EVALUATION OF ANTIMICROBIAL RESISTANCE PATTERN AGAINST AMINOGLYCOSIDES AND ITS DERIVATIVES IN HIMACHAL PRADESH

By

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Contents

CERTIFICATE

This is to certify that Ms. Kriti Vaid has carried out the undergraduate dissertation project work on "**Evaluation of Antimicrobial Resistance pattern against Aminoglycosides and its derivatives in Himachal Pradesh**" from Jaypee University of Information Technology, Solan under my supervision from August 2014 to May 2015 . This work has not been submitted partially or wholly to any other university or Institute for the award of this or any other degree or diploma.

Supervisor: Co - Supervisor

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DECLARATION

I hereby declare that the work presented in this project dissertation entitled, "**Evaluation of Antimicrobial Resistance pattern against Aminoglycosides and its derivatives in Himachal Pradesh**" submitted as partial fulfilment of Integrated UG/PG Biotechnology VIII semester was carried out at Department of Biotechnology and Bioinformatics, Jaypee University of Information and Technology, Waknaghat (H.P). The work presented in this project dissertation is original and will remain intellectual property of Department of Biotechnology and Bioinformatics, Jaypee University Of Information and Technology, Waknaghat (H.P).

Kriti Vaid (111801)

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Abstract

In this study within Himachal Pradesh we investigated the occurrence of antibiotic resistance patterns from various Clinical specimens. A total of 126 clinical specimens recovered from urine, blood, pus, stool taken from various regional hospitals were collected and were processed for the identification of bacterial isolates in these specimens. The selected bacterial isolates were examined for susceptibility to Amikacin, Gentamicin, Kanamycin, Streptomyicn, along with Aminoglycosides other antibiotics and combinations to get synergetic effect Norfloxacin, Ciprofloxacin, Streptomycin, Vacomycin, Netillin (Netilmicin sulphate), Cefotaxime, Ceftazidime, Tobramycin, Levofloxacin, Nalidixic acid, Cefepime, Ceftriaxone, Ofloxacin, a novel antibiotic entity comprising cefepime hydrochloride and amikacin sulphate referred to as Potentox, Supime comprising Cefepime, Sulbactam, Elores comprising Ceftriaxone and sulbactam, Vancoplus comprising Ceftriaxone sodium and Vancomycin.

Isolates Recovered were E. coli (67%) followed by *Shigella spp*.(14%), *Vibrio spp*.(8%), *Salmonella spp*.(6%), *Klebsiella spp*. (5%).

Our results display that Amikacin, Tobramycin and Levofloxacin are the most effective antimicrobial agents. Among the tested drugs, traditionally used antibiotics showed the maximum resistance. We suggest that that Amikacin which has been introduced recently into clinical settings would allow clinicians to overcome the aminoglycoside resistance acquired by some bacterial strains.

CHAPTER 1

Introduction

1.1 AMINOGLYCOSIDES

Aminoglycosides are highly potent, broad spectrum antibiotics that have been used for the treatment of life threatening Gram negative bacterial infections. They bind specifically to the aminoacyl site (A-site) of 16S rRNA within the prokaryotic 30S ribosomal subunits and interfere with protein synthesis. The vast majority of aminoglycosides are bactericidal, they have predictable pharmacokinetics, and they often act in synergy with other antibiotics, properties that make them valuable as anti-infectives. Furthermore, despite the potential for renal toxicity, ototoxicity, and bacterial resistance, several members of this family of antibiotics have enjoyed clinical use for several decades.

The year 1944 marked the beginning of the aminoglycoside era with streptomycin being introduced, and was followed by the discovery of a series of aminoglycosides such as kanamycin, gentamicin, and tobramycin. The semi-synthetic aminoglycosides, dibekacin, amikacin, and netilmicin, which were introduced in the seventies, allowed clinicians to overcome the anti-aminoglycoside resistance acquired by some bacterial strains against some earlier Aminoglycosides.

The most commonly encountered mechanism of resistance to Aminoglycosides is enzymatic inactivation, which is mediated by 3 classes of enzymes: acetyltransferases, adenyltransferases, and phosphotransferases They are further divided into sub-classes that are based on the site of modification and the spectrum of resistance within the class of antimicrobials. Other known

mechanisms of aminoglycoside resistance include defect in cellular permeability, active efflux, and, rarely, nucleotide substitution of the target molecule.

Aminoglycoside resistance genes are derived from bacterial genes which encode enzymes involved in normal cellular metabolism of bacteria. There are over 50 different AMEs that have been identified till date and enzymatic modification results in high-level resistance. The level of resistance produced differs significantly in various microorganisms and individual strains and depends on many factors, including the amount of enzyme produced, its catalytic efficiency, and the type of aminoglycoside being employed. Thus, in this study we explored the aminoglycoside resistance patterns of bacterial isolates of patients with different infectious diseases in Himachal Pradesh.

1.2 History

The first aminoglycoside, the antibiotic streptomycin, was discovered in 1943 by American biochemists Selman Waksman, Albert Schatz, and Elizabeth Bugie, who isolated the compound from *Streptomyces griseus*, a strain of soil bacteria. Streptomycin was found to inhibit the growth of a variety of bacterial organisms, including the organism that causes tuberculosis (*Mycobacterium tuberculosis*). Waksman later isolated a second aminoglycoside, neomycin, from another species of soil bacteria, *Streptomyces fradiae*. Gentamicin and netilmicin are derived from species of the actinomycete *Micromonospora*. Tobramycin is one of several components of an aminoglycoside complex (nebramycin) that is produced by *S. tenebrarius.* It is quite similar to gentamicin in antimicrobial activity and toxicity. In contrast to the other aminoglycosides, amikacin, a derivative of kanamycin, and netilmicin, a derivative of sisomicin, are semisynthetic products. Other aminoglycoside antibiotics have been developed (e.g., arbekacin, isepamicin, and sisomicin), but they have not been introduced into clinical practice yet.

