CHAPTER 1
Introduction
1.0 INTRODUCTION

A basic interrelationship exists between humans and microorganisms that can be mutualism, commensalism or parasitism having beneficial, neutral or harmful effects. The body surfaces are constantly exposed to microbes. Some microbes become implanted as colonists (normal flora), some are rapidly lost (transients) and others invade the tissues. Such intimate contact with microbes inevitably leads to infection, a condition in which pathogenic microorganisms penetrate host defenses enter the tissues and multiply. The cumulative effect of infection damage or disrupt tissues and organs leads to diseased condition. A disease is defined as any deviation from health. Because of numerous factors relating to host resistance and degree of pathogenicity, neither all contacts lead to infection nor all infections lead to disease (Talaro, 2004).

The type and severity of an infection depends upon pathogenicity of microbe and condition of the host. Pathogenic microorganisms have been traditionally divided into two categories, based upon nature of microbe-host relationship; true pathogens capable of causing infections and disease in healthy persons with normal immune defenses, opportunistic pathogens
become infectious in immuno-compromised hosts (Tortora, 1995). Opportunists are not generally considered pathogenic to the normal, healthy person and do not usually have well developed virulence properties. These opportunistic pathogens mostly are members of resident flora of human body.

Nosocomial infections (Greek *nosos*, disease and *komeleon*, to take care of) are produced by infectious pathogens that develop within hospital and are acquired by patients when they are in the facility. Often nosocomial infections become apparent while the patient is still in the hospital, but in some cases symptoms may not show up until after the affected patient is discharged. About one patient in ten acquires an infection as a direct result of being hospitalized (Weinstein, 1998).

Nosocomial infections are the result of three factors occurring in tandem, (i) high prevalence of pathogens (ii) high prevalence of compromised hosts and (iii) efficient mechanisms of transmission from patient to patient. All hospitalized patients are susceptible of contracting nosocomial infection. Some patients like young children, the elderly person, and persons with compromised immune systems are at greater risk for
acquiring infections. Other risk factors for acquiring a nosocomial infection are longer hospital stay, use of indwelling catheters, failure of healthcare workers to maintain the clean conditions and overuse of antibiotics (Black, 1996). Common causes of hospital-acquired infections include urinary bladder catheterization, respiratory procedures, surgical wounds and intravenous procedures (Pelczar, 1987). Besides harming patients, nosocomial infections can affect nurses, physicians, aides, visitors, delivery persons, custodians and any one who comes in contact with hospital. Infections that are incubating while patients are admitted to hospital are called as community acquired infections.

With constant source of nourishment moisture, relatively stable pH, temperature and extensive surfaces upon which to settle, the human body provides a favorable habitat for an abundance of microorganisms. The resident flora of humans includes bacteria, fungi, and protozoa. The major resident flora of human includes bacteria belonging to genus Staphylococcus, Micrococcus, Streptococci, Corynebacterium, Lactobacillus, Actinomyces etc and fungi like Candida, Protozoa like Demodix mite, Entamoeba coli and Trichomonas hominis. The genus
*Staphylococcus*, from *Staphyle*, the Greek word for “grape” (Stanier et al, 1995) is a common inhabitant of the skin and mucous membranes and accounts for a considerable proportion of human infections. Staphylococci are spherical, Gram-positive bacteria of the family *Micrococcaceae* (International subcommittee on *Staphylococci* and *Micrococci*, 1965). Currently 31 species have been identified in the genus *Staphylococcus*, but the most important human pathogens are: (1) *S. aureus* (2) *S. epidermidis*, *S. hominis*, *S. capitis*, close relatives that are common skin flora and (3) *S. saprophyticus* found in urinary tract infections (Easmon, 1983). Of these *S. aureus* is considered as most serious pathogen as it encodes many proteins that help to get colonize in humans. Other species of Staphylococcus have become increasingly associated with opportunistic infections and can no longer be regarded as harmless commensal.

*Staphylococcus aureus* is one of the major causes of hospital-acquired infection. Study conducted by Skurray and Lyon (1987) ranked it fourth in a listing of the “pathogens most frequently isolated from hospitalized patients when all anatomic sites are considered”. *S. aureus* by direct invasion and systemic dissemination produces diseases like, bacteremia, septic shock
syndrome, skin infections, abscesses, carbuncle, abscesses around hair follicle, impetigo, surgical folliculitis and deep folliculitis due to production of exotoxin. Other diseases caused by S. aureus are Staphylococcal scalded skin syndrome and toxic shock syndrome. The toxins relevant to disease causing symptoms are super-antigens and α-toxins. The α-toxins oligomerize to form pores in host cellular membrane allowing cellular contents to leak into extra cellular matrix. The super-antigens consisting of enterotoxins are responsible for S. aureus related food poisoning and toxic shock syndrome respectively. Individuals with damaged or severely compromised immune systems such as recent patients recovering from surgery or burn victims, insulin dependent diabetes and hemodialysis patients are more susceptible to staphylococcus infection than healthy individuals, (Todar, 1998). In addition, S. aureus can cause more serious ailments when it enters the bloodstream, such as pneumonia, osteomyelitis, arthritis, endocarditis, myocarditis, brain abscesses and meningitis. (Chambers, 1997).

