CHAPTER-I

1. INTRODUCTION

Antibiotics are the front-line therapeutic means for the medical intervention in an infection which plays a major role in the control and management of infectious diseases. The extraordinary progress represented by the arrival of antibiotics has changed the medical prognosis of minor and major infections. However, over the years, via several constantly changing mechanisms, many bacterial species acquired resistance to the most common classes of antibiotics. Resistance in bacteria can result from resistance genes in their chromosome or by acquisition of genes from other bacteria (Jones et al. 1998).

For several years we have been faced with the emergence and spread of microorganisms resistant to one or several antibiotics commonly used in the treatment of infections, such as respiratory tract infections, meningitis, urinary tract infections, wound infections and so on. In some cases, pathogens have become resistant to all anti-infectious drugs, leading to therapeutic failure. At the present time, this situation is not limited to the hospital ecosystem and nosocomial infections, but is spreading to the whole population and concerns community infections. Resistance to antibiotics constitutes a major threat to public health and ought to be faced, firstly by a better understanding of the numerous and “smart” mechanisms which bacteria have been developing with the passing years to escape the lethal effect of antibiotics (Lucet et al. 1996).

Microbial resistance to antibiotic is genetically determined either by genes or via mobile elements. Genes encoding for resistance mechanisms may be part of the genetic inheritance of the bacteria. In this case, microbial resistance is stable, transmitted to the descendants of the microbial cell (vertical transmission), but is generally poorly transferable from one bacteria to another (horizontal transmission). On the other hand, genes encoding for resistance may belong to mobile elements such as plasmids and transposons. In this case, resistance is transmitted to the descendants but tends to be

unstable in the absence of the selective antibiotics involved. Bacteria that have acquired such a mobile element may be cured and become susceptible again to the antibiotics. However, this type of resistance is transferable from one bacterium to another, even across different species (Tenover, 2006).

The mechanisms responsible for resistance developed towards antibiotics have been elucidated in most cases. In each class of antibiotics, there is at least one mechanism that allows the bacteria to protect themselves against antibiotic toxicity (Tenover, 2006). The most frequent mechanisms are:

- Alteration of antibiotic targets, so that the drug is no longer capable of reacting with it (ribosomes, enzymes for bacterial cell-wall synthesis, etc) as seen in *Streptococcus pneumoniae* and microorganisms producing β-lactam antibiotics.
- Defects of antibiotic penetration into the bacteria, as seen in *Escherichia coli* towards hydrophobic antibiotics (penicillins G and M, macrolides).
- Antibiotic inactivation by microbial enzymes, as seen in *Haemophilus influenzae*, which produce β-lactamases, i.e., enzymes which inactivate β-lactam antibiotics.
- Antibiotic excrusion (or active efflux) by microbial cell wall enzyme systems as seen with strains of *Escherichia coli*, *Staphylococcus aureus*, or *Pseudomonas aeruginosa*, towards cyclines and fluoroquinolones.

The defining criterion for Multi-drug Resistance (MDR) in this study was resistance to ≥ 2 of the antimicrobial agents belonging to different structural classes (Dhakal, 1999).

The term Extended Spectrum of β-lactamase (ESBL) refers to the β-lactamase enzymes produced mainly by some species of Gram-negative bacilli (*Klebsiella* spp. and *Escherichia coli*) that encode for resistance to broad spectrum of β-lactam antibiotics that normally have activity against Gram-negative bacilli, examples are the third generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime) and aztreonam. These enzymes are not active against cephamycins and are inhibited by clavulanic acid. All

ESBL producing organisms should be considered resistant to all penicillins (except temocillin), cephalosporins (except cefoxitin and cefotetan) and aztreonam. The first hospital outbreak of an ESBL-containing Gram-negative microorganism was reported in Germany in 1983 (Knothe et al. 1983). Later, outbreaks due to ESBL-producing strains were reported from various parts of the world in various studies (Arlet et al. 1990; Rice et al. 1990; Jacoby and Medeiros, 1991 and Bauernfeind et al. 1993).

Selection of resistant mutants is a major cause of hospital infection, and hence a public health threat. Commensal species that cause hospital infections in patients at risk *(Escherichia coli, Staphylococcus aureus,* or *Streptococcus pneumoniae)* are naturally susceptible to numerous antibiotics which make them poorly competitive. In addition, some of these commensal species poorly survive outside organisms. Acquired resistance also plays a pivotal role in the changing frequency of communal species responsible for hospital infections. Since they present natural resistance to antibiotics and are well adapted to the environmental conditions, saprophytic bacteria are more and more frequently responsible for hospital infections *(Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., or *Pseudomonas aeruginosa).* In addition, selection of resistant mutants in a bacterial population previously susceptible and responsible for the infection treated with antibiotics is a frequent cause of therapeutic failure. For example, hospital infections with Gram-negative bacteria and cephalosporins of the second and third generations, ureidopenicillins, carbapenems, and fluoroquinolones (Nordmann, 1998).

In the past 50 years people in both the developed and developing worlds have accepted antibiotics as their right, obtaining a prescription at the first sign of a trivial infection or treating themselves with a handful of cheap antibiotics. In the meantime, bacteria have evolved very sophisticated means of exchanging DNA, both within their own genus and species and across them. The widespread use of antibiotics will tilt the delicate balance between the bacteria and human beings (Jones et al. 1998).

This study is mainly focused on the prevalence of Multidrug Resistant (MDR) and extended spectrum beta-lactamase(ESBL) producing strains among clinical isolates of patients admitted in Shree Birendra Hospital. Similar types of studies have been done by various researchers throughout the world. According to a study by Jaiswal et al. (2007) M. L. N. Medical college in India, ESBL production was observed in 54.4% of *E. coli* and 55% of *Klebsiella* spp. isolated from urine samples. In another study performed by Pokhrel et al. (2006) in Microbiology Laboratory of TUTH, 47.57% of isolates from sputum samples were MDR and 24.27% were ESBL- producers. In case of urinary isolates, 60.40% and 16.00% were found to be MDR and ESBL respectively.

Nowadays, MDR and ESBL-producing strains are threatening the whole world. Clinically, ESBLs limit the efficacy of β-lactam including extended spectrum cephalosprins and are associated with high morbidity and mortality. Moreover, the indiscriminate use of carbapenems may select resistance to these key drugs thus sowing seeds for significant therapeutic problems to arise in the future. This type of study would help to know the present status of these organisms and hence, to minimize the therapeutic failure to some extend.

CHAPTER-II

2. OBJECTIVES

2.1 General Objective

To determine the prevalence of Multi-Drug Resistant (MDR) strains and Extended Spectrum ℘lactamase (ESBL) producing strains among the bacterial pathogens isolated from different clinical samples from patients admitted in various wards of Shree Birendra Hospital, Chhauni.

2.2 Specific Objectives

i. To isolate and identify the bacterial pathogens from urine, sputum and pus samples collected from patients admitted in different wards of the hospital.
ii. To describe the antibiotic susceptibility pattern of the isolated organisms.
iii. To find out the prevalence of Multi-Drug Resistant organisms among the total isolates.
iv. To evaluate the status of Extended Spectrum ℘lactamase producing strains from the isolates.
v. To statistically analyze the association of Multi-drug resistance with parameters such as gender and hospitalization in different wards.

CHAPTER-III

3. LITERATURE REVIEW

3.1 Introduction of Antibiotics

The word antibiotic has come to refer to a metabolic product of one microorganism that in very small amount is detrimental or inhibitory to other microorganisms (Pelczar et al. 1993). The advent of synthetic methods has, however, resulted in a modification of this definition and an antibiotic now refers to a substance produced by a microorganism, or to a similar substance (produced wholly or partly by chemical synthesis), which in low concentration inhibits the growth of other microorganisms (Hugo and Russell, 2004).

Vuillemin first defined the term “antibiosis” in 1889 from which the word “antibiotic” was derived. However, antibiotics were known by their activities long before they were given the name by which we know them. In 1881, Tyndall reported that culture media cloudy with bacterial growth became clear when mold grew on the surface. In 1877, Louis Pasteur showed that bacterial disease anthrax, which can cause respiratory failure, could be rendered harmless to animals with the injection of soil bacteria (Pelczar et al. 1993).

In 1888, a German scientist E. de Freudenreich isolated an actual product from a bacterium to have antibacterial properties. Freudenreich found that the blue pigment released in culture by bacterium *Bacillus pyocyaneus*, (as *Pseudomonas aeruginosa* was then called) arrested growth of other bacteria in the cell culture. Experimental results showed that pyocynase, product isolated from *B. pyocyaneus*, could kill a multitude of disease-causing bacteria. Clinically, though, pyocyanase proved toxic and unstable and the first natural antibiotics discovered could not be developed into an effective drug.

Alexander Fleming, a British bacteriologist in 1929, discovered the first chemo-therapeutically effective antibiotic. He noticed that an agar plate inoculated with *Staphylococcus aureus* had become contaminated with a mold (*Penicillium* sp.) and that

the mold colony was surrounded by a clear zone, indicating inhibition of bacterial growth or lysis of the bacteria (Pelczar et al.1993).

Antibiotics may be obtained from different microorganisms. For example bacitracin and polymyxin from *Bacillus* spp.; streptromycin, tetracyclines from *Streptomyces* spp. and some penicillins and cephalosporins from certain genera like *Penicillium* spp., *Acremonium* spp. etc. Synthetic antibiotics are produced in industries by using different chemical processes. For example, chloramphenicol is now usually produced by this process. Semi-synthetic antibiotics are obtained from a part of molecule that is produced by a fermentation process using the appropriate microorganism and the product is further modified by a chemical process. Many penicillins and cephalosporins are produced in this way (Pelczar et al.1993).

3.2 Classification of antibiotics

Antibiotics can be classified on various bases:

Based on their antimicrobial potency, antibiotics may be **Bactericidal-** agent that kills bacteria in usual dosages, e.g. penicillins, aminoglycosides etc. and **Bacteriostatic-** agent that doesn't kill bacteria , but stops their active multiplication in usual dosages, e.g. chloramphenicol, tetracyclines etc.

Based on spectrum of their biological action, antibiotics may be **Broad Spectrum**- e.g. tetracycline, fluoroquinolones etc. and **Narrow Spectrum**- e.g. penicillin, isoniazid, bacitracin etc (Tortora et al. 2004).

Table 1: Types of antibiotics based on their chemical structure

Types	Definition	Examples
1. β lactam antibiotics i. Penicillins ii. Cephalosporins iii. Other β lactam antibiotics	A thiazoline ring is attached to a β lactam ring that carries a free amino group.	i. Penicillin G, Cloxacillin ii. Cephalexin,Ceftaxidime iii. Aztreonam, Imipenem
2. Tetracycline group i. Tetracyclines ii. Glycylcyclines	Consists of 8 members and considered as a group of antibiotics obtained as by-products from the metabolism of various species of *Streptromyces*	Doxycycline Methacycline
3. Aminoglycoside-aminocyclitol antibiotics	Contains amino-sugars in their structures	Gentamicin Kanamycin
4. Macrolides	A large macrocyclic lactone-ring is substituted with some unusual amino-sugars linked through glycosidic bonds	Erythromycin
5. Glycopeptide antibiotics	Vancomycin has a complex tricyclic glycopeptide structure. Teicoplanin is a naturally occurring complex of five closely related tetracyclic molecules.	Vancomycin Teicoplanin
6. Miscellaneous antibacterial antibiotics	Antibiotics which cannot logically be considered in any of the other groups above.	Chloramphenicol Fusidic acid
7. Synthetic antimicrobial angents i. Sulphonamides ii. Nitrofuran compounds iii. 4-quinolone antibacterials	Antibiotics that are made in industries by various chemical means.	i. Sulphadiazine ii. Nitrofurantoin iii. Ciprofloxacin

(Source**:** Hugo and Russell, 2004)

3.3 Mechanism of Action of Antimicrobial drugs

Several key steps must be completed for an antimicrobial agent to successfully inhibit or kill the infecting microorganism. First, the agent must be in an active form. This ensured through the pharmaco-dynamic design of the drug, which takes into account the route through which the patient will receive the agent (e.g., orally, intravenously, intra-muscularly). Second, the antibiotic must also be able to achieve sufficient levels or concentrations at the site of infection so that it has a chance to exert an antibacterial effect (i.e., be in approximation with the infecting bacteria). The ability to achieve adequate levels depends on the pharmacokinetic properties of the agent. Some agents, such as ampicillin and ceftriaxone, achieve therapeutically effective levels in several body sites, while others, such as nitrofurantoin and norfloxacin are limited to the urinary tract. Therefore, knowledge of the site of infection can substantially affect the selection of antimicrobial agent for therapeutic use (Forbes et al. 2002).

The remaining steps in antimicrobial action relate to direct interactions between the antibacterial agent and the bacterial cell. When the antibiotic contacts the cell surface, adsorption results in the drug molecules maintaining its contact, with the cell surface. Next, because most targets for antibacterial agents are essentially intracellular, uptake of the antibiotic to some location within the bacterial cell is required. Once the antibiotic has achieved sufficient intracellular concentration, binding to a specific target occurs. This binding involves molecular interactions between the antimicrobial agent and one or more biochemical components that play an important role in the microorganism's cellular metabolism. Adequate binding of the target results in disruption of certain cellular processes leading to cessation of bacterial cell growth and, depending on the mode of action of antimicrobial agent, perhaps cell death (Forbes et al. 2002).

Selective toxicity: An ideal antimicrobial agent exhibits selective toxicity, which means that drug is harmful to a pathogen without being harmful to the host. Often, selective toxicity is relative rather than absolute; this implies that a drug in a concentration tolerated by the host to damage an infecting microorganism. Selective toxicity may be a function of a specific reaction required for drug attachment, or it may depend on the

inhibition of biochemical events essential to the pathogen but not to the host (Brook et al. 2004).

The mechanism of action of antimicrobial drugs can be discussed under following headings:

I. Inhibition of Cell Wall Synthesis

Bacteria have a rigid outer layer; the cell wall maintains the shape and size of microorganism, which has a high internal osmotic pressure. Injury to the cell wall or inhibition of its formation may lead to lysis of the cell. The bacterial cell wall is composed of peptidoglycan consisting of polysaccharides and a highly cross-linked polypeptide. The polysaccharides regularly contain the amino-sugars N-acetylglucosamine and N-acetylmuramic acid. To the amino sugars are attached short peptide chains. The final rigidity of the cell wall is imparted by cross-linking of the peptide chains as a result of transpeptidation. The peptidoglycan layer is thicker in the cell wall of Gram-positive than that of Gram-negative bacteria.

All $\wp$ lactam drugs are selective inhibitors of bacterial cell wall synthesis and therefore, are against growing bacteria. The inhibition of the transpeptidation enzymes by penicillins and cephalosporins may be due to structural similarity of these drugs to acyl-D-alanyl-D-alanine. The transpeptidation reaction involves loss of a D-alanine from the pentapeptide.

Examples of agents acting by inhibition of cell wall synthesis are penicillins, cephalosporins, vancomycin, cycloserine, bacitracin, and novobiocin. Since, the early stages of synthesis take place inside the cytoplasmic membrane, these drugs must penetrate the membrane to be effective (Forbes et al. 2002).

II. Inhibition of Protein Synthesis

Bacteria have 70S ribosomes, whereas mammalian cells have 80S ribosomes. The subunits of each type of ribosome (50S and 30S ribosomes; 60S and 40S ribosomes), the chemical composition and their functional specificities are sufficiently different to explain. The antimicrobial drugs can inhibit protein

synthesis in bacterial ribosomes without having a major effect on mammalian ribosomes. Examples of drugs acting by inhibition of protein synthesis are macolides, lincomycins (clindamycin), tetracyclines, aminoglycosides and chloramphenicol (Forbes et al. 2002).

III. Inhibition of Nucleic Acid Synthesis

Examples of drugs acting by inhibition of nucleic acid synthesis are the quinolones, rifampicin, actinomycin, and mitomycinC. Rifampin inhibits bacterial growth by binding strongly to DNA-dependent RNA polymerase of bacteria. Thus, it inhibits bacterial RNA synthesis (Hugo and Russell, 2004). All quinolones and fluoro-quinolones inhibit microbial DNA synthesis blocking DNA gyrase; these prevent the gyrase from supercoiling bacterial DNA, required packaging of DNA in the bacterial cell (Madigan et al. 2000).

IV. Inhibition of Synthesis of Essential Metabolites

Examples of drugs belonging to this group are sulphonamides and diamino-pyrimidines, trimethoprim). Sulphonamides are analogues of para-aminobenzoic acid and prevent condensation of this compound with dihydropteridine during the formation of folic acid. Folic acid is used in many one-carbon transfers in living cells, including the conversion of deoxyuridine and thymidine. During this process, the active form of the vitamin, tetrahydrofolate, is oxidized to dihydrofolate and this must be reduced before it can function in further reaction. Diaminopyrimidines, e.g. trimethoprim, prevent the reduction of dihydrofolate to tetrahydrofolate (Greenwood et al. 2003)

V. Disruption of Cytoplasmic Membrane

If the functional integrity of the cytoplasmic membrane is disrupted, macromolecules and ions escape from the cell, and cell damage and death ensues. Examples of this mechanism are polymyxins acting on Gram-negative bacteria and polyenes (e.g. nystatin) acting on fungi (Brook et al. 2004).

3.4 Bacterial Resistance to Antibiotics

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 (Hugo and Russell, 2004).

Bacteria, like all other life forms, are subjected to the driving force of the evolution of natural selection. On repeated use of a particular drug, bacteria are subjected to a so-called “selection pressure”. Such selective pressure on higher forms of life can take thousands of years to have effect but with bacteria it happens much more quickly. Consequently, drug resistance in bacterial colonies can develop very quickly.

The first report of bacterial drug resistance was recorded in 1887. A scientist called Kossiakoff described the acclimatization of *Bacillus subtilis* to mercuric chloride and boric acid, when the organism was grown in a medium containing these chemicals.

While working on penicillin, Florey and Chain in Oxford noted that some strains of *E. coli* produced a penicillinase enzyme that inactivated the drug. This penicillinase, quickly renamed to ℘lactamase, was soon to spread to other organisms.

In 1941, all strains of *S. aureus*, the most common post-operative infection in hospitals were susceptible to Penicillin V. By 1944, some strains of *S. aureus* were capable of destroying Penicillin V by means of ℘lactamase. Today, within hospitals and other medical facilities, in excess of 95% of *S. aureus* is resistant to penicillin and all the other ℘lactam antibiotics. The response of the pharmaceutical industry was to produce ℘lactamase resistant penicillin called methicillin. By the early 1960s, resistance to methicillin also began to emerge. This was the first emergence of the so-called superbug, MRSA (Methicillin Resistant *Staphylococcus aureus*). Today MRSA is resistant to all ℘lactam antibiotics and in addition, some strains have been reported to be resistant to erythromycin, fusidic acid, tetracycline, monocyclin, streptomycin, sulphonamides, disinfectants and toxic metals such as mercury and cadmium. *S. aureus* is not the only bacterium to have gained drug resistance. In 1967, penicillin-resistant *S. pneumoniae* surfaced in remote village in Papua New Guinea (Brook et al. 2004).

3.5 Mechanisms of Antibiotic Resistance

Successful bacterial resistance to antimicrobial action requires interruption or disturbance of one or more of the steps essential for effective antimicrobial action. These disturbances or resistance mechanisms can come about in various ways, but the end result is partial or complete loss of antibiotic effectiveness.

3.5.1 Environmentally Mediated Antimicrobial Resistance

Antimicrobial resistance is the result of nearly inseparable interactions among the drug, the microorganism and the environment in which they are brought together. Environmentally mediated resistance is defined as resistance that directly results from physical or chemical characteristics of the environment that either directly alter the antimicrobial agent or alter the microorganism's normal physiologic response to the drug. Examples of environmental factors include pH, anaerobic atmosphere, cation concentrations and thymine-thymidine content.