1.3 Aminoglycoside classes

Natural aminoglycoside antibiotics share a non-sugar 2-deoxystreptamine scaffold connected to amino sugar substituents at the 4-, 5- and 6-positions. The two most important classes of aminoglycoside antibiotics are the 4, 5- and 4, 6-disubstituted 2-deoxystreptamine derivatives. The 4, 5-disubstituted 2-

deoxystreptamine compounds include neomycin whereas the 4,6-disubstituted 2 deoxystreptamine derivatives include gentamycin, kanamycin and streptomycin. Aminoglycoside antibiotics of these three groups, 4, 5- and 4, 6-disubstituted 2-DOS derivatives share a common a target site at the decoding center (A-site) of bacterial 16S ribosomal RNA (rRNA). 2-deoxystreptamine scaffold is the key pharmacophore required for the anchoring of the drugs at the RNA target of bacteria.

1.3.1 Streptomycin

Streptomycin is the first aminoglycoside antibiotic to be discovered and was the first antibiotic to be used in treatment of tuberculosis. It was discovered in 1943, in the laboratory of Selman Waksman at Rutgers University. Streptomycin is derived from the bacterium *Streptomyces griseus*. It inhibits bacterial growth by inhibiting protein synthesis. Specifically, it binds to the 16S rRNA of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit.It is chemically stable and rapidly bactericidal, with a broad spectrum activity (apart from anaerobic bacteria).

1.3.2. Neomycin

Neomycin, an aminoglycoside antibiotic, discovered on 1949 in the lab of Selman Waksman.It has excellent activity against gram-negative bacteria, and has partial activity against gram-positive bacteria. It is produced naturally by the bacterium *Streptomyces fradiae*.

1.3.3. Kanamycin

Kanamycin is made up of 3 rings. Ring II is sugar group,while ring I and III are non-sugar group. Kanamycin B is a more potent antibiotic than either kanamycins A or C. The presence of a diamino hexose, therefore, results in a compound that is a better inhibitor of protein synthesis than one containing only one amino group. Therefore, when only one amino group is present, an antibiotic that contains a 6-amino substituent is more active than one containing a 2-amino substituent. Antibiotic activity can be related to the number and location of amino groups in the hexose moiety glycosidically linked to the 4-position of deoxystreptamine as follows (in decreasing order of potency): 2', 6'-diamino > 6'-amino > 2'-amino > no amino.

1.3.4. Gentamicin

There have 3 types of gentamicin in this class of aminoglycosides such as Gentamicin C1, Gentamicin C2 and Gentamicin C1a. Gentamicin C1 exists when both R1 and R2 are CH3. Gentamicin C2 exists when R1 is CH3 and R2 is H. Gentamicin C1a exists when both R1 and R2 are H. The structure of gentamicin is consistent with the aminoglycoside structural activity relationship(SAR), except few

minor changes. Gentamicin C1a binds in the major groove of the RNA.

1.3.5. Amikacin

Amikacin is a newly introduced semi-synthetic broad spectrum antibiotic. Amikacin is derived from Kanamyicn A and is pharmacologically kanamycin (2,3). It is on the WHO's List of Essential Medicines, regarded as the most important medication needed in a basic health system due to its great antimicrobial activity against several species. Amikacin is used to treat several infections caused by bacteria that are resistant to gentamicin and tobramycin.

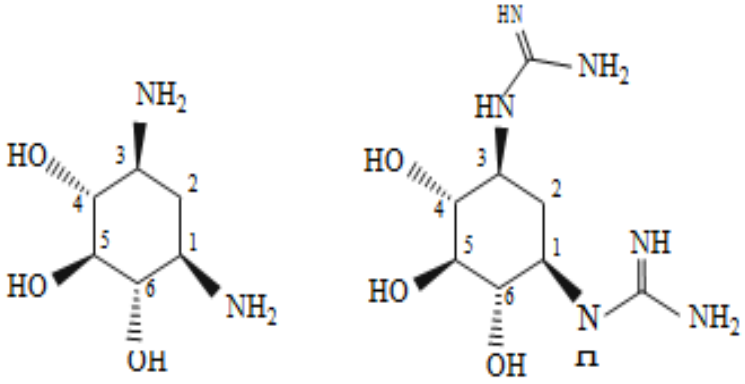
1.4 Chemical structure

As a class of antibiotics, aminoglycosides have a backbone structure consisting of an aminocyclitol ring saturated with amine and hydroxyl substitutions. In the majority of clinically useful aminoglycosides, this aminocyclitol moiety is streptamine or 2-deoxystreptamine (Fig. 1). Streptomycin, possessing a streptidine molecule, is the only exception. The aminocyclitol nucleus is connected through glycosidic linkages to various amino sugars (aminoglycosides).

The aminoglycosides can be conveniently divided into three structural types based on the position of their glycosidic linkages. These structural types include the 4,6-disubstituted 2-deoxystreptamines containing most of the clinically useful aminoglycosides such as gentamicin, tobramycin, amikacin, and netilmicin, the 4,5-disubstituted 2-deoxystreptamines (neomycin and paromomycin), and others (streptomycin and spectinomycin). Spectinomycin, although often considered an aminoglycoside, does not contain an amino sugar. Thus, several investigators have suggested that the term amino- cyclitol be used to describe this entire group of agents rather than the less precise term aminoglycoside (Ristuccia and Cunha 1982).