Over the last two decades roles of S. epidermidis and other coagulase-negative Staphylococci have been recognized and well documented, especially as causative agent in
nosocomial infections (Peters et al, 1995). The infection rate has been correlated with increase in the use of prosthetic and indwelling devices and growing numbers of immuno-compromised patients in hospitals. *S.epidermidis* is the most common cause of both foreign device infection and nosocomial bacteremia with mortality rate of 10-34 % (Archer, 2000). Three important factors contribute for infection caused by *S.epidermidis* and other coagulase-negative Staphylococcus species: namely, (i) Skin and sebaceous or apocrine gland colonization, (ii) bacterial adherence to foreign devices and (iii) production of slime (an exopolysaccharide that enhances adherence to surfaces and has anti-phagocytic properties). Since *S.epidermidis* and other coagulase-negative *Staphylococci* are part of normal microbial flora of humans they are frequently dismissed as skin contaminants. Repeated isolation of a predominant strain or a strain in pure culture is quite convincing while attempting to determine the etiologic agent (Huebner & Goldmann, 1999).

1.1 ANTIBIOTICS

The control of microorganisms is required for the prevention and treatment of disease. Modern medicine widely
depends upon chemotherapeutic agents used to treat disease. Most of these agents are antibiotics. The term antibiotic originated from the word antibiosis (Greek anti, against, and bios, life) latter firstly described by Vuillemin in 1889 in an attempt to describe the concept of survival of fittest. Antibiotics are microbial products or their derivatives that posses bacteriostatic or bactericidal effect. The modern era of chemotherapy began with the work of the German physician Paul Ehrlich (1854-1915) (Alcamo, 1999) with reasoning that a chemical with selective toxicity would kill pathogens and not human cells might be effective in treatment of disease. In 1927 German chemical industry giant, I.G. Farbenindustrie, began long-term search for chemotherapeutic agents under the direction of Gerhard Domagk. Sir Alexander Fleming’s discovery of the antibacterial properties of Penicillin in September 1928 largely credited with initiating the modern antibiotic era. In 1938, Florey and Chain introduced penicillin into therapy and practical exploitation of this discovery began to be realized. The discovery of penicillin stimulated the search for other antibiotics. Selman Waksman in 1944 announced the discovery of new antibiotic; Streptomycin produced by the actinomycete *Streptomyces griseus*. Walkman received Nobel Prize in 1952,
and his success led to a worldwide search for other antibiotic producing microorganisms from soil. Microorganisms producing chloramphenicol, neomycin, and tetracycline were isolated by 1953. The discoveries of various antibiotics during the last century are shown in figure 1.1.

![Figure 1.1: Discovery of various classes of antibiotics during the last century](image)

The ideal antimicrobial agents should possess some important properties like solubility in body fluids, selective toxicity, toxicity not easily altered, nonallergic, stability, and maintenance of a constant, therapeutic concentration in blood
and tissue fluids, difficulty for microorganisms to acquire resistance, long shelf life and reasonable cost.