1 Antimicrobial activities of erythromycin and aminoglycosides diminish with decrease in pH, while the activity of tetracycline decreases with increasing pH.
2 Aminoglycoside activity requires intracellular uptake across the cell membrane most of which is driven by oxidative processes, so that in the absence of oxygen, uptake and hence activity, is substantially diminished.
3 Aminoglycoside activity is also affected by the concentration of cations, such as Ca^{++} and Mg^{++}, in environment. These cations compete with the aminoglycoside molecules, which have a net positive charge, for the negatively charged binding sites on cell surface. For this reason, the drug activity tends to decrease as environmental cation concentrations increase.
4 Enterococci are able to use thymine and other exogenous folic acid metabolites that circumvent the activities of the sulphonamides and trimethoprim, which are folic acid pathway inhibitors (Forbes et al. 2002).

Information regarding environmentally mediated resistance is used to establish standardized testing methods that minimize the impact of environmental factors so that microorganism mediated resistance is more accurately determined. Of importance, testing conditions are established to recreate the in vivo physiology of infection, but are

set to optimize detection of resistance expressed by microorganisms. Thus, susceptibility testing results cannot be used to the clinical outcome of patients undergoing antimicrobial therapy (Forbes et al. 2002).

3.5.2 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. Organism-based resistance can be divided into the subcategories: intrinsic or inherent resistance and acquired resistance.

a. Intrinsic Resistance

Antimicrobial resistance resulting from the normal genetic, structural, or physiologic site of a microorganism is referred to as intrinsic resistance. Intrinsic resistance is usually predictable in a clinical situation and should not pose problems provided that an information and judicious choice is made of appropriate antimicrobial therapy. Intrinsic resistant profiles are useful for determining which antimicrobial agents should be included in the batter of drugs that will be tested against specific types of organisms.

For example, aminoglycosides are ineffective against enterococci due to lack of sufficient oxidative metabolism to drive uptake of aminoglycosides. Similarly, sulphonamides, trimethoprim, tetracycline or chloramphenicol are futile against *Ps. aeruginosa* due to lack of uptake resulting from inability of antibiotics to achieve effective intracellular concentration; vancomycin should not be used in the battery Gram-negative bacteria for the same reason (Forbes et al. 2002)

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. Unlike intrinsic resistance, acquired resistance may be a trait associated with only some strains of particular organism group or species, but not others. Therefore, the presence of this type of resistance in any clinical isolate is unpredictable, and this unpredictability is the primary reason why laboratory methods are necessary to detect resistance.

Because acquired resistance mechanisms are all genetically encoded, the methods for acquisition basically are those that allow for gene change or exchange. Some bacteria are notorious for rapid acquisition of resistance, e.g. Staphylococci, coliforms, tubercle bacilli, etc. Others, like *Streptococcus pyogenes* and spirochetes have not developed significant resistance to penicillin despite its widespread use for more than 40 years. Gonococci quickly develop resistance to sulfonamides, but only slow and low grade resistance to penicillin. However, in the past 30 years, highly penicillin resistant gonococci producing penicillinase have been appeared (Forbes et al. 2002).

3.1.4.3 Genetic Basis of Antimicrobial Resistance

The genetic change from drug sensitivity to resistance may come about in bacteria by following modes:

a. Mutation

It is a stable and heritable genetic change that occurs spontaneously and randomly among microorganisms. The antimicrobial agents do not induce it. Any sensitive population of a microbe contains few mutant cells, which require higher concentration of the antimicrobial agents for inhibition. These are selectively preserved and get a chance to proliferate when the agents eliminate the sensitive cells. Thus, in time it would appear that a sensitive strain has been replaced by a resistant one, e.g. when an anti-tubercular drug is used alone. Mutation and resistance may be:

(i) Single step: A single gene mutation may confer high degree of resistance; emerges rapidly, e.g. enterococci to streptomycin, *E. coli* and staphylococci to rifampin.

(ii) Multi-step: A number of gene modifications are involved; sensitivity decreases gradually in a stepwise manner. Many organisms in this manner develop resistance to erythromycin, tetracyclines and chloramphenicol.

Sometimes mutational acquisition of resistance is accompanied by decrease in virulence e.g. certain rifampin resistant staphylococci and low-grade penicillin resistant gonococci have decrease virulence (Brook et al.2004).

b. Gene transfer

Gene transfer is also referred to as infectious resistance. In this case, gene is transferred from one organism to another by following methods:

(i) Conjugation: Sexual contact through the formation of a bridge or sex pilus is common among Gram-negative bacilli of the same or another species. This may involve chromosomal or extra-chromosomal (plasmid) DNA. The gene carrying the 'resistance' or 'R' factor is transferred only if another 'resistance transfer factor' (RFT) is also present. Conjugation frequently occurs in the colon where a large variety of Gram-negative bacilli come in close contact. Even nonpathogenic organisms may transfer R factor to pathogenic organisms, which may become widespread by contamination of food or water. Chloramphenicol resistance of typhoid bacilli, streptomycin resistance of *E. coli*, penicillin resistance of *Haemophilus* spp., Gonococci and many others have been traced to this mechanism. Thus, this is a very important mechanism of resistant acquisition.

(ii) Transduction: It is the transfer of gene carrying resistance through the agency of a bacteriophage. The R factor is taken up by the phage and delivered to another bacterium, which it infects. Certain instances of penicillin, erythromycin and chloramphenicol resistance have been found to be phage mediated.

(iii)Transformation: A resistant bacterium may release the resistance carrying DNA into the medium and this may be imbibed by another sensitive organism-becoming unresponsive to the drug. This mechanism is probably not clinically significant except isolated instances of pneumococcal resistance to penicillin G due to altered penicillin binding protein, and some other cases (Forbes et al. 2002).

3.5.4 Biochemical Bases of Resistance

The following are the important possible mechanisms by which cells might resist the toxic effect of a growth-inhibiting drug.

(i) Conversion of active drug to an inert product

a. Inactivation of -lactam antibiotics: The inactivation of ℘lactam ring is catalyzed by family of related enzymes, the ℘lactamases. These enzymes are produced by many Gram-positive and Gram-negative bacteria and controlled both chromosomally and plasmids. The reaction involves the opening of the ℘lactam ring of penicillin to get penicilloic acid, and that of cephalosporins to give cephalosporic acid.

b. Inactivation of chloramphenicol by acetylation: The resistance is mediated by plasmids in Gram-negative bacteria, and is due to the presence of an enzyme chloramphenicol acetyltransferase (CAT), which acetylates the hydroxyl groups in its side chain. The resulting 1,3-diacetoxychloramphenicol is inactive.

c. Inactivation of aminoglycoside antibiotics: This takes place by following three types of inactivation reactions i.e. N-acetylation of susceptible amino groups; adenylylation (nucleotidylation); or phosphorylation of certain hydroxyl groups (Forbes et al. 2002).

(ii) Changes in the target site

1 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.

2 Emergence of methicillin resistance in *Staphylococcus aureus* appears to be due to the presence of a penicillin binding protein termed PBP2a or PBP2' which has a reduced affinity to methicillin, the gene responsible is the *mec*A gene. Penicillin resistance in *Streptococcus pneumoniae* and enterococci is due to altered PBPs.

3 Resistance to erythromycin is accomplished with alteration in the 50S subunits resulting in reduced affinity for this antibiotic. Mutation affecting proteins L4 or

L12 may be involved.

4 Bacteria can become resistant to trimethoprim, inhibitor of bacterial dihydrofolate reductase (DHFR), by coding for new types of DHFR, that are resistant to trimethoprim. In addition to this, the R-plasmids also carry a gene for one of two variants of sulphonamide target enzyme, dihydropteroate synthetase (Forbes et al. 2002).

(iii) Reduction in cellular permeability to antibiotics

In order to suppress the growth of bacteria, a drug must achieve an inhibitory concentration in its target site. Therefore, a decrease in cellular permeability to the drug may depress the drug concentration at the target site below the inhibitory level.

a. **Permeability of the outer membrane:** The complex structure of the outer envelope in Gram-negative bacteria ensures that they are intrinsically less sensitive than Gram-positive bacteria to a variety of antibiotics such as benzylpenicillin, methicillin, macrolides, lincomycin and vancomycin.

b. Antagonism of antibiotic transport process:

1 Resistance to tetracyclines is caused principally by a plasmid-mediated specific antagonism of the tetracycline accumulation process, thus the resistant strains fail to accumulate this drug within the cytoplasm. Among the Enterobacteriaceae, resistance determinants code for a membrane-located protein that actively promote the efflux of tetracycline antibiotics from the cell.

2 A form of resistance to quinolones in *E. coli* is associated with adiminution in the amount of an outer membrane porin protein (*Omp*F) and possibly an energy-depended quinolones efflux system (Forbes et al. 2002).

(iv) Development of an altered metabolic pathway

For example, some sulphonamide resistant bacteria do not require extracellular PABA, but like mammalian cells, can utilize preformed folic acid (Forbes et al. 2002).

3.6 Antibiotic Susceptibility Testing

Antimicrobial susceptibility tests measure the ability of an antibiotic or other antimicrobial agent to inhibit the growth of microorganisms. At the very best they can only indicate treatment because the mere fact that a microorganism appears sensitive to one or more antibiotics on testing in laboratory is of real guide to the ultimate result achieved in the patient. It cannot be assumed, therefore, that an antimicrobial, which kills or prevents an organism from growing in-vitro, will also be a successful treatment in-vivo.

A susceptibility test may be performed in the clinical laboratory for two main purposes:

1 To guide the clinician in selecting the best antimicrobial agent for an individual patient
2 To accumulate epidemiological information on the resistance of microorganisms of public health importance

Sensitivity is not usually indicated when the sensitivity reactions of a pathogen can be predicted, for example:

1 *Proteus* spp. are generally resistant to nitrofurantoin and tetracyclines,
2 Almost all *Klebsiella* spp. are resistant to ampicillin,
3 *Streptococcus pyogenes* is usually sensitive to penicillin and
4 Anaerobes are sensitive to metronidazoles.

For laboratory tests to accurately determine organism-based resistance, the potential influence of environmental factors on antimicrobial activity must be minimized. To control the impact of environmental factors, the conditions for susceptibility testing are extensively standardized. The components of antimicrobial susceptibility testing that are standardized and controlled include the following:

1 Bacterial inoculum size
2 Growth medium: pH, cation concentration, blood and serum supplements, thymidine content

3 Inoculation temperature, atmosphere and duration

4 Antimicrobial concentrations tested

However, the use of standard conditions has some limitations. Most notably, the laboratory test conditions in no way mimic the in-vivo environment at the infection site(s) where the antimicrobial agent and bacteria will actually interact. Factors such as bacterial inoculum size, pH, cation concentration and oxygen tension can differ substantially depending on the site of infection. Because of the lack of correlation between in-vitro test conditions and the in-vivo setting, antimicrobial susceptibility testing cannot and should not be used as predictors for therapeutic outcome for the use of particular antimicrobial agents. Additionally, several other important factors that play key roles in patient outcome are not taken into account of susceptibility testing. Some of these factors include:

1 Antibiotic diffusion in tissues and host cells

2 Serum protein binding of antimicrobial agents

3 Drug interactions and interference

4 Status of patient defense and immune system

5 Multiple simultaneous illnesses

6 Virulence and pathogenesis of infecting bacterium

7 Site and severity of infection

Despite these limitations, the goal of in-vitro susceptibility testing to detect resistance proves valuable data that are used in conjunction with other diagnostic information to optimize them (Forbes et al. 2002).

Some general considerations, which are to be made while undertaking susceptibility tests, include:

3.6.1 Inoculum Preparation

Properly prepared inoculum is a key to any of the antimicrobial susceptibility testing method. Inconsistencies in inoculum preparation often lead to inconsistencies and inaccuracies of susceptibility test results. The two important requirements for

appropriate inoculum preparation include use of a pure culture and a standardized inoculum.

Interpretation of results obtained with a mixed inoculum is not reliable and failure to use a pure culture can substantially delay reporting of results. Pure inocula are obtained by selecting four to five colonies of the same morphology, inoculating them to a broth medium, and allowing the culture to achieve good active growth (i.e., mid-logarithmic phase), as indicated by observed turbidity in the broth.

Use of standard inoculum size is as important as culture purity and is accomplished by comparison of the turbidity of the organism suspension with a turbidity standard. McFarland turbidity standards, prepared by mixing various volumes of 1% sulfuric acid and 1.175% barium chloride to obtain solutions with specific optical density comparable to the density of a bacterial suspension of 1.5×10^8 CFU/ml (Forbes et al. 2002).

3.6.2 Selection of Antimicrobial Agents for Testing

The antimicrobial agents that are chosen for testing against a particular bacterial isolation referred to as the antimicrobial battery. The content and application of each battery are based on various criteria, and the final decision regarding battery content should not be made by the laboratory alone, as input from the medical staff and pharmacy is imperative. A number of considerations are involved in selecting an appropriate antimicrobial agent to treat an infection. These include:

1 Knowledge of inherent in-vitro susceptibility of the infecting organisms to appropriate antimicrobial agents
2 The relationship of the susceptibility of the strain to that of other members of the same species
3 Pharmacological properties, including toxicity, protein binding, distribution, absorption and excretion, particularly under circumstances of existing or developing hepatic or renal failure
4 Previous clinical experience of efficacy in the treatment of infections due to the same species.

5 The nature of the underlying pathological process, its natural history, effects on patient's normal flora and its influence on chemotherapy

6 The immune status of the host.

Of these factors, the concentrations of antimicrobial agents required to inhibit or kill organisms in-vitro and those attained in the body fluids during treatment are subjected to direct measurement in the clinical laboratory (Lenette et al. 1985).

Table 2: Basic sets of drugs for routine susceptibility tests

	Staphylococcus aureus	Enterobacteriaceae			*Pseudomonas aeroginosa*
		Intestinal	Urinary	Blood and Tissue	
Set I	Benzylpenicillin	Ampicillin	Sulphonamide	Ampicillin	Piperacillin
	Oxacillin	Chloramphenicol	Trimethoprim	Chloramphenicol	Gentamicin
	Erythromycin	Co-trimoxazole	Co-trimoxazole	Co-trimoxazole	Tobramicin
	Tetracycline	Nalidixic acid	Ampicillin	Tetracycline	
	Chloramphenicol	Tetracycline	Nitrofurantoin	Cefalotin	
			Nalidixic acid	Gentamicin	
			Tetracycline		
Set II	Gentamicin	Norfloxacin	Norfloxacin	Cefuroxime	Amikacin
	Amikacin		Chloramphenicol	Ceftriaxone	
	Co-trimoxazole		Gentamicin	Ciprofloxacin	
	Clindamycin			Amikacin	

Source: Basic Laboratory Procedures in Clinical Bacteriology (WHO, 1991)

The table indicates the drugs to be tested in various situations. The drugs in the table are divided into two sets. Set I includes the drugs that are available in most hospitals and for which routine testing should be carried out for every strain. Tests for drugs in Set II are to be performed only at the special request of the physician, or when the causative organism is resistant to the first-choice of drugs. Or other reasons (allergy to the drugs, its unavailability) make further testing justified.

3.6.3 Antimicrobial Susceptibility Testing Techniques

I. Disk Diffusion

Bauer, Kirby and Sherris and Turck developed the disk diffusion test in 1966. Using this technique, antimicrobial resistance is detected by challenging bacterial isolates with antibiotic discs that are placed on the surface of an agar plate that has been seeded with a lawn of bacteria.

The antibiotic-impregnated disc absorbs moisture from the agar and antibiotic diffuses into the agar medium. The rate of extraction of the antibiotic from the disc is greater than the rate of diffusion. As the distance from the disc increases, there is a logarithmic reduction in the antibiotic concentration. The depth of the agar affects the extent of antimicrobial diffusion. Visual growth of bacteria occurs on the surface of the agar where the concentration of antibiotics fallen below its inhibitory level for the test strain (Collee et al. 1996). Following incubation, the diameter of zone of inhibition around each disk is measured in millimeters.

Mueller-Hinton is the standard agar base medium used for testing most bacterial organisms. The conditions of this medium (e.g., pH, cation concentration, thymidine content) are well controlled by commercial media manufacturers. However, media supplements or different media altogether are required to obtain good growth and reliable susceptibility profiles with relatively fastidious bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. For instance, Gram sensitivity testing agar No.2 and Iso-Sensitest agar are also used. Wilkins-Chalgren agar recommended for testing anaerobic bacteria (CLSI, 2007).

The depth of the agar medium can also affect test accuracy and must be carefully controlled. Because antimicrobial agents diffuse in all directions from the surface of the agar plate, the thickness of the agar affects the antimicrobial drug concentration gradient. If the agar is too thick the zone sizes would be smaller and if too thin the inhibition zones would be larger.

Commercially prepared discs 6mm in diameter should be used. Disc should be stored in sealed containers with a desiccant, bulk stock being kept at -20⌠C if possible, at less than 8⌠C. Working stock, also kept in sealed containers with desiccant, should be stored at less than 8⌠C. Before they are opened for use, the containers should be allowed to warm up slowly at room temperature to minimize condensation of moisture, which may lead to hydrolysis of antibiotics (Collee et al. 1996).

Before disc placement, the plate surface is inoculated using a swab that has been submerged in bacterial suspension standardized to match the turbidity of the 0.5 McFarland turbidity standard (i.e., 1.5×10^8 cfu/ml). The inoculated plates are incubated under environmental conditions to optimize bacterial growth but do not interfere with antimicrobial activity. Most commonly, the environment is air at 35⌠C. Exceptions exist for the sake of testing more fastidious bacteria (e.g., *Neisseria meningitides* optimally requires 5% to 7% Carbondioxide). Similarly, incubation time may be increased beyond 16 hours to enhance detection of certain resistance pattern. (e.g., methicillin resistance in staphylococci and vancomycin resistance in enterococci). However, prolonged incubation times beyond recommended limits should be avoided because antimicrobial deterioration may result in false resistance interpretation (Collee et al. 1996).

Before reading the results, the plates should be examined to confirm that a confluent lawn of bacterial growth has been obtained. The lack of confluent growth may be due to insufficient inoculation. Alternatively, a particular isolate may have undergone mutation so that growth factors supplied by the standard susceptibility testing medium are no longer sufficient for supporting the growth. Plate should be examined for purity because mixed cultures are most evident different colony morphologies scattered throughout the lawn of bacteria that is being tested. Mixed cultures require purification and repeat testing of the bacterial isolate of interesting instances when hazes of growth occur within more obvious inhibition zones. This may be a way which is clinically relevant to the resistance patterns that are manifested by certain bacterial isolates when tested using the disk diffusion method. Still other significant resistances may be subtly evident and appear as individual colonies within an obvious zone of inhibition. When such colonies

are seen, purity of the test isolate must be confirmed. If purity is confirmed, the individual colonies are variants of resistant mutants of the same species and the test isolate should be considered resistant (Forbes et al. 2002).

The **Kirby-Bauer method** and its modification recognize three categories of susceptibility: susceptible, intermediate susceptibility (moderately susceptible) and resistant.

Susceptible- An organism is called "susceptible" to a drug when the infection caused by it is likely to respond to treatment with this drug, at the recommended dosage.

Intermediate susceptibility covers two situations. It is applicable to strains that are "moderately susceptible" to an antibiotic that can be used for treatment at a higher dosage because of its low toxicity or because the antibiotic is concentrated in the focus of infection (e.g. urine). The classification also applies to strains that are "intermediate susceptibility" to a more toxic antibiotic that cannot be used at a higher dosage. In this situation, the intermediate category serves as a buffer zone between susceptible and resistant.

As most clinicians are not familiar with the subtle, although clinically important, distinction between intermediate and moderate susceptibility, many laboratories use the designation "intermediate" for reporting purposes.

Resistant- This term implies that the organism is expected not to respond to a given drug, irrespective of the dosage and of the location of the infection.

There are different types of diffusion sensitivity test, which vary in their methods of standardization, reading and control.

a. **Kirby-Bauer method-** In this method, the broth culture of test organism (comparable to McFarland tube no. 0.5; inoculum density 1.5×10^8 organisms per ml) is uniformly carpeted on the lawn culture of test organism. The inoculated and seeded Mueller-Hinton agar plate is then incubated at 37°C for 18 hours (or overnight). After incubation the zone diameter of each antibiotic is interpreted using

the interpretative chart and the organism is reported “resistant”, “moderately/ intermediate susceptible” or “susceptible”.

b. **Strokes disc diffusion method-** This is a comparative disc diffusion method in which both the test and control organisms are inoculated on the same plate using an inoculum which gives seeded confluent growth (neither too heavy nor too light). Thus, the inhibition zone size or the test organism can be compared directly with that of the control.