The aminoglycoside structure is important in understanding their chemical properties. These are basic, strongly polar compounds that are positively charged (cationic). They are highly soluble in water, relatively insoluble in lipids, and have enhanced antimicrobial activity in alkaline rather than acidic environments. As a result, aminoglycosides are minimally absorbed from the gut and penetrate the

blood brain barrier poorly. The cationic nature of the aminoglycosides con- tributes to their antimicrobial activity. Because of their positive charge, they are able to bind negatively charged lipopolysaccharide of the bacterial cell wall and a variety of intracellular and cell membrane anionic molecules such as DNA, RNA, and phospholipids. Unfortunately, their positive charge at physiological pH also contributes to their toxicities, e.g., nephrotoxicity, ototoxicity, and neuromuscular blockade.



2-Deoxystreptamine Streptidine

Fig. 1.1 Backbone structures of the Aminoglycosides

1.5 Mechanism of Action

Aminoglycosides can be considered as polycationic species. Because of their polycationic nature, they show binding affinity for negatively charged residues present in the outer membrane of Gram-negative bacilli and in nucleic acids. Their bactericidal activity is due to inhibition of bacterial protein synthesis through binding to prokaryotic 16S rRNA and disruption of the integrity of the bacterial cell membrane. The uptake process is involves the drug-induced disruption of Mg^{2+} bridges between adjacent lipopolysaccharide molecules. They show their bactericidal activity through a multistep process.

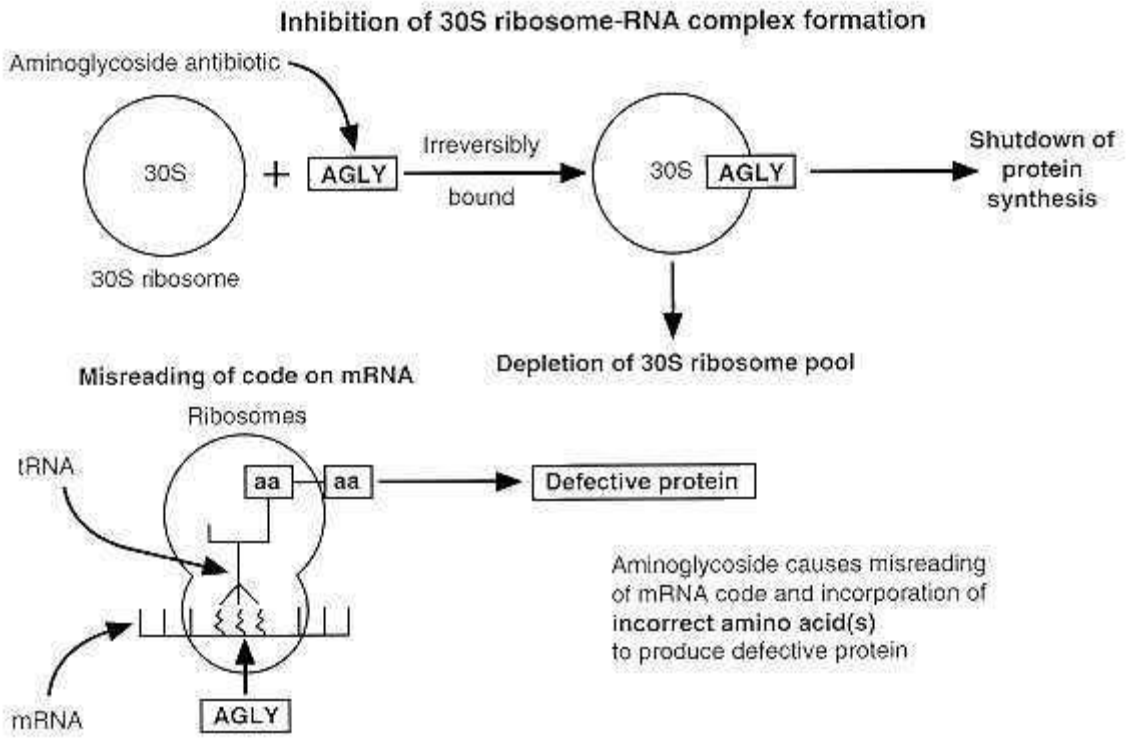


Fig 1.2 shows Inhibition of protein biosynthesis by aminoglycosides.

1.5.1 Binding

The accumulation of Aminoglycosides occurs in three consecutive phases. An initial ionic binding to cells is followed by two energy dependent phases termed energy-dependent phase I (EDPI) and energy-dependent phase II (EDPII). First, aminoglycosides bind electrostatically to negatively charged residues in the outer membrane of Gram-negative bacteria in a non energy dependent process. Then they diffuse through outer membrane porin channels and enter the periplasmic space. The subsequent transport across the cytoplasmic membrane requires metabolic energy from the electron transport system in an oxygen-dependent process. This phase of transport has been termed energy dependent phase I (EDP- I). The requirement of oxidative energy production transport explains why aminoglycosides are much less active in an anaerobic environment.

1.5.2 Misreading

Misreading implied a distortion of codon-anticodon interaction on the ribosome, (Bernard D. Davis). The binding does not prevent the formation of the initiation complex of peptide synthesis, it perturbs the elongation of the nascent chain by impairing the proofreading process which controls the translational accuracy. The aberrant proteins may be inserted into the cell membrane, leading to altered permeability and further stimulation of aminoglycoside transport.

1.5.3 Different actions on Chain elongation Process

The ribosome is a very complex structure comprising three RNA molecules and more than 50 proteins. The complex catalyzes protein synthesis with the assistance of several guanosine 5c-triphosphate hydrolyzing protein factors. Aminoglycoside antibiotics bind to the 30S ribosomal subunit, which plays a crucial role in providing high-fidelity translation of genetic material. Recently, atomic structures for both the large and the small ribosomal subunits and high-resolution crystal structures of the 30S subunit with streptomycin, spectiomycin, paromomyicn, and hygromycin B have been solved (Ban et al. 2000). Together with several available nuclear magnetic resonance (NMR) structures for the ribosomal constituents (Fourmy et al. 1998), these structures provide valuable information not only on processes during translation but also on molecular mechanisms of interaction of aminoglycosides with the bacterial ribosome.