1.2 ANTIBIOTIC CLASSES

Depending on clinical effectiveness, spectrum of activity and degree of selectivity, antibiotics that inhibit only one group of microorganisms are called as narrow spectrum antibiotics e.g., nystatin and bacitracin. These antibiotics exhibit a high degree of selectivity. Few antibiotics inhibit greater range of both Gram-positive and Gram-negative bacteria are termed as broad-spectrum antibiotics e.g., chloramphenicol and tetracyclines. Antibiotics depending upon their chemical structures have been classified as (i) β-lactams, (ii) Cephalosporins, (iii) Aminoglycosides, (iv) Tetracyclines, (v) Peptides, (vi) Macrolides, (vii) Lincomycins and (viii) Unclassified antibiotics. Each class of antibiotic has different mode of action and type of activities. The target and mode of action has been classified in Table 1.1.
<table>
<thead>
<tr>
<th>Target</th>
<th>Antibiotic</th>
<th>Process interrupted</th>
<th>Type of activity</th>
</tr>
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<tbody>
<tr>
<td>Cell wall</td>
<td>Bacitracin</td>
<td>Mucoprotein peptide synthesis</td>
<td>Bactericidal</td>
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<tr>
<td></td>
<td>Cephalosporins</td>
<td>Cell wall cross linking</td>
<td>Bactericidal</td>
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<td></td>
<td>Cycloserine</td>
<td>Synthesis of cell wall peptides</td>
<td>Bactericidal</td>
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<td></td>
<td>β-lactams</td>
<td>Cell wall cross linking</td>
<td>Bactericidal</td>
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<tr>
<td>Cell membrane</td>
<td>Amphotericin B</td>
<td>Membrane function</td>
<td>Fungicidal</td>
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<tr>
<td></td>
<td>Nystatin</td>
<td>Membrane function</td>
<td>Fungicidal</td>
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<td></td>
<td>Polymyxins</td>
<td>Membrane integrity</td>
<td>Bactericidal</td>
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<td>Ribosomes</td>
<td>Chloramphenicol</td>
<td>Protein synthesis</td>
<td>Bacteriostatic</td>
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<td>50S subunit</td>
<td>Erythromycin</td>
<td>Protein synthesis</td>
<td>Bacteriostatic</td>
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<tr>
<td></td>
<td>Lincomycins</td>
<td>Protein synthesis</td>
<td>Bacteriostatic</td>
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<tr>
<td>30S subunit</td>
<td>Aminoglycosides</td>
<td>Protein synthesis</td>
<td>Bactericidal</td>
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<tr>
<td></td>
<td>Tetracyclines</td>
<td>Protein synthesis</td>
<td>Bacteriostatic</td>
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<tr>
<td>Nucleic acids</td>
<td>Actinomycin</td>
<td>DNA and mRNA synthesis</td>
<td>PANCIDAL</td>
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<td></td>
<td>Griseofulvin</td>
<td>Cell division, microtubule assembly</td>
<td>Fungistatic</td>
</tr>
<tr>
<td>DNA Gyrase</td>
<td>Ciprofloxacin and other quinolones</td>
<td>DNA replication, transcription</td>
<td>Bactericidal</td>
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<tr>
<td>DNA and/or RNA</td>
<td>Mitomycin C</td>
<td>DNA synthesis</td>
<td>PANCIDAL</td>
</tr>
<tr>
<td></td>
<td>Rifampin</td>
<td>mRNA synthesis</td>
<td>Bactericidal</td>
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</tbody>
</table>
1.3 DRUG RESISTANCE

With the discovery of the antibiotic it was believed that man had won the war to eliminate bacteria. This thinking went wrong with the emergence of drug resistant bacterial strains. The spread of drug resistance is one of the most serious threats to successful treatment of microbial disease. The indiscriminate use of antibiotics led to microbial drug resistance, an adaptive response in which microorganisms become able to tolerate an amount of drug that would ordinarily be inhibitory. Figure 1.2 depicts the key factors playing essential role in selection and stability of antibiotic resistance. Microorganisms acquire antibiotic resistance by genetic changes, but sometimes by nongenetic mechanisms. Nongenetic resistance occurs when microorganisms such as those that cause tuberculosis persist in the tissues out of reach of antimicrobial agents. This type of resistance more properly called as evasion. Another type of nongenetic resistance occurs in certain strains of bacteria temporarily changing to L forms that lack most of cell walls. For several generations, while the cell wall is lacking, the microorganisms become resistant to antibiotics acting on cell walls. The reversion of resistance occurs with ability of microorganisms to restore cell wall synthesis (Black, 2003).
Selection of resistance is dependent upon
- Mutation rates and presence of mutator strains
- Rate and extent of horizontal transfer (including the impact of the human microbiota acting as a reservoir of

Stability of resistance is dependent upon
- The biological cost of resistance
- Compensatory evolution
- Genetic linkage with other genes undergoing selection
- Selective pressure

Figure 1.2: Overview of the main factors influencing selection and stability of antibiotic resistance
Genetic resistance in bacteria is best understood and is due to changes in bacterial chromosome or acquiring extra chromosomal DNA, i.e., plasmids or transposons. Chromosomal resistance could result due to mutation in chromosomal DNA. Mutational resistance occurs more frequently in clinical setting with prolonged therapy and use of single drug. Spontaneous mutations in bacterial chromosome though do not occur very often, make the bacteria drug resistant. Such mutations result in a change in drug receptor; thereby inhibiting the binding of antibiotic, e.g. altered streptomycin receptor protein on bacterial ribosomes. Resistance associated with inter microbial transfer originates from plasmids called resistance factors or R factors. Although specific evidence for the origin of drug resistance R plasmids is not available, circumstantial evidence suggests that plasmids with R plasmid type character existed before antibiotic era. The widespread use of antibiotics provided selective conditions for spread of R plasmids with one or more antibiotic resistance genes. A strain of *Escherichia coli* that was freeze dried in 1946 was found to contain a plasmid with genes conferring resistance to tetracycline and streptomycin even though neither of these antibiotics were used clinically until several years later (Madigan *et al.*, 2003). Clinically, plasmid associated drug resistance is most important mechanism of microbial resistance. Plasmid resistance genes often code for enzymes that destroy or modify the drug; for example hydrolysis of penicillin and aminoglycoside drugs. Plasmid associated genes
have been implicated in resistance to aminoglycosides, chloramphenicol, penicillins, cephalosporins, erythromycin, tetracyclines, sulfonamides and other antibiotics. Bacteria have a great ability to acquire new genes. Some of these genes are passed on from parents to offspring and is termed as “vertical transfer” and some are imported from other bacteria via a process called “horizontal transfer”. Bacteria have evolved three ways to share their resistance traits with one another by means of conjugation, transformation and transduction. Because single plasmid may carry genes for resistance to several drugs, pathogen population can become resistant to several antibiotics simultaneously, even though the infected patient is being treated with only one drug.

