (DIC),and death. Approximately 200,000 cases of bacteremia and fungemia occur annually, with mortality rates ranging from 20% to 50%. Common bacterial pathogens of bloodstream infection are follows (Collier *et al*., 1998 amd Forbes *et al*., 2002).

Gram -negative

E. coli, Klebsiella spp., *Enterobacter* spp., *Proteus* spp., *Salmonella typhi, Salmonella* spp., other than *S.* Typh*i, Pseudomonas aeruginosa, Neisseria meningitides, Haemophilus influenzae, Bacteoides fragilis, Brucella* spp., *Burkholderia pseudomallei*

(WHO, 2004)

Gram-positive

Staphylococcus aureus, S, epidermidis, Viridans streptococci, *Streptococcus pneumoniae, E. faecalis, S. pyogenes, S. agalactiae, Listeria monocytogenes, Clostridium perfringens , Peptostreptococcus* spp.

(WHO, 2004)

Bloodstream infection may be intravascular (those that originate within the cardiovascular system) and extravascular (those that result from bacteria entering the blood circulation through the lymphatic system from another site of infection). Intravascular infections include infective endocarditis, mycotic aneurysm, suppurative thrombophlebitis and intravenous, catheter-associated bacteremia. Extravascular infections include enteric fever, meningitis and epiglottitis. The most common portals of entry are the genitourinary tract (25%) and respiratory tract (20%) (Forbes *et al*., 2002). The likely MDR pathogens of blood stream infections are shown in table 2.

3.1.6 Other bacterial infections

There are many other bacterial infections which are equally important to diagnose in the laboratory as mentioned above like gastrointestinal tract (GIT) infection; meningitis; other central nervous system infections; genital tract infection; infections of ear, eyes and sinuses; and infections of body fluids, bone, bone marrow, and solid tissue. The common infections of GIT are gastroenteritis, food poisoning, diarrhea, esophagitis, gastritis and proctitis. Stool is frequent sample of GIT infections. If it is unavailable rectal swab and/or perianal swab may be substituted. The routine search of bacterial agents of GIT infections are for *Salmonella* spp., *Shigella* spp., Enterobacteriaceae, *V. cholerae, V. parahaemolyticus, Campylobacter jejuni* and *Clostridum difficile.*

Genital tract infections consist of lower and upper tract infections. Lower tract infections are commonly acquired by sexual or direct contact whereas upper tract infections are frequently extension of a lower tract infection. These infections include Urethritis/cervicitis, salpingitis, lymphogranuloma venereum, granuloma inguinale, syphilis, chancroid and enteritis. Most common pathogens of Genital tract are *Chlamyida trachomatis, Gardnerella vaginalis, H. ducreyi, N. gonorrhoeae* and *Treponema pllidum*.

Blepharitis, conjunctivitis, keratitis and keratoconjunctivitis are major infections of the eye involving *S. aureus, S. peumoniae* and *P. aeruginosa* as common pathogens.

Patients develop bone infection from hematogenous spread, invasion from adjacent site and breakdown of tissue from surgery *S. aureus* is common pathogen. Possible MDR pathogens associated with GIT, genital tract, CNS, eye, ear and fluid infections are shown in the table 2.

Table2. General characteristics of multidrug-resistant organisms (Alekshun *et al.,* 2007)

Organism	**Common Infections**	**Key Antibiotic Resistances**	**Drugs Considered for Treatment of MDR**
P. aeruginosa	Lung, wound	β-lactams, fluoroquinolones, aminoglycosides	Colistin
Acinetobacter spp	Lung, wound, bone, blood	. β-lactams, fluoroquinolones, aminoglycosides	Colistin, tigecycline
E. coli and *K. pneumoniae* bearing extended-spectrum β-lactamases	Urinary, biliary, gastrointestinal tracts, lung, blood	β-lactams, fluoroquinolones, aminoglycosides	Colistin (for *K. pneumoniae*), tigecycline
Vancomycin-resistant enterococci	Blood, heart, intra-abdominal	Vancomycin	Quinupristin-dalfopristin, linezolid, daptomycin
Methicillin-resistant *S. aureus*	Skin and soft tissue, Respiratory tract, blood	β-lactams, fluoroquinolones, macrolides	Quinupristin-dalfopristin, daptomycin, linezolid, tigecycline, vancomycin
Multidrug-resistant *S. pneumoniae*	Ear, lung, blood, cerebrospinal fluid	β-lactams, macrolides, tetracyclines, co-trimoxazole	Fluoroquinolones, tigecycline
Extensively drug-resistant *M. tuberculosis*	Lung	Rifampin, isoniazid, and three of the following:aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine, or para-	3rd line agents, drug combinations

		aminosalicylic acid	

3.2 ANTIBIOTIC RESISTANCE

Antibiotics are the frontline therapeutic means for the medical intervention in infection It is used as either prophylaxis, empiric therapy or pathogen directed therapy. The world Health Organization (WHO), the European Commission, and the U.S. Centers for Disease Control and Prevention (CDC) have recognized the importance of studying the emergence and determinants of resistance as well as the need for control strategies (Oteo *et al.*, 2005 and WHO, 2004).

3.2.1 Definition

When the organisms is expected not to respond to a given drug irrespective of the doses and location of infection (WHO, 2004 and Barloloni *et al.*, 2006).

Drug resistance can be defined as the temporary or permanent ability of an organism and its progeny to remain viable or multiply under environmental conditions that would destroy or inhibit other cells (Denyer *et al.*, 2004).

3.2.2 Risk factors for the development of antibiotic resistance

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

- Excessive and irrational over-utilization of antibiotics in outpatient practice and in hospitalized patients, either therapeutically or prophylactically.
- Use of antibiotics in agricultural industry, particularly in the production of food.
- Longer survival of severely ill patients.
- Longer life expectancy with increased use of antibiotics in the elderly.
- Advances in medical science have resulted in the survival of many patients with severe illness and at risk for infections:

- Critically ill patients
- Immunosuppression
- Congenital diseases (i.e., cystic fibrosis)

- Lack of use of proven and effective preventive infection control measures such as hand washing, antibiotic usage restrictions and proper isolation of patients with resistant infections.
- Increased use of invasive procedures.
- Increased use of prosthetic devices and foreign bodies amenable to super infection with resistant bacteria.

(Levi, 2001 and WHO, 2004)

3.2.3 History of antibiotic resistance

- By 1944 some strains of *S. aureus* were capable of destroying penicillin V by means of β-lactamase.
- By early 1960s, resistance to methicillin also began to emerge..
- Today, MRSA is resistant to all β-lactam antibiotics and in addition some strains have been reported to resistant to erythromycin, fusidic acid, tetracycline, monocycline, streptomycin, sulphonamides, disinfectants and toxic metals (mercury, cadmium etc).
- In 1967, penicillin resistant *Streptococcus pneumoniae* was isolated from remote village in Papua New Guinea.
- Outbreaks of vancomycin-resistant enterococci have been reported in 1988.
- In 2002, vancomycin resistant *S. aureus* (VRSA) (MIC.32 ug/ml) was isolated from a dialysis patient in Michigan

(Denyer *et al.*, 2004; Levi, 2001 and Chang *et al*. 2003).

3.2.4 Mechanisms of antibiotic resistance

Resistance mechanisms can come about in various ways, but the end result is partial or complete loss of antibiotic effectiveness. Such resistance may either be a characteristic

associated with the entire species or emerge in strains of a normally susceptible species through mutation or gene transfer. Resistance genes encode various mechanisms which allow microorganisms to resist the inhibitory effects of specific antimicrobials of the same class and sometimes to several different antimicrobial classes (Forbes *et al*., 2002 and Alekshun *et al.*, 2007).

3.2.4.1 Clinical resistance

Clinical resistance, which is dependent on outcome of administration of drug and is all too often ignored. Clinical resistance is a complex concept in which the type of infecting bacterium, its location in the body, the distribution of the antibiotic in the body and its concentration at the site of infection, and the immune status of the patient all interact. For example, most strains of *Streptococcus pneumoniae* are inhibited by 0.01 mg/l of benzylpenicillin (the minimum inhibitory concentration), whereas for *E. coli* 3264 mg/l are required to inhibit growth, a level which cannot be achieved in the human body (Forbes *et al*., 2002 and Denyer *et al.*, 2004).

3.2.4.2 Environmentally mediated antimicrobial resistance

The impact that environment has on antimicrobial activity is great because antimicrobial resistance is the result of nearly inseparable interactions among the drug, the microorganism, and the environment in which they are brought together. Examples of environmental factors include pH, anaerobic atmosphere, cation concentrations, and thymine-thymidine content For example antibacterial activities of erythromycin and aminoglycosides diminish with decreasing pH, while the activity of tetracycline decreases with increasing pH,increasing concentration of cations, such as Ca^{++} and Mg^{++} decrease activity of aminoglycoside (Forbes *et al*., 2002).

3.2.4.3 Microorganism-mediated antimicrobial resistance

Microorganism mediated resistance refers to antimicrobial resistance that is due to genetically encoded traits of the microorganism and is the type of resistance that in vitro

susceptibility testing methods are targeted to detect (Forbes *et al*., 2002). This type of resistance can be divided into two major types.

A. Intrinsic resistance (inherent;natural)

Antimicrobial resistance resulting from the normal genetic, structural, or physiologic state of a microorganism is referred to as intrinsic resistance. Intrinsic resistance is usually predictable in a clinical situation. Macrolide, lincosamide and streptogramin (MLS) are chemically distinct inhibitors of bacterial protein synthesis and their intrinsic resistance in Gram-negative bacilli is due to low permeablility of the outer membrane to these hydrophobic compounds. Similarly, sulphonamides, trimethoprim, tetracycline or chloramphenicol are futile against *P. aeruginosa* due to lack of uptake (Forbes *et al*., 2002 and Denyer *et al.*, 2004).

B. Acquired resistance

Antibiotic resistance that results from altered cellular physiology and structure caused by change in a microorganism's usual genetic makeup is known as acquired resistance. The presence of this type of resistance in any clinical isolate is unpredictable so it important to detect resistance. The many mechanisms that bacteria acquired to protect themselves from antibiotics can be classified into five basic types (Forbes *et al*., 2002 and Denyer *et al.*, 2004).

1. **Inactivation of the drug**

a. Inactivation of ß-lactam antibiotics: The inactivation of ß-lactam ring is catalyzed by a family of related enzymes, the ß-lactamases which are described in section 3.5.

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

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

2. Alteration of the target

a.Emergence of methicillin resistance in *S. aureus* appears to be due to the presence of a penicillin binding protein termed PBP2a or PBP2' which has a reduced affinity for methicillin, the gene responsible is the *mec*A gene (Denyer *et al.*, 2004).

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

3. Reduced celluar uptake and increased efflux

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

- Decreased outermembrane permeability leading to resistance to chloramphemcol has been identified in Gram-negative bacteria.
- The NorA-mediated efflux system in S.aureus was characterized in 1990 to cause resistant to fluoroquinolone antibiotics.
- The Tet efflux proteins belong to the major facilitator superfamily which exchange a proton for a tetracycline-cation (usuallyMg^{++}) complex reducing intracellular drug concentration and protecting the target ribosomes leading to tetracycline resistance**.**

(Alekshun *et al.*, 2007 and Denyer *et al.*, 2004 and Forbes *et al*., 2002)

4. Metabolic bypass

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

5. Any combination of mechanisms 1 through 4

Single bacteria may exhibit several types of resistance mechanisms leading to multiple drug resistance and this is usually mediated by of R factor (Denyer *et al.*, 2004 and Forbes *et al*., 2002).

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

1. Mutation and selection (Vertical transmission)

The spontaneous mutation occurs in hacterial population as 1 per 10^7 cells per cell division. Despite the low mutation rate when this population is exposed to antibiotics, than they mutated and grow. Development of penicillinase enzyme in *S. aureus* is due to spontaneous mutation and selection. However, the burden of drug resistance by ouccrence of such rare mutation and emergence of resistant organisms in the clinical setting may not be very large, but mutated gene pose threat in clinical condition if it is found on plasmid (Alekshun *et al.*, 2007 and Forbes *et al*., 2002).

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

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

a. Gene cassettes and integrons: Gene cassettes are mobile elements that can be captured due to flanking by specific DNA sequences that are recognized by intergrase and genes that are apparently ont expressed until they become part of an integron (Hall

and Collis, 1998). Integrons are a special case of multidrug resistance. They are genetic elements that contain the genetic determinants of a site-specific recombination system that recognizes and captures mobile gene cassettes (Alekshun *et al.*, 2007 and Denyer *et al*., 2004).

Multiple gene cassettes can be present in one integron. Integrons are coordinately expressed genes under the control of a same promoter Integrons have been identified in both gram-negative and gram-positive bacteria, and they seem to confer high-level multiple drug resistance to the bacteria that carry and express them. Four classes of integron have been described, among them class 1 integrons are the most common and at least 60 gene cassettes have been described for this which encode antibiotic disinfectant resistance; resistance to aminoglycoside, penicillins, cephalosporins, trimethoprim, tetracycline, erythromycin and chloramphenicol (Alekshun *et al.*, 2007 and Denyer *et al*., 2004).

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

c. Plasmid: R-plasmid carry genes for resistance to one and often several antiobiotic drugs which are described below.

These genetic materials can be transferred by following three mechanisms

1. Conjugation: Plasmids are the genetic element most frequently transferred by conjugation. Genetic functions required for transfer are encoded by the *tra* genes, which are carried by self-transmissible plasmids. Some self-transmissible plasmids can mobilize other plasmids or portions of the chromosome for transfer. The realization that drug resistance could be transferred during cell conjugation came during the 1950s from a case in Japan, where it was observed that a strain of *Shigella* sp. isolated during an epidemic of dysentery was resistant to severa drugs. Most of MDR strains are Gram-negative bacilli which are found in soil, gut of human and animal where they transfer their MDR traits by conjugation frequently. Multidrug resistance traits could be transferred by conjugation, often en bloc, suggesting a linkage of the corresponding resistance genes in self-transferable or mobilizable plasmids (Bartoloni *et al.,* 2004).

2. Transformation: It is a process in which a free DNA molecule is transferred from a donor to a recipient bacterium. The DNA released from the donor cell upon cell lysis may be absorbed by 'competent' cells and integrated into their genomes (Denyer *et al.*, 2004 and Snyder *et al*., 2003)

3. Transduction: Transduction is the transfer of genetic information between bacteria by bacteriophages. In the clinical setting, transduction may be more important in spreading resistance among Gram-positive bacteria than Gram-negative cells. Transduction of drug-resistance plasmids in *Staphylococcus aureus* has been observed in infected mice and on human skin (Denyer *et al.*, 2004).

3.3 MULTIDRUG RESISTANCE

Multidrug resistance (MDR) bacterial isolates have been frequently reported from different parts of the world as an emergence of treatment problem. The World Health Organization (WHO), the European Commission, and the U.S. Centers for Disease Control and Prevention (CDC) have recognized the importance of studying the

emergence and determinants of multidrug resistance as well as the need for control (Alekshun *et al.*, 2007; Oteo *et al.*, 2005 and WHO, 2004).

3.3.1 Definition

Multidrug resistance was defined as resistance to ≥two classes of antibiotics (Wright *et al*., 2000 and Bartoloni *et al.*, 2006).

The MDR strain is defined a strain that showed resistance to three or more antibiotics among the six commonly prescribed drugs (Tuladhar *et al*., 2001).

Concurrent resistance to antimicrobials of different structural classes has arisen in a multitude of bacterial species and may complicate the therapeutic management of infections and considered MDR strains if resistant to three or more antimicrobial agents (Daniel *et al.*, 2001).

3.3.2 Mechanisms of multidrug resistance

3.3.2.1 Genetic acquisition of MDR genes

This is the most common method of multiple antibiotic resistance and has paramount importance. The genetic elements that are responsible for multiple resistance are viz, R-plasmid, integrons, transposon and gene cassettes. The nature of these all elements associated with drug resistance and transfer, has been described in topic antibiotic resistance. A number of R-plasmid have now been characterized including RP4, encoding resistance to ampicillin, kanamycin, tetracycline and neomycin, found in *P. aeruginosa* and other Gram-negative bacteria; R1, encoding resistance to ampicillin, kanaycin, sulphonamides, chloramphenicol and streptomycin, found in Gram-negative bacteria and p^{SH6}, encoding resistance to gentamycin, trimethoprim and kanamycin found in *S. aureus* (Alekshun *et al.*, 2007 and Denyer *et al*., 2004).

The promiscuity of transposons jumping from DNA to DNA, whether in a plasmid, chromosome, or phage, is a major factor in dissemination of multiple antibiotic

resistance because plasmids can elaborate their own system for selftransfer between bacteria, it is not unexpected that antibiotic resistance among different groups of bacteria. Some common transposons associated with multidrug resistance are Tn4001, Tn7, and Tn21 (Alekshun *et al.*, 2007; Denyer *et al*., 2004 and Snyder *et al*., 2003).

One or more mobile gene cassettes can be integrated into a specific position on the integron and there are > 60 gene cassettes each comprising only a promoter-less single gene and 59-base element forming a specific recombination site.Integrons are coordinately express these multiple drug resistance genes under the control of a single promoter exhibiting co-resistance as one of the mechanism of multiple drug resistance (Alekshun *et al.*, 2007 and Denyer *et al*., 2004)

3.3.2.2 Chromosomal multiple-antibiotic resistance(Mar) locus

The multiple-antibiotic resistance (mar) locus was recognized in Gram-negative enteric bacteria. The locus consists of two divergently transcribed units, *mar C* and *marRAB. marR* encodes a repressor of the operon and *marA* encodes a transcriptional activator affecting expression of more than 60 genes. Increased expression of the *MarRAB* operon resulting from mutations in *marO* or *marR*, or from inactivation of MarR following exposure to inducing agents which leads to development of Mar phenotype. This Mar strains are responsible to multiple drug resistance, disinfectants and organic solvents (Alekshun *et al.*, 2007 and Denyer *et al*., 2004)

3.3.2.3 Reduced cellular uptake

Multidrug resistance can also often caused by reduced expression of porins and changes in the cell, which cause reduced uptake of several structurally unrelated antibiotics (Denyer *et al*., 2004)

3.3.2.4. Multidrug efflux pumps

In bacterial genomes 3-12% of open reading frames are predicted to encode membrane transport proteins. These proteins can be vital for antibiotic efflux, protein toxin

secretion and cell nutrition. Generally, efflux pumps excrete only one class of drugs but multidrug efflux pump can excrete wide range of antibiotics creating multidrug resistance. The majority of Gram-negative pumps spans both membranes (outer and cytoplasmic) Mutations in regulatory genes such as *nalB* cause overexpression of MexAB-OprM and consequently multidrug resistance. The QacA MDR pump is found on broad host range plasmids carry specific gentamicin and trimethoprim resistance genes (Alekshun *et al.*, 2007 and Denyer *et al*., 2004).Different types of MDR efflux pumps of MDR bacterial pathogens are shown in table 3.

Table 3: MDR Efflux Systems of Clinically Important Bacteria (Alekshun *et al.*, 2007)

Bacterial Organism	**Efflux System(s)**	**RepresentativeAntibiotic Resistance***
P. aeruginosa	MExAB-OprM MexCD-OprJ MexEF-OprN MexHI-OprD MexJK-OprM MexVW-OprM MexXY-OprM	BLA and FQ 4th gen ceph FQ, Cm, Tmp, and Tri EtBr, Nor, and Acr Cip, Tet, Ery, and Tri FQ, Cm, Tet, Ery, EtBr, and Acr AG and Tig
A. baumannii	AdeABC	AG, FQ, TET, Ctx, Cm, Ery, and Tmp
S. maltophilia	SmeABC SmeDEF	AG, BLA, and FQ MC, TET, FQ, CAR, Cm, and Ery
B. cepacia	CeoAB-OpcM	Cm, Cip, and Tmp
B. pseudomallei	AmrAB-AprA	MAC and AG
E. coli	AcrAB-TolC	FQ, BLA, TET, Cm, Acr, Tri
K. pneumoniae	AcrAB-TolC	FQ, BLA, TET, and Cm
S. aureus	MepA	Tig, Mino, Tet, Cip, Nor EtBr, and TPP
E. faecalis	EmeA Lsa	Nor, EtBr, Clind, Ery, and Nov Clind and QD
S. pneumoniae	PmrA	FQ, Acr, and EtBr

*Abbreviations: 4th gen ceph, fourth-generation cephalosporins; Acr, acriflavin; AG, aminoglycosides; BLA, â-lactams; CAR, carbapenems; Cip, ciprofloxacin; Clind, clindamycin; Cm, chloramphenicol; Ctx, cefotaxime; Ery, erythromycin; EtBr, ethidium bromide; FQ, fluoroquinolones; MC, macrolides; Mino, minocycline; Nor, norfloxacin; Nov, novobiocin; QD, quinupristin-dalfopristin; TET, tetracycline family; Tet, tetracycline; Tig, tigecycline; Tmp, trimethoprim; TPP, tetraphenylphosphonium bromide; Tri, triclosan.

3.3.3 Multidrug resistance in global context

The spread of microbial multiple drug resistance is a global public health challenge, which impairs the efficacy of antimicrobial agents and results in substantial increased illness and death rates and healthcare-associated costs. In low-resource countries, the extent and the impact of the phenomenon tend to be even larger than in industrialized countries. Moreover, in low-resource countries the impact of multiple antimicrobial drug resistance on illness and death rates tends to be greater because of the high prevalence of bacterial infections and the major role of antimicrobial agents in combating infectious diseases (Bartoloni *et al*., 2006).

Microorganisms with drug resistance that are major problems in hospitals worldwide:

Gram-positive organisms	Gram-negative organisms
MRSA	*Klebsiella* species
MRSA, VRSA	*Enterobacter* species
VRE	*Pseudomonas aeruginosa*
	Acinetobacter baumannii

(Alekshun *et al.*, 2007 and Levy *et al.*, 2001)

Microorganisms with drug resistance that are major problems in the community worldwide are follows:

Organism	Resistance
Streptococcus pneumoniae	Multiple drugs
Streptococcus pyogenes	Macrolides, tetracyclines
Mycobacterium tuberculosis	Multiple drugs
Neisseria gonorrhoeae	Penicillin, tetracycline, fluoroquinolones
Salmonella typhimurium	Multiple drugs
Escherichia coli	Multiple drugs

(Levy *et al.*, 2001)

In the study of 38, 835 urinary isolates of *E. coli* in the US that had been tested against ampicillin, cephalothin, ciprofloxacin, nitrofurantoin and trimethoprim-sulfamethoxazole 7.1% (2,763 of 38,835) were resistant to three or more agents and considered multidrug resistant. Among the multidrug-resistant isolates, 97.8% were resistant to ampicillin, 92.8% were resistant to trimethoprim-sulfamethoxazole, 86.6% were resistant to cephalothin, 38.8% were resistant to ciprofloxacin, and 7.7% were resistant to nitrofurantoin. The predominant phenotype among multidrug-resistant isolates (57.9%; 1,600 of 2,793) included resistance to ampicillin, cephalothin, and trimethoprim-sulfamethoxazole. This was the most common phenotype regardless of patient age, gender, or inpatient-outpatient status (Daniel *et al*., 2000).

Multidrug-resistant gram-negative super microbes have been emerging worldwide. Since carbapenems and fluoroquinolones are the last resort against infections caused by gram-negative bacilli, the proliferation and dissemination of such clinical isolates that produce metallo-β-lactamases and acquire mutations in *gyrA* and *parC* genes have become a global threat (Daniel *et al*., 2000).

Overall, high resistance rates were observed for ampicillin (95%), trimethoprim-sulfamethoxazole (94%), tetracycline (93%), streptomycin (82%), and chloramphenicol (70%). Lower resistance rates were observed for nalidixic acid (35%), kanamycin

(28%), gentamicin (21%), and ciprofloxacin (18%). Resistance to ceftriaxone and amikacin was uncommon (<0.5%) (Bartoloni *et al*., 2006).

3.3.4 Multidrug resistance trend in Nepal

Multiple drug resistance study in Nepal seem doesn't underscores the magnitude of the problem of antimicrobial drug resistance in low-resource settings and the urgent need for surveillance and control of this phenomenon. Inexpensive, sensitive, and simple methods to monitor antimicrobial drug resistance among different clinical isolates could be valuable tools for large-scale surveillance studies and to improve the efficacy of resistance control interventions. However, resistance trend of commonly used drugs in Nepal also depend upon the localities. The small differences observed among localities, although sometimes statistically significant, are probably of limited clinical and epidemiologic relevance (Pokhrel *et al*., 2006 and Paterson *et al*., 2005)

Nevertheless, it is obvious that percentage of MDR strains are higher in hospitalized patients, device used patient and other complications associated with the patients e.g. diabetes, microalbuminuria (Daniel *et al*., 2000).

In the study at Tribhuvan University Teaching Hospital (TUTH), out of 161 blood borne isolates 26 (16.14%) were found to be MDR. The incidence of MDR in *Salmonella* Paratyphi A, *S.* Typhi and *Staphylococcus aureus* was found to be 12.12%, 7.81% and 30.00% respectively (Wagle *et al*., 2004). In a similar study carried out at Kathmandu Model Hospital, 4 (5.19%) MDR strains were isolated. Out of 4 strains, 3 MDR strains were *S.* Typhi and 1 strain was *S.* Paratyphi A (Shrestha *et al*., 1996).

In the study carried out in poultry birds, all 259 (100.0%) isolates from poultry samples were found MDR (Pandey *et al*., 2003). In the study carried out at TUTH among different clinical samples 41.74%, 36.58%, 31.03% and 9.17% were found MDR strains (Rai *et al*., 2000). In similar study at Kanti Children Hospital and TUTH 25% was found MRSA among 52 *S. aureus* isolates (Lamichhane *et al*., 1999). However, 75.9%

and 64.5% MRSA were found among *S. aureus* isolates at Bir Hospital and TUTH respectively (Shakya *et al*., 1997).

In the nosocomial isolates, burden of multiple drug resistance was found high as compare to other settings, as in the study by Banjara *et al*. (2002) in different wards at TUTH. He found wound isolates resistant to 4 or more than 4 commonly used drugs included *S. aureus* (40.0%), *Pseudomonas aeruginosa* (46.3%), *E. coli* (56.3%), *K. pneumoniae* (62.5%), *Acinetobacter* spp. (60.0%), *Citrobacter freundii* (44.4%), *P. mirabilis* (71.4%) and *K. oxytoca* (100.0%) (Banjara *et al*. 2002).

3.4 PLASMID AND MULTIDRUG RESISTANCE

The word "plasmid" was introduced by Joshua Lederberg in 1952. They are extrachromosomal molecules of DNA that vary in size from 1kb to more than 200 kb. Most of them are double-stranded, covalently closed, circular molecules, found naturally or artificially in a wide variety of bacterial species. They have evolved a variety of mechanisms to maintain a stable copy number in bacterial hosts and dependent, to a greater or lesser extent on the enzymes and proteins encoded by their host for their replication and transcription (Sambrook *et al*., 2001 and Snyder *et al*., 2003).

3.4.1 Host range and plasmid epidemics

Most naturally occurring plasmids are either self-transmissible or mobilizable. Self-transmissible plasmids encode all the functions they need to move among cells, and can also aid in the transfer of chromosomal DNA and mobilizable plasmids. These probably exixt in all types of bacteria, but those which have been studied most extensively are from the gram-negative genera: *Escherichia* spp., *Pseudomonas* spp. and gram-positive genera – *Enterococcus* spp., *Streptococcus* spp., *Bacillus* spp., *Staphylococcus* spp.and *Streptomyces* spp. Among them, much more concerns are due to possessing and transfer of multidrug resistance traits (Mayer, 1988)

Plasmids are usually classified by their incompatibility (Inc.) group like IncF, IncP (e.g.RP4), IncW (e.g.R388) and IncN (e.g. pKM101). Some plasmids can transfer themselves between unrelated species called promiscuous plasmids e.g. R388, Rp4, pKM101 which have increasing concern developimg and transferring multidrug resistant phenotypes. Because plasmids can be transferred so readily among bacteria, and some can replicate in various species, a single plasmid has been observed in several bacterial species during a number of outbreaks and these outbreaks are called plasmid epidemics (Sambrook *et al*., 2001 and Snyder *et al*., 2003).

Plasmid transfer between staphylococci is also common (Cohen *et al*., 1982). Plasmid-mediated beta-lactamase production and aminoglycoside resistance have been reported in *Enterococcus (Streptococcus) faecalis* and other group D enterococci (Courvalin *et al*., 1978 and Murray *et al*., 1986). Antibiotic resistance transfer among streptococci can be mediated by plasmids or by other elements called conjugative transposons. Plasmid transfer between or within other gram-positive bacteria also occurs (Hachler *et al*., 1987). Transfer of resistance determinants from gram-positive to gram-negative organisms and *Mycoplasma* spp. is now being seen (Morse *et al*., 1986).

3.4.2 Types of plasmids from MDR strains:

The size of plasmid and nature of incompability groups of plasmids isolated from clinical isolates differ on the basis of area, sources and trend of antiobiotic treatment. For example plasmid R478 from *Serratia marcescens* has mol.size 286 kb exhibiting multidrug resistance phenotype The Sanger Institute Pathogen Sequencing Unit is sequencing a number of plasmids from various sources including many clinical isolates. Some are all listed below (Gilmour *et al*., 2004).

Table 4: Reference plasmids of different Inc. groups from *E. coli* (Thomas *et al.*, 2007)

Plasmid	Size	Inc group	NCTC#	Resistances
RA1	129 kb	IncA/C	50073	Su Tc
R667	168 kb	IncA/C	50463	Ap Su
TP113	86 kb	IncB=O	50084	Km Hg
TP114	62 kb	IncIbeta	50085	Km
R387	80 kb	IncK	50022	Cm Sm
R471	78 kb	IncL/M	50036	Ap Hg
pIP135	68 kb	IncL/M	50107	Gm Sm Su Tc Hg
N3	50 kb	IncN	50124	Sm Sp Su Tc Hg
R753	102 kb	IncV	50521	Ap Cm Sm Su
R7K	30 kb	IncW	50006	Ap Sm Sp
pIP71	68 kb	Com9	50106	Ap Cm Sm Sp Su Tc
R6K	39 kb	IncX	50005	Ap Sm
R485	54 kb	IncX	50040	Su

Ap - ampicillin; Su - sulphonamide; Gm - gentamycin; Cm - chloramphenicol ; Tc - tetracycline; Tp - trimethoprim; Sm - streptomycin; Sp - spectinomycin; Km - kanamycin; Hg - mercury resistance; Tel - tellurite resistance; Phi - resistance to various bacteriophages; Scr - sucrose fermentation

3.4.3 Multidrug resistance mediated by plasmid

Plasmids can carry genes that code for functions other than transfer and replication, such as antibiotic resistance and toxin, adhesin, metabolic enzyme and bacteriocin production. There are many factors responsible for MDR traits in plasmids like, transposons which promiscutity of jumping from DNA to DNA whether in plasmid or chromosome is a major factor acquiring MDR traits on plasmid which it can elaborate

among divergent groups of clinical isolates by self transfer mechanism. Plasmid also contains genes that encode antibiotic transporter which are directly involved in reduced uptake or increased efflux system of one or more different classes of antibiotics (Denyer *et al*., 2004; Mayer, 1988 and Snyder *et al*., 2003).