The 16S rRNA from E. coli is well-studied among the rRNA subunits, particularly the interactions of various aminoglycoside antibiotics with the 16S rRNA (Moazed and Noller 1987). Different classes of aminoglycoside antibiotics bind to different sites on the rRNA, depending on the structural complementarity between the two. For example, neomycin, paromomycin, gentamicin, and kanamycin are believed to bind to the A- site on the 16S rRNA in E. coli and were shown to protect bases A1408 and G1494 in chemical footprinting experiments (Noller 1991). Four bases, A1408, A1492, A1493, and G1494, in the rRNA A-site interact with tRNA, although with different affinities.

The binding of aminoglycosides to the A- site in the decoding region (i.e., the site of codon and anticodon recognition) interferes with the accurate recognition of cognate tRNA by rRNA during

translation. These interactions are also thought to interfere with the translocation of tRNA from the A-site to the peptidyl– tRNA site (P-site). The A-site makes weak contacts with the mRNA and tRNA, implying that this region plays a role in recognition of appropriate tRNA via subtle changes in the free energy. The binding of aminoglycoside near this site may affect the delicate process of interactions between codon and anticodon (Xi and Arya 2005). It was also proposed that the presence of an aminoglycoside stabilizes the complex of mRNA and tRNA at the A-site, which in turn affects the process of translation (Cate et al. 1999). It is difficult to surmise all the effects of aminoglycosides on the rRNA structure, and further structural studies with the aminoglycosides bound to the complexes, such as the 70S rRNA, will be helpful in elucidating and understanding the subtle changes that lead to the antibiotic actions of aminoglycosides.

A number of investigators have used synthetic probes to understand the interactions between RNA templates and aminoglycosides. It has been suggested that aminoglycosides bind to more than one target site in the ribozyme (Michael et al. 1999). Recently, several aminoglycoside antibiotics such as neomycin B, tobramycin, and kanamycin A have been dimerized either symmetrically or asymmetrically by using a "tether," and their binding affinities were compared to those of the monomeric parent aminoglycosides (Michael et al. 1999). It was suggested that, if there were multiple binding sites on the RNA, the dimerized aminoglycosides should bind with a higher affinity than the parent antibiotic, provided that multiple binding sites are accessible. It was indeed observed that the dimerized aminoglycosides bind to the Tetrahymena ribozyme 20 to 1,200-fold better than the parent aminoglycosides.

One explanation for the higher binding affinity could be the increased number of positively charged amino groups on the dimerized amino- glycoside, but this effect seems to be synergistic with the entropic advantage gained by dimerization (Welch et al 2005). It also indicated that the presence of multiple high- affinity binding sites for aminoglycoside antibiotics in an RNA molecule bulge in the RNA sequence is necessary to allow binding of aminoglycosides (Cho et al. 1998). By using a specific stem-loop derivative of the RNA aptamer, a series of chemical interference, chemical modification, and mutation studies was performed to understand the structural requirements for binding of tobramycin to the RNA aptamer.

This aminoglycoside appeared to interact mainly with the nucleic acid bases in the RNA aptamer but not with the phosphate backbone. The presence of a bulge, however, was proposed to be important for the high- affinity binding of tobramyicn in a stoichiometric ratio, and it was concluded that a bulge creates a cavity for interactions of the aminoglycoside and the nucleic acid base (Pilch et al. 2005). This analogy can be applied to other RNA sites, such as the hammerhead region and the A-site, where a cavity is present due to the noncanonical base- pairing, loops, or bulges that create a suitable site for the Aminoglycosides interact with the anionic phosphate groups and nucleic acid bases.

1.6 Antimicrobial activity and clinical use

Aminoglycosides are the most commonly used antibiotics for the treatment of serious infections caused by Gram-negative bacteria, including bacilli such as *Escherichia coli*, *Enterobacter, Pseudomonas* and *Salmonella* species, and Gram-positive pathogens such as *Staphylococcus* and some *streptococci* as well as *Mycobacteria* . Broad-spectrum use of aminoglycosides is limited by drug-modifying enzymes and reduced uptake in Gram-positives, which have a distinct membrane composition that prevents aminoglycoside permeation, and in anaerobes, which lack the oxygen-dependent membrane transport mechanism. Differences in the spectrum of activity among aminoglycosides are related to the presence of drug-modifying enzymes that inactivate the antibiotics and efflux pumps.

The poor oral absorption of the highly polar aminoglycoside antibiotics, which are positively charged under physiological conditions, requires administration by parenteral injection. Intravenous injection of liposome-encapsulated aminoglycosides has been investigated in animal models . Inhalation of aerosolized gentamicin and tobramycin solutions is used for the treatment of serious respiratory tract infections, including those caused by *Pseudomonas aeruginosa* in cystic fibrosis patients.

1.7 Aminoglycoside resistance

Most resistance to aminoglycosides is caused by inactivation by intracellular bacterial enzymes. Because of structural differences, amikacin is not inactivated by the common enzymes that inactivate gentamicin and tobramycin. Therefore, a large proportion of the Gram-negative aerobes that are resistant to gentamicin and tobramycin are sensitive to amikacin. In addition, with increased use of amikacin, a lower incidence of resistance has been observed compared with increased use of gentamicin and tobramycin (Watanabe et al. 2004). *P. aeruginosa* may show adaptive resistance to aminoglycosides. This occurs when formerly susceptible populations become less susceptible to the antibiotic as a result of decreased intracellular concentrations of the antibiotic. This decrease may result in colonization, slow clinical response, or failure of the antibiotic despite sensitivity on in vitro testing (Yao et al. 2004).