The ineffectiveness of certain antimicrobial drugs is due to alterations within the cell envelope. Resistance may result from alteration in porin size of cell wall, changes in cell wall configuration, alteration of a specific transport mechanism. Some pathogens have plasma membrane translocases, often called efflux pumps that expels drug. Because they are nonspecific and can pump many different drugs, these transport proteins are called multidrug-resistance pumps. Many are drug-proton antiporters, i.e., protons enter the cell as drug moves out of cytoplasm. Such systems are present in E. coli, P. aeruginosa, M. smegmatis, and S.aureus.
Many bacterial pathogens resist attack by inactivating drugs through chemical modification. The most ubiquitous drug-inactivating enzymes are β-lactamases or penicillinases. β-lactamases are found in some Gram-positive and in nearly all Gram-negative bacteria. Gram-positive bacteria produce enzyme extracellularly whereas Gram-negative bacteria retain the enzyme in periplasmic space.

As each chemotherapeutic agent acts on a specific target, resistance arises when the target enzyme or organelle is modified so that it is no longer susceptible to drug. Several components involved in replication, transcription and protein synthesis are targets of antimicrobials, but microorganisms have also evolved mechanisms for resisting antimicrobial drugs. One type of alteration is plasmid associated and is found among species of Staphylococci. These organisms possess an enzyme that methylates two adenine residues on 23S rRNA molecules of the 50S subunit of bacterial ribosome. Methylated adenine prevents binding of erythromycin and lincomycin to the 50S subunit and ultimately prevents protein synthesis. Alteration of 30S ribosome of the Gram-negative enterobacteria makes these organisms resistant to streptomycin, affinity of ribosome for erythromycin and chloramphenicol can be decreased by a change in the 23S rRNA.
The action of antimetabolites can be circumvented if a microbe develops an alternative metabolic pathway or enzyme. Some plasmids found in Enterobacteria and *Staphylococci* code for metabolic pathways that substitute for chromosome mediated enzymes. The site of inhibition of sulfonamides is enzyme dihydropteroate synthetase, required for dihydrofolate synthesis. The antimicrobial competes with PABA, the normal metabolite of the enzyme but sulfonamide resistant bacteria possess plasmids that code for a drug resistant dihydropteroate synthetase. The enzyme dihydrofolate reductase involved in folate synthesis may also be coded by plasmid borne genes. The plasmid-encoded enzyme is resistant to trimethoprim, which normally inhibits chromosome-mediated enzyme.

An infrequent mechanism of resistance involves competition between sulfa drugs and PABA for active site of enzyme. Increased resistance to sulfonamides may occur when a mutation causes an increase in metabolite, in the case of sulfa drugs resistance, PABA. The concentration of drug required to inhibit microorganism may have to be elevated considerably and is termed as quantitative resistance. Table 1.2 summarizes various mechanisms of microbial drug resistance.
<table>
<thead>
<tr>
<th>MECHANISM</th>
<th>DRUGS</th>
<th>ORGANISMS INVOLVED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Altered transport system</td>
<td></td>
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<tr>
<td>Reduced uptake of drug</td>
<td>Tetracyclines</td>
<td>Gram-negative enterobacteria</td>
</tr>
<tr>
<td>Membrane not energized</td>
<td>Aminoglycosides</td>
<td>Anaerobes</td>
</tr>
<tr>
<td>Enzymatic modification</td>
<td>Aminoglycosides, Chloramphenicol</td>
<td>Gram-negative enterobacteria, Pseudomonas</td>
</tr>
<tr>
<td>Enzymatic inactivation</td>
<td></td>
<td></td>
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<tr>
<td>β-lactamases</td>
<td>Penicillins and cephalosporins</td>
<td>Gram-positive and Gram-negative bacteria</td>
</tr>
<tr>
<td>Chloramphenicol acetyltransferase</td>
<td>Chloramphenicol</td>
<td>Gram-positive and Gram-negative bacteria</td>
</tr>
<tr>
<td>Alteration of antimicrobial targets</td>
<td></td>
<td></td>
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<tr>
<td>DNA gyrase</td>
<td>Nalidixic acid</td>
<td>Gram-negative enterobacteria</td>
</tr>
<tr>
<td>RNA polymerase</td>
<td>Rifampin</td>
<td>Gram-negative enterobacteria</td>
</tr>
<tr>
<td>Penicillin binding proteins</td>
<td>β-lactam and cephalosporins</td>
<td>Neisseria gonorrhoeae, Streptococcus pneumoniae, Staphylococcus species</td>
</tr>
<tr>
<td>Methylated 23S rRNA</td>
<td>Erythromycin and lincomycin</td>
<td>Staphylococci</td>
</tr>
<tr>
<td>30S ribosome</td>
<td>Streptomycin</td>
<td>Gram-negative enterobacteria</td>
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<tr>
<td>Synthesis of resistant metabolic pathways</td>
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<tr>
<td>Dihydrofolate reductase</td>
<td>Trimethoprim</td>
<td>Gram-negative enterobacteria</td>
</tr>
<tr>
<td>Dihydroptercate synthetase</td>
<td>Sulfonamides</td>
<td>Gram-negative enterobacteria and staphylococci</td>
</tr>
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</table>
1.4 β-LACTAM ANTIBIOTICS