Plasmid code for enzymes which are actively involve in alteration of drug by various means. Plasmid coding β-lactamase determines resistance to β-lactam drugs whereas plasmid coding enzymes that acetylate, adenylate or phosphorylate determine the aminoglycoside resistance. A single plasmid can carry different genes that are involved in particular drug resistance exhibiting multidrug resistance traits.For instance Australian MRSA isolates may harbor upto three different plasmids: small(1.6 kb) cryptic plasmids, 4.5 kb chloramphenicol resistance plasmids and 20-40 kb plasmids which variously encode resistance to antiseptics and disinfectants, trimethoprim, penicillin, gentamicin, tobramycin and kanamycin. Plasmid may carry mutated gene which is permissive carrying drug resistance (Cohen *et al*., 1982 and Mayer, 1988)

3.5 β-LACTAMS

3.5.1 Types of β-lactams

- Penams eg penicillins
- Clavams: beta-lactamase inhibitors that contain a five-membered ring with an oxygen heteroatom (eg clavulanic acid)
- Carbapenems eg meropenem, imipenem
- Cephems (cephalosporins)
- Monobactams eg aztreonam
- Oxacephems: oxygen analogues of cephems

(Rossolini, 2007)

3.5.2 Mode of action

Bacteriostatic effect is due to inhibition of cell wall synthesis by inactivation of transpeptidases. This is a crucial enzyme in the cross-linking of peptidoglycan - the basic building block of the cell wall

- act as a false substrate for D-alanyl-D-alanyl transpeptidases

- requires carboxylate or sulfonate group of beta-lactam to react the a serine residue of transpeptidases (also known as penicillin-binding proteins) to give an inactive acylated enzyme
- Bactericidal effect results from indirect mechanisms (mostly activation of autolytic enzymes)
- Only active against rapidly dividing bacteria.

(Denyer *et al*., 2004 and Rossolini, 2007)

3.5.3 Applications

a. GI tract infections

- Typhoid: cefotaxime
- Biliary tract infection: cephalosporin
- Peritonitis: cephalosporin with metronidazole
- Peritoneal dialysis associated peritonitis: cephalosporin with metronidazole.

b. Respiratory tract infections

- Throat infection: oral cephalosporin
- *Haemophilus influenzae* epiglottitis: cefotaxime
- Severe community acquired pneumonia of unknown aetiology: cefuroxime with erythromycin, or cefotaxime
- Hospital acquired pneumonia: broad spectrum cephalosporin

- Acute exacerbation of chronic bronchitis (AECB): second or third generation cephalosporins are more effective, but not less safe than amoxicillin[6]

c. CNS infections

- Meningococcal meningitis: cefotaxime
- Pneumococcal meningitis: cefotaxime
- *H. influenzae* meningitis: cefotaxime

d. Pelvis infections

- Pyelonephritis: cephalosporin for 14 days - low UTI 3 days only
- Gonorrhoea: cefotaxime

e. Blood-borne infections

- Community acquired septicaemia: broad spectrum cephalosporin
- Hospital acquired septicaemia: beropenem or imipenem
- Meningococcal septicaemia: cefotaxime.

(Brooks *et al*., 2004; Denyer *et al*., 2004 and Rossolini, 2007)

3.6 β-LACTAMASES

β-Lactamases are the principal mechanism of bacterial resistance to beta-lactam antibiotics. The ability of a beta-lactamase to cause resistance varies with its activity, quantity, and cellular location and, for gram-negative organisms, the permeability of the producer strain. Early events were an increase in their prevalence in organisms in which the enzyme was known but uncommon (such as *Staphylococcus aureus*) and spread to pathogens that previously lacked β-lactamase (namely, *Haemophilus influenzae* and *Neisseria gonorrhoeae*) (Bush *et al*., 1995).

Bacteria responded with a plethora of "new" β-lactamases — including extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC β-lactamases enzymes and carbapenem-hydrolyzing β-lactamases (carbapenemases) — that, with variable success, can confer resistance to the latest β-lactam antibiotics. There are 340 discrete beta-

lactamases that have been identified till now. The major enzymes are described below (Jacoby *et al*., 2005)

3.6.1 Classification of ß-lactamases

β-Lactamases (EC 3.5.2.6) have been designated by the Nomenclature Committee of the International Union of Biochemistry as ‘‘enzymes hydrolyzing amides, amidines and other CON bonds, separated on the basis of the substrate cyclic amides. There have been given a bewildering variety of names for β-Lactamases. Fortunately, the enzymes can be classified on the basis of their primary structure into four molecular classes (A through D), (Ambler, 1980) or on the basis of their substrate spectrum and responses to inhibitors into a larger number of functional groups (Bush *et al.*, 1995). Class A and class C β-lactamases are the most common and have a serine residue at the active site, as do class D β-lactamases. Class B comprises the metallo-β-lactamases (Bush *et al.*, 1995).The detailed schemes of β-lactamase classification are given in table 3.

3.6.2 Action of ß-lactamase

A few ß-lactamases utilize zinc ions to disrupt the ß-lactam ring, but a far greater number operate via the serine ester mechanism shown in Fig. 1. (Waley, 1992)

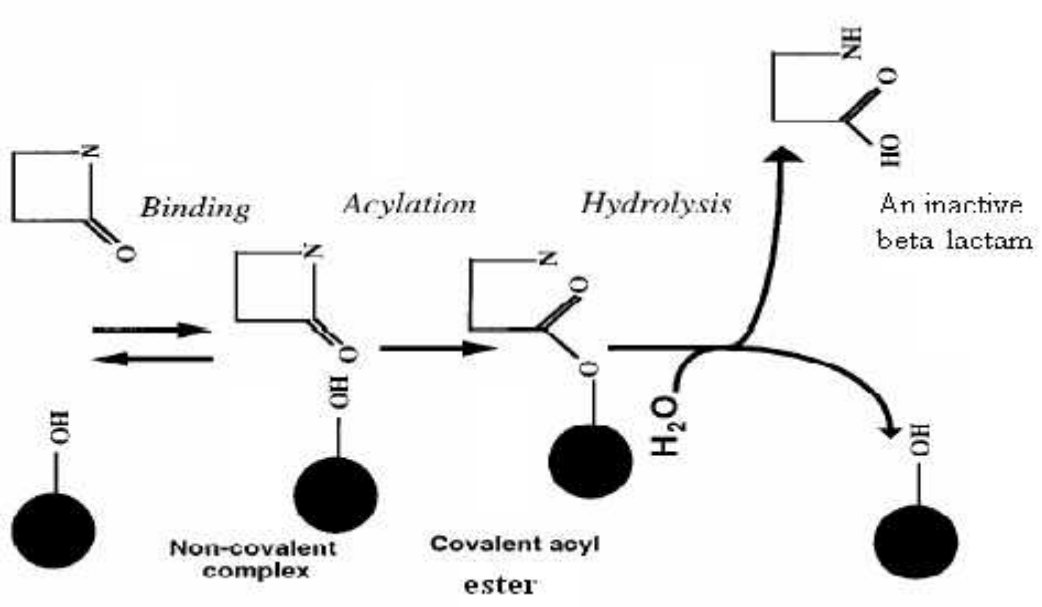

Figure 1: Action of a serine ß-lactamase (classes A, C, and D) (Waley, 1992)

Table 5: Classification schemes for bacterial β-lactamases (Bush *et al.*, 1995)

Bush-Jacoby-Medeiros group	Richmond-Sykes class	Mitsuhashi-Inoue type	Mole-cular class	Preferred substrates	Inhibited by		Representative enzymes
					CA	EDTA	
1	Ia Ib Id	CSase	C	Cephalosporins	-	-	AmpC enzymes ; MIR-1
2a	Not included	PCase V	A	Penicillins	+	-	Penicillinases
2b	III	PCase I	A	cephalosporins Penicillins	+	-	TEM-1, TEM-2, SHV-1
2be	Not included except K1 in class IV	CXase	A	Penicillins, cephalosporins, monobactams	+	-	TEM-3 to TEM-26, SHV-2, to SHV-6,
2br	Not included	Not included	A	Penicillins	±	-	TEM-30 to TEM-36, TRC-1
2c	II, V	PCase IV	A	Penicillins, carbenicillin	+	-	PSE-1,PSE-3, PSE-4
2d	V	PCase II, PCase III	D	Penicillins, cloxacillin	±	-	OXA-1 to OXA-11, PSE-2
2e	Ic	CXase	A	Cephalosporins	+	-	Inducible cephalosporinases from *P. vulgaris*
2f	Not included	Not included	A	Penicillins, carbapenems	+	-	NMC-A from *E. cloacae*
3	Not included	Not included	B	Most β-lactams	+	-	L1 ,CcrA
4	Not included	Not included	NDc	Penicillins	-	?	Penicillinase from *P cepacia*

CA, clavulanic acid. ND, not determined.

3.6.3 Extended-spectrumof Beta-Lactamase (ESBL)

Extended-spectrum beta-lactamases (ESBLs) were first reported in 1983 in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens* (Knothe *et al.*, 1983).

ESBLs are acquired class A ß-lactamase enzymes that mediate resistance to extended-spectrum, oxyimino- second and third generation cephalosporins (e.g. ceftazidime, cefotaxime, ceftriaxone, and cefuroxime); and monobactams (e.g., aztreonam); but do not affect cephamycins (e.g., cefoxitin and cefotetan) or carbapenems e.g., meropenem and imipenem (Livermore and Woodford, 2004). Typically, they derive from genes for TEM-1, TEM-2, or SHV-1 by mutations that alter the amino acid configuration around the active site of these ß-lactamases. With the exception of OXA-type enzymes (which are class D enzymes), the ESBLs are of molecular class A, in the classification scheme of Ambler.More then 100 different sequence variants of SHV and TEM genes have been so far established but TEM-1, TEM-2, TEM-10, TEM-11, SHV-1, SHV-2, CTX-M, Toho, OXA, PER and VEB-1 are common types of ESBLs (Paterson *et al.*, 2005).

Extended-spectrum beta-lactamase (ESBL) producing organisms are typically resistant to many other classes of antibiotics, including aminoglycosides trimethoprim and fluoroquinolones, as a consequence of genes for other antibiotic resistance mechanisms residing on the same plasmids as the ESBL genes, difficult to detect and treat; and associated with increased mortality. ESBLs have evolved greatly over the last 20 years. Their presence, plus the potential for plasmid-mediated quinolone and carbapanem resistance, will be sure to create significant therapeutic problems in the future. It is unlikely that many new antibiotic options will be available in the next 5 to 10 years to tackle such multiresistant infections (Jacoby *et al.*, 2005 and Paterson *et al.*, 2005).

Extended-spectrum ß-lactamases (ESBLs) are produced in *E. coli*, *Klebsiella* spp. and different species of the family Enterobacteriaceae. The genes encoding these enzymes are most often carried by multidrug-resistant plasmids and are capable of being readily transferred among different species. The CLSI (Clinical and Laboratory Standards Institute) has proposed disk diffusion methods for screening for ESBL production by *Klebsiella* spp., *Escherichia coli*, and *Proteus mirabilis* and confirmation of ESBL production (Paterson *et al.*, 2005). The detailed methods for both screening and conformation will be given in the chapter methodology.

3.6.4 AmpC β-lactamase (ABL)

AmpC beta-lactamases (ABLs) are derivatives of the chromosomally encoded, clavulanate-resistant and cephamycins resistant; and most are AmpC Group I cephalosporinases that confer resistance to a wide variety of beta-lactam drugs. Organisms producing AmpC ß-lactamases were first reported in the 1980s. ABLs, demonstrated or presumed to be chromosomally or plasmid mediated, have been described in pathogens e.g. *K. pneumoniae, E. coli, Salmonella* spp.*, Proteus rettgeri P. mirabilis, C. freundii, Hafnia alvei, Aeromonas* spp., *Morganella morganii ,Acinetobacter* spp., *S. marcescens, Enterobacte* spp.*, Chromobacterium violaceum Lysobacter lactamgenus, Providencia stuartii, Yersinia enterocolitica, Rhodobacter sphaeroides* and *P. aeruginosa* (Jacoby *et al.*, 2005).

Plasmid-mediated AmpC ß-lactamases are a heterogeneous group of enzymes that originated from the chromosomal genes of bacteria such as *Enterobacter* spp.*, Citrobacter freundii, Morganella morganii, Aeromonas* spp. and *H. alvei* (Philippon *et al*., 2002). Plasmid mediated class C AmpC beta-lactamases has been discovered most frequently in isolates of *Klebsiella pneumoniae, K. oxytoca, Salmonella* spp.*, Proteus mirabilis* and *E. coli.* Some common enzymes of this type include DHA, FOX, ACT, KPC, CMY, ACC and MIR. The genes are typically encoded on large plasmids containing additional antibiotic resistance genes, leaving few therapeutic options and treatment failure. High-level expression of AmpC ß-lactamases by Gram-negative organisms can result in organisms that are resistant to almost all β-lactam drugs (Thomson *et al*., 2000).

Although it has been over a decade since AmpC ß-lactamases were discovered, most clinical laboratories and physicians remain unaware of their clinical importance. Current detection methods for organisms producing AmpC ß-lactamases are technically demanding for clinical laboratories to perform on a routine basisCurrently, there are no recommendations available from the NCCLS or elsewhere for phenotypic detection of organisms producing AmpC ß-lactamases. Resistance to cefoxitin can indicate the

possibility of AmpC-mediated resistance but can also indicate reduced outer membrane permeability. In addition, the use of ß-lactamase inhibitors can help identify possible AmpC producing organisms eg AmpC disk test, however none of these tests are standardized and can be time consuming when screening large numbers of isolates (Black *et al*., 2005).

3.6.5 Metallo-β-lactamase (MBL)

MBLs are group 3 (Jacoby *et al.*, 1995) and Class B (Ambler, 1980) beta-lactamase that can hydrolyze almost all beta-lactam drugs. MBLs are chromosome or plasmid mediated, found on *P. aeruginosa, Acinetobacter* spp., *C. freundii* and Enterobacteriaceae. These metalloenzymes were originally found in *Klebsiella pneumoniae* isolates in Japan in 1994 (Koh *et al*., 2001).

The advent of carbapenems in the 1980s heralded a new treatment option for serious bacterial infections but carbapenem resistance can now be observed in Enterobacteriaceae and *Acinetobacter* spp. and is becoming commonplace in *P. aeruginosa,* is commonly mediated by MBL.Based on molecular studies, two types of carbapenem-hydrolyzing enzymes have been described: serine enzymes possessing a serine moiety at the active site (serine β-lactamse), and metallo-ß-lactamases (MBLs), requiring divalent cations, usually zinc, as metal cofactors for enzyme activity. Some reports indicate that nearly 30% of imipenem-resistant *Pseudomonas aeruginosa* strains possess a metallo-ß-lactamase (Jacoby *et al.*, 2005 and Walsh *et al*., 2005).

Acquisition of a metallo-ß-lactamase gene will invariably mediate broad-spectrum β-lactam resistance in *P. aeruginosa*, but the level of in vitro resistance in *Acinetobacter* spp. and Enterobacteriaceae is less dependable. Their clinical significance is further embellished by their ability to hydrolyze all ß-lactams except aztreonam and by the fact that there is currently no clinical inhibitor, nor is there likely to be for the foreseeable future (Bush *et al*., 1995). The MBL producers are major burden to combat multidrug resistance clinical isolates worldwide e.g. MRSA and Gram-negative bacilli producing

the acquired metallo-ß-lactamases (MBLs)- IMP and VIM have been increasingly reported in Asia and Europe and more recently, they have been detected in Canada and the United States (Chu *et al.*,2001).

CLSI (formerly NCCLS) documents do not yet contain a method for phenotypic detection of MBL-producing isolates. The Hodge test can be used to screen carbapenemase-producing gram-negative bacilli and that the imipenem (IPM)-EDTA double-disk synergy test (DDST) can distinguish MBL-producing from MBL-nonproducing gram-negative bacilli (Lee *et al*., 2001). A DDST using a ceftazidime (CAZ) and a 2-mercaptopropionic acid (MPA) disk (Arakawa *et al*., 2000).

We have used DDST test using ceftazidime (Ca) and EDTA disk and more about this method will be discuuss latter.Rather like the accepted ethos for the early detection of extended-spectrum β-lactamases, it is judicious to detect MBLs for precisely the same reasons.

3.7 APPROACHES TO ANALYSE R-PLASMID OF BACTERIAL STRAINS

There are many methods to exploit the study of R-plasmid. The approach to use the study of plasmid DNA depends upon the type of investigation concerned. The most frequently used methods whether to study whole plasmid DNA or genes from it are described below (Mayer, 1988).

3.7.1 Analysis of intact and digested fragments of R-plasmid

The plasmid content of most bacterial strains is usually a stable feature, although there are cases in which plasmids are lost during subculture. Plasmid profiles are relatively easy and inexpensive to perform. A number of procedures for isolating plasmid DNA have been described. Most procedures used to isolate plasmid DNA rely on the physical differences in circular plasmid molecules and linear chromosomal fragments. Alkaline lysis and denaturation of the nucleic acid form the basis of many plasmid isolation protocols. Chromosome is sheared into fragments ranging from about 20 to >100 kb. A

typical plasmid band, or other DNA that is of uniform size, has a sharply delineated leading edge. The trailing edge is often slightly diffuse due to artifacts of electrophoresis (Sambrook *et al*., 2001 and Bloom, 1996).

Various parameters should be optimized when dealing with R-plasmid of most of MDR bacterial strains such as amount of cell mass, lysis step, elution step when using gel membrane, electrophorosis running time, power supply and appropriate supercoiled ladder in order to get interpolation. Plasmids of identical size but very different sequence and function can exist in many bacteria. When only a single plasmid is present, restriction endonucleases can be used to provide further evidence of the similarities and differences between strains. Restriction endonucleases are sensitive to many of the chemicals used to isolate plasmid DNA, such as phenol, detergent, ethanol, or chelators, so care must be taken to remove these chemicals before digestion (Sambrook *et al*., 2001; Mayer, 1988 and Bloom, 1996).

3.7.2 Polymerase chain reaction (PCR) and DNA sequencing

Diiferent types of PCR technique has been used to study the genes on R-plasmid which have both diagnostic and investigational advantages**,** but most common are Multiplex PCR and randomly amplified polymorphic DNA (RAPD) PCR. Plasmid-mediated AmpC b-lactamase gene amplification was carried out using multiplex PCR by using oligonucleotide primers CMY25F1 (nt 1736–1754) and CMYDR1 (nucleotides 3167–3147) for the detection of gene of *Citrobacter freundii*-origin plasmid-mediated AmpC (blaCMY-2). The precise usage of codons, informations of mutations and polymorphisms on plasmid can only be elucidated by DNA sequencing. Early in the course of the study, *bla* genes were identified by cloning and sequencing (Sidjabat *et al*., 2006).

3.7.3 Pulsed field gel electrophoresis (PFGE)

PFGE is useful to profile when dealing with large size plasmid and it can separate DNA fragments up to 2000 kb. Following identification by multiplex PCR, 62 multidrug

resistant *E. coli* isolates were selected for PFGE and plasmid analysis (Kado & Liu, 1981) and their profiles were compared with 11 previously analysed extraintestinal clinical isolates as described by (Sidjabat *et al*., 2006).

3.7.4 Isoelectric focusing (IEF)

The method is useful to characterize certain genes associated with particular enzyme production like *bla* gene on the R-plasmid which produces different types of β-lactamase (ESBL, AmpC and MBL etc.) that have characteristic pI values. Most of AmpC β-lactamase exhibit pI values >7.0 while most of ESBL exhibit pI values <6.0 with some exceptions .By IEF the AmpC β-lactamase exhibited three pI values: 7.2 (FOX-5), 7.7 (DHA-1) and >9.0(ACT-1) (Moland *et al.*, 2000).

3.7.5 Southern hybridization analysis

Southern hybridization can be used to characterize the genes present on the R-plasmids which aid the mechanism of resistance. DNA probes were prepared from PCR products generated for the characterization of blaCMY (462 bp), blaTEM (971 bp), catA1 (581 bp) and class 1 integron associated dfrA17-aadA5 (652 bp) (Sidjabat *et al*., 2006).

3.7.6 Electron microscopy

Intact non-fragmentated plasmid DNA can be visualized by electron microscopy. Lang *et al*. (1967) used Kleinschmidt/Lang monolayer spreading technique to tease out the DNA so that its contour length can be measured assuming 1micron of DNA has 2.07 X 10^6 daltons. **Electron microscope heteroduplex technique** was described by Davis and Davidson in 1968 which help to study the molecular relationship of R-factor component units to each other, to different R-factors, and to other plasmids. This technique enables localization of regions of homology (visualized as double stranded segments) and non-homology (visualized as single DNAstrand) present in different plasmids (Land *et al*., 1967).

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 MATERIALS

The materials required for this work are listed in Appendix II

4.2 METHODS

The study was conducted at Kathmandu Model Hospital in collaboration with Central Department of Microbiology, Tribhuvan University, Kirtipur. The study was carried out from May 2007 to January 2008. During this period, a total of 1503 different samples from patients suspected of bacterial infections were collected and processed according to the standard laboratory methods. Plasmid extraction and analysis of MDR strains (Donors, transconjugants and transformants) were carried out in research lab of Central Department of Microbiology.

4.2.1 Data collection

Each patient requested for bacterial culture was directly interviewed for his/her clinical history during sample collection. The informations of patients include name, age, sex, signs and symptoms; site of infection; prior infection; duration of fever; other underlying diseases; use of medical device; trauma or surgical cases associated with infection, heredity and prior antibiotics administration or not.

4.2.2 Sample collection

Samples from the indoor and outdoor patients were collected in strictly sterile leak-proof, dry containers, free from all traces of disinfectants. The collected samples were immediately brought into Microbiology Department of KMH. The types of samples received were urine, sputum, blood, pus, fluid, tissue, CSF, throat swab, perianal swab and stool. Different procedures were followed for the collection of these samples. Each sample clearly labeled with date, patient's name, sex, bed number, time of collection
and a brief history.

4.2.2.1 Collection of urine sample

Before providing the container, each patient was instructed properly for the collection of about 5-10 ml clean catch mid-stream urine.

4.2.2.2 Collection of sputum

Adequate safety precautions were taken when collecting sputum. Patient was given with sterile clean, wide mouthed, impermeable container with a screw cap or tightly fitting cap and advised to cough deeply to produce sputum, not saliva.The patient was asked to collect the sample in the morning after the patient wake up and before any mouthwash.

4.2.2.3 Collection of throat swab

A plain sterile cotton wool swab was used to collect as much exudates as possible from the tonsils, posterior pharyngeal wall and any other area that is inflamed or bears exudates. Care was taken not to touch the tongue or buccal surfaces and duplicate swabs from the same area were taken.Then the swab was replaced in its tube and delivered to the laboratory**.**

4.2.2.4 Collection of pus sample

While collecting pus from abscesses, wounds or other sites, special care was taken to avoid contaminating the specimen with commensal organism from the skin. Pus from the abscess was collected at the time the abscess is incised and drained or after it has ruptured naturally. Five milliliter (5 ml) of pus was aspirated or collected from a drain tube and transported to a leak proof sterile container. If it was not available needle capped syringe itself was transported. A sterile cotton wool swab was used to collect a sample from the infected site in the case of undischarged pus. Usually, two deep swabs were collected.

4.2.2.5 Collection of CSF

In order to avoid iatrogenic infection, disinfection of the skin was performed before puncture. Approximately 5-10ml of CSF was collected in two sterile tubes by lumber or ventricular puncture performed by physician. The sample was dispatched to the lab at once.

4.2.2.6 Collection of blood

Following steps were carried out during collection of blood sample.

- Using tourniquet, a suitable vein was located in the arem.
- Wearing gloves, the venepuncture site was thoroughly disinfected using 70% ethanol and allowed to dry.
- Using a sterile syringe and needle, about 2-10ml blood (10ml for adults,2-5ml for children) was withdrawn from the patient and dispensed to the sterile screw capped blood culture bottle containing sodium polyanethol sulfonate (SPS) and 50-70ml tryptic soy broth.

4.2.2.7 Collection of stool sample

Following steps were carried out during collection of stool sample.

- The patient was provided with two small wooden sticks and clean container with a leakproof lid.
- The patient was instructed to collect stool sample on a piece of toilet tissue or old newspaper and to transfer it to the container, using two sticks.
- The sample of at least 5gm of faeces and, if present those parts that contain blood, mucus or pus was accepted and dispatched to laboratory.

4.2.2.8 Collection of perianal swab

Following steps were carried out during collection of perianal swab sample.

- A cotton-tipped swab was moistened with sterile water and was inserted through the perianal sphincter, rotated and withdrawed.The swab was examined for faecal staining and was repeated until sufficient staining was evident.
- The swab was placed in an empty sterile tube with a cotton plug and delivered soon.

4.2.2.9 Collection of tissue sample

The site of infection was located and superficial skin was disinfected with 70% ethanol. A piece of tissue was taken from infected site and transferred to screw –cap tube .This specimen was not allowed to dry by moistening with sterile water if not bloody.The sample was dispatched with 24 hours for processing.

4.2.2.10 Collection of fluid sample

Most common fluid samples (pleural, peritoneal, pericardial and synovial fluids) were collected by aspiration with a needle and syringe by experienced physician.

- Site of aspiration was located and skin was disinfected with 70% ethanol.
- About 1-5ml of sample was drawn and transported to the laboratory at once in a sterile tube or vial.

4.2.3 Sample evaluation

Before proceeding, the different clinical specimens were evaluated in terms of their acceptability. Considerations included proper labeling, watery sputum , visible signs of contamination and any transportation delays in getting the specimen to the laboratory. A properly labeled specimen contained patient's full name, date and time of collection.

4.2.4 Macroscopic examination

The specimen obtained in laboratory was observed for its color, odour, dryness, consistency and appearance and reported accordingly.

4.2.5 Homogenization of Sample

Larger solid tissue was homogenized in the presence of sterile saline with the help of sterile scissors and forceps into smaller pieces suitable for culturing. The sputum samples which were very thick and mucoid were treated with a mucolytic agent for homogenization (0.01% dithiothreitol).

4.2.6 Microscopic examination

The homogenized samples of sputum and pus were Gram stained for examining the presence of pus cells and studying the morphology, arrangement and Gram’s reaction displayed by bacteria. For urine sample, 10 ml urine was taken in a clean sterile centrifuge tube and centrifuged at 3000 rpm for 10 min. The supernatant was discarded. The sediment was then examined by wet mount preparation for the detection of WBC (pus cells) and RBC. Number of WBC and RBC were estimated as number per HPF. CSF and fluid were examined by Gram’s stain method. Microscopic study was recorded and reported accordingly.

4.2.7 Culture of specimen

Blood agar (BA), Mac Conkey agar (MA) and Chocolate agar (CA) were used for the routine culture of the sputum. A loopful of homogenized sputum sample was inoculated onto BA, MA and CA separately. On CA, 5 mcg of Optochin disk and 10 uints of bacitracin disk were placed on the primary and secondary incoculation respectively. Semi-quantitative culture technique was used to culture urine specimens and to detect the presence of significant bacteriuria by standard methods (Cheesbrough, 1984). An inoculating loop of standard dimension was used to take up 0.001ml urine, which was inoculated onto MA and CA.

The bacterial count was reported as:

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

Blood culture bottle was incubated for 7 days. It was routinely inspected twice a day (for the first 3 days). Growth was evidenced by:

- uniform turbidity
- a flocular deposit on top of the blood layer
- haemolysis
- coagulation of the broth
- a surface pellicle
- production of gas

Whenever visible growth appears, subcultures of broth were performed on BA, MA and Salmonella-Shigella agar (SS agar). A blind subculture was performed at the end of 7 days of incubation in the case of previous negative subculture growth. A loopful of pus and fluid samples were inoculated in the BA and MA plaetes. In the case of the swab (pus/wound) and throat swab, they were rubbed at the side of BA as well as MA plates. The specimen rubbed on the BA and MA plates, was spread in the medium with the help of a sterile inoculating loop (WHO, 2004).

Solid tissue sample, after homogenization, was inoculated in Nutrient broth (NB) and incubated at 37^0C for 4-5 hours. Then a loopful of broth was streaked on BA and MA plates. For stool and perianal swab, MA and SS agar were used. A light inoculum of faeces was placed in the middle of the agar plates and streaked it up and down and across the plate.If bacteria or fungi had been seen in the Gram-stained smear, the appropriate meida were used for culture of CSF. If no organisms had been seen, or if the interpretation of the Gram smear

was become unclear, MA, CA (with bacitracin and optochin) and BA were used (Forbes *et al*., 2002 and WHO, 2004).

All BA and MA plates used for all samples were incubated at 37^0C for 24 hours while CA was incubated in candle jar at 37^0C for 24 hours. Significant growth observed on urine was processed for indentification of isolate. Pathogenic growth was sought in stool, perianal swab, sputum and throat swab. In the case of fluid, CSF, pus, blood and tissue growth of the isolate was examined. So, single colony was assessed as offending organism (WHO, 2004).

4.2.8 Identification of isolates

Identification of isolates from different samples were done by using microbiological techniques as described in the Bergey's manual of systematic bacteriology which involves morphological appearance of the colonies, staining reactions and biochemical properties. Each of the organism was isolated in pure form before performing biochemical and othe tests.The biochemical tests used for the identification include Catalase test, Oxidase test, Sulfide indole and motility (SIM) test, Methyl red test, Voges Proskauer test, Citrate utilization test, Oxidation Fermentation test, Triple Sugar Iron (TSI) test and Nitrate reduction test (Krieg, 1984 and Sneath, 1986). The composition and preparation of these all tests are mentioned in appendix III while procedure for these tests is mentioned in appendix V.

4.2.9 Antibiotic susceptibility testing

The antimicrobial susceptibility testing of the isolates towards various antimicrobial disks was done by modified Kirby-Bauer M2-A9 disk diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI) using Mueller Hinton agar (MHA) as follows.