The recommended control strains to be tested in parallel with the culture include:

Escherichia coli NCTC 10418 or ATCC 25922 for coliform organisms

Escherichia coli NCTC 11560 for tests on ℘lactams plus inhibitors

Staphylococcus aureus NCTC 6571 or ATCC 25923 for other organisms that will grow aerobically

Pseudomonas aeruginosa NCTC 10662 for pseudomonads

Haemophilus influenzae NCTC 11931 for enterococci

Enterococcus faecalis ATCC 51299 for enterococci (testing for vancomycin resistance)

II. Broth Dilution

Broth dilution testing involves challenging the organism of interest with antimicrobial agents or a broth environment. Each antimicrobial agent is tested using a range of concentration commonly expressed as ⇑g of active drug/ml of broth (i.e., ⇑g/ml). The test concentration for drug may vary depending on the organism and its associated resistances that the test is attempted to detect. For example, to detect clinically significant resistance to cefotaxime in *Streptococcus pneumoniae*, the dilution scheme need only to go as high as 2 ⇑g/ml, but to detect cefotaxime resistance in *E. coli*, the scheme must go up to 16 ⇑g/ml or beyond (Forbes et al. 2002).

Typically, the ranges of concentrations tested for each antibiotic are a series of doubling dilution (e.g., 16, 8, 2, 1, 0.5, 0.25 ⇑g/ml); the lowest antimicrobial concentration that

completely inhibits visible bacterial growth is recorded as the minimal inhibitory concentration (MIC). Whereas, the minimum bactericidal concentration (MBC) can be estimated by sub-culturing on antibiotic-free solid media of dilutions of the antibiotic above that in which inhibition has occurred overnight. The MBC is usually taken as the lowest concentration able to reduce the original inoculum by a factor of a thousand; e.g. from 10^5 cfu/ml to 10^2 cfu/ml or below (Greenwood et al. 2003).

Broth dilution testing may be carried out as micro-dilution or macro-dilution. The only difference between these two methods is the volume of broth in which the test is performed. For micro-dilution testing, the total broth volume is 0.05 to 0.1 ml and for macro-dilution testing, the volumes are usually 1.0 ml or greater. Due to being cumbersome and labor intensive, macro-dilution is rarely used in most clinical laboratories.

Standardized bacterial suspensions that match the turbidity of the 0.5 McFarland standard (i.e., 1.5×10^8 cfu/ml) usually serve as the starting point for dilutions that ultimately allow the final standard bacterial concentration of 5×10^5 cfu/ml in each micro-litre well to be achieved (Forbes et al. 2002).

Following incubation, the micro-dilution trays are examined for bacterial growth. Each tray should contain a growth control that does not contain antimicrobial agent and a sterility control that was not inoculated. Once growth in the growth control and no growth in the sterility control well have been confirmed, the growth profiles for each antimicrobial dilution can be established as the MIC determined. The micro-dilution well containing the lowest drug concentration with completely inhibited visible bacterial growth is recorded as the MIC. Once the MICs for the antimicrobials in the test battery for a particular organism have been recorded, they are usually translated into interpretive categories of susceptible, intermediate or resistant (Collee et al. 1996).

III. Diffusion in Agar Derivations

One test, which has been developed, that combines the convenience of disk diffusion with the ability to generate MIC data is the E-test. This test uses plastic strips; one side

of which contacts the antimicrobial agent concentration gradient and the other contains a numeric scale that indicates the drug concentration. Mueller-Hinton plates are inoculated as for disk diffusion at the strips and are placed on the inoculum lawn. Several strips may be placed radially on the seeded plate so that multiple antimicrobials can be tested against a single isolate. Following overnight incubation, the plate is examined and the number present at the point at which the border growth inhibition intersects the E-strip is taken as the MIC. This method provides a means of producing MIC data in those situations in which the level of resistance can be clinically imported. (e.g., penicillin or cephalosporins against *Streptococcus pneumaniae*) (Collee et al. 1996).

3.7 Beta lactamases

3.7.1 Introduction

Beta lactamases are the commonest cause of bacterial resistance to beta lactam antimicrobial agents. Their spread destroyed the utility of benzylpenicillin against staphylococci and has a huge undermined that of ampicillin against enterobacteria, *Haemophilus* spp and *Neisseria* spp. (Vasquez et al. 1994). New enzymes and new modes of production of old enzymes now threaten the value of extended-spectrum cephalosporins against enterobacteria. Numerous chromosomal and plasmid-mediated types are known and may be classified by their sequence and phenotypic properties. The ability of a ℘lactamase to cause resistance varies with its activity, quantity, and cellular location and for Gram-negative organisms, the permeability of producer strain. ℘ lactamases sometimes cause obvious resistance to substrate drugs in round tests; often, however, these enzymes reduce susceptibility without causing resistance at current pharmacologically chosen breakpoints (Livermore, 1995).

Antibiotic resistance in isolates of Enterobacteriaceae and other Gram-negative bacilli is emerging in many parts of the world as a major threat to successful therapy of infection in hospitals. ℘lactamases of Gram-negative bacteria are the most important mechanism

of resistance against ℘lactam drugs. Other enzymes include aminoglycoside modifying enzymes, chloramphenicol acetyl-transferase, erythromycin esterases. Plasmid-mediated ℘lactamases are more important clinically as these can be transferred between various species of Gram-negative bacilli. These enzymes are called Extended-Spectrum ℘ lactamases (ESBLs). ESBLs can confer resistance against all ℘lactam drugs (penicillins, 1st, 2nd and 3rd generation cephalosporins, monobactams except carbapenems and cephamycins (Livermore, 1995).

Antibiotic inactivation by bacterial enzymes is clearly demonstrated with *H. influenza*, β-lactams, and β-lactamases. β-lactamase production is the major mechanism of resistance to β-lactams. The affinity of the antibiotic β-lactam cycle for the β-lactamases is higher than that for its target, the PBP. Among all the groups of β-lactamases, the most important is represented by TEM type β-lactamases, with more than 50 different TEMs. These are the most frequent β-lactamases produced by *H. influenzae*. However, these TEM are susceptible to inhibitors of β-lactamases (clavulanic acid, sulbactam, tazobactam), molecules which are related to β-lactams, but whose affinity for β-lactamases is higher than for PBP. They have a strong inhibitory effect on β-lactamases even at low dosage, which allows the antibiotic to act on its target, leading to fairly good restoration of the activity of the antibiotic concerned (Livermore, 1995).

Resistant bacteria are emerging world wide as a threat to the favourable outcome of common infections in community and hospital settings. ℘lactamase production by several Gram-negative and Gram-positive organisms is perhaps the most important single mechanism of resistance to penicillins and cephalosporins. In the past, it was believed that cephalosporins were relatively immune to attack by ℘lactamases. It was surprising to find cephalosporin-resistant-*Klebsiella* spp. among the clinical isolates. The mechanism of this resistance was production of extended spectrum ℘lactamases (Livermore, 1995).

3.7.2 Evolution and Dissemination of -lactamases

Fifty years ago the antibiotic era began with the discovery of penicillin. Within a few years of introduction of penicillin into clinical use, penicillinase producing *Staphylococcus aureus* started to proliferate in hospitals. To overcome this problem, penicillinase-resistant-penicillins came into picture. Shortly afterward, the broad spectrum penicillins and first generation cephalosporins were introduced. They remained a first-line of defense against microbes for over 20 years, before resistance due to ℘lactamases produced by Gram-negative bacilli became a serious problem. To counter this threat, the pharmaceutical industry marketed six novel classes of ℘lactam antibiotics (cephalosporins, oxyimino- cephalosporins, carbapenems, monobactams and clavam and penicillanic acid sulfone inhibitors) within a relatively short span of 7-8 years. Although, novel ℘lactamases had emerged gradually after the introduction of new ℘lactam agents, their number and variety accelerated at an alarming rate. More than 170 ℘lactamases have been recognized. Their growth spurt shows no signs of slowing down (Livermore, 1995) .

3.7.3 Classification Schemes

Various classification schemes have been proposed by many researchers. Classification of Sawai et al in 1968 was based on response to antisera. Richmond and Sykes scheme in 1973 was on the basis of substrate profile. Extension of Richmond and Sykes scheme by Sykes and Mathew in 1976 was based on differentiation by isoelectric focusing. In the scheme proposed by Mitushahi and Inoue in 1981, the category ‘cefuroxime hydro-lyzing ℘lactamases was added to ‘Penicillinase and cephalosporinase’ classification. The groupings proposed by Bush in 1989 were based on correlation of substrate and inhibitory properties with molecular structure (Bush et al. 1995). However, the number and variety of enzymes have proliferated beyond the boundaries of the scheme. A more modern scheme based on molecular structure classification was proposed by Ambler includes, of necessity, only those enzymes that have been characterized. Recently a new classification scheme has been developed to integrate functional and molecular

characteristics. The Bush-Jacob-Medeiros scheme puts 178 ℘lactamases from naturally occurring bacterial isolates into four groups based on their hydrolytic spectrum, susceptibility inhibitors and whether they are encoded by the chromosome or by plasmids.

Phenotypic classifications face the problem that point mutations can greatly alter substrate specificity (Phillipon et al. 1989) and inhibitor susceptibility (Vedel et al. 1992), changing group to which an enzyme is assigned. Increasingly, therefore, ℘ lactamases are classified on sequence, as was first proposed by Ambler (Ambler, 1980). Such classification is stable, as it reflects fundamental relationships and cannot be distorted by mutations. Moreover, at least at the present, sequence-based classification has the beauty of simplicity, recognizing only the classes, designed A to D. Classes A, C and D comprise evolutionarily distinct group of serine enzymes and class B contains the zinc-dependent "EDTA-inhibited" type (Waley, 1992).

3.7.4 Structure and Mechanism of Action of -lactamase

All ESBLs have serine at their active sites except for a small (but rapidly growing) group of metallo-℘lactamases belonging to class B. They share several highly conserved amino-acid sequences with penicillin binding proteins (PBPs) (Medeiros, 1997). ℘lactamases attack the amide-bond in the ℘lactam ring of penicillin and cephalosporins, with subsequent production of penicillinoic acid, respectively, ultimately rendering the compounds anti-bacteriologically inactive (Ayyagari and Bhargava, 2001). Plasmids responsible for ESBL production tend to be large (80 KB or more in size) and carry resistance to several agents, an important limitation in the design of treatment alternatives (Jacoby and Medeiros, 1991). The most frequent co-resistances found in ESBL producing organisms are aminoglycosides, fluroquinolones, tetracyclines, chloramphenicol and sulfamethoxazole-trimethoprim. Except for one brief report, none of these enzymes have been shown to be transposable. The usual transmissibility of the responsible plasmids, however, allows resistance to spread readily to other pathogens (Jacoby and Medeiros, 1991) so that extended spectrum

enzymes have been found in nearly all species of Enterobacteriaceae. Reports of ESBLs in *Proteus mirabilis* have been relatively rare. In this species, it may be due to low frequency of plasmid conjugation (Ayyagari and Bhargava, 2001). Since ESBL production is usually plasmid mediated, it is possible for one specimen to contain both ESBL producing and non ESBL producing cells of the same species. This suggests that for optimal detection, several colonies must be tested from a primary culture plate (Coudron et al. 1997).

3.8 Extended Spectrum Beta-lactamases (ESBLs)

Resistance to ℘lactam antimicrobial agents in Gram-negative bacilli is primarily mediated by ℘lactamases. Although a variety of ℘lactamases have been described, the TEM and SHV enzymes are those most frequently observed among members of the family Enterobacteriaceae. Mutations in the genes encoding the TEM and SHV ℘ lactamases can extend the spectrum of enzyme activity to include penicillins, the extended-spectrum cephalosporins (ESCs) (examples: ceftazidime, cefotaxime and ceftriaxone), and aztreonam. Such enzymes are called Extended-Spectrum ℘lactamases (ESBLs). First described in 1983 (Knothe et al. 1983), ESBLs have contributed to the dramatic increase in resistance to ℘lactam agents among Gram-negative bacteria in recent years (Coudron et al. 1997).

3.8.1 Definition of Extended Spectrum Beta-lactamases

ESBLs are enzymes that generally belong to Ambler's molecular class A (Ambler, 1980) and Bush's functional class 2be (Bush et al. 1995) mediate resistance to extended-spectrum (third generation) cephalosporins (Examples: ceftazidime, cefotaxime and ceftriaxone) and monobactams (Example: aztreonam) but do not affect cephamycins (Examples: cefoxitin and cefotetan) or carbapenems (Examples: meropenem or imipenem). ESBLs include:

• Cephalosporin-hydrolysing mutans of TEM and SHV – the common plasmid-mediated

• Penicillinases of Enterobacteriaceae- over hundred of such variants are known (http://www.lahey.org/studies/inc_wrbt.asp).

• CTX-M types- these evolved separately, at least some of them via the escape and mutation of chromosomal ℘lactamases of *Kluyvera* spp. Over 30 variants are known (Bonnet, 2004).

• Obscure types, e.g. VEB and PER, not yet of concern in the UK; also OXA (Class D) ESBLs from *Pseudomonas aeruginosa*, in Turkey.

ESBLs are not the sole ℘lactamases to confer resistance to 2nd and 3rd generation cephalosporins but are the most important. They occur mostly in Enterobacteriaceae (Examples: *E. coli*, *Klebsiella* spp. and *Enterobacter* spp.) and rarely in non-fermenters (Examples: *P. aeruginosa*). They should be distinguished from other important modes of resistance to 2nd and 3rd generation cephalosporin:

• Hyperproduced chromosomal AmpC ℘lactamases, especially in *Enterobacter* spp.

• Hyperproduced K1 or KOXY chromosomal ℘lactamases in *K. oxytoca* not *K. pneumoniae*

• Efflux-mediated resistance in *P. aeruginosa.*

• Various ill-defined mechanisms in *Acinetobacter* spp.

Guidelines on distinguishing all these resistance mechanisms from strain phenotypes have been updated recently (Livermore et al. 2000).

3.8.2 Clinical importance of ESBLs

ESBLs are clinically important because:

• They destroy cephalosposins, workhorse hospital antibiotics, given as first-line agents to many severely ill-patients including those with intra-abdominal infections, community-acquried pneumonias and bacteraemias.

• Delayed recognition and inappropriate treatment of severe infections causes by ESBL producers with cephalosporins has been associated with increased mortality.

• Many ESBL producers are multi-resistant to non-β-lactam antibiotics such as quinolones, aminoglycosides and trimethoprim, narrowing treatment options.

• Some producer strains achieve outbreak status, spreading among patients and locales, perhaps owing to particular pathogenicity traits (Paterson et al. 2000).

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3.8.3 Laboratory detection of ESBLs

a. Screening of ESBLs: Choice of indicator cephalosporin

The ideal indicator cephalosporin is one to which all ESBLs confer resistance, even when their production is scanty. Choice is predicted by the following general traits:

• TEM and SHV ESBLs- obvious resistance to ceftazidime, variable to cefotaxime

• CTX-M ESBLs- obvious resistance to cefotaxime: variable to ceftazidime

• All ESBLs – obvious resistance to cefpodoxime

Cefuroxime, cephalexin and cephradine are unreliable indicators. It follows that the logical indicator is either cefpodixime or both of cefotaxime and ceftazidime.

The CLSI (Clinical and Laboratory Standard Institute) has developed broth microdilution and disk diffusion screening tests using selected antimicrobial agents. Each *Klebsiella pneumoniae, K. oxytoca, or Escherichia coli* isolate should be considered a potential ESBL-producer if the test results are as follows:

Disc diffusion	MICs
Cefpodoxime ≤ 22mm	Cefpodoxime ≥ 2 µg/ml
Ceftazidime ≤ 22mm	Ceftazidime ≥2 µg/ml
Aztreonam ≤ 27mm	Aztreonam ≥2 µg/ml
Cefotaxime ≤ 27mm	Cefotaxime ≥2 µg/ml
Ceftriaxone ≤ 25mm	Ceftriaxone ≥2 µg/ml

b. Confirmatory tests for ESBLs

Enterobacteriaceae isolates resistant to any indicator cephalosporin in the screening tests outlined above should be subjected to confirmatory synergy between clavulanate and those indicator cephalosporin(s) to which the isolate was initially found resistant. Three methods can be used:

i. Double Disc Synergy Tests (DDST)

A plate is inoculated as for a routine susceptibility test. Discs containing cefotaxime and ceftazidime 30µg (or cefpodoxime 10 µg) are applied either side of one with amoxyclav (20+10) µg. The distance between cephalosporin disk and amoxyclav is adjusted 20-30 mm center to center depending on the species (Jarlier et al. 1988; Sanguinetti et al. 2002) or 15mm edge to edge (Coudron et al. 1997). Enhancement of inhibition zone towards the co-amoxyclav is considered suggestive of ESBL production. The method is cheap; but the optimal disc separation varies with the strain and some producers may be missed.

ii. Combination disc methods

(Oxoid or Becton Diskinson 'Combination Discs' and Mast 'MAST DO').

These compare the zones of cephalosporin discs to those of the same cephalosporin plus clavulanate. According to the supplier, either the difference in zone diameters (Oxoid) or the ratio of diameters, is compared (Mast and BD) with zone diameter which increases of >5 mm or >50% in the presence of the clavulanate implying ESBL production. These tests are cheap and do not require critical disc spacing.

iii. Etest ESBL strips

These have a cephalosporin gradient at one end and a cephalosporin + clavulanate gradient at the other. Users should follow the manufacturer's instructions, including for a heavier inoculum than in BSAC disc tests. ESBL production is inferred if the MIC ratio for cephalosporin alone: cephalosporin + clavulanate MIC is >8. These are accurate and precise, but more expensive than combination discs.

K. pneumoniae ATCC 700603 (positive control) and *E. coli* ATCC 25922 (negative control) should be used for quality control of ESBL tests (CLSI, 2007).

3.8.4 Pitfalls and problems for ESBL tests

Other isolates of Enterobacteriaceae, such as *Salmonella* species and *Proteus mirabilis*, and isolates of *Pseudomonas aeruginosa* produce ESBLs. However, at the time, methods for screening and phenotypic confirmatory testing of these isolates have not been determined by CLSI. The inhibitory-based confirmatory test approach is the most promising detection method. However, with isolates of some species, clavulanate is an unreliable agent of this test. The inhibitor-based approach is most reliable for isolates that do not co-produce an inhibitor-resistant beta-lactamase, such as AmpC. Tazobactam and sulbactam are much less likely to induce AmpC beta-lactamases and are therefore, preferable inhibitors for ESBL detection tests with these organisms (Thomson et al. 2001).

Species with inducible AmpC ß-lactamases: ESBLs are harder to detect in those Enterobacteriaceae with inducible AmpC chromosomal enzymes (e.g. *Enterobacter* spp. *Citrobacter freundii*, *Morganella morganii*, *Providencia* spp. and *Serratia* spp.). The AmpC enzymes may be induced by clavulanate (which inhibits them poorly) and may then attack the cephalosporin, masking synergy from inhibition of the ESBL.

• If ESBL tests are to be done on *Enterobacter* spp. (10-20% of cephalosporin resistance in *Enterobacter* spp. is due to ESBLs, not de-repressed AmpC). It is best to use an AmpC-stable cephalosporin (i.e. cefepime or cefpirome) in the clavulanate synergy tests (though NOT as the first indicator compound).

• Cephalosporin therapy of *Enterobacter* spp. and *C. freundii* infections is anyway not recommended, owing to the risk of selecting AmpC-derepressed mutants; and clinicians should be steered away from the use of these agents.

***K. oxytoca* :** 10-20% of *K. oxytoca* isolates hyperproduce their class A “K1” or “KOXY” chromosomal ß-lactamase. These are resistant to cefpodoxime and often cefotaxime but not to ceftazidime.

They may give positive clavulanate synergy tests with cefotaxime or cefepime (never ceftazidime), so that producers are confused with ESBL producers. K1 hyperproduction resistance should be suspected if a Klebsiella isolate is indole-positive and has high-level resistance (growth up to the disc) to piperacillin/tazobactam and cefuroxime, but has borderline susceptibility to cefotaxime and full susceptibility to ceftazidime.