The first antibiotic to be discovered was penicillin. Alexander Fleming had been culturing bacteria on an agar plate with an accidental fungal contamination, and noticed that the culture medium around the mould was free of bacteria. He had previously worked on the antibacterial properties of lysozyme and therefore made correct interpretation of what he saw; that the mold was secreting something that stopped bacterial growth. Fleming could not purify the active compound, as β-lactam ring was unstable with his isolation procedure. Since the mold was of the genus *Penicillium*, he named this compound penicillin. With the increased need for treating wound infections during second World War, resources were poured into investigating and purifying the active compound. Team led by Howard Florey succeeded in producing large quantities of the purified active ingredient. Antibiotics were soon used widespread.

Historically and medically β-lactam is the most important group of antibiotics. The β-lactam antibiotics include penicillins, cephalosporins, and cephamycins. Together penicillins and cephalosporins account for over one-half of all the antibiotics produced and used worldwide. Group includes the parent
compound Penicillin and a large, diverse group of compounds, most of which end in the suffix \(-cillin\). Initially *Penicillium chrysogenum* was the major source of the drug. All \(\beta\)-lactam consist of three parts: a thiazolidine ring, a \(\beta\)-lactam ring, and a variable side chain that dictates microbicidal activity (Vaden and Riviere, 1996) (Figure 1.3).

![Chemical structures](Image)

**Figure 1.3: Parental ring structure and various "R" groups of \(\beta\)-lactam antibiotics**

Penicillin G and V are natural forms extracted from *Penicillium notatum* and were first antibiotics to find practical use in medicine. Commercial production began in early 1940s. Penicillin G was considered as drug of choice for infections by known sensitive Gram-positive cocci (*Streptococci, Pneumococci*) and some Gram-negative
bacteria (*Meningococci* and Spirochete of syphilis). Penicillin V was similarly used against many infectious agents. In 1950s it was noted that certain strains of *Staphylococcus aureus* became resistant to penicillin provided impetus to develop semisynthetic penicillins. During development of semisynthetic penicillins, antibiotic is reduced to its basic molecular framework called nucleus and to this nucleus specially selected R groups are added. Various "R" groups are shown in figure 1.3. In β-lactam antibiotics nucleus is an inactive penicillin derivative called aminopenicillanic acid, which lacks opening on the C₆ for addition of R groups. Methicillin (figure 1.4) was first semisynthetic penicillin, effective against penicillin resistant organisms because β-lactamase enzymes produced by penicillin resistant organisms did not hydrolyze β-lactam ring the antibiotics with steric hindrance. Oxacillin is the example of the semi synthetic β-lactam antibiotic resistant to digestion by β-lactamase enzyme. The structure of oxacillin sodium salt is shown in figure 1.5.
Certain semisynthetic penicillins such as ampicillin, carbenicillin, amoxicillin and ticarcillin with broader spectra were used to treat infections by Gram-negative enteric rods in addition to Gram-positive cocci infections. Penicillinase-resistant penicillins such as methicillin, nafcillin, and cloxacillin were considered useful for treatment of infections caused by some penicillinase-producing bacteria. Mezlocillin
and azlocillin are extended spectrum and are substituted for combinations of antibiotics (Atlas, 1984).

Cephalosporins are β-lactam antibiotics isolated from *Cephalosporium* species or prepared semisynthetically. Cephalosporin C is structurally related to penicillin containing D-α-amino adipoyl side chain, which can be replaced to form various semisynthetic cephalosporins (Metzler, 2003). Cephalosporin parent ring structure is shown in figure 1.6. Interest in *Cephalosporium* fungi began in 1945 with Giuseppe Brotzu’s discovery that cultures of *C. acremonium* inhibited the growth of a wide variety of Gram-positive and Gram-negative bacteria.