- Mueller Hinton Agar was prepared and sterilized as instructed by the manufacturer.
- The pH of the medium was adjusted to 7.2-7.4 and the depth of the medium at 4 mm (about 25 ml per plate) was maintained in Petri-dish.
- Using a sterile wire loop, a single isolated colony whose susceptibility pattern is to be determined was touched and inoculated into Mueller Hinton broth tube and was incubated at 37ºC for 2-4 hrs.

- After incubation, the turbidity of the suspension was matched with the turbidity standard of McFarland tube number 0.5.
- Using a sterile swab, a plate of MHA was inoculated with the bacterial suspension using carpet culture technique. The plate was left for about 5 minutes to let the agar surface dry.
- Using sterile forceps, appropriate antimicrobial discs (6 mm diameter) was placed, evenly distributed on the inoculated plates, not more than 6 discs were placed on a 90 mm diameter Petri-dish.
- After overnight incubation, the plates were examined to ensure confluent growth and the diameter of each zone of inhibition in mm was measured and compared with standardized zone interpretative chart provided by the company.

The preparation and composition of Mueller Hinton Agar medium is mentioned in the Appendix-III. The detailed about antibiotic disks used and its interpretative chart are mentioned in the Appendix-II and VII respectively.

4.2.10 Screening and confirmation of ESBL producers

Screening of the suspected ESBL-strains was performed according to the guidelines for sceening issued by NCCLS in 2005. According to these guidelines, the zone diameter for possible ESBL strains is $\leq$ 27mm for cefotaxime (30 µg), and $\leq$ 22mm for ceftazidime (30 µg). The suspected ESBL strains were tested for confirmation by using the double disc diffusion synergy test (DDST) method, using co-amoxiclav 20+10 µg disc and cefotaxime and ceftazidime 30 µg discs placed 20-30 mm away from it. ESBL production was confirmed when the zone of either cephalosporin was expanded by the clavulanate .Detailed procedure of DDST for confirmation of ESBL production was mentioned in appendix VII.

4.2.11 Screening and confirmation of ABL producers

There is no Clinical and Laboratory Standards Institute (formerly NCCLS) guideline for the detection of plasmid mediated AmpC β-lactamase production. In present study this issue was addressed by studying the use a cefoxitin disk and Tris-EDTA mixture for the detection of AmpC β-lactamase production in different clinical isolates by **AmpC disk test** which was used by Black *et al*. (2005). The presumptive AmpC producers were detected by cefoxitin resistance and negative ESBL production. Cefoxitin resistance was detected using the disk

diffusion technique, and ESBL production was determined using double-disk diffusion methods, according to NCCLS guidelines .This phenotypic confirmatory test is based on use of Tris-EDTA to permeabilize a bacterial cell and release β-lactamases into the external environment that is detected by indentation or urinform growth of *E. coli* ATCC 25922 lawn around cefoxitin disc.The method for preparing AmpC disk and detection of production of AmpC β-lactamase is mentioned in appendix VII.

4.2.12 Screening and confirmation of MBL producers

The presumptic producer of MBL was screened by Amp C disk test negative and intermediate strains. Present study used technically simple and inexpensive method, used by Lee *et al*.(2003), **EDTA disk synergy test (EDST)** for phenotypic confirmatory detection of MBL production.This study used the metallo-beta-lactamase inhibitor EDTA owing to the fact that, all MBLs are affected by the removal of zinc from the active site. Metallo-β-lactamase producer show increased growth-inhibitory zone between the EDTA and ceftazidime while hyper AmpC producer, AmpC producer, ESBL producer and Serine β-lactamase producer show no change of growth inhibiton zone in the two disks. The method for preparing EDTA disk and detection of production of MBL was mentioned in appendix VII.

4.2.13 Purity plate

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

4.2.14 Quality control

Quality of each test was maintained by using standard procedures. The quality of each agar plates prepared was by incubating one plate of each lot on the incubator. Control strains of *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used for the standardization of the Kirby-Bauer test and also for correct interpretation of zone of diameter. Quality of sensitivity tests was maintained by maintaining the thickness of MHA at 4 mm and the pH at 7.2-7.4. Similarly antibiotics discs containing the correct amount as indicated were used. Strict aseptic conditions were maintained while carrying out all the procedures.

4.2.15 Preservation of ESBL, ABL and MBL producing isolates

All the isolates of ESBL, ABL and MBL producing strains were preserved in media containing tryptic soy broth and 20% glycerol.

4.2.16 PLASMID DNA ANALYSIS

4.2.16.1 Extraction of plasmids DNA from ESBL, ABL and MBL producing MDR *E. coli* and *C. freundii*.

The plasmid DNA was extracted from the overnight culture (mid log phase) of MDR *E. coli* and *Citrobacter freundii* grown in Lauria Bertani broth, supplemented with proper antibiotics.The plasmids were extracted according to protocols described by Sambrook *et al*. (2001) and using commercial Kits procured from Bangalore GeNei. The detailed protocols are mentioned in appendix IX. The composition of Lauria Bertani broth is mentioned in appendix III. The preparation of reagents for extraction of plasmid is mentioned in appendix III.

4.2.16.2 Conjugation between MDR *E.coli* (donor) and *E.coli* HB101

MDR *E. coli* sensitive to streptomycin were selected and grown in NB.Similarly recipient strain *E.coli* HB101 (streptomycin resistant) was also grown in NB and conjugation mixture was made by mixing both MDR *E. coli* and *E. coli* HB101. After incubation, the transconjugants were selected on suitable antibiotic plate. The detailed protocol for conjugation is mentioned in appendix IX.

4.2.16.3 Transformation of plasmid DNA in Recipient *E.coli* (TB1)

The plasmid DNA extracted were selected and transformed in *E. coli* TB1 (sensitive to all antibiotics but resistant to streptomycin) according to the protocols described by Sambrook *et al*. (2001). The detailed protocols are mentioned in appendix IX.

4.2.16.4 Extraction of plasmids DNA from transconjugants and transformants

The method used is similar as mentioned in section 4.2.16.1

4.2.16.5 Determination of minimum inhibitory concentration (MIC) of donors transconjugants and transformants

MICs were determined by two fold M7-A7 broth macrodilution method recommended by CLSI. The inocula of 4 x 10^5 CFU/ml were prepared from suitably diluted overnight broth culture at 37^0C. The detailed protocol for this test is mentioned in appendix IX.

4.2.16.6 Electrophoresis of plasmid DNA from MDR *E. coli,* transconjugant and transformant

The electrophoresis was done in standard agarose in 60 volts for 4 hours. Then the agarose slab was visualized under the UV trans-illuminator at 300 nm wavelength. The detailed protocols for electrophoresis are mentioned in appendix IX.

4.2.16.7 Decontamination of ethidium bromide

The procedure for decontamination of ethidium bromide remaining in the agarose slab is mentioned in the appendix IX.

4.2.16.8 Making standard curve

Using a ruler, distance traveled from the well (in cm) by each illuminated band was measured. The number of base pairs (of marker DNA) of each illuminated band versus the distance traveled was plotted on Semi-log paper. The data was examined to be plotted and determined the number of magnitude (i.e size of marker DNA) covered by the data to be plotted on the logarithmic axis so that 2-cycles plot was chosen. The distance of migration of each base pair in cm was plotted on the linear scale. The best fit line through these points was drawn to generate a standard curve. From this curve the size of each band was determined (Snyder *et al*., 2003 and Bloom *et al*., 1996)

4.2.16.9 Determination of size of DNA by semi log plot.

The size of unknown plasmid DNA as distinct band was determined by the extrapolation of those below the distance of migration achieved by 16.2 kb band with the help of standard curve. Those base pairs within in the range of DNA marker was determined by interpolation of distance of migration using a line of two consecutive base pairs of the DNA marker flanking the unknown base pairs.

4.2.16.10 Data analysis

Data analysis was made from the Statistical package for social science (SPSS) software (version 11.5). Chi-square test was used to determine significant association of dependent

variables like bacterial infections, MDR etc to different independent variables (gender, age, type of patients etc). Odds ratio was determined to predict the degree of association between previous antibiotic use and drug resistance as well as association of level of multidrug resistance and no.of plasmids harbored by MDR strains. Test of present work are shown in appendix- XI.

CHAPTER-V

5. RESULTS

5.1 CLINICAL PATTERN OF RESULTS

5.1.1 Number of samples, pattern of growth, MDR, ESBL, ABL and MBL producing stains

Altogether, 10 different samples in total 1503 were received in the laboratory, of which most predominant were urine samples 710 (47.24%). Out of total samples, 22.35% (336/1503) bacterial growth was found. Out of 710 urine samples, 219 (30.84%) showed significant growth. Out of total 336 isolates, 138/336 (41.07%) were found MDR isolates from all samples. Out of 138 MDR isolates, maximum isolates were isolated from urine samples as 105/138 (76.08%) isolates whereas least MDR isolates were found from fluid and tissue samples each 1/138 (0.72%) isolate. Out of total 138 MDR strains that were screened for ESBL production test, 27 isolates were found ESBL producers. Similarly, 58 MDR isolates were found ABL producers whereas 33 MDR strains were found MBL producing strains. The maximum isolates of ESBL producers (25/27), ABL producers (52/58) and MBL producers (28/33) were all isolated from urine samples. The results are shown in table 6.

5.1.2 Pattern of type of patients, isolates and MDR strains in different samples

Out of 1503 samples, most predominant were constituted from outdoor patients with 1393 (92.68%) cases. Of the total outdoor samples, 291/1393 (20.90%) samples showed growth positives, among them 36.42% (106/291) isolates were MDR strains. Highest growth positives were found from indoor samples 40.9% (45/110) and highest MDR strains were also found from indoor patients 71.11% (32/45). The results are shown in appendix X.

5.1.3 Gender and age wise distribution of patients requesting for culture and their growth pattern

Out of 1503 patients requested for culture; predominant no of samples 819 (55.5%), highest growth positivity 25.52% (209/819), and predominant MDR strains 86/209 (41.15%) were all found from female patients The age group of 21-30 years was found the maximum request of 489 (32.54%) requests for culture. Age groups of 81-90 years and over 90 years requested the

least with only 22 (1.46%) requests. Maximum isolates were found from the age group of over 90 years as 28.58% (8/22) isolates whereas least isolates were found from the age group of below 10 years with 14.52 % (9/62) isolates.The results are shown in appendix X.

5.2 PATTERN OF BACTERIAL ISOLATES

5.2.1 Pattern of bacterial growth according to Gram's stain

Among the 336 isolates, Gram-negative bacteria were found predominant constituting 294 (87.5%) of the total isolates. Among the Gram-negative bacteria, predominant isolates were obtained from urine with 209/294 (71.08 %) isolates. Out of 42 Gram-positive bacteria, predominant isolates were isolated from pus samples with 30/4 (71.42%) isolates. The results were shown in appendix X.

5.2.2 Pattern of bacterial isolates from different samples

Altogether 22 different bacteria with total 336 isolates were found from different samples. Out of total 336 isolates from different samples, *E. coli* was found the most predominant isolates with 193/336 (57.44%) isolates. Among Gram-negative bacteria, *E. coli* was found the most frequently isolated species with 193/294 (65.64%) isolates, among these, 178 (92.22%) were isolated from urine.Similarly, out of 42 isolates of Gram-positive bacteria, *Staphylococcus aureus* was the most predominant pathogen with 17/42 (40.47%) isolates, among these, maximum strains with 16 (94.12%) strains were isolated from pus samples. *Salmonella* Typhi was found predominanat pathogen isolated from the blood samples with 27/51 (52.95%) isolates. Similarly *S. aureus; E. coli* and *Neisseria gonorrhoeae; S. pneumoniae; N. meningitidis*; *E. coli* and *P. aeruginonas; S. pyogenes* and *E. coli* were found predominant pathogens with 16/56 (28.57%), 1/2 (50.0%) each, 1/1 (100.0%), 1/1 (100.0%), 1/2 (50.0%) each, 1/1 (100.0%) and 3/3(100.0%) isolates from pus, fluid, sputum, CSF, tissue, throat swab and perianal swab samples respectively.The results are shown in figure 4 and appendix X.

Table 6: Pattern of different clinical samples, status of growth, MDR strains and status of β-lactamase producers

Specimens	No. of specimens	Growth	MDR strains		No of ESBL producers	No of ABL producers	No of MBL producers
		No.	No.	%			

		(%)					
Blood	551	51 (9.25)	0	0	0	0	0
Pus	91	56 (61.53)	28	50	2	5	4
Fluid	28	2 (7.14)	1	50	0	0	1
Sputum	59	1 (1.69)	1	**100**	0	0	0
CSF	13	1 (7.69)	0	0	-	-	-
Tissue	2	2 **(100.00)**	1	50	0	0	0
Stool	16	0 (0.00)	0	0	-	-	-
Throat swab	30	1 (3.34)	0	0	-	-	-
Perianal swab	3	3 **(100.00)**	2	33.34	0	1	0
Urine	**710**	219 (30.84)	105	47.95	**25**	**52**	**28**
TOTAL	**1503**	**336 (22.35)**	**138**	**41.07**	**27**	**58**	**33**

5.2.3 Age wise distribution of pathogens and MDR strains from different samples

E. coli was found the most predominant pathogen, isolated from patients of age-group 21-30 with 61/193 (31.60%) isolates, among these 21 (34.42%) isolates were MDR. Among the different age groups, least number of *E. coli* was isolated from the age group 81-90 as 3/193 (1.55%) isolates with all MDR strains. *E.coli* from the age group 81-90 years and *Citrobacter freundii* from the age groups 21-30 years and 31-40 years were found most predominant pathogens with highest MDR strains as 3 isolates with 100.0% MDR strains among all age groups. The results are shown in appendix X.

5.2.4 Pattern of isolates and MDR strains in outdoor and indoor patients from different samples

Among the 291 isolates from the outdoor patients, the most predominant isolate was found *E. coli* with 169/291 (58.07%) isolates, among these 72 (46.6%) isolates were MDR strains. Similarly, least pathogen found from the outdoor patients were *Neisseria* spp. and *Acinetobacter* spp. with 1/291 (0.34%) isolate each. In the case of indoor patients, the most

predominant pathogen was found *E. coli* with 24/45 (53.33%) isolates, among these 17 (70.83%) were MDR strains whereas least number of pathogen isolated were *Pseudomonas aeruginosa*, *Proteus* spp., *Enterobacter* Spp., *Neisseria* spp., *Acinetobacter* spp. and *Streptococcus faecalis* with 1/45 (2.22%) isolate, among these 100.0% strain was found

Organisms	**Cases from Outpatients**	**Cases from Inpatients**	**Total**

MDR except for *Neisseria* spp. with 0.0% MDR strain. The results are shown in table 7.

Table 7: Pattern of isolates and MDR in outdoor and indoor patients from different samples

	No. of Isolates	MDR (%)	No. of Isolates	MDR (%)	
E. coli	169	72 (42.6)	24	17(70.83)	**193**
Klebsiella spp.	6	5 (83.34)	0	0(0.0)	**6**
Citrobacter spp.	10	5(50.0)	7	6(85.71)	**17**
P. aeruginosa	5	2(40.0)	1	1(100.0)	**6**
Proteus spp.	5	1(20.0)	1	1(100.0)	**6**
Enterobacter spp.	6	4 (66.67)	1	1(100.0)	**7**
Salmonella Typhi	26	1(3.84)	2	0(0.0)	**28**
Salmonella Paratyphi A	24	0(0.0)	0	0(0.0)	**24**
Neisseria spp.	1	0(0.0)	1	0(0.0)	**2**
Morganella morganii	2	0(0.0)	0	0(0.0)	**2**
Acinetobacter spp.	1	0(0.0)	1	1(100.0)	**2**
Staphylococcus aureus	15	7(46.67)	2	1(50.0)	**17**
Other *Staphyloccus* spp.	17	6(35.3)	4	3(75.0)	**21**
Streptococcus feacalis	2	2(100.0)	1	1(100.0)	**3**
Streptococcus spp.	2	1(50.0)	0	0(0.0)	**2**
Total	**291**	**106(36.43)**	**45**	**32(76.2)**	**336**

5.3 ANTIBIOTIC SUSCEPTIBILITY PATTERN OF THE ISOLATES

5.3.1 Antibiotic susceptibility pattern of Gram-negative isolates from different samples

Out of 16 different common antibiotics used against Gram-negative isolates, imipenem was found the drug of choice with a susceptibility of 98.43% (63/64) which was followed by meropenem, ofloxacin, amikacin and nitrofurantoin with susceptibility 96.88%, 94.02%, 84.88% and 82.42% respectively. Cofoxitin, ceftazidime, ceftriaxone and gentamicin were found least effective drugs against Gram negative isolates with susceptibility 12.2%, 17.18%, 50.0% and 40.69% respectively. The results are shown in table 8.

Antibiotic used	Sensitive	Intermediate	Resistant	Total

	No.of Isolates	%	No.of Isolates	%	No.of Isolates	%	
Amoxycillin	147	51.39	0	0.00	139	49.41	286
Cephotaxime	210	72.66	2	0.69	79	22.65	289
Cefixime	203	71.47	1	0.35	80	38.18	284
Chloramphenicol	89	76.72	0	0.00	17	23.28	116
Ciprofloxacin	192	66.67	3	1.04	96	32.29	288
Ceftriaxone	69	50.0	1	0.72	68	49.28	138
Ceftazidime	14	17.28	1	1.23	66	81.49	81
Cefoxitin	10	12.2	0	0.00	72	87.8	82
Co-trimoxazole	166	59.92	0	0.00	111	40.8	277
Nitrofurantoin	173	82.38	7	3.33	30	14.29	210
Norfloxacin	113	59.78	1	0.52	75	39.7	189
Ofloxacin	189	94.02	1	0.49	11	5.49	201
Amikacin	73	84.88	4	4.65	9	10.47	86
Gentamicin	35	40.69	0	0.00	51	59.31	86
Meropenem	62	96.88	0	0.00	2	3.12	64
Imipenem	**63**	**98.43**	0	0.00	1	1.57	64

Table 8: Antibiotic susceptibility pattern of Gram-negative isolates from different samples

5.3.2 Antibiotic susceptibility pattern of Gram-positive isolates from different samples

Out of 17 different common antibiotics used against Gram-positive isolates, amikacin, and vancomycin were found the drugs of choice with susceptibility of 100.0% (2/2) each, but these results were found statistically insignificant, which was followed by cefotaxime, ceftriaxone and ciprofloxacin with susceptibility 83.87%, 76.47% and 71.43% respectively. All the 2 (100%) isolates of MRSA were susceptible to vancomycin. Cefoxitin, meropenum, imipenem and oxacillin were found to be the least effective with susceptibility 0 (0.0%) each. The results are shown in table 9.

Table 9: Antibiotic susceptibility pattern of Gram-positive isolates from different samples

Antibiotic used	Sensitive		Intermediate		Resistant		Total
	No.of Isolates	%	No. of Isolates	%	No.of Isolates	%	
Amoxycillin	22	55.0	2	5	18	43.0	40
Cephotaxime	26	83.87	0	0.00	5	16.13	31
Cefixime	5	71.42	0	0.00	2	28.58	7
Erythromycin	26	65.0	0	0.00	14	35.0	40
Ciprofloxacin	30	71.43	0	0.00	12	28.57	42
Ceftriaxone	26	76.47	1	2.94	7	20.59	34
Cefoxitin	0	0.00	0	0.00	3	100.0	3

Co-trimoxazole	25	71.42	0	0.00	10	28.58	35
Cloxacillin	28	66.67	0	0.00	14	33.33	42
Norfloxacin	5	45.45	0	0.00	6	54.55	11
Meropenem	0	0.00	0	0.00	2	100.0	2
Imipenim	0	0.00	0	0.00	2	100.0	2
Ofloxacin	1	50.0	0	0.00	1	50.0	2
Amikacin	**2**	**100.0**	0	0.00	0	0.00	2
Gentamicin	1	50.0	0	0.00	1	50.0	2
Oxacillin	0	0.00	0	0.00	2	100	2
Vancomycin	**2**	**100.0**	0	0.00	0	0.00	2

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

The antibiotic susceptibility pattern of *E. coli* showed that imipenem and meropenem were the drugs of choice with susceptibility 100.0% (51), followed by amikacin with susceptibility 90.48%. Ceftazidime was found to be the least effective with susceptibility 8.62 %), followed by the cefoxitin with susceptibility 9.84 %. The results are shown in appendix X.

5.3.4 Antibiotic susceptibility pattern of *S. aureus* isolated from different samples

The antibiotic susceptibility pattern of *S. aureus* showed that doxycycline and vancomycin were the drugs of choice with susceptibilities 100.0% (1) and 100.0 % (2) respectively.100 % susceptibility of doxycycline was found statistically insignificant. Cloxacillin was found the second most effective drug with susceptibility 88.23%. Imipenem, meropenem, norfloxacin and oxacillin were found to be the least effective with susceptibility 0 (0.0%) each. The results are shown in appendix X.

5.4 ANTIBIOTIC RESISTANCE PATTERN OF THE ISOLATES

5.4.1 Antibiotics resistance pattern of the isolates from different samples

Out of the 336 isolates, 19.18% (42/219) isolates from the urine samples were resistant to >10 drugs, whereas only 7.14% (4) isolates from pus samples and 50.0% (1) isolate from fluid samples were resistant to >10 drugs used. *E. coli* was found the major organism with 19.69 % (38/193) MDR strains resistant to >10 drugs from different samples. The results are shown in table 10.

5.4.2 Distribution of MDR among gender and type of patients

Out of total 202 drug resistant isolates from different samples, MDR isolates were found as 68.31% (138/202) strains, among these, majority strains were isolated from female outpatient with 49.27% (68/138) MDR strains. Similarly, out of 202 drug resistant isolates, non-MDR isolates were found as 31.68% (64/202) strains, among these, most of strains (38/64) were isolated from female outpatients. The results are shown in table appendix X.

Table 10: Antibiotic resistance pattern of the organisms

Organism	**No of drug resistance**																						
	Blood			**Pus**			**Fluid**			**Sputum**			**CSF**			**Tissue**			**Perianal Swab**			**Urine**		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
Salmonella Typhi	4	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	1	0
S. Paratyphi A	19	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus spp.	-	-	-	-	-	-	-	-		1	0	0	-	-	-	-	-	-	-	-	-	0	3	0
E. coli	-	-		2	5	1	0	0	1	-	-	-	-	-	-	-	-	-	2	1	0	48	20	36
P. aeruginosa	-	-		0	1	0	-	-	-	-	-	-	-	-	-	1	0	0	-	-	-	0	2	0
Citrobacter spp.	-	-		1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	4	4
Enterobacter spp.	-	-		0	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	0
Proteus spp.	-	-		3	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	0	0
Staphylococcus spp.	-	-		12	2	2	-	-	-	-	-	-	-	-	-	1	0	0	-	-	-	3	0	1
Klebsiella spp.	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	1	1
Morganella morganii	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0	0
Acinetobacter spp.	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	0
Neisseria spp.	-	-		-	-	-	0	0	0	-	-	-	1	0	0	-	-	-	-	-	-	-	-	-
Total	**23**	**0**	**0**	**18**	**9**	**4**	**0**	**0**	**1**	**1**	**0**	**0**	**1**	**0**	**0**	**2**	**0**	**0**	**2**	**1**	**0**	**61**	**33**	**42**

Note:

A = 0 - 4 drug resistance

B = 5 -10 drugs resistance

C = >10 drugs resistance

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

Among the total of 138 MDR isolates from different samples, predominant MDR isolates (38/138) was belonged from the patients of age-group 21-30 years, among these 30 MDR isolates were found from urine samples whereas 8 MDR isolates were obtained from pus samples. Out of total 64 non-MDR isolates from different samples, predominant non-MDR isolates (27/64) was belonged from the patients of age-group 21-30 years, among these 11 non-MDR isolates were found from each blood and urine samples.The least number of MDR isolates were found from the patients of age-groups of over 90 years and below 10 years with 3 MDR strains, among these all strains were isolated from the urine samples. The results are shown in table 11

Table 11: Distribution of MDR among gender and type of patients

Pattern of drug resistance	Male		Female		Total
	Out-patient	In-patient	Out-patient	In-patient	
Multidrug resistance (MDR)	38	14	68	18	**138**
Non-multidrug resistance (Non -MDR)	21	4	38	1	**64**
Total	**59**	**18**	**106**	**19**	**202**

.

5.5 PATTERN OF ESBL PRODUCING STRAINS FROM DIFFERENT SAMPLES

Out of 89 MDR *E. coli* isolates, 57 were suspected of being ESBL-producers on primary screening, however only 27 (30.33%) of them were confirmed as ESBL-producers after the

confirmatory test, while 30 of the suspected isolates gave negative confirmatory test. Out of 4 *Klebsiella pneumoniae*, 1 *K. oxytoca* and 2 *P. mirabilis* MDR isolates; 1, 1 and 0 were suspected as ESBL-producers respectively and none isolates gave positive confirmatory test. The results are shown in table 12.

Table 12: Pattern of ESBL producing, suspected and confirmed cases of ESBL producing strains from different samples

S.N	Organism	Total Isolates	No.of MDR strains (%)	No.of suspected ESBL producers	No.of Confirmed Cases (%)	Negative Cases on Confirm-ation
1	*E. coli*	193	89 (46.11)	57	27	30
2	*K. pneumoniae*	4	4 (100.0)	1	0	1
3	*K. oxytoca*	2	1 (50.0)	1	0	1
4	*P. mirabilis*	5	2 (40.0)	0	0	0
Total		**204**	**96 (47.05)**	**59**	**27**	**32**

5.6 PATTERN OF ABL PRODUCING STRAINS FROM DIFFERENT SAMPLES

Out of 9 different bacteria of total 121 MDR isolates likely to produce AmpC β-lactamase (ABL), 76 MDR strains were suspected of being ABL-producers on primary screening, however only 58 (47.93 %) strains of them were confirmed as ABL-producers after the confirmatory test, while 17 of the suspected isolates gave negative confirmatory test. Among 58 ABL producers, 19/58 (32.75 %) isolates and 25/58 (43.1 %) isolates were found hyper ABL producers and ABL producers respectively. The most predominant ABL producers were found *E. coli* which gave 47 cases of positive confirmatory test. The results are shown in table 13.

Table 13: Pattern of ABL producing, suspected and confirmed cases of ABL producing strains from different samples

Organisms	Total Isolates	No. of MDR strains (%)	No. of Suspected ABL Producers	Cases of ESBL Confirmation Negative	No. of			No.of Non-ABL Producers
					Hyper ABL producers	ABL producers	Weak ABL producers	
E. coli	193	89 (46.11)	58	30	15	20	12	11
pseudomonas aeruginosa	6	3 (50.0)	3	0	1	1	0	1
Citrobacter freundii	12	9 (75.0)	7	0	2	1	0	4
C. diversus	5	2 (40.0)	2	0	1	0	1	0
Enterobacter cloacae	4	4 (100.0)	1	0	0	1	0	0
E. aerogenes	3	1 (33.33)	1	0	0	1	0	0
Staphylococcus aureus	17	8 (47.05)	2	0		0	1	1
Klebsiella oxytoca	2	1 (50.0)	1	1		1		0
K. pneumoniae	4	4 (100.0)	1	1	0	0	0	1
TOTAL	247	121 (48.9)	76	32	19	25	14	17

5.7 PATTERN OF MBL PRODUCING STRAINS FROM DIFFERENT SAMPLES

Out of 8 different bacteria likely to produce metallo-β-lactamase, altogether 117 MDR isolates likely to produce MBL were tested for MBL production. Out of 117 MDR isolates from different samples, 66 isolates were suspected of being MBL-producers on primary screening, however only 33 (28.20%) of them were confirmed as MBL-producers after the confirmatory test, while 5 (15.15%) of the MBL producers gave resistant to carbapenem.The results are shown in table 14.

Table 14: Pattern of MBL producing strains and carbapenem resistant strains

Organisms	Total isolates	No. of MDR strains (%)	No. of suspected MBL producers	Metallo-β-lactamase (MBL) producers					Cases of carbap-enem resistant
				No (%)	Cases of ESBL production		Cases of ABL production		
					+ ve	- ve	+ ve	- ve	
E. coli	193	89 (46.11)	53	25 (75.75)	8	16	18	7	0
Pseudomonas aeruginosa	6	3 (50.0)	2	1(3.03)			0	1	1
Citrobacter freundii	12	9 (75.0)	5	3(9.09)			0	3	2
C. diversus	5	2 (40.0)	1	0(0)					
Enterobacter cloacae	4	4 (100.0)	1	0(0)					
Staphylococcus aureus	17	8 (47.05)	2	2(6.06)			1	1	2
Klebsiella oxytoca	2	1 (50.0)	1	1(3.03))		1	1	0	0
Acinetobacter spp.	2	1 (50.0)	1	1(3.03)					
TOTAL	**241**	**117 (48.54)**	**66**	**33 (100)**	**8**	**17**	**20**	**12**	**5**

5.8 PATTERN OF SPECTRUM OF MDR AMONG ESBL, ABL AND MBL PRODUCING ISOLATES

Among 200 MDR isolates likely to produce ESBL, ABL and MBL, increasing proportion of no. of drug resistance was found among positive isolates of β-lactamase production with majority producers were found resistant to >10 drugs, among these 78.78% (26/33) MBL MDR producers were found most predominant and the association of MBL production with increase spectrum of MDR was also found significant ($p<0.05$). The results are shown in table 15.

Table 15: Pattern of spectrum of MDR among ESBL, ABL and MBL producing isolates

β-lactamase production	Spectrum of Multidrug resistant isolates				Total
		2-4 drugs	5-10 drugs	>10 drugs	
ESBL production	Positive	1	10	16	27
	Negative	1	8	23	32

ABL production	**Positive**	1	22	35	58
	Negative	1	6	10	17
MBL production	**Positive**	0	7	26	33
	Negative	2	11	14	33
Total		6	70	124	200

5.9 PATTERN OF MDR ISOLATES RESISTANT TO CARBAPENEMS AMONG DIFFERENT SAMPLES

Three species were responsible for the resistant of carbapenems with most predominant 25.0% (2/8) *S. aureus* were resistant to both meropenem and imipenem, followed by *C. freundii* (22.3%) with resistant to meropenem only. All these carbapenem resistant isolates were found MBL producers. The result was shown in table 16.