Aminoglycosides are often combined with a beta-lactam drug in the treatment of *Staphylococcus aureus* infection. This combination enhances bactericidal activity, whereas aminoglycoside monotherapy may allow resistant *Staphylococci* to persist during therapy and cause a clinical relapse once the antibiotic is discontinued (Davies and Wright 1997). Infective endocarditis that is due to *enterococci* with high levels of resistance to aminoglycosides is becoming increasingly common. All *enterococc*i have low-level resistance to aminoglycosides because of their anaerobic metabolism. In the treatment of bacterial endocarditis, a beta-lactam drug is also used synergistically to facilitate aminoglycoside penetration into the cell. When high-level resistance occurs, it is typically due to the production of inactivating enzymes by the bacteria. Because of the increasing frequency of this resistance, all enterococci should be tested for antibiotic susceptibility (Dworkin 1999).

As with all antibiotics, resistance to aminoglycosides is becoming increasingly prevalent. Repeated use of aminoglycosides, especially when only one type is employed, leads to an increased incidence of resistance (Fluit and Schmitz 1999). Nevertheless, resistance to aminoglycosides requires long periods of exposure or very large inoculums of organisms and occurs less frequently than with other agents, such as third-generation cephalosporins, which are also effective against Gram-negative organisms (Neu 1992). The aminoglycoside resistance genes are derived from bacterial genes, which encode

enzymes involved in normal cellular metabolism . The selective pressure of aminoglycoside usage causes mutations, which alter the expression of these enzymes, resulting in the ability to modify aminoglycosides. Bacteria can acquire foreign DNA by the mechanisms of transduction, transformation, and conjugation. This is facilitated by two types of genetic elements, self-transferable conjugative plasmids, and transposons.

1.8 Mechanism of Resistance

The mechanisms of bacterial resistance to aminoglycosides have been the subject of numerous genetic and biochemical. There are three general mechanisms of aminoglycoside resistance: (1) reduction of the intracellular concentration of the antibiotic within bacterial cells, usually via efflux of the agent out of the bacterial cell by either dedicated or general efflux pumps; (2) alteration of the molecular target of the antibiotic, usually as result of a spontaneous mutation in the gene encoding the target or substitution of the target's function by an exogenous gene; and (3) enzymatic inactivation of the aminoglycoside.

It must be acknowledged that even for these broad classifications there are some resistance mechanisms that do not really fall neatly into any of those three categories. In addition, more than one resistance mechanism is at play (often in the same strain at the same time) in the case of some classes of drug. Given the wide diversity of resistance mechanisms and the genes encoding them, it would seem a fruitless enterprise to develop agents to circumvent their activity.

1.8.1 Active efflux pump

Aminoglycoside concentration is decreased inside a target cell by reduction of drug uptake, activation of drug efflux pump, or both. This will affect the susceptibility of the strain to the whole class of aminoglycoside compounds and can be the cause of intrinsic or acquired resistance. Bacterial efflux pump is an energy-dependent (ATP) pump and is now recognized as a major cause of antibiotic resistance. This is particularly true for the multidrug-resistant opportunist pathogens responsible for nosocomial infections.

Bacterial species constitutively expressing such transporters are intrinsically resistant to low levels of various antibiotics. However, mutations in the regulatory genes of the pumps or induction of expression

in the presence of substrate, can lead to the overexpression of the originally constitutive or pump genes (Aires et al. 1999; Masuda et al. 2000). In the last several years, aminoglycosides were shown to be substrates for a number of multidrug efflux pumps, including members of the five superfamilies of bacterial transporters. The resistance nodulation cell division (RND) transporter super- family plays an important role in Gram-negative bacteria.

The transporters of the RND superfamily use the membrane proton motive force as energy source. They are localized in the cytoplasmic membrane and in Gram- negative bacteria. They interact with a membrane fusion protein, located in the periplasmic space, and an outer membrane protein to form a continuous tripartite channel able to export substrates directly out of the cell (Westbrock- Wadman et al. 1999; Livermore 2002). Several RND proteins were shown to be involved in intrinsic and/or acquired, proton motive force-dependent, aminoglycoside resistance in various gram negative pathogens including *P. aeruginosa, Burkholderia pseudomallei, Acinetobacter baumannii, and E. coli* (Poole 2005). Active efflux has been evidenced for neomycin, kanamycin, and hygromycin A in *E. coli.*

1.8.2 Target Modification

16S rRNA methylation

Many aminoglycoside producing organisms express rRNA methylases, which are capable of modifying the 16S rRNA molecule at specific positions critical for the tight binding of the drug. A number of genes encoding such enzymes have been identified from several aminoglycoside producers. The corresponding rRNA methyltransferases form the aminoglycoside resistance family of methyltransferases. Kanamycin A and B are obtained from *Streptomyces tenjimariensis* and *Streptomyces tenebrarius*, respectively. They catalyze the modification of A1408 at the N1 position and confer high-level resistance to kanamycin, tobramycin, sisomicin, and apramycin, but not gentamicin.