![Figure 1.6: Structure of Cephalosporins](image)

Important examples:
- Cephalorin ($R^1$ as ampicillin, $R^2 = \text{H}$)
- Cefazidime ($R^1$ as aztreonam, $R^2 = \text{pyridinium}$)
1.5 MODE OF ACTION OF $\beta$-LACTAM AND CEPHALOSPORIN

ANTIBIOTICS

The lethal antibacterial action of $\beta$-lactam antibiotics has been attributed to selective inhibition of bacterial cell wall synthesis. An important feature of cell wall synthesis is the transpeptidation reaction, resulting in cross-linking of two glycan-linked peptide chains catalyzed by transpeptidase enzyme. Transpeptidases binds to $\beta$-lactam ring of $\beta$-lactam or cephalosporin antibiotics and therefore are called as penicillin binding proteins (Katzung & Bactoprenol, 1998). The penicillin binding proteins (PBPs) bind tightly to penicillin and cause change in 3D confirmation of the enzyme thus unable to catalyze transpeptidation reaction. Penicillins acylate a specific serine residue of bacterial D-transpeptidase, thereby rendering it inactive for its role in forming peptide cross-links of two linear peptidoglycan strands by transpeptidation. Bacterial D-alanine carboxypeptidases are also inhibited by $\beta$-lactam antibiotics. Antibiotic-PBP complex stimulates the release of autolysins that digest existing cell wall. The result is a weakened, eventually degraded cell wall. Under normal circumstances, the osmotic pressure differences inside the cell, with that of exterior medium, cause cell lysis.
The cephalosporins have same mode of action as the penicillins. Clinically important cephalosporins are semisynthetic antibiotics that generally have a broader spectrum of antibiotic activity than penicillins (Madigan et al, 2003). Various β-lactam antibiotics differ in their affinities for PBP’s. Penicillin G preferentially binds to PBP3 whereas first generation cephalosporin binds with higher affinity to PBP1 (Delgado and Remers, 1975).

**1.6 MODE OF RESISTANCE IN β-LACTAMS**

The emergence of antibiotic established the conditions for optimizing doses for controlling *Staphylococcus aureus* infections. *S. aureus* is mostly a nosocomial pathogen and nosocomial nature of *S. aureus* exposes it to large doses of antibiotics. Optimism of controlling *S. aureus* with antibiotics was quickly quenched by the emergence of penicillin resistant strains of *S. aureus* in late 50s. In 1946 almost all strains of Staphylococcus were penicillin sensitive. Today most *S. aureus* strains of nosocomial origin are resistant to penicillin (Presscott, et al, 2003). Spread of antibiotic resistance represents one of the most serious emerging infectious disease threats. As more
and more kinds of bacteria develop resistance, human medicines are running out of options to treat the various kinds of infection. Medically, a resistant organism is one that will not be inhibited or killed by an antibacterial agent at concentrations of the drug achievable in the body after normal dosage. The increasing incidence of resistance associated with lowering ability of antibacterial agents to combat resistant strains are recognized as serious threat for treatment of life-threatening infections (Hawkey, 1998).

Shortly after penicillin became available in late 1940s for treatment of serious Staphylococcal infections resistance to this antibiotic emerged and rapidly spread among strains of *S.aureus* and coagulase-negative Staphylococci. The mechanism of resistance was production of drug inactivating β-lactamase enzyme (penicillinase) able to hydrolyze penicillin's β-lactam ring. Figure 1.7 shows the mode of action of β-lactamase enzyme. Genes located on chromosomes or on plasmids encodes β-lactamase but sometimes they are found in both types of replicons in same cell. Gram-positive bacteria produce enzyme β-lactamase extracellularly. Currently about 90% of *S.aureus* isolates are resistant to penicillin. Penicillinase-stable semi-
synthetic penicillins (e.g., methicillin, cloxacillin sodium, nafcillin sodium salt) and cephalosporins (e.g., cephalothin sodium salt and cefazolin sodium salt) developed in late 1950s and early 1960s to create β-lactam antibiotics that are not inactivated by β-lactamase (Swaren et al., 1999). Methicillin was among first of these β-lactamase resistant antibiotics to be introduced into clinical practice but strains of MRSA were reported in early 1961. Since then, MRSA has become increasingly prevalent in many countries around the world (Boyd, 1995).

**Figure 1.7: Mode of action of β-lactamase**
Apart from the production of β-lactamase enzyme that degrades β-lactam antibiotic, the other mode of resistance includes production of an altered penicillin binding protein called as PBP2' or PBP2a (Moreira et al, 1995). This altered penicillin binding protein is encoded by mecA gene. The PBP2a confers resistance in staphylococcus against all β-lactam antibiotics. Such strains of staphylococcus are historically termed as Methicillin resistant Staphylococcus aureus (MRSA) and Methicillin resistant Coagulase Negative Staphylococci (MRCNs). Today almost half of all staphylococcal infections in U.S. hospitals are caused by these methicillin-resistant S.aureus (MRSA) strains. Unlike native PBP's, PBP 2a has low affinity for β-lactams conferring resistance to all β-lactam antibiotics including β-lactamase resistant β-lactam antibiotics (nafcillin and oxacillin), β-lactamase inhibitor combinations (ampicillin/sulbactam), cephalosporins and carbapenems (Paradisi et al, 2001).