***Acinetobacter* spp., *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*:** ESBL tests were NOT developed for these species and should NOT be used for them. False positive results with *Acinetobacter* spp. are common owing to inherent susceptibility to clavulanate, whilst *S. maltophilia* may give positive results via inhibition of its chromosomal L-2 ß-lactamase. ESBLs may occur in these genera (e.g., VEB-1 in *Acinetobacter* spp. in France), but are not the common cause of cephalosporin resistance in them and should not be routinely sought.

Enzymes with marginal ESBL activity, those expressed weakly, and those produced alongside other enzymes (e.g., derepressed AmpC) are the hardest to detect. The methods outlined here will never be as precise as the best molecular analysis, but will detect most producers (Thomson et al. 2001).

3.8.5 Risk factors for ESBL infection

Risk factors for colonization or infection by ESBL-producing organisms are little different from the risk factors for other nosocomial infections (Safdar et al. 2002). Reported risks, many of which are linked, include an increased length of stay in the hospital (Mangeney et al. 2000), an increased length of stay in the intensive care unit, increased severity of illness (Pena et al. 1995), the use of a central venous or arterial catheter, the use of a urinary catheter (Lucet et al. 1996), ventilatory assistance (De Champs et al. 1991), hemodialysis (D'Agata et al. 1998), emergency abdominal surgery, the use of a gastrostomy or jejunostomy tube, gut colonization, prior administration of an oxyimino-ß-lactam antibiotic (Kim et al. 2003) and prior administration of any antibiotics (Lautenbach et al. 2001).

3.8.6 Reporting for ESBL producers

ß-lactams: Organisms inferred to have ESBLs should be reported resistant to ALL penicillins (except temocillin), cephalosporins (except cefoxitin) and to aztreonam, irrespective of routine susceptibility results. Treatment failures and death have occurred when cephalosporins were used against ESBL producers that appeared susceptible in vitro (Paterson et al. 2000).

• Carbapenems (imipenem, meropenem and ertapenem) are consistently active and are the treatment of choice in severe infections due to ESBL producers.

• Susceptibilities of ESBL producers to ß-lactamase inhibitor combinations vary with the isolate and its amount of enzyme, but should be accepted at face value.

• Mecillinam often appears active in-vitro, but its efficacy against ESBL producers remains unproven- we are cautious of advocating its use in severe infection, but further study is needed.

• Combinations of a cephalosporin with amoxiclav should work in principle, but have not been formally evaluated in treatment, and may be antagonistic *vs. Enterobacter* spp.

Non-ß-lactams: Many ESBL producers, including community isolates with CTX-M enzymes are multi-resistant to fluoroquinolones and aminoglycosides, but susceptibilities vary, and these are good options where a strain is susceptible. The predominant CTX-M-15 producing *E. coli* strains disseminating in the UK are resistant to fluoroquinolones, trimethoprim, co-trimoxazole, tetracyclines and amikacin, gentamicin resistance is variable and is absent from the predominant strain.

Oral therapy: Among oral agents suitable for community use in UTI, nitofurantion and fosfomycin (not readily available in the UK) are active verses many ESBL producers including most of the present CTX-M-15 producing *E. coli* (Paterson et al.2000).

3.9 URINARY PATHOGENS

Gram-positive	**Gram-negative**
Staphylococcus aureus	*Escherichia coli*
Staphylococcus saprophyticus	*Proteus* spp.
Other coagulase negative staphylococci(CONS)	*Pseudomonas aeruginosa*
Enterococcus faecalis	*Klebsiella* strains

Significant Bacteriuria

Presence of bacteria in urine is called bacteriuria. However, the simple demonstration of bacteria are present in the sample of urine is not proof that it has been derived from an infection in the urinary tract. Proof of a urinary tract infection requires the demonstration that the potential pathogen is present in freshly voided urine in numbers greater than those likely to result from contamination from the urethral meatus and its environs (Collee et al. 1999).

According to the Kass, Maple and Sandford criteria to interpret significant bacteriuria, Bacterial count of $<10^5$ CFU/ml corresponds to contaminations,

Whereas those showing $\geq 10^5$ CFU/ml corresponds to significant bacteriuria,

And a count of 10^4 to 10^5 CFU/ml indicates low count significant bacteriuria, which is subjected to following conditions (Pokharel, 2004):

• Urine collected before the organisms reached log phase of growth after entered into urinary tract

• Patient under antibiotic therapy

• In sexually active young women, e.g. honeymoon cystitis

• Obstruction in the ureter

• Infection with relatively slow growing organisms, e.g. *Staphylococcus saprophyticus*, streptococci other than enterococci, *Haemophilus influenzae*

3.10 LOWER RESPIRATORY TRACT PATHOGENS

Gram-positive	**Gram-negative**
Streptococcus pneumoniae	*Haemophilus influenzae*
Staphylococcus aureus	*Klebsiella pneumoniae*
Streptococcus pyogenes	*Proteus* spp.
	Pseudomonas aeruginosa
	Yersinia pestis

Also *Mycobacterium tuberculosis, Mycoplasma pneumoniae* and *Legionella pneumophila*

Commensals: Sputum, as it is being collected passes through the pharynx and the mouth; it becomes contaminated with small numbers of commensal organisms from the upper respiratory tract and mouth (Cheesbrough, 2000). These include:

Gram-positive	**Gram-negative**
Staphylococcus aureus	*Neisseria* spp.
Staphylococcus epidermidis	*Moraxella catarrhalis*
Viridians streptococci	*Haemophilus influenzae*
Streptococcus pneumoniae	Fusobacteria
Enterococci, Diphtheroids	Coliforms

Specimen culture: A semi-quantitative method of culture is recommended, so that the presence of a potential pathogen in only small numbers, e.g. less than 10^6 /ml sputum may either be ignored or be reported to the physician as probably representing contamination of the specimen from the throat (Dixon and Miller, 1965). If, however, antibiotic treatment had been given before the specimen was taken, or if special considerations apply, as in cystic fibrosis, the presence of a potential pathogen in small numbers should not be ignored (Collee et.al. 1999).

3.11 POSSIBLE PATHOGENS IN PUS SAMPLE

A wide variety of aerobic and anaerobic bacterial species may be present, either in single or in combination, in wounds and other soft tissues. In many cases, there is mixed infection with more than one bacterial species. In some cases, a pathogenic synergy may be evident with two or more species acting together to cause more damage (Collee et al. 1999).

Following are the microorganisms found in pus samples:

Gram-positive	**Gram-negative**
Staphylococcus aureus	*Pseudomonas aeruginosa*
Streptococcus pyogenes	*Proteus* spp.
Enterococci	*Escherichia coli*
Anaerobic streptococci	*Bacteroides* spp.
Other streptococci	*Klebsiella* spp.
Clostridium tetani	*Pasteurella* spp.
Clostridium perfringens and other clostridia	
Actinomycetes	
Also *Mycobacterium tuberculosis*	

CHAPTER-IV

4. METHODOLOGY

The study site for this study was the Bacteriology Laboratory, Birendra Hospital, Chhauni, Kathmandu. The study was carried out from July 2008 to December 2008.

4.1 Urine Sample

4.1.1 Specimen Collection and Transport

The patient was given a sterile, dry, wide-necked leak-proof container and requested 10 ml of first morning mid-stream urine, explaining the need to collect the urine with as less contamination as possible, i.e. a 'clean-catch' specimen. The patient was instructed to clean the area around the urethral opening with clean water, dry the area and then begin to void to collect the mid-stream urine sample. The container labeled with the date, the name and number of the patient, and the time of collection was delivered to the laboratory along with the request form as soon as possible. When immediate delivery was not possible, specimen was refrigerated at 4-6⌈ C, and when a delay in delivery of more than 2 hours is anticipated, boric acid (1.8% w/v) was added as preservative to the urine.

4.1.2 Describing the appearance of the specimen

The specimen obtained in laboratory was observed for its color and turbidity and reported accordingly.

4.1.3 Culture of Specimen

The urine sample was cultured onto the CLED agar and Blood agar medium by semi-quantitative culture technique using a standard loop.

i. After mixing the urine sample in the container thoroughly, a loopful of sample was touched to the centre of the plate, from which the inoculum was spread in a line across the diameter of the plate.

ii. Without flaming or re-entering urine, the loop was drawn across the entire plate crossing the first inoculum streak numerous times to produce isolated colonies.

iii. The plates were incubated aerobically at 35-37 ⌈C overnight.

iv. The approximate number of colonies was counted and the number of bacterial colony-forming units (CFU) per ml of urine was estimated in accordance to the volume of urine inoculated previously. For example, 100 colonies on inoculating 0.001 ml urine would correspond to 10^5 CFU/ml.

The bacterial count was reported as:

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

However, if the culture indicated the appearance of three or greater than three organism types with predominating organism, this was interpreted as due to possible contamination of specimen and asked for another specimen. In addition to the previously described guideline, pure culture of *Staphylococcus aureus* was considered significant regardless of the number of CFUs, and antimicrobial susceptibility test was performed (Forbes et al. 2002).

4.2 Sputum Sample

4.2.1 Specimen Collection and transport

Collection of sputum sample was supervised by professional personnel. Specimen were collected in a sterile, disposable, impermeable container with a screw cap or tightly fitted cap. Each sample clearly labeled with date, patient's name, sex, bed number, time of collection and a brief history was immediately transported to the bacteriology laboratory for further processing.

4.2.2 Macroscopic Examination of Sample

The received sample was macroscopically examined to see whether it consisted of only saliva or real sputum. In case if it was found only to be watery, it was discarded and a report was sent to the physician stating that the specimen was mainly saliva, requesting for another specimen.

4.2.3 Selection criteria for the acceptance of sputum sample

Inclusion criteria for a case were sputum fulfilling the criteria of American Society for Microbiology (ASM) from:

a. any person of any age and sex complaining of chest pain; and/or shortness of breath with or without wheezing; and/or coughing; and/or haemoptysis with one or more constitutional symptoms including fever, sweating, weight loss etc.

b. patients diagnosed as acute exacerbation of chronic obstructive pulmonary disease, bronchiectasis and those having radiological evidence of consolidation, pleural effusion, empyema, and chronic debilitated patient from intensive care unit suspected of aspiration and other type of pneumonia were also included.

According to ASM criteria, a reliable lower respiratory tract specimen has more than 25 leucocytes and fewer than 10 epithelial cells per low power field.

Exclusion criteria for the subject was:

a. the presence of symptoms for more than 21 days (which signifies chronic infections)

b. the specimen no fulfilling the criteria of ASM.

4.2.4 Homogenization of Sample

The sputum samples which were very thick and mucoid were treated with a mucolytic agent for homogenization (0.01% dithiothreitol). For this purpose, the commercially available sputosol, dithiothreitol power was first subjected to dilution by first adding 5 ml sterile distilled water to the vial and dissolving the powder completely, the final dilution was made 1:20 after adding this content to 95 ml sterile distilled water. Then an equal volume of diluted sputosol was added to the sputum sample aseptically, mixed gently and then incubated at 37⌈C for 30 minutes for complete homogenization of the sample.

4.2.5 Culture of Specimen

The sample was inoculated in the MacConkey agar, Blood agar and Chocolate agar plates. In the Chocolate agar plate, 5⇑g Optochin disc and a 10 U Bacitracin disc were added to screen out *Streptococcus puemoniae* and *Haemophilus influenzae* respectively.

The plates were the incubated at 37⌈C for overnight in candle jar, whereas, the MacConkey and Blood agar plates were incubated in and aerobic condition (Collee et. al. 1999).

4.3 Pus sample

4.3.1 Specimen collection and transport

The samples were collected from different wards of the hospital. Two swabs were taken for each specimen so that one could be used for the preparation of smear for microscopy and the other for the seeding of cultures. The samples were labeled with date, time and method of collection and the patient's name, age, sex number and ward. The collected specimens were transported to the bacteriological laboratory for further processing.

4.3.2 Macroscopic examination

The colour, consistency and any peculiarities such as presence or absence of granules in pus were noted.

4.3.3 Microscopic examination

Different morphological forms of gram-positive and gram-negative bacteria were studied by performing Gram staining.

4.3.4 Culture of Specimen

The samples were cultured in MacConkey agar (MA) and Blood agar (BA). Out of two swabs taken during collection, one was used for preparation of smear of microscopy and the other for culture. The swab sample was inoculated on a BA and M**A** and incubated overnight at 37º C.

4.4 Identification of the isolates

The isolates from all three specimens (Urine, Sputum and Pus) were identified by standard diagnostic procedure (Collee et al. 1999; Forbes et al. 2002). For performing biochemical tests, a pure sub-culture from the primary culture was prepared which was taken for inoculation into various biochemical media, i.e. SIM media, MRVP broth, Huge Leifson media, Urea agar, TSI media.

4.5 Susceptibility Testing

Antibiotic susceptibility testing of the isolates was performed according to the guidelines given in CLSI, recommended by Kirby-Bauer susceptibility testing method.

- Mueller Hinton agar was prepared and sterilized as instructed by the manufacturer.
- The pH of the medium 7.2-7.4 and the depth of the medium at 4mm (about 25ml media) were maintained in Petri dish.
- Using a sterile wire loop, a single isolated colony of which the susceptibility pattern is to be determined was touched and inoculated into a Nutrient broth tube and incubated for 24 hr at 37⌈C.
- After incubation in a good light source, the turbidity of the suspension was matched with the turbidity standard of McFarland 0.5 (Prepared by adding 0.6 ml of 1% w/v barium chloride solution to 99.4 ml of 1% v/v suphuric acid (Cheesbrough, 2000).

Using a sterile swab, a plate of Mueller- Hinton agar 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 (6mm diameter) was placed, evenly distributed on the inoculated plates; not more than 6 discs were placed on a 90cm diameter Petri plate.
- Within 30 minutes of applying the discs, the plates were taken for incubation at 35⌈C for 16-18 hrs.
- After overnight incubation, the plates were examined to ensure confluent growth. Using a measuring scale, the diameter of each zone of inhibition in millimeter was measured and result was interpreted accordingly (Cheesbrough, 2000).

4.6 ESBL confirmation

Confirmation of the suspected ESBL-strains was performed according to the guideline of phenotypic confirmation testing issued by CLSI in 2007. According to these guidelines, zone diameter for possible ESBL strains is ≤ 27 mm for cefotaxime (30) and ≤ 22mm for ceftazidime (30). The suspected ESBL strains were tested for confirmation by using double disc diffusion synergy test method, using amoxiclav (20+10) disc and cefotaxime and ceftazidime (30) discs placed 20-30 mm away from it. ESBL production was confirmed when the zone of either cephalosporin was expanded by the clavulanate.

- Suspected strain of ESBL producing organism was inoculated in nutrient broth incubated for 4-6 hrs.
- The standard inoculum size was carpet cultured onto MHA plates.
- After few minutes, the plates were incorporated with separate ceftazidime and cefotaxime discs and co-amoxiclav disc.
- After overnight incubation, the results were interpreted as stated above.

4.7 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 sub-cultured in respective medium and incubated. The media were then checked for the appearance of pure growth of organisms.

4.8 Quality Control for Test

Quality of each test was maintained by using standard procedures. The quality of each agar plate prepared was tested by incubating one plate of each lot on the incubator. During identification of organism, for each test ATCC control positives and control negatives were taken simultaneously. Quality of Susceptibility tests was maintained by maintaining the thickness of Mueller-Hinton agar at 4mm and the pH at 7.2-7.4.

Similarly, antibiotic discs containing the correct amount indicated were used. Strict aseptic conditions were maintained while carrying out all the procedures.

4.9 Data Analysis

The Chi-square test can be applied to find significance in the same type of data. It is most commonly used when data are in frequencies such as in the number responses in two or more categories. Therefore, the data were analyzed by the Chi-square test in this present work. Significant tests of present work are shown in Appendix VI.

CHAPTER-V

5. RESULTS

5.1 Number of samples and result pattern

Altogether, 388 clinical samples were received in the laboratory, of which 207 were urine samples, 79 were sputum samples and 102 were pus samples.

Table 1: Pattern of clinical samples and status of MDR and ESBL strains

Specimen	Total No. of processed samples	Significant growth		MDR strains		No. of ESBL producers
		No.	%	No.	%	
Urine	207	95	45.89	62	65.26	11
Sputum	77	20	25.97	9	45	3
Pus	102	75	73.53	40	39.22	5
Total	**386**	**190**	**53.87**	**111**	**31.86**	**19**

Out of 207 urine samples, 95 (45.89%) showed significant growth and among the 95 isolates, 62 (65.26%) were multi-drug resistant, additionally, 11 isolates of *Escherichia coli* among them were found to be ESBL producers. Similarly, out of 79 sputum samples received, only 77 (97.47%) samples met the ASM criteria, so only these were considered for further processing, and out of the 77 processed samples, only 20 (25.97%) showed significant growth, out of which, 9 (45%) were multi-drug resistant, 3 isolates out of 20 (3 isolates of *Klebsiella pneumoniae*) from the sputum samples were ESBL-producers. Likewise, out of 102 pus samples, 75 (73.53%) showed significant growth with 82 isolates (some samples showed more than one type of significant bacterial growth) and among 82 isolates, 40 (39.22%) were multi-drug resistant, additionally, 5 isolates (3 isolates of *Escherichia coli* and 2 isolates of *Klebsiella pneumoniae*) among 40 MDR-strains were found to be ESBL-producers. Thus, out of 386 processed samples, 209 showed significant growth and out of which 123 (31.86%) were found to be MDR-strains, and 21 of them were found to be ESBL-producers.

5.2 Urine Samples

5.2.1 Growth pattern in urine sample

Table 2: Growth pattern in urine sample

Specimen	Total No. of samples	Significant growth		No Significant Growth		No growth	
		No.	%	No.	%	No.	%
Urine	207	95	45.9	18	8.7	94	45.4

Out of 207 urine samples received, 95 (45.9%) samples showed significant growth with 95 bacterial isolates, 94 (45.4%) showed no growth and 18 (8.7%) showed growth of no significance.

5.2.2 Age and Gender-wise Distribution of In-patients requesting for Urine Culture

Table 3: Age and Gender-wise Distribution of In-patients requesting for urine culture

Age group	Surgical		Medical		ICU		NFW	Gynecological	Total (%)
	M	F	M	F	M	F	F	F	
0-10	0	0	8	0	0	0	0	0	**8 (3.86)**
10-20	0	1	5	2	0	0	1	2	**11 (5.31)**
20-30	18	0	25	1	0	0	6	8	**58 (28.03)**
30-40	15	1	6	1	2	1	6	2	**32 (15.45)**
40-50	9	1	2	1	1	0	7	7	**28 (13.53)**
50-60	11	1	2	1	1	0	3	1	**20 (9.66)**
60-70	3	2	1	1	1	3	3	1	**15 (7.24)**
70-80	12	0	5	2	2	1	2	0	**24 (11.59)**
80-90	4	1	2	0	0	1	1	1	**11 (5.31)**
Total	**70**	**7**	**56**	**10**	**7**	**6**	**29**	**22**	**207 (100.00)**

Where, M= Male; F= Female

The age group 20-30 years had the maximum requests of 58 (28.03%) for urine culture, while the age group 30-40 was second with 32 (15.45%) requests. Age group below 10 years requested the least with 8 (3.86%) requests. For both the age groups 20-30 and 30-40, male requests were more than female in all the wards except NFW and Gynecological (no male patients are admitted in these wards).