Gentamicin A is obtained from the gentamicin producer *Micromonospora purpurea* and kasugamycin is obtained from *S. tenebrarius*. They catalyze the modification of G1405 at the N7 position and conferring high-level resistance only to the 4,6-disubstituted deoxystreptamines including gentamicin (Doi et al. 2004). Methylation of these nucleotides presumably abolishes the intermolecular con- tacts

that they make with the drug. There are also reports available on genes encoding a 16S rRNA methyltransferase in 2003 and 2004, which described the characterization of methyltransferase genes in clinical isolates of human Gram- negative pathogens. The rmtA and rmtB genes were found in clinical isolates of *P. aeruginosa* and *Serratia marcescens*, respectively. These strains were found in Japan, where arbekacin has been used extensively since 1990. The two genes share 82% sequence identity and the encoded Rmt enzymes confer high-level resistance [minimal inhibitory concentrations (MICs) >1024 μg/ml] to almost all clinically useful aminoglycosides including arbekacin (Galimand et al. 2003). The considerable primary sequence similarity was observed between the Rmt proteins and the 16S rRNA methylases of Actinomycetes. They show the high G + C content of the gene (55%). This suggests a possible gene transfer from the producing organisms to Gram-negative pathogens. Another 16S rRNA methylase was characterized from *Klebsiella pneumoniae*. The structural gene, armA, was located on plasmid containing several other resistant genes including those conferring resistance to beta-lactams, trimethoprim, sulfonamides, and other aminoglycoside resistance determinants.

Ribosomal Mutations

Aminoglycoside resistance can also occur by mutation of the ribosomal target. It is clinically relevant only for streptomycin in *M. tuberculosis*. Mycobacterium is the only genus of eubacteria with species that contain a single copy of the ribosomal operon. This implies that a single mutation can lead to the production of a homogeneous population of mutant ribosomes and, thus, can result in resistance (Meier et al. 1994). The mutations in the rrs gene, encoding the 16S rRNA and associated with streptomycin resistance in *M. tuberculosis*, affect two highly conserved regions. These are the 530 loop and the nucleotide 912, resulting in a decrease in affinity for streptomycin. Mutations in genes encoding ribosomal proteins can also alter the activity of aminoglycosides. Notably, mutations in protein S12 are the other major cause of streptomycin resistance in *M. tuberculosis*.

Enzymatic modifications

Enzymatic modification is one of the most important mechanisms of aminoglycoside resistance, resulting in a loss of antibacterial activity due to a diminished affinity for the ribosomal A-site target (Llano-Sotelo et al. 2002). There are three classes of these enzymes: aminoglycoside acetyltransferases, aminoglycoside nucleotidyltransferases, and aminoglycoside phosphotransferases.

Aminoglycoside acetyltransferases

AACs catalyze the acetylation of one of the four amino groups of aminoglycoside antibiotic. Acetylation reduces the affinity of these compounds for the acceptor tRNA site on the 30S ribosome by four orders of magnitude. The acetylation of aminoglycosides occurs after the random binding of both acetyl-CoA and amino group of aminoglycosides to the enzyme and was proposed to proceed via a direct nucleophilic attack by the amine on the thioester (Levings et al. 2005).

The AACs are classified based on their regiospecificity of acetyl transfer on the aminoglycoside structure. For example, the AAC(6') N- acetylate aminoglycoside on the amine group that is frequently found on position 6' of the aminohexose linked to position 4 of the central 2-deoxystreptamine ring, while AAC(3) N-acetylate linked to position 3 of the 2- deoxystreptamine ring (Wright and Serpersu 2004). The first to be identified was the 178 amino acid AAC(2')-Ia from Providencia stuartii. The AAC(3) family of amino- glycoside acetyltransferases is one of the largest. It includes four major types, I–IV, based on the pattern of aminogly- coside resistance that they confer (Sunada et al. 1999; Draker and Wright 2004).

The first aminoglycoside- modifying enzyme to be purified to homogeneity was the E. coli R-plasmid-encoded gentamicin acetyltransferase. This allowed for the first studies of the substrate specificity of these enzymes. The bifunctional AAC(6')-Ie APH(2")-Ia enzyme (Hegde et al. 2001) confers broad spectrum and high-level aminoglycoside resistance in enterococci and staphylococci. It differs from the two AAC(6') described above in its genetic localization and catalytic mechanism. The structural gene of the enzyme is generally found on transposable elements and frequently carried on R plasmids (Vetting et al. 2004). These mobile supports account for the intergenus transfer of the resistant determinant, originally isolated from *Enterococcus faecalis*. The enzyme is monomeric in solution and

the acetyltransferase activity exhibits exceptionally broad substrate specificity for aminoglycosides including fortimcin A and aminoglycosides possessing a hydroxyl group at the 6'-position. Three-dimensional structures for four members of the class have been reported. These show structural homology to the GCN5 superfamily of acyltransferases. There are no common active site catalytic residues among all AACs. Analysis of the active site region where aminoglycosides bind, though, reveals a highly negatively charged surface that serves as a docking platform for these basic antibiotics.

Aminoglycoside nucleotidyltransferases

The ANTs represent the smallest class of aminoglycoside- inactivating enzymes. The clinically important aminoglycosides, such as gentamicin and tobramycin, are both modified by ANT("). The gene encoding this enzyme is widely distributed among pathogenic bacteria and its local prevalence is clearly selected by aminoglycoside usage in different clinical environments. There are 10 ANTs identified to date. These are of both chromosomally encoded and plasmid-encoded enzymes. The ant (2") and ant(3") genes encoding adenylyltransferases are often identified on mobile genetic elements in resistant Gram-negative organisms.