Cephalosporins have a bactericidal effect by inhibiting the synthesis of the bacteria cell wall resulting in cell lysis. Cell lysis occurs due to deteriorated cell wall causing cell to be exposed unprotected to the environment and subjected to burst under
pressure. Cephalosporins bind to penicillin-binding proteins (PBP's), enzymes responsible for synthesis of peptidoglycan, a component of cell wall. Because \( \beta \)-lactam agents are structurally similar to D-alanyl-D-alanine (component of peptidoglycan) they are able to react chemically with native PBP's and inactivate them.

1.7 CAUSES OF ANTIBIOTIC RESISTANCE

The production and sale of antibiotics continuously increased since their discovery. Because of the massive quantities of antibiotics being prepared and used, an increasing number of diseases are resisting to the treatment due to the spread of drug resistance. The most frequent practice contributing to development of drug resistant bacteria is unnecessary prescription of antibiotics to patients exhibiting symptoms of maladies that are not caused by bacterial infections. This is especially common for treatment of colds and flu, caused by viruses. Because antibiotics have no effect against viruses the only function that they perform in these cases is to promote rate at which bacteria in body develop mutations conferring antibiotic resistance. The prescription and reliance on broad-spectrum antibiotics as a primary course of treatment also
contributes to the problem. As opposed to specificity of narrow-spectrum antibiotics that are targeted at a small range of bacteria, broad-spectrum antibiotics indiscriminately kill large number of bacteria.

Extent of antibiotic use is another cause of increase in antibiotic resistance. When an antibiotic attacks a group of bacteria the, growth of susceptible bacteria is controlled and bacteria that have resistance from the start or those acquire resistance later through gene exchange or mutation survive especially if small doses of antibiotic is given. These cells facing reduced competition from susceptible bacteria then proliferate. Thus confronted with an antibiotic, the most resistant cells in a group inevitably out compete others. Simultaneously antibiotic encourages growth of resistant bacteria. Propagation of these resistant bacteria increases the reservoir of resistance traits in bacterial population as a whole.

Based on study of 15,000 individuals, the Health Care Payers Coalition (HCPC) of New Jersey estimated that 40 to 60% of common respiratory infections caused by viruses were treated by prescription of antibiotics. The CDC estimates that 1 of every
3 prescriptions for antibiotics each year are unnecessary (Maiden et al, 1998).

Additionally, patients who do not complete their prescription of a certain antibiotic promote the resistance. Treatment is often stopped because a patient begins to feel better. Antibiotic treatment kills the most susceptible and exposed bacteria. However, feeling better often indicates that the antibiotics are working, not that they have completed their job. Incomplete treatment leaves behind bacteria that are less susceptible and, by mutation, have a certain level of resistance. When the bacterial population reestablishes itself, a large number of bacteria are again produced, but with less susceptibility to the drug. These survivors can also share their genetic material (resistance) across different strains of bacteria (Kingman, 1994).

Approximately 40% of all antibiotics manufactured in United States are given to animals. Some of these antibiotics are used to treat infections because animals are often raised in unhealthy conditions. Majority of these antibiotics are mixed into animal feed because they typically promote growth
approximately by 5%. The antibiotic and growth hormones act to speed growth while reducing amount of feed necessary to achieve a particular weight. Small amounts of the drugs therefore are given to animals for long time periods. Long-term exposure to low doses of antibiotics also develops antibiotic resistance. These resistant bacteria may then be passed to people who prepare or consume undercooked meat.

Antibiotics are often sprayed onto large plots of land containing fruit trees to control and prevent bacterial infections. A high concentration of these antibiotics is effective in killing all of bacteria on trees but as dosage decreases over time and residual antibiotic encourages growth of resistant bacteria during processes of shipping and handling. Additionally applying antibiotic as an aerosol leads to growth of resistant bacteria on trees and plants other than those initially targeted because they only receive a small dosage of drug. After produce is eaten antibiotic resistant bacteria can reside in intestinal tract of host. A study in France showed that volunteers who eat only bacteria-free foods had a 1,000-fold decrease in number of resistant bacteria in their feces. This finding suggests that there is a significant amount of resistant bacteria introduced to intestinal
tract through agents of raw or undercooked foods. Evidence has been discovered to support theory that genes conferring antibiotic resistance can be transferred from genetically modified plants to disease-causing germs in a process called horizontal gene transfer (Cohen, 1992).