5.2.3 Pattern of bacterial isolates from Urine Sample

Table 4: Pattern of Bacterial isolates from urine sample

Bacteria	No. of isolates (%)	MDR (%)	ESBL (%)	% of total isolates
Gram-positive bacteria				
Staphylococcus aureus	2 (50)	------	------	2.10
Enterococcus faecalis	2 (50)	1 (50)	------	2.10
TOTAL	**4 (100)**	**1 (25)**	------	**4.20**
Gram-negative bacteria				
Escherichia coli	68 (74.70)	47 (69.12)	11 (16.18)	71.58
Morganella morganii	3 (3.30)	1 (33.33)	------	3.16
Klebsiella pneumoniae	6 (6.60)	2 (33.33)	------	6.31
Citrobacter freundii	3 (3.30)	2(66.66)	------	3.16
Proteus mirabilis	3 (3.30)	3 (100.00)	------	3.16
Proteus vulgaris	3 (3.30)	1 (33.33)	------	3.16
Pseudomonas aeruginosa	5 (5.50)	5 (100.00)	------	5.26
TOTAL	**91 (100.00)**	**61 (67.03)**	**11 (16.18)**	**95.79**

Among the 95 isolates, Gram-negative bacteria were predominant constituting 91 (95.79%) of the total isolates, among them, 61 (67.03%) were MDR whereas 11 (12.09%) were found to be ESBL-producers. Among Gram-negatives, *Escherichia coli* was the most frequently isolated species with 68 (74.70%) isolates, among them, 47 (69 .12%) were found to be MDR and 11 (16.18%) were ESBL-producers. Significant growth of *E. coli* on CLED media has been shown on Photograph 1. Among 6 isolates of *Klebsiella pneumoniae*, 2 isolates were found to be MDR but none of them were ESBL-producers. Other bacteria like, *Morganella morganii*, *Citrobacter freundii, Proteus mirabilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* were also isolated during the study. Gram-positive organisms constituted only 4(4.20%) of total isolates i.e. 2 isolates of *Staphylococcus aureus* and 2 isolates of E*nterococcus faecalis*, among them, only one strain of *Enterococcus faecalis* was found to be MDR.

5.2.4 Distribution of Pathogens among In-patients from Urine Sample

Table 5: Distribution of Pathogens among In-patients from Urine Sample

Bacterial isolates	Surgical		Medical		ICU		NFW		Gynecological		Total (MDR)
	F	%	F	%	F	%	F	%	F	%	
S. aureus	2(0)	100	0	0	0	0	0	0	0	0	**2(0)**
E. faecalis	1(1)	50	0	0	0	0	0	0	1(0)	50	**2(1)**
E. coli	34(26)	50	14(10)	13.23	3(3)	4.41	9(5)	13.23	8(3)	11.76	**68(47)**
M. morganii	1(1)	33.33	2(0)	33.33	0	0	0	0	0	0	**3(1)**
K. pneumoniae	3(1)	50	0	0	0	0	1(1)	16.66	2(0)	33.33	**6(2)**
C. fruendii	1(1)	33.33	1(1)	33.33	0	0	1(0)	33.33	0	0	**3(2)**
P. mirabilis	3(3)	100	0	0	0	0	0	0	0	0	**3(3)**
P. vulgaris	2(1)	66.66	0	0	0	0	1(0)	33.33	0	0	**3(1)**
P. aeruginosa	3(3)	60	2(2)	40	0	0	0	0	0	0	**5(5)**
Total	**50(37)**	**52.63**	**19(13)**	**13.68**	**3(3)**	**3.15**	**12(6)**	**12.63**	**11(3)**	**11.58**	**95(62)**

Note: Figures in parentheses indicate the number of MDR-strains isolated for that species. Where, F= Frequency of the isolates

E. coli was the most predominant pathogen isolated from different wards of the hospital. Out of 68 *E. coli* isolates, 34 (50%) were from patients admitted in Surgical ward, from whom other types of pathogens were also isolated in predominant numbers than from other wards.

5.2.5 Age-wise Distribution of Pathogens and MDR-strains from Urine Sample

Table 6: Age-wise Distribution of Pathogens and MDR-strains from Urine Sample

Bacterial isolates	Age Group							Total
	<20	20-30	30-40	40-50	50-60	60-70	>70	
S. aureus	0	1 (0)	1 (0)	0	0	0	0	**2(0)**
E. faecalis	0	0	1 (1)	1(0)	0	0	0	**2(1)**
E.coli	7 (5)	16 (13)	10 (7)	10(6)	10(5)	7(5)	8(6)	**68(47)**
M. morganii	1 (0)	0	0	0	0	1(1)	1(0)	**3(1)**
K. pneumoniae	0	1 (0)	1(0)	1(1)	1(0)	1(0)	1(1)	**6(2)**
C. freundii	0	1 (0)	1(1)	0	0	0	1(1)	**3(2)**
P. mirabilis	0	0	1(1)	0	0	0	2(2)	**3(3)**
P. vulgaris	0	0	0	1(0)	0	1(1)	1(0)	**3(1)**
P. aeruginosa	0	1(1)	0	1(1)	0	2(2)	1(1)	**5(5)**
Total	**8 (5)**	**20 (14)**	**15(10)**	**14(8)**	**11(5)**	**12(9)**	**15(11)**	**95(62)**

Note: Figures in parentheses indicate the number of MDR-strains isolated for that species.

Among the total of 95 isolates, 20 were from age-group 20-30, out of which 14 were found to be MDR. Likewise, 15 isolates were isolated from samples collected from patients of age-group 30-40, out of which 10 were found to be MDR-strains. *Escherichia coli* were the most predominant MDR-strain, found in all age-groups.

5.2.6 Antibiotic Susceptibility Pattern of Gram-positive isolates from Urine Sample

Among the common antibiotics used against all Gram-positive isolates, erythromycin was the drug of choice with susceptibility of 100% (4/4). Other drugs like, amoxycillin and ciprofloxacin can be used as the alternatives for the treatment of infections caused due to Gram-positive bacteria. The results are shown in Figure 4.

5.2.7 Antibiotic Susceptibility Pattern of Gram-negative isolates from Urine Sample

Among the Gram-negative isolates of urine, nitrofurantoin seems to be the most effective drug with susceptibility of 69.23% (63/91), followed by ceftazidime. Amikacin and imipenem can be used and second line drugs if the isolated bacteria showed resistance to the first line drugs (amoxycillin, nitrofurantoin, etc.). The results are shown in Figure 5.

5.2.8 Antibiotic Susceptibility Pattern of Bacterial isolates from Urine Sample

Table 7: Antibiotic Susceptibility Pattern of Bacterial isolates from Urine Sample

No. of bacterial isolates	Antibiotic used	Susceptibility pattern						Total
		Sensitive		Moderately Sensitive		Resistant		
		No.	%	No.	%	No.	%	
Staphylococcus aureus (N=2)	Amoxycillin	2	100	0	0		0	**2**
	Ciprofloxacin	2	100	0	0	0	0	**2**
	Cephalaxin	2	100	0	0	0	0	**2**
	Cloxacillin	2	100	0	0	0	0	**2**
	Erythromycin	2	100	0	0	0	0	**2**
Enterococcus faecalis (N=2)	Amoxycillin	1	50	0	0	1	50	**2**
	Ciprofloxacin	1	50	0	0	1	50	**2**
	Cephalaxin	1	50	0	0	1	50	**2**
	Cloxacillin	0	0	0	0	0	0	**2**
	Erythromycin	2	100	0	0	0	0	**2**
Escherichia coli (N=68)	Amoxycillin	12	17.64	1	1.47	55	80.88	**68**
	Ciprofloxacin	19	27.94	4	5.88	45	66.17	**68**
	Cephalexin	9	13.23	7	10.29	52	76.47	**68**
	Nalidixic acid	14	20.59	0	0	54	79.41	**68**
	Nitrofurantoin	51	75	9	13.23	8	11.76	**68**
	Norfloxacin	18	26.47	0	0	50	73.53	**68**
	Ceftazidime	35	51.47	1	1.47	32	47.06	**68**
	Amikacin	25	69.44	2	5.55	9	25	**36**
	Imipenem	14	100	0	0	0	0	**14**
Morganella morganii (N=3)	Amoxycillin	1	33.33	0	0	2	66.66	**3**
	Ciprofloxacin	2	66.66	0	0	1	33.33	**3**
	Cephalexin	0	0	0	0	3	0	**3**
	Nalidixic acid	1	33.33	0	0	2	66.66	**3**
	Nitrofurantoin	2	66.66	1	33.33	0	0	**3**
	Norfloxacin	1	33.33	0	0	2	66.66	**3**
	Ceftazidime	2	66.66	0	0	1	33.33	**3**
Klebsiella pneumonaie (N=6)	Amoxycillin	3	50	1	16.66	2	33.33	**6**
	Ciprofloxacin	3	50	0	0	3	50	**6**
	Cephalexin	1	16.66	1	16.66	4	66.66	**6**
	Nalidixic acid	3	50	0	0	3	50	**6**
	Nitrofurantion	4	66.66	0	0	2	33.33	**6**
	Norfloxacin	3	50	0	0	3	50	**6**
	Ceftaxidime	3	50	0	0	3	50	**6**
	Amikacin	2	33.33	0	0	0	0	**6**

No. of bacterial isolates	Antibiotic used	Susceptibility pattern						Total
		Sensitive		Moderately Sensitive		Resistant		
		No.	%	No.	%	No.	%	
Citrobacter freundii (N=3)	Amoxycillin	0	0	0	0	3	100	**3**
	Ciprofloxacin	2	66.66	0	0	1	33.33	**3**
	Cephalexin	0	0	0	0	3	100	**3**
	Nalidixic acid	1	33.33	0	0	2	66.66	**3**
	Nitrofurantoin	1	33.33	1	33.33	1	33.33	**3**
	Norfloxacin	1	33.33	0	0	2	66.66	**3**
	Ceftazidime	1	33.33	1	33.33	1	33.33	**3**
	Amikacin	2	66.66	0	0	0	0	**3**
Proteus mirabilis (N=3)	Amoxycillin	0	0	0	0	3	100	**3**
	Ciprofloxacin	1	33.33	0	0	2	66.66	**3**
	Cephalexin	0	0	0	0	3	100	**3**
	Nalidixic acid	2	66.66	0	0	1	33.33	**3**
	Nitrofurantoin	3	100	0	0	0	0	**3**
	Norfloxacin	1	33.33	0	0	2	66.66	**3**
	Ceftazidime	0	0	0	0	3	100	**3**
Proteus vulgaris (N=3)	Amoxycillin	0	0	0	0	3	100	**3**
	Ciprofloxacin	2	66.66	1	33.33	0	0	**3**
	Cephalexin	0	0	1	33.33	2	66.66	**3**
	Nalidixic acid	2	66.66	1	33.33	0	0	**3**
	Nitrofurantion	1	33.33	1	33.33	1	33.33	**3**
	Norfloxacin	2	66.66	0	0	1	33.33	**3**
	Ceftaxidime	2	66.66	0	0	1	33.33	**3**
Pseudomonas aeruginosa (N=5)	Ciprofloxacin	2	40	1	20	2	40	**5**
	Cephalexin	0	0	0	0	5	100	**5**
	Nalidixic acid	0	0	0	0	5	100	**5**
	Nitrofurantoin	1	20	0	0	4	80	**5**
	Norfloxacin	0	0	0	0	5	100	**5**
	Ceftazidime	2	40	0	0	3	60	**5**
	Amikacin	5	100	0	0	0	0	**5**

Altogether 95 bacteria were isolated from urine samples collected from in-patients of the hospital. The Gram-positive isolates (*Staphylococcus aureus* and *Enterococcus faecalis*) were found to be most sensitive towards erythromycin while most of the Gram-negative isolates (*Escherichia coli, Morganella morganii, Klebsiella pneumonaie, Proteus mirabilis, Proteus vulgaris*) showed the susceptibility towards nitrofurantoin while others like, *Pseudomonas aeruginosa, Citrobacter freundii* were most sensitive to amikacin.

5.2.9 Antibiotic Susceptibility Pattern of MDR-strains from Urine Sample

Table 8: Antibiotic Susceptibility Pattern of MDR-strains from Urine Sample

No. of bacterial isolates	Antibiotic used	Susceptibility pattern					
		Sensitive		Moderately Sensitive		Resistant	
		No.	%	No.	%	No.	%
Enterococcus faecalis (N=1)	Amoxycillin	0	0	0	0	1	100
	Ciprofloxacin	0	0	0	0	1	100
	Cephalexin	0	0	0	0	1	100
	Cloxacillin	0	0	0	0	1	100
	Erythromycin	1	100	0	0	0	0
Escherichia coli (N=47)	Amoxycillin	0	0	0	0	47	100
	Ciprofloxacin	0	0	4	8.51	43	91.14
	Cephalexin	0	0	1	2.12	46	97.87
	Nalidixic acid	3	6.38	0	0	44	93.61
	Nitrofurantoin	32	68.08	8	17.02	7	14.89
	Norfloxacin	0	0	1	2.12	46	97.87
	Ceftazidime	13	27.65	1	2.12	33	70.21
	Amikacin	25	69.44	2	5.55	9	25
	Imipenem	14	100	0	0	0	0
Morganella morganii (N=1)	Amoxycillin	0	0	0	0	1	100
	Ciprofloxacin	0	0	0	0	1	100
	Cephalexin	0	0	0	0	1	100
	Nalidixic acid	0	0	0	0	1	100
	Nitrofurantoin	1	100	0	0	0	0
	Norfloxacin	0	0	0	0	0	0
	Ceftazidime	0	0	0	0	0	0
Klebsiella pneumonaie (N=2)	Amoxycillin	0	0	0	0	2	100
	Ciprofloxacin	0	0	0	0	2	100
	Cephalexin	0	0	0	0	2	100
	Nalidixic acid	0	0	0	0	2	100
	Nitrofurantion	1	50	0	0	1	50
	Norfloxacin	0	0	0	0	2	100
	Ceftaxidime	0	0	0	0	2	100
	Amikacin	2	100	0	0	0	0
Citrobacter freundii (N=2)	Amoxycillin	0	0	0	0	2	100
	Ciprofloxacin	1	50	0	0	1	50
	Cephalexin	0	0	0	0	2	100
	Nalidixic acid	0	0	0	0	2	100
	Nitrofurantoin	0	0	1	50	1	50
	Norfloxacin	0	0	0	0	2	100
	Ceftazidime	0	0	1	50	1	50
	Amikacin	2	100	0	0	0	0

Bacteria (No. of isolates	Antibiotic used	Susceptibility pattern					
		Sensitive		Moderately Sensitive		Resistant	
		No.	%	No.	%	No.	%
Proteus mirabilis (N=3)	Amoxycillin	0	0	0	0	3	100
	Ciprofloxacin	1	33.33	0	0	2	66.66
	Cephalexin	0	0	0	0	3	100
	Nalidixic acid	2	66.66	0	0	1	33.33
	Nitrofurantoin	3	100	0	0	0	0
	Norfloxacin	1	33.33	0	0	2	66.66
	Ceftazidime	0	0	0	0	3	100
Proteus vulgaris (N=1)	Amoxycillin	0	0	0	0	1	100
	Ciprofloxacin	0	0	1	100	0	0
	Cephalexin	0	0	0	0	1	100
	Nalidixic acid	0	0	1	100	0	0
	Nitrofurantion	0	0	0	0	1	100
	Norfloxacin	0	0	0	0	1	100
	Ceftaxidime	0	0	0	0	1	100
Pseudomonas aeruginosa (N=5)	Ciprofloxacin	2	40	1	20	2	40
	Cephalexin	0	0	0	0	5	100
	Nalidixic acid	0	0	0	0	5	100
	Nitrofurantoin	1	20	0	0	4	80
	Norfloxacin	0	0	0	0	5	100
	Ceftazidime	0	0	2	40	3	60
	Amikacin	5	100	0	0	0	0

The antibiotic susceptibility pattern of the MDR-strains among the bacterial isolates from urine sample is shown in Table 8. The efficiency of drugs against the MDR-strains was found to be similar to that seen against the total bacterial isolates. The MDR strain of *E. coli* on MHA media has been shown on Photograph 2.

5.2.10 Antibiotic Resistance Pattern of the Bacterial isolates from Urine Sample

Table 9: Antibiotic Resistance Pattern of the Bacterial isolates from Urine Sample

Bacteria	Total strains isolated	Resistant to						
		0 Drug	1 Drug	MDR strains				
				2 Drugs	3 Drugs	>3 Drugs	Total	%
S. aureus	2	0	2	0	0	0	**0**	**0**
E. faecalis	2	0	1	0	0	1	**1**	**50**
E.coli	68	6	15	0	0	47	**47**	**69.12**
M. morganii	6	0	4	0	0	2	**2**	**33.33**
K. pneumoniae	3	0	2	0	0	1	**1**	**33.33**
C. freundii	3	0	1	0	0	2	**2**	**66.66**
P. mirabilis	3	0	0	0	0	2	**3**	**100**
P. vulgaris	3	0	2	0	0	1	**1**	**33.33**
P. aeruginosa	5	0	0	0	1	5	**5**	**100**
Total	**95**	**6**	**27**	**0**	**1**	**61**	**62**	**43.15**

Out of the 95 isolates, 61 were resistant to more than 3 drugs. Only 6 isolates of *Escherichia coli* were sensitive to all the antibiotics used. All the isolates of *Proteus mirabilis* and *Pseudomonas aeruginosa* were found to be MDR-strains.

5.3 Sputum Samples

5.3.1 Percentage of sputum samples meeting ASM criteria

Table 10: Percentage of sputum samples meeting ASM criteria

Specimen	Total Samples Received	Accepted Samples		Rejected Samples	
		Number	%	Number	%
Sputum	79	77	97.47	2	2.53

Out of 79 sputum samples received, 77 (97.47%) met the ASM criteria and thus were considered for further processing, whereas 2 (2.53%) of the samples didn't meet the criteria and were not included in this study.

5.3.2 Growth pattern in Sputum sample

Table 11: Growth pattern in sputum sample

Specimen	Total No. of processed samples	Significant growth		No Significant Growth		No growth	
		No.	%	No.	%	No.	%
Sputum	77	20	25.97	52	67.53	5	6.50

Out of 77 processed samples, 20 (25.97%) samples showed significant growth, whereas 5 (6.50%) showed no growth and 52 (67.53%) showed non significant growth.

5.3.3 Age and Gender-wise Distribution of In-patients requesting for Sputum Culture

Table 12: Age and Gender-wise Distribution of In-patients requesting for Sputum Culture

Age group	Surgical		Medical		ICU		NFW	Total (%)
	M	F	M	F	M	F	F	
0-10	2	0	0	0	0	0	0	**2(2.6)**
10-20	0	0	1	0	0	0	1	**2(2.6)**
20-30	0	0	13	0	0	0	1	**14(18.18)**
30-40	2	1	4	0	2	0	0	**11(14.28)**
40-50	1	1	1	0	2	0	1	**6(7.92)**
50-60	0	0	5	0	2	1	4	**12(15.58)**
60-70	4	0	6	1	0	1	5	**16(20.78)**
70-80	2	1	3	0	1	1	1	**9(11.69)**
80-90	1	1	2	1	0	0	1	**5(6.5)**
Total	**12**	**4**	**35**	**2**	**7**	**3**	**14**	**77(100)**

Where, M= Male; F= Female

The age group 60-70 years had the maximum requests of 16 (20.78%) for sputum culture, while the age group 20-30 was second with 14 (18.18%) requests. Age group below 10 years requested the least with 2 (2.6%) requests, which were from pediatric ward. For all the age groups, male requests were more than female, except NFW (no male patients are admitted in this ward).

5.3.4 Pattern of bacterial isolates from Sputum Sample

Table 13: Pattern of Bacterial isolates from Sputum Sample

Bacteria	No. of isolates (%)	MDR (%)	ESBL (%)	% of total isolates
Gram-positive bacteria				
Streptococcus pnuemonaie	2 (100)	0	------	10
TOTAL	**2 (100)**	**0 (0)**	------	**10**
Gram-negative bacteria				
Klebsiella pneumoniae	6(33.33)	3(50)	3(50)	30
Proteus mirabilis	1(5.55)	0(0)	------	5
Pseudomonas aeruginosa	11(61.11)	6(54.54)	------	55
TOTAL	**18(100)**	**9(50)**	**3(16.66)**	**90**

Among the 20 isolates, Gram-negative bacteria were predominant constituting 18 (90%) of the total isolates, among them, 9 (50%) were MDR whereas 2 (11.11%) were found to be ESBL-producers. Among Gram-negatives, *Pseudomonas aeruginosa* was the most frequently isolated species with 11(61.11%) isolates, among them, 6 (54.54%) were found to be MDR. Among 6 isolates of *Klebsiella pneumoniae*, 3 were found to be MDR and all were confirmed as ESBL-producers. Other bacteria like, *Proteus mirabilis* were also isolated during the study. Gram-positive organisms constituted only 2(10%) of total isolates i.e. 2 isolates of *Streptococcus pneumoniae.*

5.3.5 Distribution of Pathogens among In-patients from Sputum Sample

Table 14: Distribution of Pathogens among In-patients from Sputum Sample

Bacterial isolates	Surgical		Medical		ICU		NFW		Total (MDR)
	F	%	F	%	F	%	F	%	
S. pneumoniae	0	0	2(0)	100	0	0	0	0	**2(0)**
K. pneumoniae	3(2)	50	1(1)	16.66	1(0)	16.66	1(0)	16.66	**6(3)**
P. mirabilis	0	0	0	0	1(0)	100	0	0	**1(0)**
P. aeruginosa	2(1)	18.18	4(2)	36.36	3(3)	27.27	2(0)	18.18	**11(6)**
Total	**5(3)**	**25**	**7(3)**	**35**	**5(3)**	**25**	**3(0)**	**15**	**20(9)**

Note: Figures in parentheses indicate the number of MDR-strains isolated for that species. Where, F= Frequency of the isolates

P. aeruginosa was the most predominant pathogen isolated from different wards of the hospital. Out of 11 *P. aeruginosa*, 4 (36.36%) were from patients admitted in Medical ward, from whom other types of pathogens were also isolated in predominant numbers than from other wards.