The ant(4'), ant(6), and ant(9) genes are also found on plasmids or integrated into transposons in Gram-positive organisms. These enzymes catalyze the reaction between Mg–ATP and aminoglycoside to form the O-adenylylated aminoglycoside and the magnesium chelate of inorganic pyrophosphate. Enzymes that regioselectively adenylylate the 6 and 3" positions of the streptomycin and the 9 and 3" positions of the spectinomycin have been identified. The reactions catalyzed by the ANT(2") and ANT(4') are most significant and have been the most thoroughly mechanistically and structurally studied (Gates and Northrop 1988). The two investigators found the kinetic mechanism to be sequential and an ordered mechanism of substrate binding, with nucleotide (ATP) binding before aminoglycoside. The structure of aminoglycoside substrates bound to the enzyme has been characterized by NMR methods, although a 3-D structure of the entire enzyme remains elusive. The

3-D structure of only one ANT has been reported, that of ANT(4') from S. aureus (Pedersen et al. 1995). The enzyme functions as a dimer, with the active site at the interface and with both monomers contributing residues to stabilize the substrates. The positioning of the substrates supports independent mechanistic evidence for direct attack of the nucleophilic hydroxyl on the α-phosphate of ATP.

Aminoglycoside phosphotransferases

Aminoglycoside kinases are known as aminoglycoside phosphotransferases. These are widely distributed among bacterial pathogens. Phosphorylation of the antibiotics results in a dramatic effect on their ability to bind to their target on the A-site of the ribosome. The genes encoding these enzymes are frequently found on multidrug resistance R plasmids, transposons, and integrons. APHs are classified based on their regiospecificity of phosphoryl transfer, their substrate specificity, and the specific gene sequence in question. APHs catalyze the regiospecific transfer of the γ-phosphoryl group of ATP to one of the hydroxyl substituents present on the aminoglycoside. They include a large number of aminoglycoside-modifying enzymes and are most relevant to clinical resistance to aminoglycosides in Gram-positive organisms .

The APH (3') family is especially ubiquitous and has been widely used as resistance marker in molecular biology research (example, the neo cassette). The best- studied APH is APH (3')-IIIa, which has both 3' and 5"- regiospecific phosphoryl transfer capacities. The enzyme is primarily found in Gram-positive cocci such as *Staphylococci and Enterococci*, and confers resistance to a broad range of aminoglycosides but not to gentamicin or tobramycin. Both antibiotics lack the critical 3'-hydroxyl groups that accept the phosphate group donated by ATP.

The 3-D structure of the enzyme revealed a remarkable similarity with Ser, Thr, and Tyr protein kinases, which was not evident from the primary amino acid sequence (Nurizzo et al. 2004). Other aminoglycoside kinases include the spectinomycin-modifying enzyme APH(9) and APH(3") (StrA) and APH(6) (StrB), both of which modify streptomycin. The bifunc- tional enzyme, AAC(6')–APH(2') is widely distributed among pathogenic bacteria and confers high level resis- tance to virtually all aminoglycosides except streptomycin and spectinomycin. The AAC(6') domain of this bifunc- tional enzyme has overlapping aminoglycoside modifica- tion capacity with APH(2") domain, and aminoglycosides can be doubly modified (Boehr et al. 2005). As a result, this enzyme has shown very high MICs.

CHAPTER 2

Materials and Methods

2.1 Clinical Isolates Collection:

Clinical specimens from various hospitals of Himachal Pradesh were collected. The specimens were isolated from Urine, Stool, Sputum and Pus samples. Among these, Gram negative bacterial isolates that include *Escherichia coli, Pseudomonas species and Klebsiella species and Shigella spp.* were recovered and identified using standard microbiological procedures.

S. No	Region	No. of Samples
1.	Dr. R.P. Govt. Medical College, Tanda, Kangra (Urine samples)	60
2.	IGMC Shimla (Urine & Sputum Samples)	19
3.	CRI, Kasauli (Pus samples)	05
4.	IGMC Shimla (Stool samples)	42

Table 2.1. shows No. of samples collected from various hospitals of Himachal Pradesh.

2.2 Isolation of bacteria from different infectious samples

Samples were streaked on nutrient agar and MacConkey agar and the plates were kept in incubation for overnight at 37˚C, and further sub culturing was done.

2.3 Biochemical Tests

2.3.1 Triple Sugar Iron Agar

Principle: Triple sugar iron agar (TSI) is a differential medium that contains lactose, sucrose, a small amount of glucose (dextrose), ferrous sulfate, and the pH indicator phenol red. It is used to differentiate enterics based on the ability to reduce sulfur and ferment carbohydrates.

As with the phenol red fermentation broths, if an organism can ferment any of the three sugars present in the medium, the medium will turn yellow. If an organism can only ferment dextrose, the small amount of dextrose in the medium is used by the organism within the first ten hours of incubation. After that time, the reaction that produced acid reverts in the aerobic areas of the slant, and the medium in those areas turns red, indicating alkaline conditions.

Procedure for Triple Sugar Iron Agar (TSI) Test

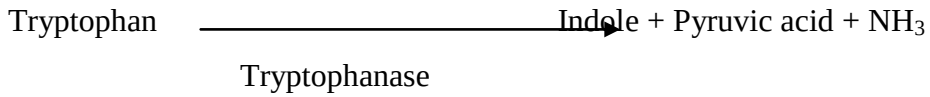
1. With a sterilized straight inoculation loop touch the top of a well-isolated colony.
2. Inoculate TSI Agar by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant.
3. Incubate the tube at 37˚C in for 18 to 24 hours.

Interpretation of Triple Sugar Iron Agar Test

1. Alkaline slant/ no change in butt (K/NC) i.e Red/Red = glucose, lactose and sucrose nonfermenter
2. Alkaline slant/Alkaline butt (K/K) i.e Red/Red = glucose, lactose and sucrose nonfermenter
3. Alkaline slant/acidic butt (K/A); Red/Yellow = glucose fermentation only, gas (+ or -), H_2S(+ or -)
4. Acidic slant/acidic butt (A/A); Yellow/Yellow = glucose, lactose and/or sucrose fermenter gas (+ or -), H_2S (+ or -).