1.8 PHENOTYPIC DETECTION OF ANTIBIOTIC RESISTANCE

Microorganisms vary in their susceptibility to different chemotherapeutic agents and susceptibilities can change over time. Ideally, the appropriate antibiotic to treat any particular infection should be determined before any antibiotics are prescribed. Sometimes an appropriate agent can be prescribed as soon as causative organism is identified from a laboratory culture. Several methods like - disk diffusion, dilution, and automated methods are available for detection of susceptibility to appropriate antibiotics (Black, 2003).

The disk diffusion test represents the qualitative antimicrobial sensitivity test developed by Bauer et al (1966) is an agar diffusion technique recommended by the Food and Drug Administration (FDA) and is used widely by clinical laboratories for determining antimicrobial activity. In disk diffusion test the
standard concentration of microorganisms is seeded on agar plate and an antibiotic disk of known concentration is placed. Those microorganisms inhibited by antimicrobial following suitable incubation will show a zone of inhibition the diameter of, which is proportional to susceptibility of microorganism. This proportionality is based on standardization of certain variables: agar, type of disk, antimicrobial and microorganisms tested (Prescott, 2003).

Quantitative *in vitro* susceptibility tests are used to determine more exactly the concentration of drug that will inhibit growth of microorganisms (the minimal inhibitory concentration, or MIC) or will kill microorganisms (the minimal bactericidal concentration, or MBC). Quantitative testing more application in pharmaceutical laboratory where exact concentrations of drug must be known to establish levels of drug for future animal and human studies. Two *in vitro* tests are available for determining MIC of a drug: (i) agar dilution test and (ii) broth dilution test. In agar dilution technique antibiotic is mixed into a liquefied agar medium (45°- 50°C), and poured into an agar plate. A series of plates are set up with increasing concentration. Seeded plates are incubated overnight at appropriate temperature and MIC is
read as lowest concentration of drug that completely inhibits growth of seeded microorganism. MICs obtained from these assays are used in clinical laboratory to establish dosage that will be effective in treatment of human infections.

Commercial antimicrobial susceptibility testing systems are also available for clinical laboratories. A micro dilution procedure has been developed commercially in which micro trays are filled with antibiotics. Some clinical laboratories use this technique instead of disk diffusion technique. A second type of test uses growth curve kinetics by measuring the turbidity of antibiotic-treated and untreated cell suspensions. These measurements are evaluated by a built-in computer system (Boyd, 1995).

1.9 NEED FOR ADAPTING RAPID GENOTYPIC BASED METHODS FOR DETECTION OF GENES RESPONSIBLE FOR ANTIBIOTIC RESISTANCE

Although phenotypic techniques of susceptibility tests are relatively simple, it requires bacterial isolation, time required to grow and isolate bacterium in pure culture depends on type of bacterium. Rapidly growing aerobic organisms such as Staphylococci are isolated in laboratory within 24 to 48 hr after receipt of clinical specimen. On other hand other organisms such
as *M. tuberculosis* require prolonged incubation (4 to 6 weeks) on solid medium or lengthy incubations (7 to 21 days) in liquid medium (Notle and Metchock, 1995). Many days are required for confirmation of cultivation dependent bacterial identification of *Mycobacterium tuberculosis* result in delays in critical treatment and other management decisions for these patients. The phenotypic approach has some shortcomings; since different bacterial species have different susceptibilities to same antibiotics, breakpoints of different values must be tested. Several of the presently performed susceptibility tests are highly dependent on experimental conditions, and often, more than one method needs to be performed to obtain an accurate susceptibility profile. Speed is the essence when one deals with treatment of bacterial infections. Presently, diagnosis in clinical laboratory is only confirmatory because a clinical decision has been made long before the identity of the organism responsible for infection and its susceptibility to antibiotics become available. The lack of timely response by the laboratory has consequences on antibiotic usage and prescription. Patients must be treated empirically. When severe or nosocomial infections are suspected, they are often treated with broad-spectrum antibiotics. If physicians could have in hand the identity of the microorganism,
Its resistance profile from microbiology laboratory at the same time that they have the biochemistry, hematology results, antibiotic prescription rates could go down dramatically and when antibiotics are needed more targeted and inexpensive antibiotics could be used.

To increase the rapidity and accuracy of resistance testing, use of genotypic approach has been advocated (Courvalin, 1991). Molecular methods can rapidly detect antimicrobial-drug resistance in clinical settings; have substantially contributed to our understanding of the spread and genetics of resistance. Molecular methods for drug resistance detection may be applied directly to the clinical specimen, providing simultaneous detection and identification of the pathogen plus resistance characterization obviating the need for isolation of the organism by culture (Tenover, 1992). Genetic approaches are useful in detecting resistance in viruses, slow growing or nonviable organisms, or organisms with resistance mechanisms that are not reliably detected by phenotypic methods. Genetic methods tend to lessen the biohazard risk, which occur with the propagation by culture of a microorganism and requirement for conventional test methods (Uhl, 1997).