5.3.6 Age-wise Distribution of Pathogens and MDR-strains from Sputum Sample

Table 15: Age-wise Distribution of Pathogens and MDR-strains from Sputum Sample

Bacterial isolates	**Age Group**						**Total**
	20-30	30-40	40-50	50-60	60-70	>70	
S. pneumoniae	1(0)	0	0	0	0	1(0)	**2(0)**
K. pneumoniae	0	2(1)	1(1)	1(1)	2(0)	0	**6(3)**
P. mirabilis	0	0	0	0	0	1(0)	**1(0)**
P. aeruginosa	1(0)	2(2)	5(4)	0	3(0)	0	**11(6)**
Total	**2(0)**	**4(3)**	**6(5)**	**1(1)**	**5(0)**	**2(0)**	**20(9)**

Note: Figures in parentheses indicate the number of MDR-strains isolated for that species.

Among the total of 20 isolates, 6 were from age-group 40-50, out of which 5 were found to be MDR. Likewise, 5 isolates were isolated from samples collected from patients of age-group 60-70. *Pseudomonas aeruginosa* were the most predominant MDR-strain, found in all age-groups.

5.3.7 Antibiotic Susceptibility Pattern of Gram-positive isolates from sputum

Among the common antibiotics used against all Gram-positive isolates, cloxacillin was the drug of choice with susceptibility of 100% (5/5). Other drugs like, amoxycillin, erythromycin and ciprofloxacin can be used as the alternatives for the treatment of infections caused due to Gram-positive bacteria. The results are shown in Figure 9.

5.3.8 Antibiotic Susceptibility Pattern of Gram-negative isolates from sputum

Among the Gram-negative isolates of sputum, gentamicin seems to be the most effective drug with susceptibility of 66.66% (22/33), followed by ciprofloxacin. Amikacin and imipenem can be used as second line drugs if the isolated bacteria showed resistance to the first line drugs (amoxycillin, gentamicin, etc.). The results are shown in Figure 10.

5.3.9 Antibiotic Susceptibility Pattern of Bacterial isolates from Sputum Sample

Table 16: Antibiotic Susceptibility Pattern of Bacterial isolates from Sputum Sample

No. of bacterial isolates	Antibiotic used	Susceptibility Pattern						Total
		Sensitive		Moderately Sensitive		Resistant		
		No.	%	No.	%	No.	%	
Streptococcus pneumonaie (N=2)	Amoxycillin	2	100	0	0	0	0	**2**
	Ciprofloxacin	2	100	0	0	0	0	**2**
	Cephalexin	0	0	0	0	2	100	**2**
	Cloxacillin	2	100	0	0	0	0	**2**
	Erythromycin	1	50	1	50	0	0	**2**
Klebsiella pneumonaie (N=6)	Amoxycillin	3	50	0	0	3	50	**6**
	Ciprofloxacin	4	66.66	1	16.66	1	16.66	**6**
	Cephalexin	3	50	0	0	3	50	**6**
	Gentamicin	5	83.33	1	16.66	0	0	**6**
	Ceftriaxone	4	66.66	0	0	2	33.33	**6**
	Ceftazidime	3	50	0	0	3	50	**6**
	Amikacin	3	100	0	0	0	0	**3**
Proteus mirabilis (N=1)	Amoxycillin	1	100	0	0	0	0	**1**
	Ciprofloxacin	1	100	0	0	0	0	**1**
	Cephalexin	1	100	0	0	0	0	**1**
	Gentamicin	1	100	0	0	0	0	**1**
	Ceftriaxone	1	100	0	0	0	0	**1**
	Ceftazidime	1	100	0	0	0	0	**1**
Pseudomonas aeruginosa (N=11)	Ciprofloxacin	9	81.81	0	0	2	18.18	**11**
	Cephalexin	2	18.18	0	0	9	81.81	**11**
	Gentamicin	8	72.72	0	0	3	27.27	**11**
	Ceftriaxone	5	45.45	1	9.09	5	45.45	**11**
	Ceftazidime	5	45.45	1	9.09	5	45.45	**11**
	Amikacin	9	81.81	0	0	2	18.18	**11**
	Imipenem	0	0	0	0	2	100	**2**

Altogether 20 bacteria were isolated from sputum samples collected from in-patients of the hospital. The Gram-positive isolates (*Streptococcus pnuemoniae*) were found to be most sensitive towards cloxacillin while most of the Gram-negative isolates (*Klebseilla pneumonaie*, *Proteus mirabilis*) showed susceptibility towards ciprofloxacin while others like, *Pseudomonas aeruginosa*, were most sensitive to amikacin.

5.3.10 Antibiotic Susceptibility Pattern of MDR-strains from Sputum Sample

Table 17: Antibiotic Susceptibility Pattern of MDR-strains from Sputum Sample

No. of bacterial isolates	Antibiotic used	Susceptibility Pattern					
		Sensitive		Moderately Sensitive		Resistant	
		No.	%	No.	%	No.	%
Klebsiella pneumonaie (N=3)	Amoxycillin	0	0	0	0	3	100
	Ciprofloxacin	3	100	0	0	0	0
	Cephalexin	0	0	0	0	3	100
	Gentamicin	2	66.66	1	33.33	0	0
	Ceftriaxone	1	33.33	0	0	2	66.66
	Ceftazidime	0	0	0	0	3	100
	Amikacin	3	100	0	0	0	0
Pseudomonas aeruginosa (N=6)	Ciprofloxacin	4	66.66	0	0	2	33.33
	Cephalexin	0	0	0	0	6	100
	Gentamicin	3	50	0	0	3	50
	Ceftriaxone	0	0	1	16.66	5	83.33
	Ceftazidime	0	0	1	16.66	5	83.33
	Amikacin	4	66.66	0	0	2	33.33
	Imipenem	2	100	0	0	0	0

The antibiotic susceptibility pattern of the MDR-strains among the bacterial isolates from sputum samples is shown in Table 17. The efficiency of drugs against the MDR-strains was found to be similar to that seen against the total bacterial isolates.

5.3.11 Antibiotic Resistance Pattern of the Bacteria Isolated from Sputum Sample

Table 18: Antibiotic Resistance Pattern of the Bacteria Isolated from Sputum Sample

Bacteria	Total strains isolated	Resistant to						
		0 Drug	1 Drug	MDR strains				
				2 Drugs	3 Drugs	>3 Drugs	Total	%
S. pneumonaie	2	0	2	0	0	0	0	0
K. pneumoniae	6	2	1	0	0	3	3	50
P. mirabilis	1	1	0	0	0	0	0	0
P. aeruginosa	11	2	3	2	1	3	6	54.54
Total	**20**	**5**	**6**	**2**	**1**	**6**	**9**	**45**

Out of the 20 isolates, 9 were resistant to more than 3 drugs. Only 5 isolates were sensitive to all the antibiotics used. Most of the *P. aeruginosa* (6/11) isolates were found to be MDR.

5.4 Pus Samples

5.4.1 Growth pattern in Pus Sample

Table 19: Growth pattern in Pus Sample

Specimen	Total No. of samples	Significant growth		No growth	
		No.	%	No.	%
Pus	102	75	73.53	27	26.47

Out of 102 pus samples received, 75 (73.53%) samples showed significant growth with 82 bacterial isolates (more than one type of bacteria were isolated in some specimen), while 27 showed no growth.

5.4.2 Age and Gender-wise Distribution of In-patients requesting for Pus Culture

Table 20: Age and Gender-wise Distribution of In-patients requesting for Pus Culture

Age group	Surgical		Medical		ICU		Gynecological	Total (%)
	M	F	M	F	M	F	F	
0-10	1	0	0	0	0	0	0	**1 (0.98)**
10-20	0	1	0	2	2	2	0	**8 (7.84)**
20-30	16	1	3	6	1	0	1	**27 (26.47)**
30-40	11	1	2	0	1	1	0	**18 (17.65)**
40-50	8	0	1	0	0	0	2	**11 (10.78)**
50-60	8	0	2	0	1	0	2	**12 (11.76)**
60-70	7	2	2	1	0	0	1	**13 (12.74)**
70-80	1	1	2	0	2	0	1	**7 (6.86)**
80-90	1	0	2	0	2	0	1	**5 (4.90)**
Total	**53**	**6**	**14**	**9**	**9**	**3**	**8**	**102 (100)**

Where, M= Male; F= Female

The age group 20-30 years had the maximum requests of 27 (26.47%) for pus culture, while the age group 30-40 was the second, with 18 (17.64%) requests. Age group below 10 years requested the least, with only 1 (0.98%) requests. For both the age groups 20-

30 and 30-40, male requests were more than female, in all the wards except Gynecological (no male patients are admitted in this ward).

5.4.3 Pattern of bacterial isolates from Pus Sample

Table 21: Pattern of Bacterial isolates from Pus Sample

Bacteria	No. of isolates (%)	MDR (%)	ESBL (%)	% of total isolates
Gram-positive bacteria				
Staphylococcus aureus	22 (91.66)	4 (18.18)	------	26.83
CONS	2 (8.33)	0 (0)	------	2.44
TOTAL	**24 (100)**	**4 (16.66)**	------	**29.27**
Gram-negative bacteria				
Escherichia coli	23 (39.66)	16 (69.56)	3 (13.04)	28.05
Citrobacter freundii	6 (10.34)	4 (66.66)	------	70.32
Pseudomonas aeruginosa	14 (24.14)	6 (42.86)	------	17.07
Klebsiella pneumoniae	8 (13.80)	4 (50.00)	2(25.00)	9.76
Klebsiella oxytoca	2 (3.45)	2 (100.00)	------	2.44
Acinetobacter spp.	1 (1.72)	1 (100.00)	------	1.22
Proteus mirabilis	3 (3.66)	3 (100.00)	------	3.66
Proteus vulgaris	1 (1.72)	0 (0)	------	1.22
TOTAL	**58 (100.00)**	**36 (62.07)**	**5 (8.62)**	**70.73**

Among the 82 isolates, Gram-negative bacteria were predominant constituting 58 (70.73%) of the total isolates, among them, 36 (43.90%) were MDR whereas 5 (8.62%) were found to be ESBL-producers. Among Gram-negatives, *Escherichia coli* was the most frequently isolated species with 23 (39.66%) isolates, among them, 16 (69.56%) were found to be MDR and 3 (13.04%) were ESBL-producers. Among 8 isolates of *Klebsiella pneumoniae*, 4 were found to be MDR and 2 of them were ESBL-producers. Other bacteria like, *Klebsiella oxytoca, Citrobacter freundii, Proteus mirabilis, Proteus vulgaris, Acinetobacter* spp. and *Pseudomonas aeruginosa* were also isolated during the study. Gram-positive organisms constituted 24 (29.27%) of total isolates i.e. 22 *Staphylococcus aureus* and 2 Coagulase negative Staphylococci (CONS), among them, 4 strains of *Staphylococcus aureus* were found to be MDR.

5.4.4 Distribution of Pathogens among In-patients from Pus Sample

Table 22: Distribution of Pathogens among In-patients from Pus Sample

Bacteria	Cases from patients of different wards								Total
	Surgical		Medical		ICU		Gynecological		
	F	%	F	%	F	%	F	%	
S. aureus	12(3)	54.54	5(0)	22.7	3(1)	13.63	2(0)	9.09	**22(4)**
CONS	1(0)	50	0	0	0	0	1(0)	50	**2(0)**
E. coli	17(11)	73.91	3(3)	13.04	2(1)	8.69	1(1)	4.35	**23(16)**
C. fruendii	3(3)	50	2(1)	33.33	0	0	1(0)	16.66	**6(4)**
P. aeruginosa	10(4)	71.43	1(0)	7.14	3(2)	21.43	0	0	**14(6)**
K. pneumoniae	2(2)	25	2(0)	25	3(2)	37.50	1(0)	12.50	**8(4)**
K. oxytoca	2(2)	100	0	0	0	0	0	0	**2(2)**
Acinetobacter spp.	0	0	0	0	1(1)	100	0	0	**1(1)**
P. mirabilis	1(1)	33.33	2(2)	66.66	0	0	0	0	**3(3)**
P. vulgaris	1(0)	100	0	0	0	0	0	0	**1(0)**
TOTAL	**49(26)**	**59.75**	**15(6)**	**18.29**	**12(7)**	**14.63**	**6(1)**	**7.32**	**82(40)**

Note: Figures in parentheses indicate the number of MDR-strains isolated for that species. Where, F= Frequency of the isolates

E. coli was the most predominant pathogen isolated from different wards of the hospital. Out of 23 *E. coli* isolates, 17 (73.91%) were from patients admitted in Surgical ward, from whom other types of pathogens were also isolated in predominant numbers than that from other wards.

5.4.5 Age-wise Distribution of Pathogens and MDR-strains from Pus Sample

Table 23: Age-wise Distribution of Pathogens and MDR-strains from Pus Sample

Bacterial isolates	Age Group							Total
	<20	20-30	30-40	40-50	50-60	60-70	>70	
S. aureus	1(0)	6(1)	6(2)	1(0)	3(0)	3(1)	2(0)	**22(4)**
CONS	0	2(0)	0	0	0	0	0	**2(0)**
E. coli	0	7(4)	3(2)	2(2)	4(3)	3(2)	4(3)	**23(16)**
C. fruendii	0	2(2)	1(1)	1(1)	0	0	2(0)	**6(4)**
P. aeruginosa	3(1)	4(2)	2(1)	2(1)	1(0)	0	2(1)	**14(6)**
K. pneumoniae	3(2)	1(1)	0	1(1)	0	1(0)	2(0)	**8(4)**
K. oxytoca	0	0	0	1(1)	0	0	1(1)	**2(2)**
Acinetobacter spp.	0	0	0	0	0	0	1(1)	**1(1)**
P. mirabilis	0	1(1)	0	1(1)	0	1(1)	0	**3(3)**
P. vulgaris	0	1(0)	0	0	0	0	0	**1(0)**
Total	**7(3)**	**24(11)**	**12(6)**	**9(7)**	**8(3)**	**8(4)**	**14(6)**	**82(40)**

Note: Figures in parentheses indicate the number of MDR-strains isolated for that species.

Among the total of 82 isolates, 24 were from age-group 20-30, out of which 11 were found to be MDR. Likewise, 14 isolates were isolated from samples collected from patients of age-group above 70, out of which 6 were found to be MDR-strains. *Escherichia coli* were the most predominant MDR-strain, found in all age-groups.

5.4.6 Antibiotic Susceptibility Pattern of Gram-positive isolates from Pus Sample

Among the common antibiotics used against all Gram-positive isolates, erythromycin was the drug of choice with susceptibility of 95.83% (23/24). Other drugs like, cloxacillin and ciprofloxacin can be used as the alternatives for the treatment of infections caused due to Gram-positive bacteria. The results are shown in Figure 14.

5.4.7 Antibiotic Susceptibility Pattern of Gram-negative isolates from Pus Sample

Among the Gram-negative isolates of pus, ciprofloxacin seems to be the most effective drug with susceptibility of 55.17% (32/58), followed by gentamicin. Amikacin can be used as second line drug if the isolated bacteria showed resistance to the first line drugs (ciprofloxacin, gentamicin etc.). The results are shown in Figure 15.

5.4.8 Antibiotic Susceptibility Pattern of Bacterial isolates from Pus Sample

Table 24: Antibiotic Susceptibility Pattern of Bacterial isolates from Pus Sample

Bacteria (No. of isolates	**Antibiotic used**	**Susceptibility Pattern**						**Total**
		Sensitive		**Moderately Sensitive**		**Resistant**		
		No.	**%**	**No.**	**%**	**No.**	**%**	
Staphylococcus aureus (N=22)	Amoxycillin	7	31.81	8	36.36	7	31.81	**22**
	Ciprofloxacin	15	68.18	2	9.09	5	22.72	**22**
	Cephalexin	12	54.54	3	13.64	7	31.81	**22**
	Cloxacillin	21	95.45	1	4.54	0	0	**22**
	Ceftriaxone	16	72.72	2	9.09	4	18.18	**22**
	Erythromycin	21	95.45	1	4.54	0	0	**22**
CONS (N=2)	Amoxycillin	1	50	0	0	1	50	**2**
	Ciprofloxacin	2	100	0	0	0	0	**2**
	Cephalexin	1	50	0	0	1	50	**2**
	Cloxacillin	2	100	0	0	0	0	**2**
	Ceftriaxone	2	100	0	0	0	0	**2**
	Erythromycin	2	100	0	0	0	0	**2**
Escherichia coli (N=23)	Amoxycillin	6	26.09	0	0	17	73.91	**23**
	Ciprofloxacin	12	52.17	1	4.35	10	43.48	**23**
	Cephalexin	4	17.39	1	4.35	18	78.26	**23**
	Ceftriaxone	8	34.78	3	13.04	12	52.17	**23**
	Gentamicin	12	52.17	5	21.74	6	26.09	**23**
	Ceftaxidime	9	39.13	0	0	14	60.87	**23**
	Amikacin	6	26.09	0	0	0	0	**6**
Citrobacter fruendii (N=6)	Amoxycillin	1	16.66	1	16.66	4	66.66	**6**
	Ciprofloxacin	6	100	0	0	0	0	**6**
	Cephalexin	1	16.66	0	0	5	83.33	**6**
	Ceftriaxone	2	33.33	0	0	4	66.66	**6**
	Gentamicin	3	50	0	0	3	50	**6**
	Ceftazidime	3	50	0	0	3	50	**6**
Pseudomonas aeruginosa (N=14)	Ciprofloxacin	10	71.43	0	0	4	28.57	**14**
	Cephalexin	3	21.43	1	7.14	10	71.43	**14**
	Ceftriaxone	7	50	1	7.14	6	42.86	**14**
	Gentamicin	8	57.13	0	0	0	0	**14**
	Ceftaxidime	12	85.71	2	14.28	0	0	**14**
	Amikacin	7	50	1	7.14	6	42.86	**14**

Table 24: Antibiotic Susceptibility Pattern of Bacterial isolates from Pus Sample

Bacteria (No. of isolates	**Antibiotic used**	**Susceptibility Pattern**						**Total**
		Sensitive		**Moderately Sensitive**		**Resistant**		
		No.	**%**	**No.**	**%**	**No.**	**%**	
Klebsiella pnuemoniae (N=8)	Amoxycillin	3	37.5	1	12.5	4	50	**8**
	Ciprofloxacin	8	100	0	0	0	0	**8**
	Cephalexin	1	12.5	0	0	7	87.50	**8**
	Ceftriaxone	4	50	0	0	4	50	**8**
	Gentamicin	5	62.5	1	12.5	2	25	**8**
	Ceftazidime	4	50	0	0	4	50	**8**
Klebsiella oxytoca (N=2)	Amoxycillin	0	0	0	0	2	100	**2**
	Ciprofloxacin	0	0	0	0	2	100	**2**
	Cephalexin	0	0	0	0	2	100	**2**
	Ceftriaxone	0	0	0	0	2	100	**2**
	Gentamicin	2	100	0	0	0	0	**2**
	Ceftazidime	1	50	0	0	1	50	**2**
Acinetobacter sp. (N=1)	Amoxycillin	0	0	0	0	1	100	**1**
	Ciprofloxacin	0	0	0	0	1	100	**1**
	Cephalexin	0	0	0	0	1	100	**1**
	Ceftriaxone	0	0	0	0	1	100	**1**
	Gentamicin	0	0	0	0	1	100	**1**
	Ceftazidime	0	0	0	0	1	100	**1**
	Amikacin	1	100	0	0	0	0	**1**
Proteus vulgaris (N=1)	Amoxycillin	1	100	0	0	0	0	**1**
	Ciprofloxacin	1	100	0	0	0	0	**1**
	Cephalexin	1	100	0	0	0	0	**1**
	Ceftriaxone	1	100	0	0	0	0	**1**
	Gentamicin	1	100	0	0	0	0	**1**
	Ceftazidime	1	100	0	0	0	0	**1**
Proteus mirabilis (N=3)	Amoxycillin	3	100	0	0	0	0	**3**
	Ciprofloxacin	2	66.66	1	33.33	0	0	**3**
	Cephalexin	0	0	0	0	3	100	**3**
	Ceftriaxone	2	66.66	0	0	1	33.33	**3**
	Gentamicin	1	33.33	0	0	2	66.66	**3**
	Ceftazidime	0	0	0	0	3	100	**3**

Altogether 82 bacteria were isolated from pus samples collected from in-patients of the hospital. The Gram-positive isolates (*Staphylococcus aureus* and CONS) were found to be most sensitive towards erythromycin while most of the Gram-negative isolates (*Escherichia coli, Klebsiella pneumonaie, Proteus mirabilis, Proteus vulgaris*) showed

the susceptibility towards ciprofloxacin and gentamicin while others like, *Pseudomonas aeruginosa* were most sensitive to amikacin.