Table 16: Pattern of MDR isolates resistant to carbapenem

Organisms	**Sample**	**MDR Isolates**	**No.(%)**	**No.of drug resistant**	**Type of carbapenem resistance**	**β-lactamase production**
S. aureus	Urine	8	2(25)	>10	Meropenem, Imipenem	ABL, MBL
C. freundii	Urine,Pus	9	2(22.3)	>10	Meropenem	MBL
P. aeruginosa	Urine	3	1(33.4)	>10	Imipenem	ABL, MBL

5.10 PLASMID DNA ANALYSIS

5.10.1 Plasmid DNA profiles from ESBL, ABL and MBL producing MDR *E. coli* and *C. freundii.*

Plasmids were extracted from 30 MDR *E. coli* and 3 MDR *C. freudii* that produce different types of β-lactamases (i.e.ESBL, ABL and MBL) on the basis of judgement selection and run in agarose gel which showed 16 types of plasmid profiles of size ranging from 2 to 51kb. Twelve isolates showed single plasmid and 2 isolates showed double plasmids. Similarly 6, 6, 1 and 1 isolates contained 3, 4, 5 and 7 plasmids respectively.One isolate KMH_{4131} with no all three β-lactamase production has single differerent plasmid size of 45 kb from other β-lactamase producing strains.The most repetitive plasmid was found 32.5 kb. The results are given in appendix X and photograph 8.

5.10.2 Plasmid DNA profiles and resistance patterns of donors and transconjugants

Different 10 MDR *E. coli* isolates which were resistant to commonlly used antibiotics and produce the any one or more than one types of β-lactamase but sensitive to streptomycin were tested their transfer mechanism by conjugation experiment. Most of the transconjugants seemed to acquire the β-lactamase production phenotype during transfer study through conjugation. It was seen that all (10) isolates possesses transferrable type of plasmid and most strains were transferred plasmid mediated β-lactamase traits and MDR phenotypes successfully to recipient *E. coli* HB101. In most of the transconjugants, plasmids of size 32.5 kb were found to be transferred more frequently. KMH_{877} isolate transferred 51 kb plasmid while KMH_{1140}, $KMH_{1133,}$ and KMH_{2974} transferred 38 kb plasmid. The results are shown in table 17 and photograph 9

Table 17: Plasmid profiles and resistance patterns of donors and transconjugants

	Resistance pattern of Donors	**Resistance pattern of transconjugants (TC)**	**β-lactamase Production by TC**	**Size of pla-smid of donors**	**Size of Plas-mid of TCs**
KMH_{533}	Am,Cf,Ce,Cfx,Co,Of, Nx, Na,Ca,Ci	Am,Cf,Co,Of,Nx, Na, Cz	MBL	32.5	32.5
KMH_{2176}	Am,Cf,Ce,Cfx,Co,Of, Nx,Ca,C,G,Ci	Am,Cf,Of,Nx, Ce,Ca,Cn,Na, Cz	ESBL,ABL	32.5	32.5
KMH_{1140}	Am,Cf,Co,Nx,Of,Ce, Ca,Ci	Am,Cf,Co,Of,Ce,Ca,C nNx, Na, Cz	ABL	38	38
KMH_{1133}	Am,Cf,Ce,Cfx,Co,Of, Nx,Ca,G,Ci	Am,Cf,Co,Ce,CaOf, Nx, Na, Cz	ESBL	38,5.5, 2.5	38
KMH_{759}	Am,Cf,Ce,Cfx,Co,Of, ,Ca,C,G,Ci	Am,Cf,Co,Of,Ce,Ca, Nx, Na, Cz	ESBL, MBL	32.5, 4.5, 2.5, 1.8	32.5,4.5
KMH_{877}	Am,Cf,Ce,Cfx,Co,Of,Nx,	Am, Cz	-	51	51

	Ca,G,Ci				
KMH_{85}	ı,Cf,Ce,Cfx,Co,Of,Nx, Ak,Ca,G,Ci	Am,Cf,Co,Of,CeCa, Cn,Nx, Na, Cz	ESBL, ABL	29, 8	29
KMH_{2974}	Am,Cf,Co,Nx,Ca,Ci	Am,Cz	-	38	38
KMH_{3263}	Am,Cf,Ce,Cfx,Co,Of, Nx,C,Ca,G,Ci	Am,Cf,Co,Of,Nx, Ce,Ca,Cn, Cz	ESBL, ABL	29, 8, 3.9	29,8
KMH_{1569}	Am,Cf,Ce,Cfx,Co,Of,Nx, Ca,Ci	Am,Cf,Co,Of,NxCe, Ca,Cn,, Na,,Cz	ABL	32.5, 10.1, 3.9	32.5,101, 3.9

5.10.3 CONJUGATION FREUUENCY

The conjugation frequencies of different *E. coli* transconjugants were determined in terms of per ml of donors used. It was found that the chances of transferring trimethoprim and norfloxacin resistance through conjugation were higher compared to the other antibiotics used. Most common conjugation frequency was found 10^{-7}. The results are shown in table 19.

5.10.4 TRANSFORMATION

Transformation was carried out using purified 0.5µg plasmids extracted from different MDR *E. coli* strains which produce the one or more than one types of β-lactamase. Four out of five plasmids used were successfully transformed the competent cells *(E. coli* TB1). The transformed cells showed resistance to fewer antibiotics but none of them are able to produce any type of plasmid mediated β-lactamase. The plasmids were not found from these transformants. Plasmid with trimethoprim drug resistance showed higher transformation efficiency than other, upto transformation frequency 10^9 while transformation frequency 10^8 was found most common among all transformants selected. The results are shown in table 18.

Table 18: Transformation Study with *E. coli* TB1 using supercoiled plasmid DNA

Donors plasmid from	**Recipient**	**Resistance pattern of transformants**	**No. of transformants on selective plates containing streptomycin**	**Transformation frequency (Transformants/µg of supercoiled plasmid DNA**

			Cf	Tr	
KMH_{877}	*E.coli* TB1	A,G,Nf,Cz	7	97	3.5×10^7(Cf) 4.85×10^8(Tr)
KMH_{890}		A, Co,Cz	57	312	2.85×10^8(Cf) 1.56×10^9(Tr)
KMH_{759}		A,G,Nf,Cz	30	70	1.5×10^8(Cf) 3.5×10^8(Tr)
$KMH_{1490\ (pus)}$		A,G,Nf,Cz	5	60	2.5×10^7(Cf) 3×10^8(Tr)

Table 19: Conjugation Frequency of transconjugants on Different selective media

Donors	**Approximate no. of donors (CFU/ml)**	**No. of transconjugants on selective plates containing Streptomycin**						**Conjugation frequency (Transconjugants /ml of Donor cells)**
		Tr	**Nx**	**A**	**Cf**	**G**	**C**	
D_{533}	5×10^8	112	324	5	400	0	0	2.24×10^{-7} (Tr) 0.648×10^{-8}(Nx) 1×10^{-7}(A) 0.8×10^{-8} (Cf)
D_{2176}	5×10^8	0	5	0	40	0	0	0.1×10^{-7}(Nx) 0.8×10^{-7}(Cf)
D_{1140}	5×10^8	125	132	16	176	0	0	2.5×10^{-7} (Tr) 1.32×10^{-7} (Nx) 0.32×10^{-7} (A) 1.76×10^{-7} (Cf)
D_{1133}	5×10^8	55	20	0	25	0	0	1.1×10^{-7} (Tr) 0.4×10^{-7} (Nx) 0.5×10^{-7} (Cf)
D_{759}	5×10^8	60	105	0	60	0	0	1.2×10^{-7} (Tr) 2.1×10^{-7} (Nx) 1.2×10^{-7} (Cf)
D_{877}	5×10^8	0	0	TNTC	0	0	0	
D_{85}	5×10^8	20	15	100	25	0	0	0.4×10^{-7} (Tr) 0.3×10^{-7} (Nx) 2×10^{-7} (A) 0.5×10^{-7} (Cf)
D_{2974}	5×10^8	50	0	TNTC	0	0	0	1×10^{-7} (Tr)
D_{3263}	5×10^8	30	5	TNTC	15	0	0	0.6×10^{-7} (Tr) 0.1×10^{-7} (Nx) 0.3×10^{-7} (Cf)
D_{1569}	5×10^8	552	568	TNTC	30	100	100	1.10×10^{-6} (Tr) 1.136×10^{-6} (Nx) 0.6×10^{-7} (Cf) 2×10^{-7} (C) 2×10^{-7} (G)

5.10.5 MINIMUM INHIBITORY CONCENTRATION (MIC) OF DONORS, TRANSCONJUGANTS AND TRANSFORMANTS

The donors and transconjugants showed high degree of resistance to the common antibiotics. The resistance transferred through conjugation was stronger than resistance transferred through transformation. Most strains of donors and transconjugants had highest MICs value of >1024 µg/ml. Maximum MIC value for transformant was found as 256 µg/ml. The results are shown in appendix X.

6. STATISTICAL PATTERN OF RESULTS

Statistical differences in the aetiologies of various dependent variables were determined by the $\chi 2$ test, OR and other pattern. The higher percentage of growth seen in our result among female patients of different bacterial infections were found statistically significant ($p<0.05$, $p<0.001$). Similarly, higher percentage of growth seen among indoor patients suffering different bacterial infections were also found statistically significant ($p<0.05$, $p<0.001$). Prevalence of MDR were found higher among indoor patients, which also significant ($p<0.05$, $p<0.001$).

ESBL and MBL production among different MDR bacteria that are likely to produce these enzymes were found strongly associated with the increasing spectrum of multidrug resistance ($p<0.05$, $p<0.001$). The sensitivity and specificity of urine culture test on the basis of pyuria were found significantly higher as 91.66% and 94.32% respectively.

Chances of antibiotic resistance among the different bacterial pathogens, prior antibiotic exposure was found 1.15 times (OR = 1.15) stronger than patients without antibiotic exposure.The chances of increasing level of multiple drug resistance among β - lactamase producing MDR isolates was found 1.52 more likely to harbor more than 2 plasmid than those of harboring less than 2 plasmid. The results are tabulated in table 20 and shown in appendix XI.

Table 20: Statistical pattern of the results

N=1503 / Independent Variables	Bacterial infection			X^2 value	p value
	Positive (%)	**Negative (%)**			
Gender (female)	25.51	74.49		10.37	< 0.05 < 0.001
Types of patients (in – patients)	40.9	59.1		23.53	< 0.05 < 0.001
	Multidrug resistance				
	MDR strains (%)	**Non-MDR strains (%)**			
Type of patients (in-patients)	86.48	13.52		6.9	< 0.05 < 0.001
Gender (female)	39.06	40.04		0.035	> 0.05 > 0.001
	No. of isolates	**MDR strains**			
Gender (female)	209	86		0.0006	>0.05
	Range of multidrug resistance				
	2-4 drugs(%)	5-10 drugs(%)	>10 drugs(%)		
ESBL production (positive)	3.7	37.03	59.25	320.7	< 0.05 < 0.001
ABL production (positive)		37.93		0.24	> 0.05 > 0.001
MBL production (positive)			78.78	9.14	< 0.05 < 0.001
B-lactamase production (ESBL,ABL and MBL)		40.7, 41.37, 21.1	59.3, 58.6, 78.8	4.11	< 0.05

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

This study was conducted during May 2007 to January 2008.in the Kathmandu Model Hospital, Pradarshani Marg and Research Lab of Central Department of Microbiolog, T.U. to determine the status of multiple durg resistance among different bacterial infections and underlying production of different types of β-lactamases with their likely transfer mechanism with MDR traits by R-plasmid DNA analysis.Altogether one thousand five hundred and three different clinical samples were collected, examined and subjected to culture to isolate the bacterial pathogen and allowed further analysis.

Out of total 1503 different clinical samples, major portion was contributed by the urine samples with 710 (47.24%), followed by blood samples with 551 (36.66%). Dhungel (2001) showed similar pattern of clinical samples distribution. Among the total samples, 819 (54.5%) were from female, while 684(45.5 %) were from male. Similarly, 1393 (92.68%) of the total samples were from outdoor patients, whereas 110 (7.32%) samples were from indoor patients. Age group of 21-30 years had the maximum requests of 489 (32.53%) for culture of specimen. Out of total samples, 336 (22.35%) showed growth positive, among which 62.20% were from female whereas 37.80% from male. A similar study carried out by Dhakal (1999) showed growth positivity of 25.16% and in his study, among the total requests for urine culture; 53.46% were from female patients. Our findings also compatible with the findings from Bomjan (2005) with growth positivity 35.4% from urine and sputum samples carried out in TUTH.

Out of total 710 urine samples, 272 (43.23%) urine samples showed no growth, 184 (25.92%) showed no significant growth and 219 (30.85%) samples showed significant growth. In similar studies carried out by Chhetri *et al*. (2001), Dhakal (1999), Obi *et al*., (1996) in Harare, Levett (1993), Gautam (1997), Manandhar (1996) and Ling *et al*., (1992) showed low number of growth positivity.The low growth positive rate observed in this study might be due

to inclusion of every patients requesting for urine culture regardless of their illness and symptoms. As stated by Manandhar (1996), the possible cause of low rate of growth positivity is that the samples might be from patients under treatment, infection due to slow growing organisms or due to those organisms that were not able to grow on the routine culture media used.

Out of total 551 blood samples, 51 (9.25%) showed positive growth, this result was similar from the findings of bacteraemic episodes of 154/1000 (15.4%) in TUTH by Shakya (2001) and with 14.83% growth positive by Shrestha (1996) in KMH. Out of 91 pus samples, 56 (61.53%) showed positive growth which was similar with the findings of 67.4% growth positivity by Dhungel (2001) and 61.97% growth positivity by Rai *et al*. (2000). In the case of 2 tissue samples, 100.0% growth positive was found though it is not statistically significant.This also indicates higher growth positivity from skin, soft tissue and solid tissue sample is obvious as found by many researchers.

Similarly, out of 28 fluid samples, 2 (7.14%) showed positive growth. A study carried out in TUTH showed 41.32% growth among body fluid samples by Joshi (1997).The underlying reasons of low positivity could be described in different ways. Among sexually active people *Neisseria gonorrhoeae* and *Chlmydia trachomatis* are common etiologic agents of peritoneal infection which could not recovered or died during culture. Other etiologic agents like *M. tuberculosis, Mycoplasma pneumoniae*, fungal agents and viral agents are also equally ressonsible of pericarditis, peritonitis and pleural infections. The major factor is also transportation delay which decrease detection of true positive cultures. Runyon *et al.* (1990) had been mentioned that fluid collected by percutaneous needle aspiration or at the time of surgery should be inoculated into aerobic and anaerobic blood culture broth bottles immediately at the bedside to avoid transportation delay.

Out of 13 CSF samples, 1 (7.69%) showed positive growth which is similar with the finding of 10.79% growth positive among suspected patients of meningitis in TUTH by Tiwari (2002). The significant pathogenic growth as 1.69% from 59 sputum samples was found from suspected patients of LRTI whereas the 3.34% pathogenic growth was found from 30 suspected patients of URTI. The low growth yield from sputum and throat samples sample may be due to factors such as patients already under antibiotic therapy, or infection of upper and lower respiratory tract by agents like viruses or other organisms such as *Mycoplasma*

spp., Chlamydiae, fungi, Legionellae etc which are not cultured by routine methods (Smith and Easmon, 1990).

Out of 16 stool samples, no growth was seen but 100.0% pathogenic growth was found from 3 perianal swabs. Not using transport medium which is required for delicate pathogens like *Shigella* spp., *Vibrio* spp. and chances of infections caused by other potential GIT pathogens like *Campylobacter* spp.,*Clostridium difficile* which are not intended to diagnose in the lab, supports the facts of no growth at all in the stool samples. Most of the patients giving pathogenic growth of perianal swabs, were severely infected with recurrent MDR pathogens from the older age group of 61-70 yrs. which supports the 100.0% positivity.

The growth pattern of pathogens from different samples were increased from the age group 41-50 years to >90 years with highest isolation of pathogens as 28.58%. This pattern of result indicates that the chances of bacterial infections increases with the increase in age which also supports immunity deterioration associated with the higher infection rate. Higher bacterial pathogens isolation was found from indoor patients with 40.9% as compare to the outdoor patients with 20.9% isolates. The prevalence of MDR among different samples was found 41.07 %, among them, predominant rate of drug resistance was found from 100.0% MDR from sputum but it was not statistically significant. This was followed by pus and fluid each 50.0% multidrug resistance. Higher prevalence of MDR strains from sputum, urine, pus and fluid were similar findings by Bomjan (2005) and Banjara (2000). Similarly, higher proportion of multiple drug resistance was also found in indoor patients with 71.11% than outdoor patients as 36.2% among the total isolates from different samples. This findings supports that nosocomial pathogens are more virulent and more to cause multiple drug resistance as compare to community acquired bacterial pathogens. These facts were also supported by the findings of 45.9% acquired wound infection in the burn ward of TUTH by Banjara (2002).

Among the total isolates, 87.5% were Gram-negative bacteria. In a similar study performed by Blomberg *et al.*(2005) in Tanzania in 1996, 66.36% constituted Gram-negative isolates, Bomjan (2005) found 83.33% Gram-negative isolates in UTI at TUTH. In this study, the most predominant pathogen were *Escherichia coli* (57.44%) of the total isolates, followed by *Salmonella* Typhi (8.33%). While *S. aureus* was the most predominant pathogen among Gram-positive bacteria with 40.47 % isolates and followed by *Staphylococcus saprophyticus*

constituting 35.71 %. These pattern of results are compatible with findings from Manandhar (1996) and Bomjan (2005) that had been found that *Escherichia coli* was the most predominant bacterial isolate whereas *Staphylococcus aureus* was the predominant Gram-positive species.

Among the uropathogens, *E. coli* (81.28%) was found to be the most predominant organism followed by *Citrobacter* spp. (5.02%), *Klebsiella* spp. (2.74%). Higher prevalence of *E. coli* seen in this study also resembled the study done by various other workers viz: Shrestha (2007), Chhetri *et al*. (2001), Sharma *et al.* (1983), Tuladhar *et al*. (1989), Jha and Yadav (1992), Manandhar (1996) and Dhakal (1999) in Nepal and Steenberg *et al.* (1969), Kahlmeter (2000) and Farrell *et al*. (2003) in the international context. *E. coli* have special virulent properties to cause UTI, being the major uropathogen throughout the world. *E. coli* can bind to the glycoconjugate receptor (Gal α 1| 4 Gal) of the uroepithelial cells of human urinary tract so it can initiate infection itself. *E. coli* is isolated in 90.0% of infections and strains are characterized by unique virulence determinant, the p pilus (Gal-Gal receptor) (Johnson, 1991). *E. coli* is the most predominant organism to colonize the urethral meatus (Schaeffer and Chmiel, 1983) and perineum (Leigh, 1990) before ascending to the bladder. *C. freundii* as second principal uropathogens were also found from findings of Puri (2006) with 27.2% of total isolates carried out at OM Hospital and Research Centre, Kathmandu, Nepal.

Among the blood stream infections, *Salmonella* Typhi (52.94%) was the most predominant pathogen, followed by *S.* Paratyphi A (47.06%). Higher prevalence of *S.* Typhi found in this study also resembled the study done by various other workers viz: Wagley (2004) and Shrestha (1996). Globally, typhoid is far more common than paratyphoid (Levine *et al*., 1983) and the pictures of enteric fever in only rarely caused by other serovars of *Salmonella enterica* (Rubin and Weinstein, 1977). In the lamina propria, after ingestion of contaminated food or water, *S.* Typhi stimulates a predominantly mononuclear response and when engulfed by the predominant macrophages,many of the bacteria are able to survive and multiply with more than 30 *Salmonella* proeteins being induced during invasion of the macrophages. The *phaP-phoQ* gene complex in salmonellas appears to determine survival within macrophages. These are the reasons for higher incidence of *S. typhi* enteric fever.

Out of 56 soft tissues and wound infections, *Staphylococcus aureus* (28.57%) was the most predominant pathogen, followed by *Staphylococcus saprophyticus* (21.42%). Higher prevalence of *Staphylococcus aureus* seen in this study also harmony with the study done by various other workers viz: Banjara (2002) with 25.3% among wound isolates; Dhungel (2001) with 70.97% isolates in pus samples; Aryal (2001) with 49.40% in ear discharges and Tuladhar (1999) with 57.66% isolates in pus samples. *S.aureus* is responsible for about 70% of all soft tissue infections in humans but the proportion due to specific organism is very site specific; e.g. half of all axillary abscess contain *S.aureus* but only 8% of vulvovaginal abscesses do so (Collier *et al.,* 1998). Because of their rigid body, resistance to dry conditions and high salt concentration, *S. aureus* are well suited for skin, 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.

Out of 2 isolates from fluid samples, *E. coli* and *Neisseria gonorrhoeae,* 50.0% each was was isolated. *E. coli* infection may suggest peritoneal infection which gain access to the peritoneum through the perforation of the bowel, through infection within abdominal viscera or by external inoculation as in surgery or trauma. *N. gonorrhoeae* infection indicates the peritoneal infection in sexually active women. One *S. pyogenes* pathogen was isolated from throat swab culture.*S. pyogenes* as major pathogen of URTI was also resembled with findings of Shrestha (2002). Similarly, one *S. pneumoniae* pathogen was found from sputum and this finding of *S. pneumoniae* as major pathogen of LRTI was also found similar with the findings of Sharma (2004) and Shrestha (1995). From the CSF samples, one (100.0%) *Neisseria meningitidis* was found.

Out of total 336 isolates, major pathoges were isolated from the age group of 21-30 years (33.92%) as *E. coli* (54.46%) predominant contributer, follwed by the *S.* Paratyphi A (12.28%). Out of 193 *E. coli* isolates, (87.56%) were isolated from the outpatients. Age group 81-90 years contributes lowest pathogenss (1.78%) as *E. coli* predominant pathogen. These findings are compatible with the findings by Bomjan (2005). Out of total isolates, 100.0% *Morganella morganii, Klebsiella* spp., *Salmonella* Paratyphi A and *Streptooccus* spp. were found from the outdoor patients whereas 50.0% *Neisseria* spp., 50.0%, *Acinetobacter* spp. and 41.17% *Citrobacter* spp. were found from the indoor patients.

Among the common antibiotics used against all Gram-negative isolates, imipenem was the drug of choice with susceptibility 98.43% but this are used, if there were no alternative second line drugs of choice. This was followed by meropenem, ofloxacin, amikacin and nitrofurantoin with susceptibility 96.88%, 94.02%, 84.88% and 82.42% respectively. Ofloxacin, amikacin and nitrofurantoin were most effective drugs for Gram-negative isolates. This finding was also supported by findings of Puri (2006); Bomjan (2005); Paneru (2002); Oteo *et al.* (2001) and Dhakal (1999).

Among the common antibiotics used against all Gram-positive isolates, amikacin and vancomycin were the drugs of choice with susceptibility 100.0% each but these findings were not statistically significant, followed by cefotaxime, ceftriaxone and ciprofloxacin with susceptibility 83.87%, 6.47% and 71.43% respectively. Cefotaxime, ceftriaxone and ciprofloxacin were most effective drugs for Gram-positive isolates. Similar findings were also found by Tuladhar (1999) and Dhungel (2001).

Out of the 336 isolates, 42/219 (19.18%) isolates from the urine samples were resistant to >10 drugs, whereas only 4 (7.14%) isolates from pus samples and 1 (50.0%) isolate from fluid samples were resistant to >10 drugs used. The high level of drug resistance from urine, and pus samples were also compatible with the findings by Dhakal (1999); Tuladhar (1999) and Rai (2000).

Out of total 202 drug resistant isolates from different samples, MDR isolates were found as 68.31% (138/202) strains, among these, majority strains were isolated from female outpatient with 49.27% (68/138) MDR strains. These findings suggest the stronger propensity of drug resistance towards multiple drug in female patients.The burden of multiple drug resistance was found higher among hospital admitted patients as 71.11%(32/45) than outpatients (36.42%)(106/291). Similar findings were found by Bomjan (2005) and Manandhar (2005). Antibiotic treatment and hospital infection control are intimately entwined. The prophylactic or empirical treatment of antibiotics alters the prevailing pathogens in the hospital setting and lead to establish drug resistant pathogens. So, the higher prevalence of MDR strains found among inpatients was obvious and found statistically significant ($p<0.05$).

Out of 336 isolates, all(3) *Streptococcus faecalis* from both outpatients and inpatients were found MDR whereas single isolate of *Pseudomonas aeruginosa*, *Proteus* spp., *Enterobacter* spp., *Neisseria* spp. and *Acinetobacter* spp. were found MDR which all isolated from inpatients. These findings indicate these are associated with nosocomial superbugs. These findings are supported by Yamane *et al.* (2005). The age group 21-30 gave maximum 27.53% (38/138) MDR strains though highest isolation of MDR strains was found from the age group 81-90 years (83.33%).There were no burden of MDR in blood stream infections and central nervous system infections as 0.0 % MDR strain in each case. These pattern of multiple durg resistance in these infections have similarities with findings by Shrestha (1996), Wagle (2004) and Tiwari (2002).

Out of 96 MDR isolates likely to produce extended-spectrum β-lactamase from different samples, 27 (28.12%) cases of confirmed ESBL producers were found. Out of 89 *E. coli*, 4 *Klebsiella pneumoniae*, 1 *K. oxytoca* and 2 *P. mirabilis* MDR isolates from diffeent samples, only 27 isolates of *E. coli* (30.33%) were found to be ESBL-producers. In a similar study carried out by Sharma *et al*.(2004) in Nepal, 8% *Klebsiella pneumoniae*, 12.5% *E. coli*, 12.5% *Citrobacter freundii*, 25% *Acinetobacter calcoaceticus* and 5% *Pseudomonas aeruginosa* were found to be ESBL-producing strains. A study done in Saudi Arabia showed, 197 (6%) isolates were multidrug resistant (MDR), and 156 (4.8%) were positive for ESBL. Seventy nine percent of the MDR strains were positive for ESBL in this study. The ESBL was detected in 72 (6.5%) *E. coli* isolates (Kader *et al.*, 2004).

By the early 1990s, 25 to 35% of nosocomially acquired *Klebsiella pneumoniae* isolates in France were ESBL producing (Marty *et al*, 1998). *E. coli* only pathogens to produce ESBLs in this study have many meanings. There is_considerable geographical difference in the occurrence of ESBLs. Among countries, territories, within countries, hospital-to-hospital variability in occurrence may also be marked (Babini *et al*., 2000). A common environmental source of ESBL-producing organisms has occasionally been discovered. These findings were analysed whether similar or dissimilar *E. coli* strains are responsible for the ESBL production with similar drug resistance traits by plasmid DNA profiles and are mentioned in next pages. Since, isoelectric focusing and sequencing of plasmid DNA was not conducted to analyze the types of ESBL producing genes. The types of ESBL may be TEM, SHV, OXA, CTX-M, PER or other but their production in solely *E. coli* with similar sources indicate SHV types because SHV-type ESBLs have been detected in a wide range of Enterobacteriaceae and

outbreaks of SHV-producing *Pseudomonas aeruginosa* and *Acinetobacter* spp.(Paterson, 2005). The spread of the bla_{SHV} genes throughout the bacterial world has probably been initiated by repeated events of the IS*26*-mediated transposition that mobilizes these genes to plasmids from the chromosome of *Klebsiella pneumoniae.*

Major 92.6% (25/27) ESBL producers were found from urine sample while rest were found from pus samples. This indicates stronger propensity of ESBL producers towards uropathogens. Bomjan *et al.* (2005) had been found similar prevalence of ESBL producers (29.26%) among *E. coli* and *K. pneumoniae* isolates of UTI. Most of ESBL producers (85.18%) were found resistant to >10 common drugs used.This findings indicates stronger propensity of ESBL production towards multiple drugs resistant limiting few therapeutic options for the treatment of ESBL producing offending bacteria.

Out of total 121 MDR isolates from different samples likely to produce AmpC β-lactamase, 58 (47.93%) isolates were found to produce ABL, however 76 (62.8%) suspected strains to produce ABLs were found from different samples. Out of 58 isolates, *E. coli* strains with 81.03% (47/58) were the major pathoges to produce ABL, followed by *C. freundii* (5.17%). Out of 47 *E. coli* isolates, 42.55% (20/47) were AmpC β-lactamase producers, followed by 31.91% (15/47) hyper AmpC β-lactamase producers. High-level AmpC production is typically associated with in vitro resistance to all beta-lactam antibiotics except for carbapenems. A similary study by Subha *et al* (2003) showed that 28 isolates (24.1%) of *Klebsiella* spp. and 12 (37.5%) of *E. coli* were found to be AmpC beta-lactamase producers; 66.6 % and 81 % of *Klebsiella* spp. and *E. coli* isolates respectively showed resistance to all the third generation cephalosporin (3GCs). All the strains were found to be sensitive to imipenem.

Plasmid mediated AmpC β-lactamases have been shown to be now widespread. For example, during the period 1992 to 2000 in United States, plasmid-mediated AmpC-producing *K. pneumoniae* isolates were reported, had a prevalence of 8.5% among clinical isolates of this species. Plasmid-mediated AmpC-producing *Salmonella* spp. and *E. coli* isolates are also a veterinary problem, affecting livestock, pets, and their human contacts (Bradford *et al*., 1999). Out of 30 negative cases of ESBL production by DDST, most strains (44.82 %) were found producing ABLs with major strains producing AmpC β-lactamase.These facts supports the characteristics of ABLproducing strains as resistance to cephamycin (in this study

cefoxitin), β-lactamase inhibitor (e.g. augmentin) and 3GCs. In clinical isolates of *E. coli*, cephamycin resistance can be due to promoter or attenuator gene mutations and acquisition of plasmids with *ampC* genes.

Out of total 117 MDR isolates from different samples likely to produce metallo-β-lactamase, 33 (28.2%) isolates were found to produce MBL, however 66 (56.41%) suspected strains producing MBLs were found from different samples. Out of 33 isolates, *E. coli* strains with 75.75% (25/33) were the major pathogens to produce MBL, followed by *C. freundii* (9.09%). Out of 33 MBL isolates, 2 *C. freundii*, 1 *P. aeruginosa* and 2 *S. aureus* were found carbapenem resistant. Two carbapenem resistant metallo-β-lactamase producing *S. aureus* were found MRSA. Findings of 100.0% carbapenem resistant isolates as MBL producers were compatible with many other findings throughout world and emergence of such superbug have many clinical implications. MBLs, like all ß-lactamases, can be divided into those that are normally chromosomally mediated and those that are encoded by transferable genes but it could not be said about the type and position of MBL genes of 33 isolates. However, in the past 3 to 4 years many new transferable types of MBLs have been studied and appear to have rapidly spread. In some countries, *P. aeruginosa* possessing MBLs constitute nearly 20% of all nosocomial isolates (Walsh *et al.* 2005).