5.4.9 Antibiotic Susceptibility Pattern of MDR-strains from Pus Sample

Table 25: Antibiotic Susceptibility Pattern of MDR-strains from Pus Sample

Bacteria (No. of isolates	**Antibiotic used**	**Susceptibility pattern**					
		Sensitive		**Moderately Sensitive**		**Resistant**	
		No.	**%**	**No.**	**%**	**No.**	**%**
Staphylococcus aureus (N=4)	Amoxycillin	0	0	0	0	4	100
	Ciprofloxacin	1	25	0	0	3	75
	Cephalexin	1	25	0	0	3	75
	Cloxacillin	3	75	1	25	0	0
	Ceftriaxone	1	25	0	0	3	75
	Erythromycin	3	75	1	25	0	0
Escherichia coli (N=16)	Amoxycillin	0	0	0	0	16	100
	Ciprofloxacin	5	31.25	1	6.25	10	62.5
	Cephalexin	0	0	0	0	16	100
	Ceftriaxone	1	6.25	3	18.75	12	75
	Gentamicin	5	31.25	5	31.25	6	37.50
	Ceftaxidime	2	12.5	0	0	14	100
	Amikacin	6	100	0	0	0	0
Citrobacter fruendii (N=4)	Amoxycillin	1	25	0	0	3	75
	Ciprofloxacin	4	100	0	0	0	0
	Cephalexin	0	0	0	0	4	100
	Ceftriaxone	0	0	0	0	4	100
	Gentamicin	1	25	0	0	3	75
	Ceftazidime	1	25	0	0	3	75
Pseudomonas aeruginosa (N=6)	Ciprofloxacin	2	33.33	0	0	4	66.66
	Cephalexin	1	16.66	1	16.66	4	66.66
	Ceftriaxone	0	0	0	0	6	100
	Gentamicin	0	0	0	0	6	100
	Ceftaxidime	0	0	0	0	6	100
	Amikacin	4	66.66	2	33.33	0	0
Klebsiella pnuemoniae (N=4)	Amoxycillin	0	0	0	0	4	100
	Ciprofloxacin	4	100	0	0	0	0
	Cephalexin	0	0	0	0	4	100
	Ceftriaxone	0	0	0	0	4	100
	Gentamicin	1	25	1	25	2	50
	Ceftaxidime	0	0	0	0	4	100

Bacteria (No. of isolates)	Antibiotic used	Susceptibility pattern					
		Sensitive		Moderately Sensitive		Resistant	
		No.	%	No.	%	No.	%
Klebsiella oxytoca (N=2)	Amoxycillin	0	0	0	0	2	100
	Ciprofloxacin	0	0	0	0	2	100
	Cephalexin	0	0	0	0	2	100
	Ceftriaxone	0	0	0	0	2	100
	Gentamicin	2	100	0	0	0	0
	Ceftaxidime	1	50	0	0	1	50
Acinetobacter spp. (N=1)	Amoxycillin	0	0	0	0	1	100
	Ciprofloxacin	0	0	0	0	1	100
	Cephalexin	0	0	0	0	1	100
	Ceftriaxone	0	0	0	0	1	100
	Gentamicin	0	0	0	0	1	100
	Ceftaxidime	0	0	0	0	1	100
	Amikacin	1	100	0	0	0	0
Proteus mirabilis (N=3)	Amoxycillin	0	0	0	0	3	100
	Ciprofloxacin	2	66.66	1	33.33	0	0
	Cephalexin	0	0	0	0	3	100
	Ceftriaxone	2	66.66	0	0	1	33.33
	Gentamicin	1	33.33	0	0	2	66.66
	Ceftazidime	0	0	0	0	3	100

The antibiotic susceptibility pattern of the MDR-strains among the bacterial isolates from pus sample is shown in Table 25. The efficiency of drugs against the MDR-strains was found to be similar to that seen against the total bacterial isolates.

5.4.10 Antibiotic Resistance Pattern of the Bacterial isolates from Pus Sample

Table 26: Antibiotic Resistance Pattern of the Bacterial isolates from Pus Sample

Bacteria	Total strains isolated	Resistant to						
		0 Drug	1 Drug	MDR strains				
				2 Drugs	3 Drugs	>3 Drugs	Total	%
S. aureus	22	12	4	2	1	3	6	27.27
CONS	2	1	1	0	0	0	0	0
E. coli	23	5	2	0	2	14	16	69.56
C. fruendii	6	1	1	1	0	3	4	66.66
P. aeruginosa	14	2	6	0	0	6	6	42.85
K. pneumoniae	8	1	3	0	0	4	4	50.00
K. oxytoca	2	0	0	0	0	2	2	100
Acinetobacter spp.	1	0	0	0	0	1	1	100
P. mirabilis	3	0	0	0	0	0	3	100
P. vulgaris	1	1	0	0	0	0	0	0
Total	**82**	**23**	**17**	**3**	**3**	**36**	**42**	**51.21**

Out of the 82 isolates, 42 were resistant to more than 3 drugs. Only 5 isolates of *Escherichia coli* were sensitive to all the antibiotics used. All the isolates of *Klebsiella oxytoca, Acinetobacter* spp. And *Proteus mirabilis* were found to be MDR-strains.

5.5 Pattern of Suspected and Confirmed cases of ESBL strains from Urine, Sputum and Pus samples

Table 27: Pattern of Suspected and Confirmed cases of ESBL strains from Urine, Sputum and Pus samples

Sample	Bacterial isolates	Total Isolates	No. of Suspected ESBL producers	No. of Confirmed Cases (%)	Cases Negative After Confirmation
Urine	i. *E .coli*	68	34	11(16.17)	23
	ii. *K. pneumoniae*	6	2	0(0)	2
Sputum	i. *K. pneumoniae*	6	3	3(50)	0
Pus	i. *E.coli*	23	14	3(60.87)	11
	ii. *K. pneumoniae*	8	4	2(25)	2
Total		111	57	19(17.11)	38

Altogether 111 isolates of *E. coli* and *K. pneumoniae* were isolated from urine, sputum and pus samples, out of which 57 were suspected to be ESBL producers. But only 19(17.11%) were confirmed as ESBL producing bacteria. The positive and negative ESBL-confirmation test of *E.coli* on MHA media has been shown on Photograph 3 and 4 respectively.

CHAPTER-VI

6. Discussion

This study was conducted during July 2008 to December 2008 in the Bacteriology laboratory of Shree Birendra Hospital, for the purpose of evaluating the status of MDR-strains and ESBL-producing strains from urine, sputum and pus samples received in the laboratory from different wards of the hospital.

Out of 207 urine samples processed, 133 (63.77%) were from male, while 74 (36.23%) were from female. During the study, the samples were collected from different wards. 77 (37.2%) urine samples were collected from Surgical ward, 51 (24.63%) were from Medical ward, 3 (6.30%) from ICU (Intensive Care Unit), 15 (7.20%) from Pediatric ward, 29 (14%) from NFW (New Female Ward) and 22 (10.62%) from Gynecological ward. The age group of 20-30 years had the maximum requests of 58 (28.03%). Out of total samples received, 95 (45.89%) showed significant growth, among which 61 (64.21%) were from male, whereas 34 (35.79%) were from female. A similar study carried out by Dhakal (1999) showed positive growth of 25.16% and in their study, among the total requests for urine culture, 53.46% were from female patients.

Among the total isolates, 91 (95.79%) were Gram-negative bacteria. In the similar study performed by Blomberg et al. (2005), out of 107 urinary isolates 66.36% constituted Gram-negative isolates. In our study, *Escherichia coli* was the most predominant bacteria with 68 (74.70%) isolates and the second predominant one was *Klebsiella pnuemoniae* with 6 (6.60%) isolates. Other Gram-negatives isolates were *Morganella morganii, Citrobacter freundii, Proteus mirabilis, Proteus vulgaris* and *Pseudomonas aeruginosa.* Gram positive organisms constituted 4.20%, out of which 2.10% were *Staphylococcus aureus* and 2.10% were *Enterococcus faecalis.* Manandhar (1996) also found that *E. coli* was the most predominant bacterial isolate from urine constituting 53.30% of total isolates and *Staphylococcus aureus* was the frequently found Gram-positive isolates constituting 12% of the total isolates.

Nitrofurantoin seems to be the most effective drug against Gram-negative urinary isolates, with susceptibility of 69.23% (63/91), followed by ceftazidime with the susceptibility of 48.35% (44/91). In a study carried out by Dhakal (1999), 84.21% of urinary isolates were susceptible to nitrofurantoin. In our study, amoxycillin was found to be less effective drug with resistance of 80.22% (73/91) towards Gram-negative isolates whereas, 75% (3/4) of Gram-positive isolates were susceptible to amoxycillin and 100% (4/4) were sensitive towards erythromycin.

Among 68 *Escherichia coli* isolates, 47 were found to be MDR-strains. Nitrofurantoin was most effective against this bacterium with susceptibility of 75% (51/68) followed by ceftazidime 51.47% (35/68). The isolates which were less effective towards these drugs were found to be sensitive towards amikacin 69.44% (25/36) and imipenem 100% (14/14) which can be used as drug of choice for the multi-drug resistant (MDR), ESBL-producers.

Since the use of fluoroquinolone antibiotic in clinical practice was introduced about a decade ago, quinolone-resistant *E. coli* (QREC) strains are being isolated with increasing frequency. Eom et al. in 2002 reported that the incidence of QREC increased steadily from 14.4% to 21.3% during 5 years from 1996 to 2000 in Korea. In their study, they found that the multi-drug resistance rate of QREC was much higher (38.3%) than those of quinolone susceptible isolates (18.8%). Mutations at the target site appear to be the major mechanism for fluoroquinolone resistance in *E. coli*; point mutations conferring resistance are localized to particular portions of *gyrA*, which is a gyrase subunit gene and *parC*, which encodes a topoisomerase subunit. In the case of *E. coli*; mutation in *gyrA* are predictive of major differences in the level of resistance, irrespective of mutations in *parC* (Ozeki et al. 1997).

In our study, 80.88% of *E. coli* isolates were resistant to amoxycillin. Resistance to ß-lactam antimicrobial agents in *E. coli* is primarily mediated by ß-lactamase enzyme, which hydrolyses the ß-lactam ring and thus inactivates the antibiotic. The classical

TEM-1, TEM-2 and SHV-1 enzymes are the predominant plasmid-mediated lactamases of Gram-negative rods (Livermore, 1995). In addition to this mechanism, there are more than seven efflux systems in *E. coli* that can export structurally unrelated antibiotics; these multi-drug resistance efflux pump (MDR pump) systems contribute to intrins resistance for toxic compounds such as antibiotics, antiseptics, detergents and dyes (Sulavik et al. 2001).

Out of 6 *Klebsiella pneumoniae* isolates, 4 (66.66%) were sensitive to nitrofurantion and 50% to ceftazidime, whereas four isolates were resistant to cephalexin. Numerous case-control studies have examined risk factors for the isolation of fluoroquinolone-resistant Enterobacteriaceae from clinical specimens. All have identified previous fluoroquinolone exposure- either as prophylaxis (Ortiz et al. 1999) or as treatment (Parterson et al. 2000) or as a significant risk factor. Notably, all of these prior studies compared cases with fluoroquinolone resistant organisms to controls with susceptible organisms. A study conducted by Bolon et al. 2004, reported that out of 51 *Klebsiella pnuemoniae* isolates from various clinical samples, 16% were resistant to levofloxacin, a fluoroquinolone. Moreover, various clinical isolates show alteration of nonspecific porins associated with the presence of active drug efflux in these bacteria; both processes maintain a very low intracellular concentration of drugs and contribute to a high resistance level for structurally unrelated molecules including ß-lactam antibiotics, quinolones, tetracyclines, and chloramphenicol (Martinez-Martinez et al. 2002).

In our study, all the five isolates of *Pseudomonas aeruginosa* were found to be MDR-strains. Infection with *P. aeruginosa* is a serious problem affecting hospitalized patients, particularly those who are critically ill and immuno-compromised, such as patients with cystic fibrosis, neutropenia, iatrogenic immunosuppression, or disrupted anatomical barriers (Kiska et al. 1999). Rates of colonization with *P. aeruginosa* increase in hospitalized patients, particularly, in those who have been hospitalized for extended periods of time and / or have received broad-spectrum antimicrobial therapy or cancer

chemotherapy. These increasing resistance rates have greatly limited the number of therapeutic choices (Livermore, 1995).

In a study by Flamm et al. (2004), data from The Surveillance Network (TSN) Database-USA from 1999 to 2002 were analyzed to evaluate the activities of several anti-pseudomonal agents, and they found that MDR-strains (resistance to three or more antimicrobial agents) accounted for 24.9% of all isolates.

In our study, 2 isolates of *P. aeruginosa* were resistant to ciprofloxacin whereas as all the isolates were resistant to cephalexin. Li et al. in 1994 showed that active efflux played a role in the resistance, to various non- ß-lactam agents, of *P. aeruginosa* strains, and de-energization by the addition of proton conduction increased the accumulation level to that expected for equilibration across the cytoplasmic membrane. Their study also suggested the involvement of an active efflux mechanism also in the resistance to ß-lactams; the hydrophilic ß-lactams with more than one charged group did not cross the cytoplasmic membrane readily, yet one such compound, ceftriaxone, appeared to be extruded from the cells of more-resistant strains. They postulated that these strains of *P. aeruginosa* pumped out such hydrophilic ß-lactams either from the periplasm or from the outer leaflet of the lipid bilayer of the cytoplasmic membrane.

Three mechanisms of resistance are known to cause quinoline resistance in *P. aeruginosa* (Cambau and Gutmann, 1993): alteration in DNA gyrase by mutation in *gyrA* or *gyrB* genes decreased drug accumulation by decreased permeability of the cell wall, and enhanced efflux (Li et al. 1994). *GyrA* mutations appear to be the most prominent cause of resistance in clinical strains (Yoshida et al. 1994). The *gyrA* (*nfxA*, *nalA* or *cfxA*) mutation causes an alteration in the subunit A of DNA gyrase (Robillard and Scarpa, 1988). The *nalB* (*cfxB*) (Masuda and Ohya, 1992), *nfxB* and (Hirai et al. 1997), and *nfxC* (Fukuda et al. 1990) mutations cause a decrease in the level of accumulation of norfloxacin, and strains with these mutations show cross-resistance to structurally unrelated antimicrobial agents.

In our study, out of three *Proteus mirabilis* isolates, two isolates were resistant to norfloxacin and all three isolates were resistant to cephalexin, amoxycillin and ceftazidime while all three were sensitive to ciprofloxacin. Bret et al. in 1998 had reported a chromosomally encoded class C ß-lactamase produced by a clinical strain of *P. mirabilis* resistant to all penicillins and cephalosporins including cephamycins and aztreonam in France. In a recent study in France conducted by Neuwirth et al. in 2001, a clinical strain of *Proteus mirabilis* (strain Pm 631) was reported to have produced a complex mutant ß-lactamase: TEM-89 (CMT-3), which is an Inhibitor–resistant TEM (IRT) ß-lactamase.

Among the Gram-positive isolates in our study, out of two isolates of *Enterococcus faecalis*, one was found to be MDR-strain. The bacteria were 100% sensitive to erythromycin. Two mechanisms of ß-lactam resistance in *E. faecalis* have been reported, i.e. the production of ß-lactamase and the over production of penicillin-binding proteins (PBPs). A recent study in 2005 by Ono et al. in Japan, suggested that development of high level resistance to penicillins and imipenem in *Enterococcus faecalis* depends on point mutations (amino acid substitutions) of PBP4 at positions 520 and 605. Three different mechanisms account for the acquired resistance to Macrolide-lincosamide streptogramin (MLS) antibiotics in Gram-positive bacteria: modification of the drug target (23S rRNA); inactivation of the drug and active efflux of the antibiotic. Erythromycin resistance by *erm* methylases of the *ermB-ermAM* hybridization class has been described in Enterococci isolates (Berryman and Rood, 1995).

In our study, *Staphylococcus aureus* were sensitive to all the antibiotics used i.e. amoxycillin, ciprofloxacin, cephalexin, cloxacillin and erythromycin.

In our study, of the 79 sputum samples received, 77 (97.47%) were considered for further processing since only this number of samples met the ASM criteria. Out of 77 patients, 35.06% were male, and 15.58% were female. The age group of patients ranged from 9 years to 89 years with the highest number of requests (20.78%) from age group

of 60-70. Of the total processed sample, 25.97% showed significant growth. In a similar study carried out by Joshi et al. 1998, growth positivity was found to be 57.35% in 204 processed samples. The antibiotic therapy, or infection of lower respiratory tract by agents like viruses or other organisms such as Mycoplasma, Chlamydiae, fungi, Legionellae etc. which are not cultured by routine methods (Smith and Easmon,1990).
In our study, *Psuedomonas aeruginosa* were the common isolate with 61.11% of total Gram-negative isolates followed by *Klebsiella pneumonaie* with 33.33% of total isolates. Among Gram-positive bacteria, *Streptococcus pneumoniae* had been isolated.

Gentamicin was found to be most effective among the Gram-negatives, with a susceptibility of 66.66%, followed by ciprofloxacin and amikacin, each with a susceptibility of 63.63%. Amikacin and imipenem can be used as second line drugs if the isolated bacteria showed resistance to the first line drugs (amoxycillin, gentamicin, etc.). Cephalexin was found to least effective with a susceptibility of only 27.27%. A study carried out by Sharma et al. 2004, showed that amikacin was the most effective drug against Gram-negative sputum isolates with susceptibility of 70.68%. Among the Gram-positives, 72.73% of them were susceptible to cloxacillin and 63.64% were susceptible to erythromycin. Ciprofloxacin was found to be the least effective as 54.55% of isolates were resistant.

In our study, gentamicin, ciprofloxacin seems to be most effective drugs against *Klebsiella pneumoniae* isolates from sputum with sensitivity of 83.33% and 66.66% respectively.

There are three known mechanisms of resistance to aminoglycosides in bacterial pathogens: (i) decreased intracellular accumulation of the antibiotic by alteration of membrane permeability diminished inner membrane transport or active efflux (ii) modification of the target by mutation ribosomal proteins or 16S RNA; and (iii) enzymatic modification of the drug, which is the most common (Prammananan et al. 1998).

Out of eleven isolates of *Pseudomonas aeruginosa* from sputum samples, 81.81% were sensitive to ciprofloxacin and amikacin while 81.81% were resistant to cephalexin. Previous work has demonstrated that adaptively resistant *P. aeruginosa* cells accumulate significantly less aminoglycoside than controls (Parr and Bayer, 1988). However, the mechanism of, and the cellular structure(s) responsible for, this transient impermeability have remained enigmatic. The natural resistant of *P. aeruginosa* to aminoglycosides in part relies on inducible expression of *mexXY* efflux system (Masuda et al. 2000).