Out of 33 MBL isolates, 24.24% strains also co-produce ESBL whereas 62.5% MBL producing isolates also co-produce weak AmpC β-lactamase. The simultaneous production of these β-lactamases did not alter resistance towards carbapenem. But findings by Rodrigues *et al.* (2004) are contrary with our findigs.They showed *Pseudomonas* spp. producing plain ESBL were also sensitive to imipenem but eight (61.53%) out of 13 *Pseudomonas* spp. producing both ESBL and high level AmpC and eight out of 20 producing plain high level AmpC were resistant to imipenem.

In similar study, among ceftazidime resistant *P. aeruginosa* 100.0 % isolates produce MBL as detected by EDTA-Imipenem disk synergy test (Lee *et al.,* 2003). Of the 214 MDR isolates from different hospitals from Japan, 213 (99.5%) produced MBL and all except one were MDR isolates by double-disk synergy test with disks containing sodium mercaptoacetic acid as described by Arakawa *et al.* (2007). In recent years MBL genes have spread from *P. aeruginosa* to Enterobacteriaceae, and a clinical scenario appears to be developing that could simulate the global spread of extended-spectrum ß-lactamases. Moreover, given that MBLs will hydrolyze virtually all classes of ß-lactams and that we are several years away from the

implementation of a therapeutic inhibitor, their continued spread would be a clinical catastrophe (Walsh *et al.*, 2005). So emergence of 2 carbapenem resistant MBL producing *C. freundii* will be harmony with these facts.

Out of 30 *E. coli* and 3 *C. freundii* isolates that produce different types of β-lactamase, 100.0% strains harbored the plasmids. Sixteen types of plasmid profiles of size ranging from 2 to 51 kb were found. Among them, 12 isolates showed single plasmid, 2 showed double plasmids. Similarly 6, 6, 1 and 1 isolates contained 3, 4, 5 and 7 plasmids respectively.One isolate KMH_{4131} with no all three β-lactamase production has single differerent plasmid of 45 kb from other β-lactamase producing strains.The plasmid profiling showed the largest plasmid was 51 kb which was followed by 38 kb, and 32.5 kb. This result is in agreemetnt with the findings of Shehabi *et al.* (2002); Enabulele *et al.*(2006) and Wang *et al.*(2004). Large self-conjugative plasmids was mentioned by different researchers as Aibinu *et al.* (2004), KC *et al.* (1997) and Olukoya *et al.* (1997) are highly associated with the different types of β-lactamse production. In present study, plasmids having smaller size (2, 2.6, 3.2, 3.7, 4.5, 4.7, 6.5, 7.5, 10 Kb) were also found. Results of present study were compatible with the findings from Australia that MRSA isolates may harbor up to three different plasmids: small (1.6kb) cryptic plasmids,4.5 kb chloramphenicol resistance plasmids,and 20 to 42 kb plasmids which variously encode to different classes of antibiotics.

There are also well characterized different types of medium size self-transmissible or mobilizable plasmids with MDR traits and β-lactamase production like p^{KM101} (35.4kb). Occurrence of small plasmids have also been described by KC *et al.* (1997); Shakya *et al.* (2001); Ghimire *et al.*, (2004) and Paneru *et al.*, (2002). The number of plasmid per isolate did not reflect the nature of resistant markers. The resistant transfer study showed successful transfer of resistance to the recipients. This result is further supported by the findings of previous researchers (KC *et al.*,1997; Mandal *et al.*,2004 and Aibinu *et al.*,2004). The transfer of comparably small plasmids along with large plasmids with similar MDR traits were also encountered in this study and these facts also supported by Bartoloni *et al.*,(2006) that mentioned resistance traits could be transferred by conjugation, often en bloc, suggesting a linkage of the corresponding resistance genes in self-transferable or mobilizable plasmids. Stronger propensity of ESBL, ABL and MBL production towards increasing level of MDR found in this study with dependency of majority of strains upon transferable plasmids supports the findings of Bartoloni *et al.* (2006).

Most of ABL producers haing MDR phenotypes (Am, Cf, Ce, Cfx, Co, Nx, Ca, Ci) conatained 3 or 4 different plasmids while most of typical MBL producers harbored single plasmid of size 32.5 kb having MDR phenotypes (Am, Cf, Ce, Cfx, Co, Nx, Ca, Ci, G) but carbapnem hydrolyzing MBL producers showed MDR phenotype (Am, Cf, Ce, Cfx, Co, Nx, Ca, Ci, C, Nf, G).Such pattern of MDR phenotypes with ABL or MBL production with more than two types of plasmidic content has similar the findings by
Horii *et al*.(1993). *K pneumoniae* NU2936 was isolated from a patient with bladder cancer who suffered from urinary tract infection and who had been treated with various β-lactam antibiotics (moxalactam, cefmetazole, and piperacillin). This strain showed resistance to these antibiotics producing ABL of type MOX-1 harbored 2 large plasmids with size of the larger plasmid (pRMOX1) of 180 kb (Horii *et al*., 1993).

Similar MDR phenotypes with production of ABL in this study have been found by Church *et al.* (2007).Among the 369 AmpC β-lactamase-producing isolates, the principal site of isolation was the urinary tract for 333 (90%) patients, of these same 369 isolates, 73 (20%) were not susceptible to trimethoprim-sulfamethoxazole, 32 (9%) to tobramycin, 54 (14%) to gentamicin, and 33 (9%) to ciprofloxacin. No resistance to imipenem was detected. (Church *et al.* 2007)

All β-lactamase-producing MDR isolates tested for transfer of their phenotypic traits, transferred drug resistance traits by conjugation. Most strains showing plasmid mediated MDR and β-lactamase production with transfer upto 51 kb R-plasmid. Most of MDR donors and transconjugants were susceptible to carbapenems. Similar findings were also found from conjugation experiments by Horii *et al*., (1993).They had been found that the larger plasmid, pRMOX1 (180 kb), was transferred into *E. coli* CSH2 at a frequency (5×10^{-7}), with the simultaneous transfer of resistance to tetracycline, broadspectrum β-lactams including moxalactam, cefotaxime, ceftazidime, and ceftizoxime but susceptible to imipenem (Horii *et al*., 1993).

6.2 CONCLUSION

Major findings of this study were concluded as follows: Out of 336 (22.35%) isolates from different types of bacterial infections, higher proportion 138 (41.07%) MDR strains were

found. Among MDR strains likely to produce β-lactamases, 28.12% (27/96) ESBL producers, 47.93% (58/121) ABL producers and 28.2%(33/117) MBL producers were found. Altogether 22 different bacteria were isolated, among them *E. coli* was found the most predominant (57.44%) organisms. The most effective antibiotic against Gram-negative bacteria was found imipenem with a susceptibility of 98.43% whereas against Gram-positive bacteria amikacin and vancomycin were found with a susceptibility of 100.0% each.

Burden of multidrug resistance was found higher among LRTI (100.0%), soft tissue infections (50.0%), solid tissue infection (50.0%) and UTI (47.01%). Similarly, the burden of MDR pathogens infections were found higher as the age of the patients increases and found highest among the patients of age-group 81-90 years (83.34%). All the isolates of 30 MDR *E. coli* and 3 MDR *C. freudii* that produce different types of β-lactamases (i.e.ESBL, ABL and MBL) harbored small size and mega plasmids ranging 2kb to 51kb. Majority of MDR strains showed plasmid mediated ESBL, ABL and MBL productions by the conjugation study with similar patten of MDR traits as donors among transconjugants with simultaneous transfer of ESBL, ABL and MBL production.

In conclusion, this study underscored the magnitude of the problem of multiple drug resistance among clinical bacterial pathogens and the urgent need for surveillance, control measures and exploration of this phenomenon.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 SUMMARY

1. Out of 1503 different clinical samples, 336 (22.35%) were bacterial growth positive with 138 (41.07%) MDR strains. Among MDR strains likely to produce β-lactamases, 28.12 % (27/96) ESBL producers, 47.93% (58/121) ABL producers and 28.2% (3/117) MBL producers were found.
2. The infection rate was found to be higher in females (25.51%) than in males (18.56%). Association of bacterial infections and gender of patients was found to be statistically significant ($P<0.05$).
3. Of total 336 isolates, 45 were isolated from in-patients and 71.11% (32/45) of these were MDR-strains.
4. The predominant pathogen causing bacterial infections were the Gram-negatives which constituted 87.5% (294/336) and among them, most 71.08% (209/294) were found from the patients of UTIs. Gram-positive bacteria constituted only 2.5% (42/336) and most of them 71.42% (30/42) were MDR-strains.
5. Altogether 22 different bacteria were isolated from growth positive different samples. *E. coli* 193/336 (57.44%) was found the most predominant organisms followed by *Salmonella* Typhi 28/336 (8.33%) among different clinical samples.
6. The most effective antibiotic against Gram-negative bacteria was imipenem with a susceptibility of 98.43% (63) which was followed by Meropenem, Ofloxacin, Amikacin and Nitrofurantoin with susceptibility 96.88%, 94.02%, 84.88% and 82.42% respectively.
7. Similarly, the most effective antibiotic against Gram-positive bacteria was amikacin and vancomycin with a susceptibility of 100.0% (2) each ($p>0.05$), which was followed by cefotaxime, ceftriaxone and ciprofloxacin with susceptibility 83.87%, 76.47% and 71.43% respectively.
8. Out of 336 isolates from different clinical samples, 41.07% (138/336) MDR isolates were found and burden of multidrug resistance was found higher among LRTI 100.0% (1/138) ($p>0.05$), soft tissue infections50.0% (28/56), solid tissue infection 50.0% (1/2) ($p>0.05$) and UTIs 47.01% (105/219).
9. Out of the 336 isolates, 19.18% (42/219) isolates from the urine samples were resistant to >10 drugs, whereas only 7.14% (4) isolates from pus samples and 50.0% (1) isolate from fluid samples were resistant to >10 drugs used.

10. Higher status of MDR was found in females 40.94% (52/127) than in males 41.14% (86/209) which was found statistically insignificant ($p>0.05$) but higher status of MDR found in males than females cases of UTIs was found significant ($p<0.05$).
11. Of total 193 *E. coli* isolates, 46.12% (89/193) were found to be MDR-strains; while all the isolates of *Enterococcus faecalis* 100% (3/3) were found to be MDR- strains.
12. The burden of MDR pathogens infections were found higher as the age of the patients increases and found highest among the patients of age-group 81-90 years (83.34%).
13. Out of 96 MDR isolates likely to produce ESBL, 27 (28.12%) were found ESBL producers, among them 100.0% were *E. coli* isolates.
14. Out of 121 MDR isolates likely to produce ABL, 58 (48.76%) were found ABL producers, among them most predominant ABL producers were *E. coli* (81.03%) while 100.0% ABL production was found from *Citrobacter diversus, Enterobacter aerogenes* and *K. oxytoca*.
15. Out of 117 MDR isolates likely to produce MBL from different samples, 33(28.20%) of them were confirmed as MBL-producers, among them most predominant were *E. coli* (75.75%) while 100.0 % MDR *K. oxytoca* and *Acinetobacter* spp. were produced MBL. Five (15.15%) of the MBL producers gave resistant to carbapenem.
16. Out of 33 MBL postive isolates, 24.24 % strains also co-produce ESBL whereas 62.5 % MBL postive isolates also co-produce weak AmpC β-lactamase.
17. Among 200 MDR isolates likely to produce ESBL, ABL and MBL , increasing rate of no. of drug resistance was found among positive isolates of β-lactamase production with majority producers were found resistant to >10 drugs, among these 78.78 % MBL MDR producers were found most predominant.
18. All the isolates of 30 MDR *E. coli* and 3 MDR *C. freudii* that produce different types of β-lactamases (i.e.ESBL, ABL and MBL) harbored plasmids which showed 16 types of plasmid profiles of size ranging from 2 to 51 kb with most common plasmid was 32.5 kb.
19. Most of hyper AmpC and AmpC β-lactamase producing strains harbored the four plasmid with frequently associated with 32.5 kb and 4.5 kb plasmids while most strains producing only MBL harbored single plasmid.

20. Majority of MDR strains showed plasmid mediated ESBL, ABL and MBL productions by the conjugation study with similar patten of MDR traits as donors among transconjugants with conjugation frequency 10^{-7}.
21. Chances of transfer of MDR traits found in plasmid to other competent pathogen were found least by transformation study but transfer of certain drug resistance was found good with most of transformation efficiency 10^{8}.

7.2 RECOMMENDATIONS

1 The practices of empiric therapy when an infectious syndrome has been identified (e.g. UTI, high fever, pneumonia) should be discouraged, only pathogen directed therapy should be practiced.

2 As there are no guidelines or recommendations for the detection of AmpC β-lactamase and metallo-β-lactamase by CLSI or other standard institutions, so AmpC disk test for the detection of AmpC β-lactamase and EDTA-synergy disk test for the detection of MBL should be practiced in the clinical laboratoroy.

3 Several phenotypic and molecular typing methods should be used to investigate the origin of infection, route of spread and prevalence of isolates producing ESBL, ABL, MBL or other β-lactamase in a bacterial population.

4 The MBLs efficiently hydrolyze all ß-lactams, except for aztreonam, in vitro and associated with resistance to other classes of antibiotics, therefore, detection of MBL-producing gram-negative bacilli is crucial for the optimal treatment of patients and to control the spread of resistance.

5 High-level expression of AmpC may prevent recognition of an ESBL in species that produce a chromosomally encoded inducible AmpC beta-lactamase so, other microbiological, biochemical and molecular methods should be used to characterize ESBL production with co-producing ABL .

6. The vast majority of Enterobacteriaceae, including ESBL, ABL and MBL producers, remain susceptible to carbapenems, and these antibiotcs are considered preferred empiric therapy as well as pathogen directed therapy for serious Enterobacteriaceae infections.

7. This type of study should be carried out on larger scale throughout the country and results should be disseminated to monitor multidrug resistance.

8 Better antibiotic stewardship and infection control are needed to prevent further spread of extended-spectrum, AmpC and metallo β-lactamase mediated MDR in particularly Enterobacteriaceae, MRSA and *P. aeruginosa* throughout the country.

9. Plasmid profiles are most useful when combined with other methods for screening or typing multidrug resistance traits among Gram-negative bacterial outbreaks.

10. Further characterization of ESBL (i.e. TEM, SHV, CTX-M or other), ABL (e.g. MIR, DHA or other) and MBL (eg VIM, IMP or other novel types) by isoelectric focusing, PCR and DNA sequencing, or other methods should be done.

CHAPTER-VIII

8. REFERENCES

Aibinu I, Adenipekun E and Odugbemi (2004) Emergence of quinolone resistance amongst *Escherichia coli* strains isolated from clinical infections in some Lagos state hospitals, in Nigeria. Nigerian Journal of Health and Biomedical Science 3 (2):73–78

Alekshun MN and Levy SB (2007) Molecular Mechanisms of Antibacterial Multidrug Resistance. Cell 128, Elsevier Incorporation 1037-1050

Ambler RP (1980) The structure of ß□-Lactamases. Philosophical Transactions of the Royal Society of London. 289: 321-331

ArakawaY, Shibata N, Shibayama K, Kurokawa H, Yagi T, Fujiwara H, and Goto M (2000) Convenient Test for Screening Metallo-β-Lactamase-Producing Gram-Negative Bacteria by Using Thiol Compounds. Journal of Clinical Microbiology 38(1): 40-43

Ardanuy C, Liñares J, Domínguez MA, Hernández-Allés S, Benedí VJ, and Martínez-Martínez L. (1998) Outer membrane profiles of clonally related *Klebsiella pneumoniae* isolates from clinical samples and activities of cephalosporins and carbapenems. Antimicrobial Agents and Chemotherapy 42:1636-1640

Arlet G, Sanson-Le Pors MJ, Rouveau M, Fournier G, Marie O, Schlemmer B and Philippon A. (1990) Outbreak of nosocomial infections due to Klebsiella pneumoniae producing SHV-4 ß-lactamase. European Journal of Clinical Microbiology and Infectious Disease 9:797-803

Arpin C, Dubois V, Coulange L, André C, Fischer I, Noury P. (2003) Extended-Spectrum ß-Lactamase-Producing Enterobacteriaceae in Community and Private Health Care Centers. Antimicrobial Agents and Chemotherapy 47: 3506-3514

Aryal C (2001) Bacteriology of ear discharges. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Babini GS and Livermore DM (2000) Antimicrobial resistance amongst *Klebsiella* spp. collected from intensive care units in Southern and Western Europe in 1997-1998. Journal of Antimicrobial Chemotherapy 45:183-189

Banjara MR (2002) Study of air, water and wound infection in different wards of T.U. Teaching Hospital. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Barthelemy M, Peduzzi J, Bernard H, Tancrede C and Labia R (1992) Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum beta-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoc,*. Biochimestry and Biophysics Acta 1122:15-22

Bartoloni A, Pallecchi L, Benedetti M, Fernandez C, Vallejos Y, Guzman E, Villagran AL, Mantella A, Lucchetti C, Bartalesi F, Strohmeyer M, Bechini A, Gamboa H, Rodríguez H, Falkenberg T, Kronvall G, Gotuzzo E, Paradisi F and Rossolini GM (2006) Multidrug-resistant Commensal *Escherichia coli* in Children, Peru and Bolivia. Emerging infectious disease, Center for Disease Control (CDC), 12(6)

Bauer AW, Kirby WMM, Sherris JC and Turck M (1996) Antibiotic susceptibility testing by a standardized single disc method. American Journal of Clinical Pathology 45 : 493-6

Bauernfeind A, Chong Y and Schweighart S. (1989) Extended broad spectrum ß-lactamase in *Klebsiella pneumoniae* including resistance to cephamycins. Infection, 17:316-321

Brinboim HC and Doly J (1979) Arapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic acids research, 7: 1513-1516

Black JA,. Thomson KS and. Pitout JDD (2004) Use of ß-Lactamase Inhibitors in Disk Tests To Detect Plasmid-Mediated AmpC ß-Lactamases. Journal of Clinical Microbiology 42 : 2203-2206

Blomberg B, Jureen R, Manji KP, Tamim BS, Mwakagile DSM, Urassa WK, Fataki M, Msangi V, Tellevik MG, Maselle SY and Langeland N (2005) High Rate of Fatal Cases of Pediatric Septicemia Caused by Gram-Negative Bacteria with Extended-Spectrum Beta-Lactamases in Dar es Salaam, Tanzania. Journal of Clinical Microbiology 43(2): 745-749

Bloom MV, Freyer GA and Micklos DA (1996) Laboratory DNA Science. The Benjamin/Cummings Publishing Company, Incorporation,Ca

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

Bradford PA (2001) Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clinical Microbiological Reviews 14:933–951

Bradford PA, Urban C, Mariano N, Projan SJ, Rahal JJ and Bush K (1997) Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC beta-lactamase, and the foss of an outer membrane protein. Antimicrobial Agents and Chemotherapy 41:563-569

Brooks GF, Butel JS and Morse SA (2004) Jawetz, Melnick, & Adelberg's Medical Microbiology, 23rd edition, International edition, McGraw Hill, USA

Bush K, Jacoby GA and Medeiros AA. (1995) A functional classification scheme forß-lactamases and its correlation with molecular structure. Antimicrobial Agents and Chemotherapy 39:1211-1233

Centers for Disease Control and Prevention (2000) National nosocomial `infections surveillance (NNIS) system report, data summary from January 1992-April 2000. American Journal of Infections and Control, 28:429-448

Centers for Disease Control and Prevention (2000) Multidrug-Resistant Organisms in Non-Hospital Healthcare Settings(www.cdc.gov), Atlanta

Chakraborty P.(1998) A Textbook of Microbiology, 1st edition, New Central Book Agency (P) Ltd., India

Chang S, Sievert DM, Hageman JC, Boulton ML, Tenover FC and Downes FP (2003) Infection with vancomycin-resistant Staphylococcus aureus containing the vanA resistance gene. New England Journal of Medicine. 348:1342-1347

Cheesbrough M. (2000) District Laboratory Practice in Tropical Countries. Part 2, Cambridge University Press

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

Chu DTW (1999) Recent progress in novel macrolides, quinolones, and 2-pyridones to overcome bacterial resistance. Medicinal Research Reviews. 19(6): 497-520

Chu, Y-W, Afzal-Shah M, Houang ETS, Palepou MFI, Lyon DJ, Woodford N and Livermore DM (2001) IMP-4, a novel metallo-β-lactamase from nosocomial *Acinetobacter* spp. collected in Hong Kong between 1994 and 1998. Antimicrobial Agents and Chemotherapy 45:710-714

Church DL. Gregson, DB, Laupland KB and Pitout JDD (2007) Population-based laboratory surveillance for AmpC [beta]-lactamase-producing *Escherichia coli*, Calgary. (RESEARCH) (ampicillin) (Clinical report) Emerging Infectious Diseases 56-59

Clinical and Laboratory Standards Institute (2007) Methods for dilution antimicrobial susceptibility testing for bacteria that grow aerobically. Seventeenth Informational Supplement. M7-A7, Wayne, PA

Clinical and Laboratory Standards Institute (2007) Performance Standards for Antimicrobial Susceptibility Testing; Approved standards-ningth edition ,M2- A9 , Wayne, PA

Cohen ML, Wong ES and Falkow S (1982) Common R plasmids in Staphylococcus aureus and Staphylococcus epidermidis during a nosocomial Staphylococcus aureus outbreak.Antimicrobial Agents and Chemotherapy 21:210-215

Collee JG, Fraser AG, Marmion BP and Simmons A. (1996) Mackie and McCartney Practical Medical Microbiology.14th edition. Churchill Livingstone, USA

Collier L, Balows A and Sussman M (1998) Topley & Wilson's Microbiology and microbial infections. 9th edition. Arnold, Great Britain. 3:319-322

Courvalin PM, Shaw WV and Jacob AE (1978) .Plasmid-mediated mechanisms of resistance to aminoglycoside-aminocyclitol antibiotics and to hloramphenicol in group D streptococci. Antimicrobial Agents and Chemotherapy 13:716-725

Dahal RK, Koirala J, Khadka P, Pokhrel BM and Tuladhar NR. (2005) The Status of Multidrug Resistant and Extended-spectrumß-lactamase producing Salmonella Isolated from Blood Culture. Journal of Nepal Association for Medical Laboratory Sciences 7: 24-29

Daniel FS, Thornsberry C, Mayfield DC, Jones ME and Karlowsky JA (2001) Multidrug-Resistant Urinary Tract Isolates of *Escherichia coli:* Prevalence and Patient Demographics in the United States in 2000. Journal of Antimicrobial Chemotherapy 45.5.1402–1406

Davis RW, Somon M and Davidson N (1968) Electron-Microscope Hetroduplex methods for mapping regions of base sequence homology in nucleir acids, IN:Methods in Enzymology, 21(Nucleir acids): 413-428

Denyer SP, Hodges NA and Gorman SP (2004) Hugo and Russell's Pharmaceutical Microbiology. 7th edition. Blackwell Scientific Publications, UK

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

Dhungel R (2001) Prevalence of common bacterial pathogens in different clinical samples submitted at Tribhuvan University Teaching Hospital and their antibiotics sensitivity test profiles. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu

Edelstein M, Pimkin M, Palagin I, Edelstein I and Stratchounski L (2003) Prevalence and Molecular Epidemiology of CTX-M Extended-Spectrum ß-Lactamase-Producing Escherichia coli and Klebsiella pneumoniae in Russian Hospitals. Antimicrobial Agents and Chemotherapy 47: 3724-3732

Enabulele IO, Yah SC, Yusuf EO, Eghafona NO (2006) Emerging quinolones resistant transfer genes among gram-negative bacteria, isolated from faeces of HIV/AIDS patients attending some Clinics and Hospitals in the City of Benin, Edo State, Nigeria. Online Journal of Health Allied Science 3:3

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

Forbes BA, Sahm DF and Weissfeld AS (2002) Bailey and Scott's Diagnostic Microbiology.11th edition. Mosby, Inc. USA

Fowler JE and Mariano M (1990) Immunologic response of the prostate to bacteriuria and bacterial prostates: Antigen specific immunoglobulin in men with bacterial prostatitis. Journal of Urology 131:363

Gautam K (1997) Prevalence of urinary tract infection on children. M sc dessertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

George WL (1989) Other infections of skin, soft tissue and muscle.Anaerobic infection in humans. Academic press, San Diego

Ghimire M (2004) Evaluation of antimicrobial resistance status in Kirtipur community bacterial isolates. M.Sc. dessertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Gilmour A (2004) Plasmid (parkhill@sanger.ac.uk), 52: 182-202

Goldstein EJC 91992) Bite wounds and infection. Clinical Infectious Disease,14:633

Greenwood D, Slack R and Peutherer J. (2000) Medical Microbiology. A Guide to Microbial Infections: Pathogenesis, Immunity, Laboratory Diagnosis and Control. 15th edition. Churchill Livingstone, USA

Gupta V, Datta P and Singla N (2008) Skin and Soft Tissue Infection: Frequency of Aerobic Bacterial Isolates and Their Antimicrobial Susceptibility Pattern. Journal of association of physicians of India 56:25-27

Gustafson TL, Band JD and Hutcheson RH Jr (1983) *Pseudomonas* folliculitis: an outbreak and review, Review of Infection and Disease. 5:1

Hachler H., Berger-Bachi B and Kayser FH. (1987) Genetic characterization of a *Clostridium difficile* erythromycin-clindamycin resistance determinant that is transferable to *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 31:1039-1045

Hall RM and Collis CM (1998) Antibiotic Resistance in Gram-negative bacteria: the role of gene cassettes and integrons. Drug Resistance Update, 1:109-119

Heinemann Hs and Radano RR (1979) Acceptability and cost savings of selective sputum microbiology in a community teaching hospital. Journal of Clinical Microbiology 10:567-573

Hodge W, Ciak J and Tramont EC (1978) Simple method for detection of penicillinase-producing *Neisseria gonorrohoeae*. Journal of Clinical Microbiolog. 7: 102-3

Horii T, Arakawa Y, Ohta M, Ichiyam S, Wacharotayankun R and Kato N (1993) Plasmid-Mediated AmpC-Type 1-Lactamase Isolated from Klebsiella pneumoniae Confers Resistance to Broad-Spectrum ,B-Lactams,Including Moxalactam. Antimicrobial Agents and Chemotherapy 37(5) : 984-990

Jacoby GA and Medeiros AA (1991) More extended-spectrum ß-lactamases. Antimicrobial Agents of Chemotherapy 35:1697-1704

Jacoby GA, and Munoz-Price LS (2005) The New β-Lactamases. The New England Journal of Medicine 352(4):380-391

Jesudason MV, Kandathil AJ & Balaji V (2005) Comparison of two methods to detect carbapenemase & metallo-β-lactamase production in clinical isolates. Indian Journal of Medical Reseach 121: 780-783

Jha VC and Yadav JN (1992) Bacterial species isolated from urine of UTI suspected patients and their sensitivity to commonly available antibiotics. Journal of Nepal Medical Association 30:222-225

Johnson AP, Sanyal D, George RC, Cookson BD and Williams AJ (1991) Peritonitis due to vancomycin-resistant *Staphylococcus epidermidis*. Lancet, 337:54

Joshi S (1997) Microbiological study of body fluids. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Joshi K, Basnyat SR, Pokhrel BM and Tuladhar NR (1998) A prospective study on bacteriology of lower respiratory tract infection among the patients visiting T.U. Teaching Hospital, Kathmandu. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu

Kader AA and Kumar AK (2004) Prevalence of Extended-spectrumbeta-lactamase among multidrug resistant gram-negative isolates from a general hospital in Saudi Arabia. Saudi Medical Journal 25(5):570-4

Kahlmeter G (2000) The ECO-SENS project: a prospective, multinational, multicenter epidemiological survey of the prevalence and antimicrobial susceptibility of urinary tract pathogens–interim report. Journal of Antimicrobial Chemotherapy 46 (Suppl. S1):15–22

Kass EH, Savage W, Santamarina BA (1965) The significance of bacteriuria preventing medicine.Progress of pyelonephritis, Philadelphia: FA Davis Co.

KC K (1997) Isolation of antibiotic resistant enteric bacteria from community ponds and their antibiotic transfer mechanism in such environment. M.Sc. dessertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Knothe H, Shah P, Kremery V, Antal M and Mitsuhashi S (1983) Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection 1:315-317

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

Koneman EW, Allen SD, Janda WM, Schreckenberger PC and Winn WC Jr. (1997) The non fermentative Gram-negative bacilli. In: color atlas and textbook of diagnostic microbiology. 5th edition. Lippincott, Philadelphia . 293-303

Krieg NR (1984) Bergey's Manual of Systematic Bacteriology. Vol 1, Williams and Wilkins

Kwa AL, Low JG, Lee E, Kurup A, Chee HL and Tam VH (2007) The impact of multidrug resistance on the outcomes of critically ill patients with Gram- negative bacterial pneumonia. Diagnostic Microbiology and Infectious Diseases 58(1):99-104

Lamichhane R (1999) Study of methicillin resistant *Staphylococcus aureus* (MRSA) isolated from different clinical samples. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Lang D (1970) Molecular weights of colipahges and coliphage DNA III, Contour length and molecular weight of DNA from bacteriophages T4, T5 and T7 and from bovine papilloma virus. Journal of Molecular Biology 54:557

Lee K, Chong Y, Shin HB, Kim YA, Yong D and Yum JH (2003) Modified Hodge and EDTA - disk synergy tests to screen metallo-β-lactamase-producing strains of *Pseudomonas* and *Acinetobacter* species. Journal of Clinical Microbiology 41: 4623-4629

Leigh DA (1990) UTI In: Smith GR and Easmon CSF, In (eds.) Topely and Wilson's Principles of Bacteriology, Virology and Immunity, Bacterial Diseases, 8th ed. Frome and London: Butler and Tanner Ltd. 3:197-214

Levett PN (1993) Analysis of pathogens isolated from urinary tract infection in Barbados. West Indian Medical Journal, 4(4):372-374

Levi SB (2001).Antibiotic Resistance:Consequences of inaction. Clinical Infectious Diseases. 33(Suppl 3):S124–9

Levine MM and Kaper JB (1983) New knowledge on the pathogenesis of bacterial enteric infections as applied to vaccine development.Microbiological Reviews 47: 510-550

Ling JM, Cheng AF and Ho EM (1992) Bacteriology of UTI in two general hospitals in Hong Kong. Journal of Hong Kong Medical association, 44:105-107

Livermore DM and Woodford N (2004) Laboratory Detection & Reporting of Bacteria with Extended-Spectrum ß-Lactamases. Health Protection Agency

Livermore DM. (1995) ß-Lactamases in laboratory and clinical resistance. Clinical Microbiological Reviews 8:557-584

Livermore DM.(2002) ultiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare? Clinical Infectious Disease 34: 634-640

Madigan MT, Martinko JM and Parker J. (2002) Brock Biology of Microorganisms. 9th edition. Prentice-Hall Inc. NJ, USA

Manandhar S (1996) Microbiology of Urinary Tract Infection: a hospital based study. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu

Manandhar T (2005) Antibiotic susceptibility profile of bacterial pathogens in urinary tract infection with special reference to Extended-spectrumbeta lactamase (ESBL) producing strains. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu

Mandal S, Mandal MD and Pal NK (2004) Plasmid-Encoded Multidrug Resistance of *Salmonella typhi* and some Enteric Bacteria in and around Kolkata, India: A Preliminary Study. Online Journal of Health and Allied Science 4:2

Manges AR, Johnson JR, Foxman B, O'Bryan TT, Fullerton KE and Riley LW (2001) Widespread Distribution of Urinary Tract Infections Caused by a Multidrug-Resistant *Escherichia coli* Clonal Group. 345(14):1007-1013

Marty L and Jarlier V (1998). Surveillance of multiresistant bacteria: justification, role of the laboratory, indicators, and recent French data. Pathol Biol (Paris) 46:217-226

Mayer LW (1988) Use of Plasmid Profiles in Epidemiologic Surveillance of Disease Outbreaks and in Tracing the Transmission of Antibiotic Resistance. Clinical Microbiology Reviews (1) 2: 228-243

Moland ES, Black JA, Ourada J, Reisbig MD, Hanson ND and Thomson KS (2002) Occurrence of newer beta-lactamases in *Klebsiella pneumoniae* isolates from 24 U.S. hospitals. Antimicrobial Agents and Chemotherapy 46:3837-3842

Morse SA, Johnson SR, Biddle JW and Roberts MC (1986) High-level tetracycline resistance in Neisseria gonorrhoeae. Antimicrobial Agents and Chemotherapy 30:664-670

Murray BE, Church DA, Wagner A, Zscheck K, Levison ME, Ingerman MJ, Abrutyn E, and Mederske-Samoraj B (1986) Comparison of two beta-lactamase-producing strains of Streptococcus faecalis. Antimicrobial Agents and Chemotherapy 30:861-864

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

Obi CL, Tarupiwa A and Simango C (1996) Scope of urinary pathogens isolated in the public Health Bacteriology Laboratory. Harare: Antibiotic susceptibility patterns of isolates and incidence of haemolytic bacteria. Central African Journal of Medicine. 42(8):244-249

Olukoya DK, Olasupo NA (1997) Drug resistance and plasmids profiles of Diarrhoeagenic bacteria isolates in Nigeria (1988-1996). Nigeria Quaternary Journal of Hospital Medicine, **7**:26- 32

Opal SM, Mayer KH and Medeiros AA (2000) Mechanisms of bacterial antibiotic resistance. Mandell, Douglas and Bennett's principles and practice of infectious diseases. 5th edition. Churchill Livingstone. 1: 236-52

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

Oteo J, Lázaro E, de Abajo FJ, Baquero F, Campos J and Spanish members of EARSS (2005) Antimicrobial-resistant Invasive *Escherichia coli*, Spain. Emerging Infectious Diseases (www.cdc.gov/eid) 11(4): 25-30

Pandey M (2003) Study on the prevalence of multiple drug resistant *Salmonella* in poultry birds. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Paneru TP (2002) Antibiotic sensitivity profile of *E. coli*, *Klebsiella* and *Pseudomonas* of patients visiting TUTH Kathmandu. M.Sc. dessertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Paterson DL and Bonomo RA (2005) Extended-Spectrum ß-Lactamases: a Clinical Update. Clinical Microbiology Reviews, 18(4): 657-686

Paterson DL, Ko WC and von Gottberg A. (2001) Outcome of cephalosporin treatment for serious infections due to apparently susceptible organisms producing extended-spectrum ß-lactamases: implications for the clinical microbiology laboratory. Journal of Clinical Microbiology 39:2206-2212

Payne DJ, Cramp R, Bateson JH, Neale J and Knowles D (1994) Rapid identification of metallo- and serine beta-lactamases. Antimicrobial Agents and Chemotherapy 38(5): 991-996

Pelczar MJ, Chan ECS and Krieg NR (1983) Microbiology. 5th edition. Tata McGraw-Hill edition

Philippon A, Arlet G and Jacoby GA (2002) Plasmid-determined AmpC-type ß-lactamases. Antimicrobial Agents and Chemotherapy 46:1-11

Pokhrel BM (2004) A Handbook of Clinical Microbiology. 1st edition. Gorakhnath Desktop and Printing Supports, Kathmandu, Nepal

Phillips IE and King S (1988) A Epidemic multiresistant Escherichia coli infection in West Lambeth Health District. Lancet 1:1038-1041

Pokhrel BM, Koirala J, Mishra SK, Dahal RK, Khadga P and Tuladhar NR (2006) Multidrug resistance and extended spectrumbetalactamase producing strains causing lower respiratory tract and urinary tract infection. Journal of Institute of Medicine 8(3): 30-34

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

Rai CK (2000) A prospective study on antibiotic sensitivity profiles of the organisms associated with clinical infections among the patients attending T.U. Teaching Hospital: A Hospital based study. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Rodrigues C, Joshi P, Jani SH, Alphonse M, Radhakrishnan R and Mehta A (2004) Detection of β-Lactamases in nosocomial Gram-negative clinical isolates. Indian Journal of Microbiology 22(4) : 247-250

Rossolini GM (2007) Redesigning beta-lactams to combat resistance: summary and conclusions. Clinical Microbiological Infections 13 Suppl 2:30-3. [abstract]

Rubin RH and Weinstein L (1977) Salmonellosis: Microbiologic, Pathologic and Clinical features, Stratton Intercontinental, New York, 46-58

Runyon B, Antillon MR and Akriviadis EA (1990) Bedside inoculation of blood culture bottles with ascitic fliud is superior to delayed inoculation in the detection of spontaneous bacterial peritonitis. Journal of Clinical Microbiology 28:2811

Sambrook J and Russell DW (2001) Molecular cloning: a laboratory manual, 2nd ed. Volume 1, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schaeffer AJ and Chmiel J (1983) Urethral meatal colonization in the pathogenesis of catheter associated bacteriuria .Journal of Urology 130:1096-1099

Sekiguchi JI, Asagi T, Akiyama TM, Kasai A, Mizuguchi Y, Araake M, Fujino T,Kikuchi H, Sasaki S, Watari H, Kojima T, Miki H, Kanemitsu K, Kunishima H, Kikuchi Y, Kaku M, Yoshikura H, Kuratsuji T and Kirikae T (2007) Outbreaks of Multidrug-Resistant *Pseudomonas aeruginosa* in Community Hospitals in Japan. Journal of Clinical Microbiology 45: 979-989

Shakya MS (1997) Microbial analysis of burn injuries at burn unit of different hospitals

Shakya R (2001) Study on bacterial flora in blood specimen collected from hospitalized and out patients services of TUTH. M.Sc. dessertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Sharma J (2004) Prospective study on Microbiology of Lower Respiratory Tract Infections and antibiotic sensitivity profile with special interest in Multidrug Resistant and Extended-spectrumof ß-lactamase strain. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Sharma PR (1983) Urinary infection- The infection that matters. Journal of infectious Medicine, 5:19-22

Shehabi AA, Mahafzah AM and Al-Khalili KZ (2002) Antimicrobial resistance and plasmid profiles of urinary *Escherichia coli* isolates from Jordanian patients. Eastern Mediterranean Health Journal 10(3): 322 – 328

Shrestha A (2002) Bacteriological study of upper respiratory tract infection in pediatric patients at Kanti Children's Hospital. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Shrestha B (1995) Epidemiology and microbiology of lower respiratory tract infection among patients in Nepal. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Shrestha B (1996) Isolation of Salmonella Species from Blood and Study its Antibiotic sensitivity Pattern. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Shrestha P (2007) Study of bacteria causing urinary tract infection and their antimicrobial resistance trend at National Public health Laboratory. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Sidjabat HE, Townsend KM, Hanson ND, Bell JM, Stokes HW, Gobius KS, Moss SM and Trott DJ (2006) Identification of blaCMY-7 and ssociated plasmid-mediated resistance genes in multidrug-resistant *Escherichia coli* isolated from dogs at a veterinary teaching hospital in Australia. Journal of Antimicrobial Chemotherapy 57: 840–848

Singh DS (1991) Urinary examination, its importance in pediatric medicine. Indian Journal of pediatric 58:717-723

Smith GR and Easmon CSF **(**1990) Topley and Wilson's Principles of Bacteriology, Virology and Immunity, 8th edition, Volume III Bacterial Disease, BC Decker Inc., Philadelphia, USA

Sneath PHA (1986) Bergey's Manual of Systematic Bacteriology. Vol 2 ,Williams and Wilkins

Snyder L and Champness W (2003) Molecular Genetics of Bacteria. ASM press

Steenberg J, Bartels ED and Bay-Nielsen H (1985) Epidemiology of urinary tract diseases in general practice. British Medical Journal 4:390

Subha A, Devi VR and Ananthan S (2003) AmpC beta-lactamase producing multidrug resistant strains of *Klebsiella* spp. & *Escherichia coli* isolated from children under five in Chennai. Indian Journal of Medical science 117:13-8

Suman E, Jose J, Varghese S and Kotian MS (2007) Study of biofilm production in *Escherichia coli* causing urinary tract infection. Indian Journal of Medical Microbiology 25(3): 305-306

Takakura S, Senda K, Saito T, Fujihara N, Kudo T, Ichiyama S, Komori T and Fujita N (2002) Prospective Surveillance Study of Metallo-beta-lactamase Producing Gram-Negative Bacteria: Possible Transmission of MBL Gene within an Individual Patient. Antimicrobial Agents and Chemotherapy. 42: abstract no. C2-1887

Thomas C, Wain J and the broader plasmid biology community (2007) Plasmid sequencing**.** The Sanger Institute Pathogen Sequencing Unit (parkhill @ sanger .ac.uk), UK

Thomson K, Black JA, Buynak JD and Pitout J (2003) Detection of Plasmid-Mediated AmpC beta-Lactamases (pAmpCs) in Disk Tests Based on beta-Lactamase Inhibitors (BLIs) Ro 48-1220 (RO) and LN-2-128 (LN).Antimicrobial Agents a nd Chemotherapy 43: abstract no. D-258

Tiwari KB (2002) Study of meningitis in patients visiting Tribhuvan University Teaching Hospital. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Tuladhar NR (2001) A Report: Surveillance of Multiple Drug Resistant (MDR) bacterial infection among the patients attending to different Out Patient Departments (OPD) and Hospitalized Patients in Tribhuvan University Teaching Hospital

Tuladhar NR, Nakanishi M, Shrestha HG, Duffy J, Tomiyoshi Y, Pandey G and Sharma SP (1990) Nosocomial infections in surgical patients at Tribhuvan University Teaching Hospital. Journal of Nepal Medical Association 28: 152-156

Tuladhar P (1999) A prospective study on bacteriology of wound infection at T.U. Teaching Hospital. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Wagle Y (2004) Microbiology of bacteraemia and septicaemia in patients visiting Tribhuvan University Teaching Hospital (TUTH), Kathmandu. M.Sc. dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal

Waley SG (1962) β-Lactamase: mechanism of action.In M.I. Page (ed.).The chemistry of β-lactams. A.and P. Blackie, London. 198-228

Walsh TR, Toleman MA, Poirel L and Nordmann P (2005) Metallo-ß-Lactamases: the Quiet before the Storm?. Clinical Microbiology Reviews18 (2):306-325

Wang M, Daniel FS, George AJ, David CH (2004) Emerging Plasmid-Mediated Quinolone Resistance Associated with the *qnr* Gene in *Klebsiella pneumoniae* Clinical Isolates in the United States**.** Antimicrob Agents and Chemotherapy 48(4):1295-1299

Waugh A and Grant A (2001) Ross and Wilson Anatomy and Physiology in Health and Illness.9th edition. Churchill Livingstone.361-367

WHO (2004) Basic laboratory procedures in clinical bacteriology. 2nd edition . World Health Organization, Geneva

WHO (2004) Bulletin of World Health organization. The antibiotic resistance containment and Surveillance approach – a public health tool.World Health Organization, 82 : 928-934

Wright SW, Wrenn KD, Haynes M and Haas DW (2000) Prevalence and risk factors for multidrug resistant uropathogens in ED patients. The American Journal of Emergency Medicine. 18 (2):143-146

Yamane K, Wachino J, Doi Y, Kurokawa H and Arakawa Y (2005) Global Spread of Multiple Aminoglycoside Resistance Genes Emerging Infectious Diseases, 11(6): 951-953

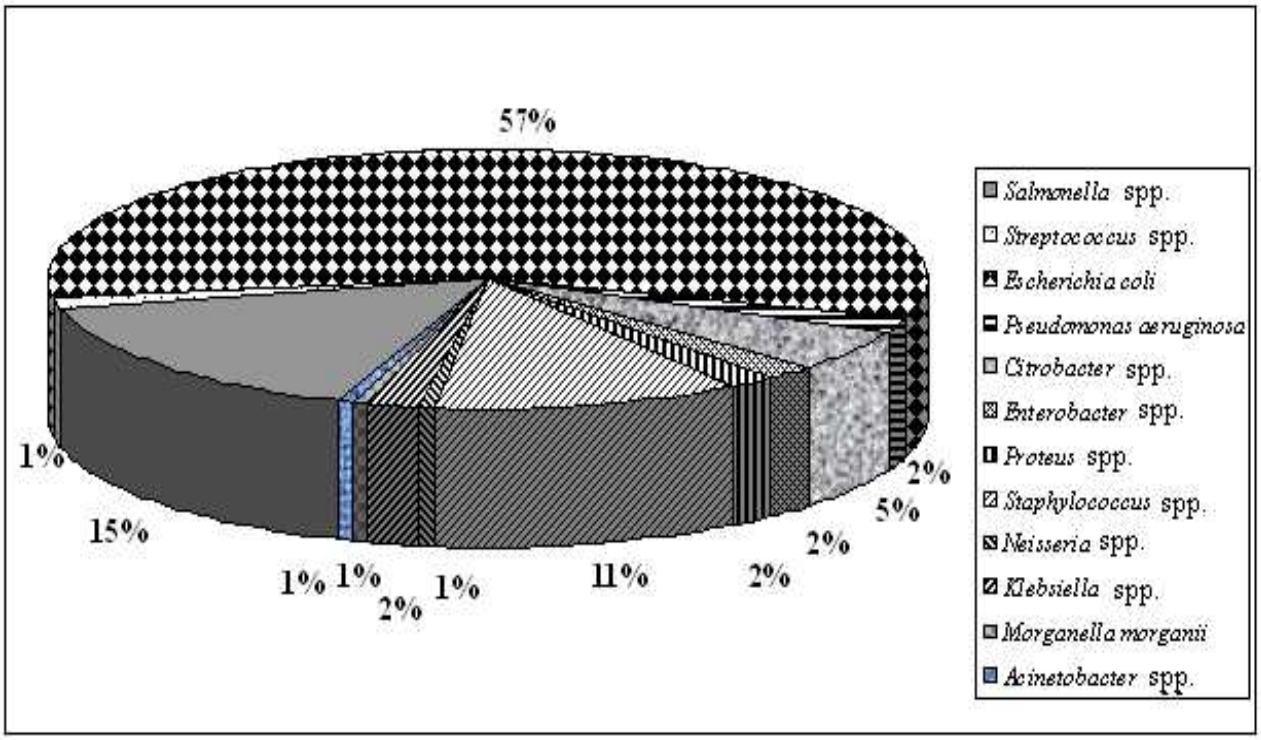

Figure 3: Percentage pattern of pathogens isolated from different samples

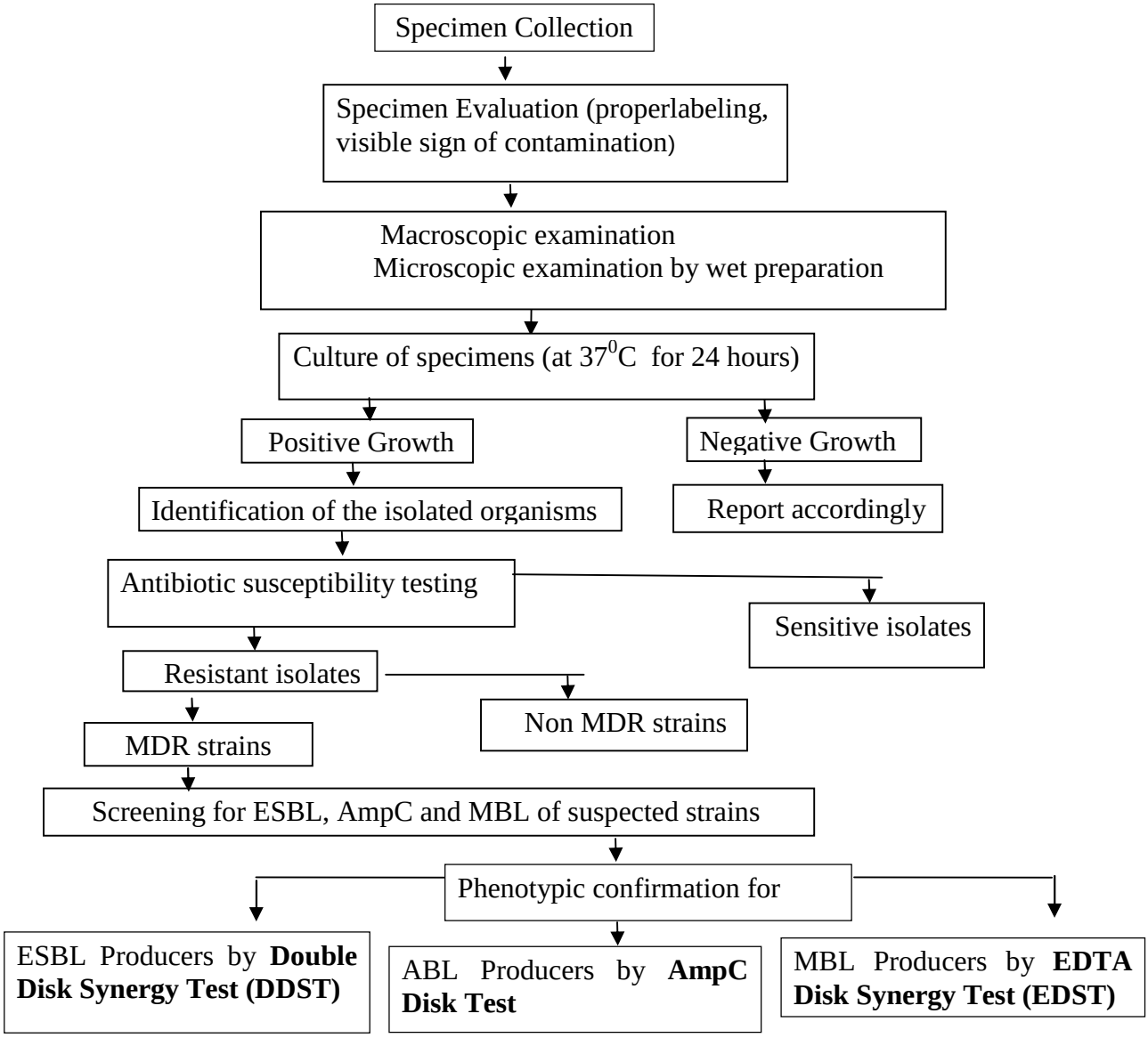

Figure 2: Flow chart of processing of different specimens and identification of ESBL, ABL and MBL.

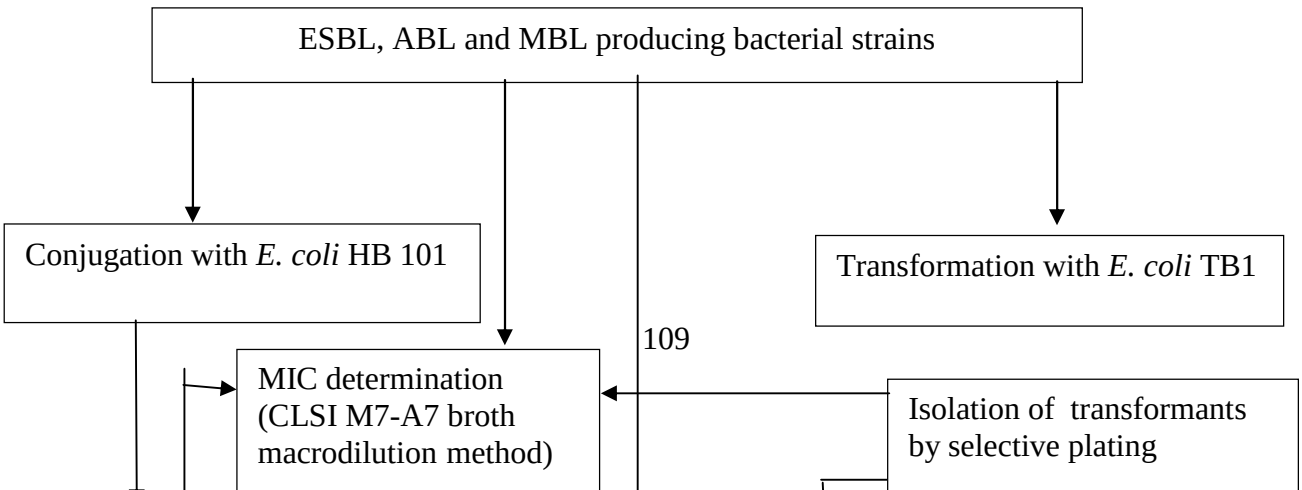

Figure 3: Plasmid DNA analysis from ESBL, ABL and MBL producing strains

Photograph 1:Significant growth of *E. coli* isolated from urine sample on Blood agar plate

Photograph 2: Biochemical tests of *E. coli*

Legend: From left A/A gas, H_2S negative; H_2S negative, Indole positive, Motile; Citrate negative; MR positive; VP negative; O/F–fermentative; Urease negative; Nitrate reduction positive

Photograph 3: Antibiotic susceptibility test of *E. coli*: MDR strain

Photograph 4: Positive ESBL –confirmation test on Mueller-Hinton agar

Photograph 5: Positive hyper AmpC and AmpC β–lactamase production test on on Mueller-Hinton agar (A =hyper AmpC β–lactamase producer, B and C = AmpC β–lactamase producer)

Photograph 6: Positive weak and non- AmpC β-lactamase production on MHA (A = weak AmpC β-lactamase producer and B = non- AmpC β-lactamase producer)

Photograph 7; Positive MBL producing MRSA isolate resistant to carbapenem

Photograph 8: Plasmid DNA profiles from different β-lactamase producing MDR strains

Lane M1 and M2- Supercoiled Plasmid DNA ladder

Lane 1, 5, 9, 11, 15, 19 and 21 – ABL producers (KMH_{2102}, KMH_{3028}, KMH_{1830}, KMH_{1140}, KMH_{3128}, KMH697 and KMH_{1569})

Lane 2, 7, 10, 12, 13, 14, 16, and 18 – ESBL and ABL producers (KMH_{3263}, KMH_{6261}, KMH_{1490}, KMH_{4137}, KMH_{55}, KMH_{1133}, KMH_{890} and KMH_{2466}),

Lane 6, 8, 20, 22, 23 and 24 – MBL producers (KMH_{3957}, KMH_{686}, KMH_{915}, KMH_{533}, KMH_{3625} and KMH_{3675})
Lane 3 and 4 – ESBL and MBL producers (KMH_{879} and KMH_{877})
Lane 17 – ESBL, ABL and MBL producer (KMH_{759})

Photograph 9: Plasmid profiles of MDR *E. coli* and their transconjugants

Lane M1 and M2 - Supercoiled Plasmid DNA ladder
Lane 1 to 20 – Plasmid DNA profiles of MDR *E. coli* and their transconjugants ($KMH_{1569,}$ $KMH_{877,}$ $KMH_{1133,}$ $KMH_{85,}$ $KMH_{1140,}$ $KMH_{2974,}$ $KMH_{2176,}$ $KMH_{3263,}$ KMH_{533} and KMH_{759})

APPENDIX-I

QUESTIONNAIRE

Name:
Age: -
Sex:-
Address:-

Lab no:-
Date:-
Occupation:-
Patient: OPD/IN
Ward:-
Bed no:-

Clinical profile of patient:

1. What are you suffering from prospective disease, illness or syndrome?

2. Are you suffering from any chronic disease?
   - Yes
   - No If, yes than type of disease……..

3. Are you taking any drug for this chronic disease?

4. Have you taken any antibiotic before? if yes, than

   - Complete course
   - Incomplete course

5. History of hereditary disease?
   - Yes
   - No If, yes than type of disease……..

A. Blood sample

Lab tests:

Day 1

- Inoculation of blood to the BHI broth/Blood culture broth

Day 2

Observation:

- Flocular deposit on top of the blood layer
- Uniform or subsurface turbidity.
- Haemolysis
- Cogulation of broth or surface turbidity.
- Production of gas
- White grains on the surface or deep in the blood layer.

Subculture on BA / MA / SS agar

Day 3

If growth occurs on agar plate on subculture ,identification of organisms by

- Gram staining ;

Gram-positive	cocci / rod
Gram-negative	cocci / rod

- Catalase
- Oxidase:

Identification by biochemical tests:

Biochemical tests:

S.N.	Tests	Results
1	Sulphide	
2	Indole	
3	Motility	
4	MR	
5	VP	
6	Citrate Utilization	
7	TSI	
8	O/F	
9	Urease	
10	Nitrate reduction	

Organism identified as .....

Antibiotic susceptibility testing :

For Gram-negative bacteria:
1. Ampicillin/Amoxycillin
2. Chloramphenicol
3. Co-trimoxazole
4. Cefotaxime
5. Ciprofloxacin
6. Cefixime
7. Ceftriaxone
8. Ofloxacin

For Gram-positive bacteria:
1.Ampicillin/Amoxycillin
2.Ciprofloxacin
3.Cephalexin
4.Cephotaxime
5.Cloxacillin
6.Erythromycin
7.Co-trimoxazole

B.Urine sample

Do you currently have any of the following symptoms?

Dysuria	
Frequency	
Urgency (compelling need to urinate)	
Tenderness and pain above bladder	
Flank pain	
Fever	
Vomiting	
Diarrhea	
Chills	
Lower abdominal pain	

Lab tests

Day-1

a. Appearance of specimen

- Clear
- Cloudy

b. Microscopic examination of specimen

- Bacteria
- Pus cells
- Red cells
- Yeast cells
- Epithelial cells
- Casts
- Crystals

c. Gram smear

Bacteria

- Gram-positive cocci / rod
- Gram-negative cocci / rod

Culture on Blood agar and Mac-conkey agar by semi- quantitative method

Day-2

a. Examine the culture on

- BA Haemolytic /Non-haemolytic
- MA LF / NLF

b. Observe for growth

Recommendation for reporting (Kass, Marple and Sandford)

S.N.	Number of organisms/ml	result	Remarks
1.	$<10^4$ CFU/ml	Contaminants	
2.	Equal to or more than 10^5 CFU/ml	Significant bacteriuria	
3.	10^4-10^5 CFU/ml	Doubtful significant bacteriuria	

Day-3 **Biochemical test:** As mentioned above

Organism identified as**……**

S.N.	**Gram-positive bacteria**	**Sensitive**	**Resistant**	**Gram-negative bacteria**	**Sensitive**	**Resistant**
1.	Amoxycillin			Amoxicillin		
2.	Cephalexin			Cefixime		
3.	Ciprofloxacin			Cefotaxime		
4.	Cloxacillin			Ciprofloxacin		

5.	Co-trimoxazole			Co-trimoxazole		
6.	Erythromycin			Nalidixic acid		
7.	Norfloxacin			Nitrofurantoin		
8.				Norfloxacin		
9.				Ofloxacin		

Antibiotic susceptibility test

C. Sputum sample

Day 1:

- Sputum meeting ASM criteria ( )
- Inoculation on BA ,CA , MA

Day 2:

Observation -
Bacitracin sensitivity
Optochin sensitivity
Gram staining
Oxidase
Catalase

Day 3:

Biochemical tests:- As mentioned above
Organism identified as .....

Antibiotic susceptibility test:-

S. pneumoniae	*H. influenzae*	Other isolate (.....)
1 Penicillin	1. Ampicillin	1
2. Ceftriaxone	2. Cefotaxime	2.
3. Cefotaxime	3. Co-trimoxazole	3.
4. Cephalexin	4. Tetracycline	4.
5 Azithromycin.	5. Chloramphenicol	5.
6. Erythromycin	6. Erythromycin	6.
7. Ciprofloxacin	7. Cirpoflozacin	7.

D.Cerebrospinal fluid (CSF) sample

Day 1:

1 Macroscopic appearance:-

- Clear
- Hazy
- Turbid
- Yellow
- Blood tinged

2. Direct Microscopic examination:-

Presence of
Leucocytes
Erythrocyte
Bacteria
Yeast
Gram staining-
Z-N staining -

India -Ink preparation –

3 Culture

BA

CA

MA

Day 2: Observation of culture growth

Day 3:

4. Biochemical tests- As mentioned above

Organism identified as .....

5. Antibiotic susceptibility test:-

N. meningitidis	Other isolate(.....)
1.Penicillin	1.
2.Ampicillin	2.
3.Chloramphenicol	3.
4. Cefotaxime	4.
5. Ceftriaxone	5.
6. Ceftizoxime	6.

E. Pus and fluid sample

Physical examiniation:

•Colour • Consistency •Turbidity •Odour •Blood tinged

Day 1

Inoculation on BA, MA

Day 2

Observation-

Gram staining-

Catalase

Oxidase

Inoculation on biochemical set

Day 3

Identification by biochemical test: As mentioned above

Organism identified as .....

Antibiotic susceptibility test:-

P. aeruginosa	*S. aureus*	*S. pyogenes*
1 Piperacillin	1.Penicillin	1.Penicillin
2 Genatamicin	2. Oxacillin	2.Tetracycline
3 Tobramycin	3. Vancomycin	3. Gentamicin
4 Ceftazidime	4. Erythromycin	4. Cephalexin
5 Aztreonam	5. Chloramphenicol	5. Erythromycin
6 Amikacin	6. Tetracycline	6. Vancomycin

7. Ciprofloxacin 7. Ciprofloxacin

E. coli	Other isolate(.....)
1 Cefotaxime	1.
2.Ceftazidime	2. .
3 Ceftriaxone	3.
4.Gentamicin	4.
5. Ciproflozacin	5
6.Cefixime	6
7.Amikacin	7.
8.Co-trimoxazole	8. .