In our study, *Streptococcus pneumonaie* isolates were 100% sensitive to amoxycillin, ciprofloxacin and cloxacillin while 50% of the isolates were resistant to erythromycin.

Macrolide resistance in *S. pneumonaie* is usually caused by the presence of the *erm*(B) or *mef*(E)[renamed *men*(A)] resistance determinants. The *erm*(B) protein encodes a 23S rRNA methylase and the *mef*(A) protein encodes an efflux pump (Sutcliffe et al. 1996). In a study carried out in France, 17.4% of *Streptococcus pneumonaie* were found resistant to erythromycin (Reinert et al. 2003).

Although rare, clinical isolates of pneumococci with mutations in the quinolone resistance in determining regions (QRDRs) of the DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV gene (*parC* and *parE*) have been recognized (Tankovic et al. 1996).

The first isolation of penicillin resistant pneumococci was reported in Australia (Hansman and Bullen, 1967). In the United States in 1999 and 2000, of all *S. pneumoniae* isolates tested, 12.7% were intermediately resistant to penicillin (MICs, 0.12µg/ml to 1µg/ml) while 21.5% were highly penicillin resistant (MICs,≥2µg/ml) (Doern et al. 1999). Pneumococcal resistance to ß-lactams is the result of altered PBPs with decreased antibiotic affinities. Pneumococci contain a set of six PBPs (Hakenbeck et al. 1980). High-level penicillin resistance can be established by alteration in only three of these PBPs, that is, PBPs 2X, 2B and 1A (Barcus et al.1995), while only altered PBPs 2X and 1A are required for high-level cefotaxime resistance (Munoz et al. 1992).

More recently, further mechanisms for ß-lactam resistance in pneumococci have been described, i.e., mutations in the histidine protein kinase CiaH (Guenzi et al. 1996) and mutations in the glycosyltransferase CpoA (Grebe et al. 1997). These non-PBP mechanisms have been identified only in laboratory mutants and account for low-level resistance (Smith and Klugman, 2001).

In our study, 102 pus samples were processed, out of which 75 (73.53%) samples showed significant growth with 82 bacterial isolates (more than one type of bacteria were isolated in some specimen), while 27 showed no growth. According to a ten-year survey program on wound infection carried out by Gongol et al. 1994, growth was reported in 97.5% of the infected wound specimens.

Out of 102 patients, 78 (76.47%) were male, while 24 (23.53%) were female. The samples were collected from different wards, the highest number being from Surgical, 50 (49.02%). The age group 20-30 years had the maximum requests of 27 (26.47%) for pus culture, while the age group 30-40 was the second, with 18 (17.64%) requests. Out of 82 isolates from 75 positive cases with an average of 1.09 isolates per patient, 67 (81.71%) were from male, whereas, 15 (18.29%) were from female. Among the 67 isolates from male, 35 (42.68%) were MDR-strains. Likewise, 5 (6.10%) MDR-strains were isolated from pus samples of female patients.

According to a study of Brook et al. 1990, on aerobic and anaerobic bacteriology of wounds, 38% isolates were aerobic or facultative bacteria, 30% were anaerobes and 32% were mixed flora. However, anaerobic cultures were not attempted in this study due to available circumstances. According to their study, from 584 wounds, there were 1470 isolates with an average of 2.5 isolates per wound.

In our study, infection by Gram-negatives was predominant (70.73%) than the Gram-positive ones (29.23%) which is comparable to the study conducted by Gongol et al. 1994. According to the study, majority of organisms causing wound infection in post operative patients in Bir Hospital were Gram-negatives (75%). In another study

conducted by Sologus et al. 1985 in Moscow, Russia on clinical bacteriological of Gram-negative infection on patients with burns found greater incidence of wound infection by Gram-negatives most commonly caused by enterobacteria and pseudomonas.

Among the Gram-negatives, *Escherichia coli* was the most frequently isolated species with 23 (39.66%) isolates and *Klebsiella pneumonaie* was the second one with 13.8% isolates. Other bacteria like, *Klebsiella oxytoca, Citrobacter freundii, Proteus mirabilis, Proteus vulgaris, Acinetobacter* sp. and *Pseudomonas aeruginosa* were also isolated during the study. Gram-positive organisms constituted 24 (29.27%) of total isolates i.e. 22 isolates of *Staphylococcus aureus* and 2 isolates of Coagulase negative Staphylococci (CONS).

Different surveys reveal that the predominating bacteria in wounds depend upon the nature of wound and type of operation in surgical infections.

In a study conducted in Milwalkee (1975), on the sequential analysis of Staphylo-coccal colonization of body surfaces of patients undergoing vascular surgery, most of the CONS recovered colonization of body surfaces of patients undergoing vascular surgery. The isolated CONS were *S. epidermidis, S. homiletics, S. hominis*.

According to the study of Barber et al. in 1995, in the pus samples drained or swabbed from clean wounds (Class I) *Staphylococcus aureus* was predominant isolate (22.6%) while Enterococci was predominant in clean contaminated (Class II), contaminated and dirty infected (Class III) wounds. *E. coli* was the second predominating organism in Class I (12.9%), Class II (13.0%) and Class IV wounds (12.6%). But in Class III wounds, CONS was the second predominating bacteria (17.6%).

S. aureus is the most prevalent aerobe in abscesses and skin wounds and is usually found alone. This bacterium has a well recognized property for abscess formation, both

in local and visceral infection. In contrast to anaerobes, its potential for abscess formation is not as dependent on synergistic bacterial mixtures.

According to Brook et al. 1990, *S. aureus* was most common in abscess from all body sites, but predominant in abscess of legs. Brook's study reported 386 *S. aureus* from 1260 pus samples drained from wounds and abscesses.

In our study, *E. coli* were the most predominant bacteria which is comparable with the study of Gongol et al. 1994 in Bir Hospital, which showed that *E. coli* was the most common etiopathogen from surgical wound infections (23%) and the second one was *Pseudomonas* sp. (18%) followed by *Proteus* sp. (14%). According to their study, 64% of the specimens yielded single isolate and 26% yielded multiple isolates. Barber et al. 1995 reported 12.6% *E. coli* from surgical wounds 7.6% by Brook et al. 1990.

E. coli isolated from pus specimens showed 52.17% sensitivity to ciprofloxacin and gentamicin. In a study carried by Gongol et al. 1994, most effective antibiotic for Gram-negative isolates from wound infection was gentamicin with 95% susceptibility towards *E. coli*. Similarly, Esuvaranathan et al. 1992, found 89% of isolated *E. coli* from wound infection, sensitive towards gentamicin.

6 isolates of *Citrobacter freudii* from pus sample in our study, was found to be sensitive to ciprofloxacin (100%), gentamicin and ceftazidime (50%). In the *Citrobacter* genus, resitance to ß-lactam antibiotics is mainly mediated by production of chromosomally encoded ß-lactamases: *C. freundii* produces an inducible Ambler class C ß-lactamase (Jones and Bennett, 1995). The AmpC ß-lactamases of *C. freundii* are primarily cephalosporines that mediate resistance to cephalosprins oxyiminocephalosporins, and aztreonam (Nordmann, 1998). Because of their location on whole chromosomes, ampC genes were not initially subject to the rapid dissemination allowed by the horizontal transmission of plasmid-borne genes. The first plasmid-borne ampC gene for CMY-1 was reported in 1989 (Bauernfeind et al. 1989).

In our study, the only isolate of *Acinetobacte*r spp. from pus was found to be sensitive only to amikacin, whereas it was resistant to amoxycillin, cephalexin, ciprofloxacin, gentamicin. In 1997, detection of PER-1 type extended spectrum ß-lactamase in 14% of nosocomial *Acinetobacter* spp. and 11% *Pseudomonas aeruginosa* isolates was reported in Turkey; the PER-1-type enzyme producers were highly resistant to ceftazidime and gentamicin, intermediately resistant to amikacin and susceptible or moderately susceptible to imipenem and meropenem (Vahaboglu et al. 1997). In 2000, a multiresistant *Acinetobacter baumannii* strain with a carbapenem-hydrolysing enzyme was characterized (Bou et al. 2000).

Erythromycin was found to be most effective drug (100% sensitive) for Gram-positive isolates (i.e. *S. aureus* and CONS) in our study which is comparable with the study conducted by Gongol et al. 1994 showing 79.5% sensitivity.

In our study, out of 68 *Escherichia coli* and 6 *Klebsiella pneumoniae* isolates from urine, 11 *E. coli* isolates were found to be ESBL-producers while none of the *K. pneumoniae* was detected as ESBL producing organism. Similarly, out of 6 *K. pneumonaie* from sputum samples 3 *K. pneumonaie* were found to be ESBL-producers. Among 23 *E. coli* and 8 *K. pneumoniae* isolates isolated from the pus samples, 2 *E. coli* and 3 *K. pneumonaie* isolates were confirmed as ESBL-producers. Thus out of 111 total *Escherichia coli* and *Klebsiella pneumoniae* isolates, 19 (17.11%) were found to be ESBL-producers. No other Gram-negative isolates were suspected of being ESBL-producer in the primary screening test. In a similar study carried out by Sharma et al. in Nepal, 8% *Klebsiella pneumoniae*, 12.5% *Escherichia coli*, 12.5% *Citrobacter freundii*, 25% *Acinetobacter calcoacericus* and 5% *Pseudomonas aeruginosa* were found to be ESBL-producing strains (Sharma et al. 2004).

In 1999, 39 out of 2,559 isolates of the family Enterobacteriaceae (1.5%) collected by eight private laboratories in the Aquitance region in France produced an extended-spectrum ß-lactamase (Arpin et al. 1999). ESBLs have been observed in virtually all

species of the family Enterobacteriaceae. Most of the ESBLs found so far in this family are Ambler classA ℘lactamases. They are plasmid encoded and the enzymes most commonly observed in *E. coli* are TEM derivatives and to lesser extent SHV derivatives (Medeiros, 1997). In addition to these ESBLs, non-SHV, non-TEM derivative enzymes have been detected in *E. coli*: FEC-1 (Matsumoto et al. 1989), CTX-M1 (MEN-1), CTX-M2, PER-2 (Bauernfeind et al. 1996) and TOHO-1 (Ishii et al. 1995). In a study carried out in Russia in 2003, a total of 900 consecutive nosocomial isolates of *Escherichia coli* and *Klebsiella pneumoniae* collected were screened for production of ESBLs. The ESBL phenotype was detected in 78 (15.8%) *E. coli* and 248 (60.8%) *K. pneumoniae* isolates. One hundred fifteen isolates carried the genes for CTX-M-2 (7%)-related enzymes (Edelstein et al. 2003).

Klebsiella pneumoniae strains producing ESBL are more frequently resistant to fluoroquinolones than *K. pneumoniae* strains lacking these enzymes (Paterson et al. 2000). An explanation for ℘lactam-fluoroquinolone co-resistance in ESBL-producing *K. pneumoniae* is a decrease in the permeability of the outer membrane to both classes of agents because of porin alterations. Porin loss in ESBL-producing *K. pneumoniae* causes resistance to cefoxitin and increased resistance to decreased susceptibility to all oxyimino-cephalosporins, zwitter-ionic cephalosporins and the lactam-℘lactamase combinations (Ardanuy et al. 1998). In a study carried out in Spain in 2002, porin loss was significantly more common among ESBL-positive strains than among ESBL-negative strains of *Klebsiella pneumoniae* (Martinez-Martinez et al. 2002).

In our study, altogether 111 isolates of *E. coli* and *K. pneumoniae* were isolated from urine, sputum and pus samples, out of which 57 were suspected to be ESBL producers, however, only 19 were confirmed as ESBL producing bacteria. ESBLs are harder to detect in those enterobacteriaceae with inducible AmpC chromosomal enzymes e.g. *Enterobacter* spp., *Citrobacter freundii*, *Morganella morganii*, *Providencia* spp. and *Serretia* spp. (Philippon et al. 1989). The AmpC enzymes may be induced by clavulanate (which inhibits them poorly), might then attack the cephalosporin, masking

synergy arising from inhibition of the ESBL (Bauernfein et al., 1993). Enzymes with marginal ESBL activity, those expressed weakly and those produced alongside other enzymes (e.g. de-repressed AmpC) are the hardest to detect (M'Zail et al. 2000).

ESBL tests were not developed for species such as *Acinetobacter* spp., *P. aeruginosa*, *Steotrophomonas maltophilia* and should not be used for them. False positive results in *Acinetobacter* spp. are common owing to inherent susceptibility to clavulanate, whilst *S. maltophilia* may give positive results via inhibition of its chromosomal L-2 β-lactamase. ESBLs may occur in these genera (e.g. VEB-1 in *Acinetobacter* spp. in France) but are not the common cause for cephalosporin resistance in them and should not be routinely used (Livermore and Woodford, 2004).

On performing the chi-square test at 5% level of significance, significant association was found between multidrug resistance and hospitalization of patients in different wards of the hospital ($P<0.05$). In a survey carried out by Sahm et al. 2001, rates of multidrug resistance were demonstrated to be higher among inpatients (7.6%) than outpatients (6.9%).

The health care system has been greatly impacted by the emergence of antibiotic-resistant Gram-negative infections (Jones and Pfaller, 1998), and according to the Centers for Disease Control and Prevention's National Nosocomial Infections Surveillance (NNIS) System, the incidence of nosocomial infections caused by antibiotic-resistant Gram-negative pathogens is increasing (NNIS, 2001). Antibiotic-resistant pathogens compromise the treatment of hospitalized patients with serious infections (Jones and Pfaller, 1998) and the literature is replete with evidence that the presence of antibiotic-resistant Enterobacteriaceace is associated with longer hospital stays, type of hospital wards, greater use of antibiotics and higher mortality (Dworzack et al. 1987). In a study carried out by Kim et al. 2002, the risk factors for Ceftriaxone-resistant *Citrobacter freundii* that were found to be statistically significant included length of stay in the hospital and ICU stay, they also found that exposure to antibiotics

in general posed a significant risk. Flamm et al. 2004 reported that rates of antibiotic resistance in clinical isolates of *Pseudomonas aeruginosa* were highest for the isolates from patients in ICU, followed by patients in non-ICU hospital wards, then nursing homes and finally outpatients.

In our study, no association was seen between multidrug resistance and gender ($P>0.05$).

Conclusion

For several years we have been faced with the emergence and spread of microorganisms resistant to one or several antibiotics commonly used in the treatment of infections, such as respiratory tract infections, meningitis, urinary tract infections, wound infections and so on. Nowadays, pathogens have become resistant to almost all anti-infectious drugs, leading to therapeutic failure. The present study was mainly focused on the prevalence of multi-drug resistant organisms isolated from various clinical samples which revealed that the drug resistance is significantly associated with the hospitalization of the patients in different wards. This type of study helps to minimize the drug resistance in the hospital ecosystem and hence to minimize the therapeutic failure to some extent.

CHAPTER-VII

7. Summary and Recommendations

7.1 Summary

Altogether, 388 samples were received in the laboratory, of which 207 were urine samples, 79 were sputum samples (but only 77 sputum samples met the ASM criteria) and 102 were pus samples. Thus total of 386 samples were processed, out of which 190 (49.22%) samples showed positive growth with 197 (51.03%) of total isolates (in case of pus samples, there were growth of more than one bacterium from some of the specimen) and among those bacterial isolates, 111 (57.21%) were found to be MDR-strains whereas 19 of them were found to be ESBL-producers.

Urine sample

- Out of 207 urine samples, 95 (45.89%) showed significant growth and among the 95 isolates, 62 (65.26%) were multi-drug resistant, additionally, 11 isolates of *Escherichia coli* among them were found to be ESBL-producers.
- Of the total 95 isolates, 61 (64.21%) were from male, whereas, 34 (35.79%) were from female. The samples were collected from different wards, among which 77 (37.2%) were from Surgical and 51 (24.63%), 13 (6.3%), 15 (7.2%), 29 (14%) and 22 (10.62%) were from Medical, ICU, Pediatric, New Female Ward (NFW) and Gynecological respectively.
- The predominant bacteria causing UTI were Gram-negatives which were constitutively 95.79% (91/95) and among them 67.03% (61/91) were MDR-strains. Gram positive bacteria constituted only 4.20% (4/95) and of them 25% (1/4) were MDR-strains.
- Altogether 9 different bacterial species were isolated from the positive growth culture. *Escherichia coli* (N=68) was found to be the most predominant Gram negative isolate (74.70%). Among Gram positives, *Staphylococcus aureus* (N=2)

and *Enterococcus faecalis* (N=2) were isolated, out of which one strain of *Enterococcus faecalis* was MDR.

- Of total 68 *E. coli* isolates, 69.12% (47/68) were MDR-strains, while all strains of *Pseudomonas aeruginosa* (5/5) were found to be MDR.
- Erythromycin was found to be the most effective drug with susceptibility of 100% against Gram positive bacteria while Nitrofurantoin was effective against most of Gram negative bacteria.
- Out of total *E. coli* isolates 16.18% (11/68) were found to be ESBL-producers while no strain of ESBL producing *K. pneumoniae* was found. All the ESBL-producers were also found to be MDR-strains.

Sputum samples

- Of total 79 sputum samples received, 77 (97.47%) samples met the ASM criteria and among them 20 (25.97%) samples showed significant growth.
- Out of 20 isolates, 5 (25%) were obtained from Surgical, 7 (35%) from Medical, 5 (25%) from ICU and 3 (15%) from NFW.
- The predominant bacteria isolated from the culture positive samples were the Gram negatives which constituted 90% (18/20), among them 50% (9/18) were found to be MDR; whereas 3 isolates were ESBL-producers. Among Gram positives no MDR strains were found.
- Of the four different bacterial species isolated, *Pseudomonas aeruginosa* (N=11) was found to be the most frequent (55%), out of which 54.54% (6/11) were MDR-strains. Among 6 isolates of *Klebseilla pneumoniae*, 3 were found to be MDR and all were confirmed as ESBL-producers. Other bacteria like, *Proteus mirabilis* were also isolated during the study. Gram-positive organisms constituted only 2(10%) of total isolates i.e. 2 isolates of *Streptococcus pneumoniae.*
- Cloxacillin was effective against Gram positive isolates with susceptibility of100% while Gram negative isolates were most sensitive towards ciprofloxacin.

- Out of 6 *Klebsiella pneumoniae* isolates, 3(50%) were found to be MDR and all 3 isolates were confirmed as ESBL-producers.

Pus Samples

- Out of 102 pus samples received, 75 (73.53%) samples showed significant growth with 82 bacterial isolates (more than one type of bacteria were isolated in some specimen), among them 40(48.78%) were MDR-strains and 5 isolates were found to be ESBL-producers.
- Out of 82 isolates 52 were from Surgical ward, among which 24 (46.15%) were MDR-strains. Similarly, out of 13 isolates from Medical, 13 isolates from ICU and 4 isolates from Gynecological, 6, 9 and 1 isolates were MDR-strains, respectively.
- The predominant bacteria isolated from the culture positive samples were the Gram negatives which constituted 58 (70.73%), among them 36 (43.90%) were found to be MDR; whereas 5 (8.62%) isolates were ESBL-producers. Out of 24(29.27%) Gram positive isolates 4(16.66%) were MDR-strains.
- Altogether 10 different species of bacteria were isolated from the growth positive culture. *E. coli* (N=23) was found to be the most predominant Gram negative isolate (39.66%). Among Gram positive isolates, *Staphylococcus aureus* (N=22) was the most predominant one.
- Out of 23 *E. coli* isolates, 16 (69.56%) were found to be MDR-strains, while out of 14, 6 isolates of *Pseudomonas aeruginosa* were MDR-strains. Four, out of 22 isolates, of *S. aureus* were found to be MDR-strains.
- Erythromycin was found to be the drug of choice for Gram-positive isolates, with susceptibility of 95.83% (23/24). Among the Gram-negative isolates, ciprofloxacin seems to be the most effective drug with susceptibility of 55.17% (32/58), followed by gentamicin.
- 3 out of total 23 *E. coli* isolates and 2 out of 8 *K. pneumoniae* isolates were confirmed as ESBL-producers. All the 5 ESBL-producers were also found to be MDR-strains.

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