F.Throat swab

Day 1

Inoculation on BA {with bacitracin) MA and **CA**

Day 2

Observation:
Gram staining -
Haemolysis -
Catalase -
Oxidase -

Day 3

Identification by biochemical test: As mentioned above

Antibiotic susceptibility test

Streptococcus pyogenes	Other isolate (.....)
1.Gentamycin	1.
2 Vancomycin	2
3.Tetracycline	3
4 Cephlexin	4
5 Penicillin	5
6 Erythromycin	6

APPENDIX –II

LIST OF EQUIPMENTS AND MATERIALS USED DURING THE STUDY

A. EQUIPMENTS

Hot air oven	Universal (India)
Microwave oven	LG
Incubator	Memmert (Germany)
Autoclave	
Refrigerator	Sanyo (Japan)
Microscope	Olympus (Japan)
Centrifuge	Remi (India),Eppendorf (Germany)
Weighing Machine	Scaltec instruments (Germany)
Water bath shaker	Grant, OLS 200 (UK)
UV transilluminator	Alpha Innotech Corporation (Germany)
Thermal printer	Alpha Innotech Corporation (Germany)
Electrophoretic apparatus	International Biotechnologies Inc, USA
Voltguard	Fisher Scientific ,USA
AC automatic voltage regulator	Matsunaga (Japan)
Spectrophotometer	Alpha Innotech Corporation (Germany)

B. MICROBIOLOGICAL MEDIA

Blood agar	Mueller Hinton broth
MacConkey agar	Simmons Citrate agar
Salmonella shigella agar	
Chocolate agar	
Thiosulfate citrate bile salt	Hugh Leiffson media
sucrose agar (TCBS)	Nitrate broth
MR-VP medium	Sulphur Indole Motility (SIM) agar
Mueller Hinton agar	Triple Sugar Iron agar
Nutrient agar	Urea agar
Luria-Berteni agar / broth	Bile esculin agar

C. CHEMICALS AND REAGENTS

3 % Hydrogen peroxide	Barritt's reagent	Barium chloride
Crystal violet	Absolute (95%) alcohol	Kovac's reagent
Gram's iodine	Phenol (equlibriated)	Sulphuric acid
Safranine	Tris base	Chloroform
Sodium hydroxide	Ethylene diamine	Sodium Chloride

Glacial acetic acid
Glucose
Bromophenol blue
Sucrose
Agarose
tetra acetate(EDTA)
Ethidium bromide
SDS
Whatmann filter paper no1
Tris buffer
Potassium acetate
Glycerol

D. ANTIBIOTIC DISKS

The antibiotics disks used for the susceptibility tests were from Hi-Media Laboratories Pvt. Limited, Bombay, India were as follows

Amoxycillin (30mcg)
Ceftriazone(30mcg)
Ceftazidime(30mcg)
Cefixime (30mcg)
Ciprofloxacin (5mcg)
Cloxacillin (1mcg)
Cofoxitin (30mcg)
Amikicin (30mcg)
Cotrimoxazole (1.25/23.75mcg)
Erythromycin (15mcg)
Gentamicin (10mcg)
Nitrofurantoin (300mcg)
Norfloxacin (10mcg)
Ofloxacin (5mcg)
Gentamycin(10mcg)
Vancomycin(30mcg)
Oxacillin (1mcg)

Antibiotics used from Oxoid Limited, Basingstoke, Hampshire, England were
Ceftazidime(30mcg)
Cefotaxime(30mcg)
Co-amoxyclav(20mcg:10mcg)

Antibiotics used from Becton,Dickinson andCompany, Sparks, MD21152,USA were
Imipenem (10mcg)
Meropenem (10mcg)

E. ANTIBIOTIC POWDER

The antibiotic powders used for the determination of MICs were from Hi-Media Laboratories Pvt. Limited, Bombay, India and National Drug Laboratory, Bijulibazar, Kathmandu , Nepal were as follows

Ampicillin
Ciprofloxacin
Cefixime
Chloramphenicol
Norfloxacin
Streptomycin
Trimethoprim
Gentamicin

E. MISCELLANEOUS

E.coli ATCC 25922 culture,Punching machine(with 6 mm hole),Conical flasks, Cotton, Distilled water, Droppers, Forceps, Glass slides and cover slips, Immersion oil, Inoculating loop, Inoculating wire, Lysol, Measuring cylinder,Erlenmeyer flask, Petri dishes, Pipettes, Plastic containers, Spatula, Test tubes, Wooden applicator sticks

APPENDIX-III

I. Composition and preparation of different culture media

The culture media used were from Hi-Media Laboratories Pvt. Limited, Bombay, India.
(All compositions are given in grams per liter and at 25^0C temperature)

1. Blood agar (BA)

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

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

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

2. MacConkey Agar (MA)

(With sodium taurocholate, with salt and crystal violet)

Ingredients	gm/liter
Peptone	20.0
Lactose	10.0
Sodium taurocholate	5.0
Crystal violet	0.015
Sodium chloride	5.0
Neutral Red	0.04
Agar	20.0
Final pH (at 25^0C) 7.4±0.2	

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

3. Mueller- Hinton Agar (MHA)

Ingredients	gm/liter
Beef, Infusion form	300.0
Casein Acid Hyrolysate	17.5
Starch	1.5
Agar	17.0

Final pH (at 25^0C) 7.4±0.2

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

4. Nutrient Agar (NA)

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

Final pH (at 25^0C) 7.4±0.2

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

5. Nutrient Broth (NB)

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

Final pH (at 25^0C) 7.4±0.2

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

6. Chocolate Agar

The sterilized blood agar was poured in Petri plates and was allowed to solidify and was heated at 750C in an oven for 30 minutes. By this time, the color changes to chocolate brown.

7. Thiosulfate citrate bile salt sucrose (TCBS) agar

Ingredients	gm/litre
Yeast extract	5
Bacteriological peptone	10
Sodium thiosulfate	10
Ox bile	8
Sodium citrate	10
Sucrose	20
Sodium chloride	10
Ferric citrate	1
Bromothymol blue	0.04

Thymol blue	0.04
Agar	15
P^H 8.4 – 8.8 at 37^0C	

To 1000 ml of distilled water 8.8 grams of the medium was suspended and then boiled to dissolve completely

8. Mannitol Salt agar

Ingredients	gm/litre
Protease peptone	10
Beef extract	1
NacL	75
D-Mannitol	10
Phenol red	0.025
Agar	15
P^H	7.4 ± 0.2

One hundred and eleven grams (111 g) of the medium was boiled in 1000 ml of distilled water to dissolve completely and then atuclaved.

9. Salmonella Shigella (SS) agar

Ingredients	gm/litre
Peptic digest of animal tissue	5
Beef extract	5
Lactose	10
Bile salt mixture	8.5
Sodium citrate	10
Sodium thiosulfate	8.5
Agar	15
Neutral red	0.025
Ferric citrate	1
Brilliant green	0.00033
Final P^H	7.0± 0.2

Sixty three (63 g) of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely.

10. Lauria- Bertani Broth/ Agar

Tryptone	10g
Yeast extracts	5g
Nacl	10g
*[Agar	15g (for agar plates)]

* 15 grams agar added in the case of preparation of LB agar

11. LB broth

All the ingredients were added to a clean 2 L flask that has been rinsed with deionised or distilled water.1 l deionized or distilled water was added to the flask. 0.5 ml of 4-M NaOH was added.The dry ingredients were stirred to dissolve, preferably using a magnetic stir bar.The preparation was autoclaved for 20 minutes at 121^0C.

12. LB broth + antibiotic (can be stored at 4^0C for 3 months)

1 ml of 10 mg/ml antibiotic was sterilely added to 100 ml of cooled LB broth.The preparation was swirled to mix.

13. LB agar + antibiotic (can be stored at 4^0C for 2 months) (1000 ml)

LB agar solution was made from the ingredients listed above and autoclaved.When the agar flask was cool enough to hold, 10ml of 10mg /ml antibiotic solution was added sterilely. The flask was swirled to mix the antibiotic.The media solution was poured on the sterile Petri-plates.

14. Tryptic Soy broth + 20 % Glycerol

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

Fourty gram (40 g) of the powder was suspended in 800 ml distilled water containing 200 ml glycerol and mixed thoroughly. It was heated with frequent agitation and boiled for 1 minute to completely dissolve the powder. If was autoclaved at 121°C for 15 minutes.

15. Bile esculin agar

Ingredients	gm/litre
Peptone	5.0
Beef extract	3.0
Oxgall (bile)	40.0
Esculin	3.0
Ferric citrate	0.5
Agar	15
P^H (at 25^0C) 6.6 ± 0.2	

To 1000 ml of distilled water, 64.5 g of the medium was suspended then boiled to dissolve completely and autoclaved.

II. Biochemical Test Media

1. MR-VP Medium

Ingredients	gm/litre

Buffered Peptone	7.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 25^0C)	6.9±0.2

Seventeen (17 g) was dissolved in 1000 ml distilled water. 3 ml of medium was distributed in each test tube and autoclaved at 121^0C for 15 minutes.

2. Hugh and Leifson's Medium

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

To 1000 ml cold distilled water 9.4 grams of the medium was rehydrated and then heated to boiling to dissolve completely and autoclaved for 15 minutes at 15 lbs pressure (121^0C).

3. Sulphide Indole Motility (SIM) medium

Ingredients	gm/litre
Beef Extract	3.0
Peptone	30.0
Peptonized Iron	0.2
Sodium Thiosulphate	0.025
Agar	3.0
Final pH (at 25^0C)	7.3±0.2

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

4. Simmon Citrate Agar

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

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

5. Triple Sugar Iron (TSI) Agar

Ingredients	gm/litre
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Beef Extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0
Final pH (at 25^0C)	7.4±0.2

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

6. Christensen Urea Agar

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

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

III. Staining and Test Reagents

1. For Gram's Stain

(a) Crystal Violet solution

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

Distilled Water (D/W) to make 1 litre

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

(b) Lugol's Iodine

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

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

(c) Acetone-Alcohol Decoloriser

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

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

(d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

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

2. Normal saline

Sodium Cholride	0.85 g
Distilled Water	100 ml

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

3. Test Reagents

a. For Catalase test

Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

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

b. For Oxidase Test

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

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

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

c. For Indole Test

Kovac's Indole Reagent

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

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

d. For Methyl Red Test

Methyl Red Solution

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

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

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

Solution A

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

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

Solution B

Potassium hydroxide	40.0 g
Distilled Water	1000 ml

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

5. Chemicals of plasmid DNA analysis

a.Tris- acetate EDTA buffer (50X) (Electrophoresis buffer)

Tris base	242g

Glacial acetic acid	57.1ml
0.5 M EDTA	100 ml (p^H 8.0)
Working solution 1X.	

b. 6X gel loading buffer (100 ml)

Bromophenol blue	0.25%
Sucrose in water	40% (w/v)

c. 0.5 M Ethylene Diamine Tetra acetic acid (EDTA) (p^H 8.0) 100ml (can be stored at room temperature indefinitely).

18.6 g of EDTA (disodium salt, m. w. 372.24) was added to 80 ml of deionized or distilled water.The p^H was adjusted to 8.0 by slowly adding the sodium hydroxide.The preparation was mixed vigorously using a magnetic stirrer.

d. 5 M potassium acetate (200 ml, store at room temperature.)

98.1 g potassium acetate was added to 160 ml of distilled water.Distilled water was added to make final volume of 200ml.

e.10% Sodium Dodecyl Sulfate (100ml, store at room temperature.)

10 g of electrophoresis grade SDS (m.w. 288.37) was dissolved in 80 ml of distilled water.the final volume was made 100 ml.

f. Solution I (Glucose / Tris / EDTA (GTE) (100 ml) store at room temperature)

Glucose	0.9g (m.w. 180.16)
Tris	2.5 ml of 1 M (p^H 8.0)
EDTA	2 ml of 0.5M
Water	94.5 ml

g. Solution II (1% SDS/ 0.2 N NaOH, for 10ml)

SDS	1 ml (10%)
NaOH	0.5ml (4N)
Water	8.5 ml

h. Solution III (Potassium acetate / acetic acid 100ml, store at room temperature)

11.5 ml of glacial acetic acid was mixed with 60 ml of 5M potassium acetate and 28.5 ml distilled water.

i. Solution IV (phenol/chloroform (v/v))

Phenol	50 ml (equilibrated)
Chloroform	50 ml

j. Solution V(Tris EDTA buffer 100 ml store at room temperature)

Tris	1 ml of 1M (p^H 8.0)	
EDTA	200µl of 0.5M Water	99 ml

APPENDIX-IV

A. Gram-staining Procedure

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

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

APPENDIX-V

1. BIOCHEMICAL TESTS FOR IDENTIFICATION OF BACTERIA

METHODOLOGY OF BIOCHEMICAL TESTS USED FOR IDENTIFICATION OF BACTERIA

A. Catalase test

This test is performed to demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide.

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

B. Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (Whatman's No. 1 filter paper impregnated with 1% tetramethyl-p-phenylene diamine dihydrochloride). Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

C. 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 indole acetic acid.

Procedure: A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37°C for 24 hours. After 24 hours incubation, 2-3 drops of Kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

D. Methyl Red test

This test is performed to test the ability of an organism to produce and maintain stable acid end product from the fermentation of glucose to give a red color with the indicator methyl red and to overcome the buffering capacity of the system.

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

E. Voges-Proskauer (VP) test

The principle of this test is to determine the ability of some organisms to produce a acetyl methyl carbinol, a neutral end product (acetoin) or its reduction product 2, 3-butanidiol during fermentation of carbohydrates.

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

F. Citrate Utilization test

This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity.

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

G. Motility test

This test is done to determine if an organism was motile or non-motile. Bacteria are motile by means of flagella. Flagella occur primarily among the bacilli; however a few cocci forms are motile .Motile bacteria may contain a single flagella. The motility media used for motility test are semisolid, making motility interpretations macroscopic.

Procedure: Motility of organism was tested by hanging drop and cultural method. In cultural method, the test organism was stabbed in the SIM medium and incubated at 37ºC for 48 hours. Motile organisms migrate from the stabline and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stabline, and the surrounding media remains colorless and clear.

H. 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). **Procedure:** The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours .Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

a. **Yellow (Acid)/ Yellow (Acid), Gas, H_2S → Lactose/ Sucrose fermenter, H_2S producer.**
b. **Red (Alkaline) / Yellow (Acid), No Gas, No H_2S → Only Glucose, not lactose/ Sucrose fermenter, not aerogenic, No H_2S production.**
c. **Red (Alkaline) / No Change → Glucose, Lactose and Sucrose non-fermenter.**
d. **Yellow (Acid)/ No Change → Glucose- Oxidiser.**
e. **No Change / No Change →Non-fermenter.**

I. Urea Hydrolysis test:

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

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

J. Coagulase test

This test is used specifically to differentiate species within the genus *Staphylococcus*: *S. aureus* (usually positive) from *S. saprophyticus, 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 possess both free and bound coagulase.

Slide Coagulase Test

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

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

Tube Coagulase Test

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

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

K. Optochin Sensitivity test

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

L. Bacitracin Sensitivity test

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

M. Mac Farland standard 0.5

It is prepared by adding 0.6 ml of 1% w/v barium chloride solution to 99.4 ml of 1% v/v solution of sulphuric acid.

APPENDIX-VI

Table: Distinguishing reactions of the commoner and pathogenic Enterobacteriaceae

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

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

[c] *Yersinia* are motile at 22°C.{Key: +, ≥85% of strains positive; -, ≥ 85% of strains negative; 16-84% of strains are positive after 24-48 hour at 36°C}(Source: Collee *et al.* 1996)**.**

APPENDIX-VII

ZONE SIZE INTERPRETATIVE CHART

Antimicrobial Agents used	**Symbol**	**Disc Content**	**Resistant (mm or less)**	**Intermediate (mm)**	**Susceptible (mm or more)**
Amikacin	Ak	30 µg	14	15-16	17
Amoxycillin When testing gram-negative enteric organisms When testing Staphylococci	Amp	30 µg	13 28	14-16 -	17 29
Ceftazidime	Ca	30 µg	14	15-17	18
Ceftriaxone	Ci	30 µg	13	14-20	21
Cephalexin	Cp	30 µg	14	15-17	18
Chloramphenicol	C	30 µg	12	13-17	18
Ciprofloxacin	Cf	5 µg	15	16-20	21
Cloxacillin	Cx	5 µg	12	12-13	14
Cotrimoxazole	Co	1.25/23.75µg	10	11-15	16
Erythromycin	E	15 µg	13	14-22	23
Imipenem	I	10 µg	13	14-15	16
Gentamicin	G	10 µg	12	13-14	15
Meropenem	Mr	10µg	13	14-15	16
Nitrofurantoin	Nf	300µg	14	15-16	17
Norfloxacin	Nx	10 µg	12	13-16	17
Ofloxacin	Of	5 µg	12	13-15	16
Oxacillin	Ox	1 µg	10	11-12	13
Vancomycin When testing Staphylococci When testing Streptococci	Va	30 µg	- -	- -	15 17

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

APPENDIX-VIII

A. Doubel Disk Synergy Test (DDST) for ESBL confirmation

Confirmation of the suspected ESBL-strains was performed according to the guidelines for phenotypic confirmatory testing issued by NCCLS in 2005. According to these guidelines, the zone diameter for possible ESBL strains is $\leq$ 27mm for cefotaxime (30 µg), and $\leq$ 22mm for ceftazidime (30 µg). The suspected ESBL strains were tested for confirmation by using the double disk diffusion synergy test method, using co-amoxiclav 20+10 µg disk and cefotaxime and ceftazidime 30 µg disks placed 20-30 mm away from it. ESBL production was confirmed when the zone of *either* cephalosporin was expanded by the clavulanate

1. Suspected strain of ESBL producing organism was inoculated in nutrient broth and I ncubated for 4-6 hours.
2. The standard inoculum size was carpet cultured onto MHA plates.
3. After few minutes the plates were incorporated with separate ceftazidime and cefotaxime disks and co-amoxiclav dikc.
4. After overnight incubation, the results were interpreted as stated above.

B. AmpC Disk test for confirmation of ABL

1 A lawn of ATCC 25922 *E. coli* was made on MHA plate.
2 A cefoxitin disk was placed on the lawn of MHA plates.
3. The two AmpC disks were inserted almost touching the cefoxitin after first moistening with normal saline and then inoculating with the heavy inocula of test organism grown in MHA.
4. The plate was incubated at 37^0C for 24 hours.
5. The zone of distortion, indentation, uniform growth or zone of inhibition of cefoxitin towards *E. coli* ATCC 25922 lawn was observed.

C. Resut Interpretation

S.N..	Change around the Cefoxitin disk	Remarks
1	Uniform growth	Hyper AmpC β-lactamase producer
2	Indentation towards Cefoxitin	AmpC β-lactamase producer
3	Flattening of Zone of inhibition of Cefoxitin	Weak AmpC β-lactamase producer
4	No distortion or indentation	Non AmpC β-lactamase producer

D. Preparation of AmpC disk

1. 6 mm disks were made from Whatmann filter paper no.1
2. All disks were sterilized by autoclaving.
3. A mixture of solution of 100X Tris and EDTA was made.

4. 12µl of mixture was loaded to each disk (Volume was determined by absorbance of disk).
5. Discs were dried in oven and placed them in a sterile vial and kept at 2-8^{0}C until use.

C. EDTA Disk Synergy Test (EDST) for MBL confirmation

EDST technically simple and inexpensive method for detection of MBL production.We used the metallo-beta-lactamase inhibitor EDTA. Given the fact that all MBLs are affected by the removal of zinc from the active site, in principle, their detection should be straightforward, and studies have seized upon this principle and used a variety of inhibitor-ß-lactam combinations to detect strains possessing these clinically important enzymes.

Method:

1. Amp C disk test negative and intermediate strains were selected and studied their meropenem and imipenem hydrolyzing pattern.
2 At least one carbapenem resistant, intermediate or hydrolyzing isolate is subjected for Disk inhibition test.
3 A colony of each bacterial strain was suspended and diluted with Mueller-Hinton (MH) broth to 10^6 CFU/ml and spread on an MH agar plate with a cotton swab according to the protocol recommended by the National Committee for Clinical Laboratory Standard.
4 Two commercially supplied Kirby-Bauer (KB) disks, each containing 30 µg of CAZ (HiMedia Laboratories Pvt Lt.,Mumbai,India),are then placed on the plate.
5 The distance between the two CAZ disks are kept at about 4 to 5 cm and a filter disk is placed near one of the CAZ disks within a center-to-center distance of 1.0 to 2.5 cm
6. Five microliters of inhibitor solution (100 mM EDTA) added to the filter disk on the agar.
7 The agar plate is incubated at 37°C overnight.

Result interpretation:

Metallo β-lactamase producer ⟶ Increased growth-inhibitory zone between the two disks (EDTA disk and CAZ disk)

Hyper AmpC producer, ESBL producer ⟶ No change of growth –inhibitory zone
Serine β- lactamase producer between the two disks

APPENDIX-IX

A. Extraction of plasmid DNA

1. 1.5 ml of MDR *E. coli* culture was transferred to a clean microfuge tube and centrifuged at 10000 rpm.
2. The supernatant was discarded and again the *E. coli* culture was transferred to the same tube and centrifuged to collect cells from 3 ml of culture.
3. The supernatant was discarded and any residual media was blotted away.
4. 100 µl of ice cold GTE (solution I) was added to the tube. The cells pellet was resuspeded by pipetting ins and pipetting out or by vortexing.
5. 200 µl of SDS/NaOH (solution II) was added. The contents were mixed by closing and rapidly inverting the tubes for five times. The tube was incubated on ice for 5 minutes.
6. 150 µl of ice cold Potassium acetate was added. The contents were mixed by closing and rapidly inverting the tubes for five times.White precipitate appeared.
7. The tube was incubated on ice for 5 minutes.
8. The tubes were centrifuged at 13000 rpm for 10 minutes at 4^0C.
9. 400 µl of supernatant was transferred to a clean microfuge tube avoiding the precipitate.
10. An equal volume of phenol:chloroform was added to the tube and shaked vigorously to denature the protein and form an emulsion.
11. The tube was centrifuged at full speed for 2-3 min to separate the phases.
12. The upper, aqueous phase was decanted to a clean microfuge tube, taking care to avoid carryout of the lower organic phase.
13. Two volumes of ice cold 95% ethanol was added, mixed well and placed on the ice for 30 min.
14. The preparation was centrifuged in the refrigerated centrifuge at full speed for 10 min.
15. The supernatant was poured off and immediately washed the pellet by adding 1000µl cold 70% ethanol and gently inverted tube 5-10 times.
16. It was centrifuged at full speed for 2 minutes and poured off supernatant.
17. The tube was left open for 10-20 min. in room temperature to permit ethanol evaporation .
18. About 50-100 µl TE buffer was added to tube and gently dissolved the pellet by making tip up and down. The neutralized plasmid DNA solution was stored at 4^0C until use.

B. Agarose gel electrophoresis:

i Preparation of agarose gel (0.5%)

1. 0.5g of agarose was weighed out into a 250mL conical flask. 100mL of 1X TAE was added and swirled to mix.
2. The preparation was microwaved for about 1 minute to dissolve the agarose.
3. It was left to cool on the bench for 5 minutes down to about 60°C.

4. 5µL of ethidium bromide was added (10mg/mL) and swirled to mix.
5. The gel was poured slowly into the tank. Any bubbles were pushed away to the side using a disposable tip. The comb was inserted and double checked that it is correctly positioned.
6. It was left to set for at least 30 minutes, preferably 1 hour, with the lid on if possible.
7. 1X TAE buffer was poured into the gel tank to submerge the gel to 2–5mm depth. This is the running buffer.

ii. preparation of sample

15 µL of each sample was transferred to a fresh microfuge tube.An appropriate amount (3 µL) of 6X gel loading buffer was added into each tube and left the tip in the tube.Similarly the DNA markers were prepared.

iii.Loading of sample in the wells

The first well was loaded with 15 µL marker.The samples (15 µL) were loaded in the adjacent wells.

iv. Electrophoresis

1. The gel tank was closed, the power-source was switched on and the gel was run at 60V for 4 hours.
2. Current flowing was checked.

v. Monitoring

1. The progress was monitored on the gel by reference to the marker dye.
2. The gel was stopped when the bromophenol blue had run $3/4^{th}$ the length of the gel.
3. The power was switched off.

vi Photodocumentation (Visualization)

1. The gel was carried (in its holder if possible) to the room to look at on the UV trans-illuminator.
2. The gel was illuminated at 300nm UV and the picture was saved for analysis.

vii.Decontamination of ethidium bromide

Sufficient water was added to reduce the concentration of EtBr to < 0.5 ug/ml.One volume of 0.5 M $KMnO_4$, one of volume of 2.5 N HCl was mixed and it was allowed to stand for several hours at room temperature.1 volume of 2.5 N NaOH was mixed and the solution was discarded.

C. Transformation

1. A single bacterial colony *(E. coli* TB1) was picked from a plate that had been incubated for 16-20 hours at 37^0C.The colony was transferred into 10 of LB broth.

The culture was incubated for 3 hours at 37°C with vigorous shaking monitoring the growth of the culture until OD_{600} ~0.4 was achieved.

2. The bacterial cells were transferred to sterile eppendorf tube. The cultures were cooled to 0^0C by storing the tubes on ice for 10 minutes.

i. **Preparation of competent cells**

1. 1.5 ml of the chilled bacterial culture was transferred to a sterile microfuge tube prechilled on ice.
2. The tube was centrifuged at 0^0C for 90 seconds at 10000 rpm.
3. The supernatant was poured off and, while the tube was inverted, any residual growth medium was quickly blotted away. The tube was immediately returned to the ice.
4. Steps 1-3 above were repeated using the same microfuge tube.
5. Cell pellet was re-suspended gently in 1000µl of ice cold competent cell buffer1 (20mM $CaCl_2$ and 80mM Mg Cl_2) and then centrifugeed for 90 seconds at 10000 rpm.
6. The supernatant was poured off and the tube was returned immediately to the ice.
7. Cell pellet was re-suspended gently in 200µl of ice cold competent cell buffer 2 (100mM $CaCl_2$).
8. The cell suspension was returned to the ice bath until use in the transformation.

ii. **Transformation of plasmid DNA**

1. 75µl of the competent cell preparation was transferred to the bottom of the sterile plastic tube that had been pre-chilled on ice. The tube was marked for identification.
2. 10µl of the plasmid DNA (0.5ug/ml) preparation was pipetted into the competent cell suspension in the bottom of the tube.
3. The tube was incubated on ice for 10 minutes (to permit binding of the DNA to the cells).
4. The tube was transferred to 42^0C water bath for 90 seconds and then immediately placed it on ice.
5. 900µl sterile Lauria Bertani broth was added to the tube and incubated with shaking at 37^0C for 30-40 minutes.
6. 100µl of the transformation mixture was pipetted onto the surface of L+AMP+S, L+CIP+S, L+TR+S agar. The inoculum was spread over the plate surface using a glass spreader sterilized with flaming ethanol.
7. The plates were incubated at 37^0C for 16-18 hours and the antibiotic resistant colonies were counted.

D. CONJUGATION

1. The resistant isolates were grown in 5 ml nutrient broth for $4^{1/2}$ with constant shaking (250 rpm).
2. At the same time the recipient strain was also grown in the nutrient broth at 37^0C for $4^{1/2}$ hours with constant shaking.
3. Then the conjugation mixture was prepared having following composition:
   0.2 ml Donor culture
   2.0 ml Recipient culture
   2.5 ml Warm nutrient broth
4. Then the mixture was incubated at 37^0 C for 24 hours.

5. Transconjugants were selected by plating in suitable media containing appropriate antibiotics.

E. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

1. Preparation of antibiotic stock solutions

- Informations regarding expiry date, potency, solubility, stability as a powder and in solution, storage conditions and any relevant COSHH (Control of Substances Hazardous to Health) information were obtained from the supplier.
- Appropriate solvent, diluents and storage conditions for different antibiotics used viz.ampicillin, chloramphenicol, cefixime, ciprofloxacin, trimethoprim, norfloxacin, gentamycin and streptomycin were chosen.
- Suitable range of antibiotic concentrations for the test organisms (Drug resistant donor strains, transconjugants and transformants) were made.

- Stock solutions were prepared using the formula

$$\frac{1000}{P} \times V \times C = W$$

Where, P = Potency given by the manufacturer (µg/mg), V = volume required (mL), C = final concentration of solution (multiples of 1000) (mg/L), and W = weight of antibiotic (mg) to be dissolved in volume V (mL).

2. Preparation of antibiotic dilution range

Dilution range :- 16 -1024 µg/ml

- Ten screw capped tubes were labeled according to antibiotic dilution. A parallel set of tubes containing dilution range for different antibiotics were taken to examine the potency of antibiotics using the reference strain *E. coli* ATCC 25922. Antibiotic ranges should be prepared one step higher than the final dilution range required for the coimpensation of the addition of an equal volume of inoculum.
- One (1ml) Mueller-Hinton broth was substituted for each tubes i.e. tube 1 to tube 10.

3. Preparation of inoculum

Bacterial suspensions that have been matched the turbidity of the 0.5 McFarland standard (i.e., 1.5 X 10^8 CFU/ml) served as the inoculum. Preparation of 0.5 McFarland standard is mentioned in appendix V.

Ten microliter (10 µl) inoculum were added to each tube so that final concentration of inocula used was made 5 X 10^5 CFU/ml.
All the tubes were incubated for 16-20 hrs at 35^0C .MICs end point for test organisms were determined by examining lowest concentration of antibiotic at which there is no visible growth. The MIC of the reference strain was found within one, two- fold dilution of expected MIC as given by CLSI.

APPENDIX-X

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

Type of patients	Male			Female			TOTAL (Isolate %) (Isolate)	MDR (Isolate %)
	No. (%)	Isolates (%)	MDR (%)	No. (%)	Isolates (%)	MDR (%)		
Outdoor	628 (41.78)	104 (7.47)	38 (36.54)	765 (50.9)	187 (13.43)	68 (65.38)	1393 (20.9) (291)	106 (36.42)
Indoor	56 (3.72)	23 (20.9)	14 (60.87)	54 (3.6)	22 (20)	18 **(78.26)**	110 (40.9) (45)	32 **(71.11)**
TOTAL	684 (45.5	127 (8.45)	52 (40.94)	819 (54.5)	209 (13.9)	86 (41.15)	1503 (22.35) (336)	138 (41.07)

Table 2: Age and gender wise distribution of patients requesting for culture and their growth pattern

Age group (in years)	Outdoor Patients		Indoor Patients		TOTAL (Isolates)	(Isolate%)
	Male (Isolates)	Female (Isolates)	Male (Isolates)	Female (Isolates)		
0-10	33(4)	24(3)	1(1)	4(1)	62(9)	**14.52**
11-20	126(21)	97(18)	9(4)	3(1)	235(44)	**18.72**
21-30	189(37)	269(65)	13(5)	18(7)	489(114)	**23.31**
31-40	81(11)	105(29)	10(7)	7(4)	203(51)	**25.12**
41-50	62(7)	84(18)	1(0)	0(0)	147(25)	**17.00**
51-60	45(7)	65(14)	3(2)	4(3)	117(26)	**22.22**
61-70	50(6)	65(23)	8(1)	15(5)	138(35)	**25.36**
71-80	26(7)	33(9)	7(1)	2(1)	68(18)	**26.48**
81-90	9(1)	9(3)	4(2)	0(0)	22(6)	**27.28**
>90	7(3)	14(5)	0(0)	1(0)	22(8)	**28.58**
TOTAL	**628 (104)**	**765 (187)**	**56 (23)**	**54 (22)**	**1503 (336)**	**22.35**

Table 3: Pattern of bacterial isolates from different samples

anisms	Blood	Pus	Fluid	Sputum	CSF	Tissue	Throat swab	Perianal swab	Urine	Total
Salmonella Typhi	27	0	0	0	0	0	0	0	1	**28**
S. Paratyphi A	24	0	0	0	0	0	0	0	0	**24**
Streptococcus pneumoniae	0	0	0	1	0	0	0	0	0	**1**
S. pyogens	0	0	0	0	0	0	1	0	0	**1**
Escherichia coli	0	11	1	0	0	0	0	3	178	**193**
Pseudomonas aeruginosa	0	3	0	0	0	1	0	0	2	**6**
Citrobacter freundii	0	4	0	0	0	0	0	0	8	**12**
C. diversus	0	2	0	0	0	0	0	0	3	**5**
Enterobacter cloacae	0	3	0	0	0	0	0	0	1	**4**
E. aerogenes	0	0	0	0	0	0	0	0	3	**3**
Proteus vulgaris	0	1	0	0	0	0	0	0	0	**1**
P.mirabilis	0	2	0	0	0	0	0	0	3	**5**
Staphylococcus aureus	0	16	0	0	0	0	0	0	1	**17**
Neisseria meningitidis	0	0	0	0	1	0	0	0	0	**1**
Neisseria gonorrheae	0	0	1	0	0	0	0	0	0	**1**
Staphylococcus saprophyticus	0	12	0	0	0	1	0	0	2	**15**
S. epidermidis	0	2	0	0	0	0	0	0	4	**6**
Klebsiella oxytoca	0	0	0	0	0	0	0	0	2	**2**
K. pneumoniae	0	0	0	0	0	0	0	0	4	**4**
Streptococcus faecalis	0	0	0	0	0	0	0	0	3	**3**
Morganella morganii	0	0	0	0	0	0	0	0	2	**2**
Acinetobacter spp.	0	0	0	0	0	0	0	0	2	**2**
TOTAL	**51**	**56**	**2**	**1**	**1**	**2**	**1**	**3**	**219**	**336**

Table 4: Pattern of bacterial growth according to Gram's stain

Isolated organisms	Blood	Pus	Fluid	Sputum	CSF	Tissue	Throat swab	Perianal swab	Urine	TOTAL (%)
Gram-negative bacteria	51	26	2	1	1	1	0	3	209	**294 (87.5)**
Gram-positive Bacteria	0	30	0	0	0	1	1	0	10	**42 (12.5)**
Total	**51**	**56**	**2**	**1**	**1**	**2**	**1**	**3**	**209**	**336**

Table 5: Age wise distribution of pathogens and MDR strains from different samples

Organisms	**Age of the patient (in years)**									
	<10	**11-20**	**21-30**	**31-40**	**41-50**	**51-60**	**61-70**	**71-80**	**81-90**	**>90**
Salmonella Typhi	2(0)	11(0)	10(1)	4(0)	1(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Paratyphi A	0(0)	6(0)	14(0)	3(0)	1(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Streptococcus pneumoniae	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(1)	0(0)	0(0)
S. pyogenes	0(0)	0(0)	0(0)	1(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Escherichia coli	6(2)	15(5)	61(21)	24(14)	16(8)	21(14)	29(13)	12 (7)	3(3)	5(2)
Pseudomonas aeruginosa	0(0)	0(0)	2(1)	2(1)	1(1)	1(0)	0(0)	0(0)	0(0)	0(0)
Citrobacter freundii	0(0)	2(1)	3(3)	3 (3)	1(1)	0(0)	0(0)	1(0)	1(1)	1(0)
C. diversus	0(0)	2(0)	0(0)	0(0)	0(0)	1(0)	1(1)	1(1)	0(0)	0(0)
Enterobacter cloacae	0(0)	0(0)	2(2)	2(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. aerogenes	0(0)	1(1)	1(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(0)
Proteus vulgaris	0(0)	0(0)	1(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
P. mirabilis	0(0)	0(0)	3(1)	2(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Staphylococcus aureus	0(0)	3(1)	8(4)	3(2)	1(0)	0(0)	0(0)	1(1)	1(0)	0(0)
Neisseria meningitidis	0(0)	1(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Neisseria gonorrheae	0(0)	0(0)	0(0)	1(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Staphylococcus saprophyticus	0(0)	3(1)	5(1)	1(0)	1(1)	2(1)	1(0)	1(0)	1(1)	0(0)
S. epidermidis	1(1)	0(0)	1(1)	3(2)	0(0)	0(0)	1(0)	0(0)	0(0)	0(0)
Klebsiella oxytoca	0(0)	0(0)	0(0)	0(0)	0(0)	1(0)	0(0)	0(0)	0(0)	1(1)
Streptococcus faecalis	0(0)	0(0)	1(1)	0(0)	0(0)	0(0)	2(2)	0(0)	0(0)	0(0)
Morganella morganii	0(0)	0(0)	0(0)	2(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Acinetobacter spp.	0(0)	0(0)	1(1)	0(0)	0(0)	0(0)	0(0)	1(0)	0(0)	0(0)
K. pneumoniae	0(0)	1(1)	1(1)	2(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

Total	**9(3)**	**45(10)**	**114(38)**	**53(27)**	**23(11)**	**26(15)**	**34(16)**	**18(10)**	**6(5)**	**8(3)**

Table 6: Antibiotic susceptibility pattern of Gram-negative bacterial pathogens

Organisms		**Ak**	**Am**	**Cfx**	**Cn**	**Ca**	**Ci**	**Ce**	**C**	**Cf**	**Co**	**G**	**I**	**Mr**	**Na**	**Nf**	**Nx**	**Of**
S. Typhi	S	-	27	28	-	-	27	28	27	26	28	-	-	-	20	0	0	27
	R	-	1	0	-	-	0	0	0	2	0	-	-	-	6	1	1	0
S. Paratyphi A	S	-	24	24	-	-	24	24	24	13	24	-	-	-	4	-	-	24
	R	-	0	0	-	-	0	0	0	11	0	-	-	-	20	-	-	0
E. coli	S	57	85	132	6	5	8	131	36	120	105	21	51	51	1	162	114	116
	I	3	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
	R	3	108	61	55	53	55	62	13	73	88	42	0	0	1	14	66	64
Citrobacter freundii	S	3	2	3	1	3	3	5	1	4	2	3	5	3	-	3	3	4
	I	0	0	0	0	0	0	0	0	2	0	0	0	0	-	1	0	1
	R	3	10	8	7	6	6	7	3	6	9	3	0	2	-	4	4	3
C. diversus	S	3	3	3	0	3	2	3	1	3	2	2	1	1	-	2	1	2
	I	0	0	0	0	0	0	0	0	1	0	0	0	0	-	0	0	0
	R	0	2	2	2	1	1	2	0	1	2	1	0	0	-	1	2	1
Enterobacter cloacae	S	1		1	0	0	0	1	-	4	0	1	1	1	-	1	1	1
	I	1	0	0	0	0	0	0	-	0	0	0	0	0	-	0	0	0
	R	1	4	3	1	2	3	3	-	0	4	2	0	0	-	0	0	0
E. aerogenes	S	-	2	2	0	-	-	2	-	3	3		1	1	-	2	3	3
	R	-	1	1	1	-	-	1	-	0	0		0	0	-	1	0	0
Proteus vulgaris	S	1	0	-	-	-	1	1	-	1	1	1	-	-	-			1
	R	0	1	-	-	-	0	0	-	0	0	0	-	-	-		3	0
P. mirabilis	S	2	3	4	1	0	0	4	-	4	3	2	-	-	-	1	4	4
	I	0	0	0	0	0	1	1	-	1	0	0	0	0	-	0	0	0
	R	0	2	0	0	1	1	0	-	0	2	0	-	-	-	2	0	0
K. pneumoniae	S	-	0	3	1			3	-	1			-	-	-	0		-
	R	-	4	1	1			1	-	0			-	-	-	4		-
Klebsiella oxytoca	S	1	0	1	1	0	0	1	1	1	1	0	1	1	-	1	1	1
	I	0	0	0	0	0	0	0	0	0	0	0	0	0	-	0	1	0
	R	0	2	1	1	1	1	1	0	1	1	1	0	0	-	0	1	1
Neisseria gonorrheae	S	-	-	1			1	1	-	1	1	1	-	-	-	-	-	1
	R	-		0			0	0	-	0	0	0	-	-	-	-	-	0
Neisseria meningitidis	S	1	-	1		1	1	1	-	1	0	1	-	-	-	-	-	-
	R	0		0		0	0	0	-	0	1	0	-	-	-	-	-	-
P. aeruginosa	S	5		2	0	2	3	3	0	4	3	4	2	3	-	1	0	0
	I	0	0	0	0	1	0	2	0	0	0	0	0	0	-	0	0	0
	R	0		4	3	2	2	1	1	2	3	1	1	0	-	0	1	1
Acinetobacter spp.	S	-	1	0	0	-	-	1	-	1	1	-	1	1	-	0	1	1
	I	-	0	1	1	-	-	0	-	0	0	-	0	0	-	0	0	0
	R	-	1	1	0	-	-	1	-	0	1	-	-	-	-	2	1	1
Morganella morganii	S	-		1	-	-	-	1	-	1	1	-	-	-	-	0	1	1
	R	-	1	0	-	-	-	0	-	0	0	-	-	-	-	1	0	0

Abbreviations: Ak-amikacin; Am-amoxycillin; Cfx-cefixime; Cn- cefoxitin; Ca- ceftazidime; Ci- ceftriaxone; Ce-cefotaxime; C-choramphenicol; Cf-ciprofloxacin; C0-co trimoxazole; G-gentamicin; I-imipenem; Mr-meropenem; Na-nalidixic acid;Nf-nitrofurantoin; Nx-norfloxacin and Of – ofloxacin.

Table 7: Antibiotic susceptibility pattern of *E. coli* isolated from different samples

Antibiotic used	Sensitive		Moderately Sensitive		Resistant		Total
	No. of isolates	%	No. of isolates	%	No. of isolates	%	
Amoxycillin	85	44.04	0	0.00	108	55.96	**193**
Cephotaxime	131	67.88	0	0.00	62	32.12	**193**
Cefixime	132	68.4	0	0.00	61	31.6	**193**
Chloramphenicol	36	73.47	0	0.00	13	26.53	**49**
Ciprofloxacin	120	62.18	0	0.00	73	37.82	**193**
Ceftriaxone	8	12.7	0	0.00	55	87.3	**63**
Ceftazidime	5	8.62	0	0.00	53	91.38	**58**
Cefoxitin	6	9.84	0	0.00	55	90.16	**61**
Co-trimoxazole	105	54.4	0	0.00	88	45.6	**193**
Nalidixic acid	1	50.0	0	0.00	1	50.0	**2**
Nitrofurantoin	162	90.0	4	2.23	14	7.77	**180**
Norfloxacin	114	63.33	0	0.00	66	36.67	**180**
Ofloxacin	116	64.44	0	0.00	64	35.56	**180**
Amikacin	57	90.48	3	4.76	3	4.76	**63**
Gentamicin	21	33.33	0	0.00	42	66.67	**63**
Meropenem	**51**	**100.0**	0	0.00	0	0.00	**51**
Imipenem	**51**	**100.0**	0	0.00	0	0.00	**51**

Table 8: Antibiotic susceptibility pattern of Gram-positive bacterial pathogens

Organisms		Ak	Am	Az	Cfx	Ci	Ce	Cf	Co	Cp	Cx	Do	E	G	I	Mr	Nx	Ox	Va
S. pyogenes	S	-	1	1	-	-	-	1	1	1	0	-	1		-	-		-	-
	R	-	0	0	-	-	-	0	0	0	1	-	0		-	-		-	-
S. pneumoniae	S	-	0	1	-	1	1	1	0	1	1	-	1		-	-		-	-
	R	-	1	0	-	0	0	0	1	0	0	-	0		-	-		-	-
S. aureus	S	1	6	-	3	14	14	12	10	3	15	2	14		0	0	0	0	2
	I	0	1	-	0	0	0	0	0	1	0	0	0		0	0	0	0	0
	R	0	10	-	2	3	3	5	7	3	2	0	3		2	2	1	2	0
S. saprophyticus	S	1	11	-	2	10	9	13	13	9	10	-	10	1	-	-	2	-	-
	I	0	1	-	0	0	0	0	0	2	0	-	0	0	-	-	0	-	-
	R	0	3	-	0	3	2	2	2	2	5	-	5	0	-	-	0	-	-
S. epidermidis	S	1	4	-	-	1	3	4	4	3	3	-	4	0	-	-	3	-	-
	I	0	0	-	-	1	0	0	0	0	0	-	0	0	-	-	0	-	-
	R	0	2	-	-	1	1	2	2	3	3	-	2	2	-	-	1	-	-
Streptococcus faecalis	S	-	1	-	0	0	0	0	3	0	0	-	0	-	-	-	0	-	3
	R	-	2	-	3	3	3	3	0	3	3	-	3	-	-	-	3	-	0
Total		3	43	2	10	37	36	43	43	31	43	2	43	3	2	2	10	2	5

Abbreviations: Ak-amikacin; Am-amoxycillin; Az-azithromycin; Cfx-cefixime; Cn-cefoxitin; Ca- ceftazidime; Ci- ceftriaxone; Ce-cefotaxime; C-choramphenicol; Cf-

Age Group	Clinical Samples							
	Blood	**Pus**	**Fluid**	**Sputum**	**CSF**	**Tissue**	**Perianal**	**Urine**

ciprofloxacin; C0-co trimoxazole; Cp- cephalexin; Cx-cloxacin; Do- doxycycline; E-erythromycin; G-gentamicin; I-imipenem; Mr-meropenem; Na-nalidixic acid;; Nx-norfloxacin; Ox-oxacillin and Va-vancomycin

Table 9: Pattern of MDR strains from different samples among different age groups of patients

(in years)																								
	I	M	N	I	M	N	I	M	N	I	M	N	I	M	N	I	M	N	I	M	N	I	M	N
<10	2	-	0	0	0	0	0	0	-	0	0	0	0	-	0	0	0	0	0	0	0	7	3	1
11-20	17	-	6	11	5	1	0	0	-	0	0	0	1	-	1	0	0	0	1	1	0	14	4	4
21-30	22	-	11	16	8	4	0	0	-	0	0	0	0	-	0	1	0	1	0	0	0	75	30	11
31-40	7	-	5	10	7	1	1	0	-	0	0	0	0	-	0	0	0	0	0	0	0	33	20	3
41-50	3	-	1	3	1	0	1	1	-	0	0	0	0	-	0	0	0	0	0	0	0	17	9	2
51-60	0	-	0	7	3	1	0	0	-	0	0	0	0	-	0	1	1	0	0	0	0	18	11	4
61-70	0	-	0	2	0	0	0	0	-	0	0	0	0	-	0	0	0	0	2	1	1	31	15	2
71-80	0	-	0	5	3	0	0	0	-	1	1	0	0	-	0	0	0	0	0	0	0	12	6	4
81-90	0	-	0	2	1	0	0	0	-	0	0	0	0	-	0	0	0	0	0	0	0	4	4	0
>90	0	-	0	0	0	0	0	0	-	0	0	0	0	-	0	0	0	0	0	0	0	8	3	0
Total	**51**	**-**	**23**	**56**	**28**	**7**	**2**	**1**	**-**	**1**	**1**	**-**	**1**	**-**	**1**	**2**	**1**	**1**	**3**	**2**	**1**	**219**	**105**	**31**

Note:-

I = No. of Bacterial Isolates N = No. of Non-MDR strains

M = No. of MDR strains

Table 10: Plasmid DNA profiles of ESBL, ABL and MBL producing MDR *E. coli* and *C. freundii*

Organism	Antibiotic Resistance pattern	β-lactamase Production	No.of Plasmid	Size of plasmid (kb)
E. coli				
KMH_{3128}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,G,Ci	ABL	3	4.5,3.9, 2.1
KMH_{1740}	Am,Cf,Ce,Cfx,Co,Of,Nx,C,Nf,Ca,G,Ci	ABl, & MBL	3	29,3.9, 2.1

KMH_{2466}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,G,Ci	ESBL & ABL	1	8
KMH_{55}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ak,C,Ca,G,Ci	ESBL & ABL	3	29, 8, 2.1
KMH_{686}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,G,Ci	MBL	3	32.5,5.2, 4.5
KMH_{533}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,Ci	MBL	1	32.5
KMH_{697}	Am,Ce,Cf,Co,Cfx,Nx,Of,Ci,Ca,G	ABL	4	32.5, 4.5, 2.5, 1.8
KMH_{3261}	**Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,Ci**	**ESBL & ABL**	**7**	**38, 10.1, 8, 6.7, 5.8, 3.9, 3.4**
KMH_{759}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,C,G,Ci	ESBL,ABL & MBL	4	32.5, 4.5, 2.5, 1.8
KMH_{877}	**Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,G,Ci**	**ESBL & MBL**	**1**	**51**
KMH_{4137}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,G,Ci	ESBL & ABL	4	29, 8, 3.9, 3.4
KMH_{1569}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,Ci	ABL	3	32.5,10.1, 3.9
KMH_{3503}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,Ci	ABL	1	32.5
KMH_{2176}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,C,G,Ci	ESBL & ABL	1	32.5
KMH_{2102}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,C,G,Ci	ABL	4	38, 4.5, 2.8, 2.5
KMH_{3957}	Am,Cf,Co,Of,Nx,Nf	MBL	1	38
KMH_{915}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,G,Ci	MBL	1	32.5
KMH_{879}	Am,Cf,Ce,Cfx,Co,Of,Nx,Nf,C,Ca,G,Ci	ESBL & MBL	3	32.5, 4.5, 2.5
KMH_{4131}	**Am,Cf,Co,Of,Nx**	**Nil**	**1**	**45**
KMH_{3030}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,G,Ci	ESBL & ABL	3	32.5, 4.2, 3.9
KMH_{890}	Am,Cf,Ce,Cfx,Co,Of,Nx, Nf,Ca,G,Ci	ESBL & ABL	1	32.5
KMH_{3733}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,Ci	ESBL & ABL	1	32.5
KMH_{1830}	Am,Ce,Cf,Co,Cfx,Nx,Of,Ci,Ca,G	ABL	4	32.5, 8, 4.2, 2.8
KMH_{1133}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,G,Ci	ESBL & ABL	3	5.5, 2.5, 1.8
KMH_{3263}	Am,Cf,Ce,Cfx,Co,Of,Nx,C,Ca,G,Ci	ESBL & ABL	3	29, 8, 3.9
KMH_{85}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ak,Ca,G,Ci	ESBL & ABL	2	29, 8
KMH_{1140}	Am,Cf,Co,Nx,Of,Ca,Ci	ABL	1	38
KMH_{1680}	Am,Cf,Ce,Cfx,Co,Of,Nx,Ca,C,Ci	ESBL	2	32.5, 8
KMH_{2974}	Am,Cf,Co,Of,Nx,Ca,Ci,G	ABL & MBL	1	38
KMH_{1490}	Am,Cf,Co,Of,Ca,Ci,G,Ce,C,Cfx	ESBL & ABL	1	38
C.freundii				
KMH_{838}	Am,Cf,Co,Nx,Of,Ce,Cfx,Ca,Ci,C,Ak,G	MBL*	1	32.5
KMH_{3625}	Am,Cf,Co,Ce,Cfx,Ca	MBL	1	32.5
KMB_{3675}	Am,Cf,Co,Of,Ce,G,Ak,Ca,C,Ci,M	MBL*	1	32.5

MBL* is the MDR strain with resistant to carbapenem

Table 11: Minimum inhibitory concentration (µg/ ml) of donors, transconjugants and transformants

Organisms	A	C	Cf	Cfx	G	Nx	Tr	S

533d	>1024		>1024	>1024		1024	1024	
533t	>1024		128	>1024		1024	1024	
1569d	>1024		>1024	>1024		>1024	>1024	
1569t	>1024		1024	>1024		128	1024	
2176d	1024		>1024	1024		>1024	>1024	
2176t	1024		>1024	1024		1024	1024	
1140d	>1024		>1024	>1024		>1024	>1024	
1140t	>1024		>1024	>1024		1024	1024	
1133t	1024		>1024	1024		1024	1024	
890d	>1024		>1024	>1024	>1024	>1024	>1024	
877d	1024		>1024	1024		512	1024	
877t	256							
2974d	1024		1024	>1024		1024	1024	
2974t	256							
85d	>1024		>1024	>1024		>1024	>1024	
85t	>1024		128	>1024		1024	>1024	
3263d	1024		1024	>1024		1024	>1024	
3263t	1024		1024	>1024		1024	>1024	
759d	>1024	1024	>1024	>1024		>1024	>1024	
759t	>1024	1024	>1024	1024	>1024	>1024	>1024	
Transformants								
759	256				256		128	
877	128						64	
890	128		32		32		32	
E. coli TB1								512
E. coli HB101								512

APPENDIX-XI

1. Association of culture positive and negative among male and female patients.

	Culture positive	Culture negative	Total
Male	127	557	684
Female	209	610	819
Total	336	1167	1503

Test statistics is χ^2

H_0: There is no significant association of culture positive and culture negative among male and female patients.

H_1: There is significant association of culture positive and culture negative among male and female patients.

From $\chi^2 = \Sigma \frac{(O - E)^2}{E}$ we find from $\chi^2 = 10.37$

Thus χ^2_{cal} (10.37) > χ^2_{tab} at α =0.05(3.84) and α =0.01(6.63) at d.f =1 Hence, H_0 is rejected.

Result: There is significant association of culture Positive and negative among male and female patients i.e. the higher proportion of culture positive cases seen among female cases is statistically significant

2. Association of Culture positive and negative among out-patients and in-patients.

	Culture positive	Culture negative	Total
Out-patients	291	1102	1393
In-patients	45	65	110
Total	336	1167	1503

Test statistics is χ^2

H_0: There is no significant association of Culture positive and negative among out-patients and in-patients.

H_1: There is significant association Culture positive and negative among out-patients and in-patients.

From $\chi^2 = \Sigma \frac{(O - E)^2}{E}$ we find from $\chi^2 = 23.53$

Thus χ^2_{cal} (23.53) > χ^2_{tab} α =0.05(3.84) and α =0.01(6.63) at d.f =1 Hence, H_0 is rejected.

Result: There is significant association of Culture positivity among types of patiets i.e. higher proportion of bacterial infections found among in-patients was statistically significant.

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

	MDR strains	Non-MDR strains	Total
Out-patients	106	59	165
In-patients	32	5	37
Total	138	64	202

Test statistics is χ^2

H_0: There is no significant association of multi-drug resistance among out-patients and in-patients among different pathogens

H_1: There is no significant association of multi-drug resistance among out-patients and in-patients among different pathogens

From $\chi^2 = \Sigma \frac{(O - E)^2}{E}$ we find from $\chi^2 = 6.9$

Thus χ^2_{cal} (6.9) > χ^2_{tab} at α =0.05(3.84) and α =0.01(6.63) at d.f =1 Hence, H_0 is rejected.

Result: There is no reason that there is no significant association of multi-drug resistance among types of patients suffering bacterial infections i.e. the higher percentage of MDR strains found in in-patients was statistically significant.

4. Association of MDR and Non-MDR strains among Male and Female patients.

	MDR strains	**Non-MDR strains**	**Total**
Male	52	25	77
Female	86	39	125
Total	138	64	202

Test statistics is χ^2

H_0: There is no significant association of MDR and non-MDR strains among male and female patients.

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

From $\chi^2 = \Sigma \frac{(O - E)^2}{E}$ we find from $\chi^2 = 0.035$

Thus χ^2_{cal} (0.035) < χ^2_{tab} α =0.05(3.84) and α =0.01(6.63) Hence, H_0 is accepted.

Result: There is no significant association of MDR and non MDR strains among male and female patients i.e. higher proportion nf multidrug resistance seen among female patient was not found statistically significant.

5. Association of Culture Isolates and MDR strains among Gender.

	No. of Isolates	**MDR strains**	**Total**
Male	127	52	179
Female	209	86	295
Total	336	138	474

Test statistics is χ^2

H_0: There is no significant association of Culture Isolates and MDR strains among Gender.

H_1: There is significant association of Culture Isolates and MDR strains among Gender.

From $\chi^2 = \Sigma \frac{(O - E)^2}{E}$ we find from $\chi^2 = 0.0006$

Thus χ^2_{cal} (0.0006) < χ^2_{tab} α =0.05(3.84) and α =0.01 (6.63) Hence, H_0 is accepted.

Result: There was no significant association of Culture Isolates and MDR strains among gender i.e. higher proportion of MDR strains seen among female patients was found statistically insignificant.

6. Association of ESBL production and the level of multidrug resistance.

	Range of multidrug resistance (No. of drugs)	**Total**

	2-4	5-10	>10	
ESBL positive	1	10	16	27
ESBL negative	1	8	23	32
Total	2	18	39	59

Test statistics is χ^2

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

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

.

From $\chi^2 = \Sigma \frac{(O - E)^2}{E}$ we find from $\chi^2 = 320.7$

Thus χ^2_{cal} (320.7) > χ^2_{tab} α =0.05(5.99) and α =0.01(9.21) as d.f. = 2 Hence, H_0 is rejected.

Result: There is strong significant association of ESBL production and the level of multidrug resistance among MDR ESBL producing isolates i.e. increasing level of multidrug resistance seen among ESBL positive MDR isolates were found highly significant.

7. Association ABL production and the level of multidrug resistance

	Range of multidrug resistance (No. of drugs)			**Total**
	2-4	5-10	>10	
ABL positive	2	22	34	58
ABL negative	1	6	11	18
Total	3	28	45	76

Test statistics is χ^2

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

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

.

From $\chi^2 = \Sigma \frac{(O - E)^2}{E}$ we find from $\chi^2 = 0.24$

Thus χ^2_{cal} (0.24) < χ^2_{tab} α =0.05(5.99) and α =0.01(9. 21) as d.f. = 2 Hence, H_0 is accepted.

Result: There is no significant association of ABL production and the level of multidrug resistance among MDR ABL producing isolates i.e. increasing level of multidrug resistance seen among ABL positive MDR isolates were found statistically insignificant.

8. Association MBL production with the level of antibiotic resistance

	Range of multidrug resistance (No. of drugs)		Total
	2-10	>10	
MBL positive	7	26	33
MBL negative	19	14	33
Total	26	40	66

Test statistics is χ^2

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

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

.

From $\chi^2 = \Sigma \frac{(O - E)^2}{E}$ we find from $\chi^2 = 9.14$

Thus χ^2_{cal} (9.14) > χ^2_{tab} α =0.05(5.99) at d.f. = 2 Hence, H_0 is rejected.

Result: There is significant association of MBL production and the level of multidrug resistance among MDR MBL producing isolates.i.e. increasing level of multidrug resistance seen among MBL positive MDR isolates were found statistically significant.

9. Association β-lactamase production and the level of multidrug resistance

	Range of multiple drug resistance (No.of drugs)		Total
	5-10	>10	
ESBL production	11	16	27
ABL production	24	34	58
MBL production	7	26	33
Total	42	76	118

Test statistics is χ^2

H_0: There is no significant association of type of β-lactamase production and the level of multidrug resistance among MDR isolates.

H_1: There is significant association of type of β-lactamase production and the level of multidrug resistance among MDR isolates.

.

From $\chi^2 = \Sigma \frac{(O - E)^2}{E}$ we find from $\chi^2 = 4.87$

Thus χ^2_{cal} (4.87) > χ^2_{tab} α =0.05(5.99) at d.f. = 2 Hence, H_0 is accepted.

Result: There is no significant association of type of β-lactamase production and the level of multidrug resistance among MDR isolates.i.e. increasing level of multidrug resistance seen among MBL positive MDR isolates then other ESBL and ABL positive isolates were found statistically insignificant.

10. Evaluation of urine culture test to detect UTI on the basis of pyuria.

Screening test results	**Culture positive**	**Culture negative**	**Total**
(≥5WBC/HPF)(Cases)	44	8	52
(<5WBC/HPF) (Control)	4	133	137
Total	48	141	189

a. Sensitivity = (44/48) x 100 = 91.66 %

b. Specificity = (133/141) x 100 = 94.32%

c. Positive predictive value = (44/52) x 100 = 84.61%

d. Negative predictive value = (133/137) x 100 = 97.08 %

11. Association between previous antibiotic exposure and drug resistance among bacterial infected patients.

		Resistance to antibiotic		Total
		Present	**Absent**	
Exposure to antibiotic	**Yes**	24	11	35
	No	21	11	32
Total		45	22	67

Odds ratio = $\frac{24\times11}{21\times11}$ = 1.15

This means the cases were 1.15 times more likely than the controls to have drug resistance.

12. Association between level of multi drug resistance and no. of plasmid among MDR strains harboring plasmid/s.

		No. of plasmid		Total
		>2	**≤2**	
Level of MDR (No. of drug resistance)	**>10**	4	6	23
	2-10	7	16	10
Total		11	22	33

Odds ratio = $\frac{4\times16}{7 \times 6}$ = 1.52

This means the higher level of multidrug resistance was 1.52 times more likely than the lower level of multidrug resistance to have multiple plasmids.