2.3.2 INDOLE TEST

Principle: Some bacteria can produce indole from amino acid tryptophan using the enzyme typtophanase. Production of indole is detected using Kovac's reagent. Indole reacts with the aldehyde in the reagent to give a red color. An alcoholic layer concentrates the red color as a ring at the top.



Procedure:

1. Bacterium to be tested is inoculated in peptone water, which contains amino acid tryptophan and incubated overnight at 37˚C.
2. After incubation few drops of Kovac's reagent are added. Kovac's reagent consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol and conc. HCl. Formation of a red or pink coloured ring at the top is taken as positive.

2.3.3 METHYL RED (MR) TEST:

Principle: This test detects the ability of an organism to produce and maintain stable acid end products from glucose fermentation. Methyl Red is a pH indicator, which remains red in color at a pH of 4.4 or less.

Procedure:

1. The colony to be tested is inoculated into glucose phosphate broth, which contains glucose and phosphate buffer and incubated at 37˚C for 48 hours.

2. The pH of the medium is tested by the addition of 5 drops of MR reagent.

3. Development of red color is taken as positive. MR negative organism produce yellow color.

2.3.4 VOGES PROSKAUER (VP) TEST:

Principle: VP test detects butylene glycol producers. Acetoin is an intermediate in the production of butylene glycol. In this test two reagents, 40% KOH and alpha-naphthol are added to test broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone, in the presence of alphanaphthol to produce red color. Role of alpha-naphthol is that of a catalyst and a color intensifier.

Procedure:

1. Bacterium to be tested is inoculated into glucose phosphate broth and incubated for at least 48 hours.

2. 0.6 ml of alpha-naphthol is added to the test broth and shaken. 0.2 ml of 40% KOH is added to the broth and shaken. The tube is allowed to stand for 15 minutes.

3. Appearance of red color is taken as a positive test. The negative tubes must be held for one hour, since maximum color development occurs within one hour after addition of reagents.

2.3.5 CITRATE UTILIZATION TEST:

Principle: This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which is utilized as sole source of nitrogen.

Utilization of citrate involves the enzyme citritase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO_2. Production of Na_2CO_3 as well as NH_3 from utilization of sodium citrate and ammonium salt respectively results in alkaline pH. This results in change of medium's color from green to blue.

Procedure:

1. Bacterial colonies are picked up from a straight wire and inoculated into slope of Simmon's citrate agar and incubated overnight at 37˚C.

2. If the organism has the ability to utilize citrate, the medium changes its color from green to blue.

2.4 Antibacterial Agents

In view of increasing incidence of aminoglycoside resistance and failure of monotherapy, a combination therapy may be the only notable therapeutic approach to treat infections caused by aminoglycoside resistant organisms . The combination of aminoglycosides with β-lactams have been documented to be synergistic. Therefore along with Aminoglycosides other antibiotics and combinations have also been employed such as Amikacin, Gentamicin, Kanamycin, Norfloxacin, Ciprofloxacin, Streptomycin, Vacomycin, Netillin (Netilmicin sulphate), Cefotaxime, Ceftazidime, Tobramycin, Levofloxacin, Nalidixic acid, Cefepime, Ceftriaxone, Ofloxacin, A novel antibiotic adjuvant entity (AAE) comprising cefepime hydrochloride and amikacin sulphate referred to as Potentox, Supime comprising Cefepime, Sulbactam, Elores comprising Ceftriaxone and sulbactam, Vancoplus comprising Ceftriaxone sodium and Vancomycin hydrochloride (Venus Remedies Limited, Baddi, India).

2.5 Aminoglycoside Susceptibility Testing:

Antimicrobial susceptibility test was carried out using Kirby–Bauer disk diffusion method.

Procedure:

1. Select a pure culture plate of one of the organisms to be tested.
2. Emulsify the colony in the nutrient broth with a tip.
3. Allow it to incubate for around 6-8 hrs until it reaches the log phase.
4. After incubation, allow the tubes to reach room temperature.
5. Use the culture with inoculation loop to streak MHA (Mueller-Hinton agar) plate.
6. After the streaking is complete, allow the plate to dry for 5 minutes.
7. Antibiotic discs can be placed on the surface of the agar using sterilized forceps.

8. Gently press the discs onto the surface of the agar using flame sterilized forceps or inoculation loop.

9. Carefully invert the inoculated plates and incubate for 24 hours at 37° C.

10. After incubation, use a metric ruler to measure the diameter of the zone of inhibition for each antibiotic used.

11. Compare the measurement obtained from the individual antibiotics with the standard table to determine the sensitivity zone.

12.Compare the measurement obtained from the individual antibiotics to the standard table to determine whether the tested bacterial species is sensitive or resistant to the tested antibiotic.

2.6 DNA Isolation:

1. DNA from all isolates was extracted

2. Five ml of each at concentration of 10^{10} colony forming unit (cfu)/ml was used for the DNA isolation.

3. DNA purity and concentrations were measured with spectrophotometer (260/280).

Procedure for DNA isolation

1. Inoculate 5 ml Nutrient broth bacterial culture and incubate at 37˚C for 6-8 hrs.

2. Pellet down bacterial culture at 7000 rpm for 5-10 mins at 4˚C.

3. Discard the supernatant.

4. Add SDS lysis buffer (200-400µl) to pellet.

5. Incubate at 60˚C for 1 hr.

6. Add phenol: Chloroform: Isoamylalcohol (25:24:1).

7. Centrifuge at 13000 rpm for 10 mins.

8. Transfer supernatant to another tube ad add equal volume of Isopropanol and 1/20th volume of Sodium acetate 0.5M/ 0.5M NaCl. DNA threads would be visible by now.

9. Keep it at 4˚C for 1 hr.

10. Centrifuge at 13000 rpm for 10 mins.

11. Wash with 70% ethanol for 5 mins at 13000 rpm twice.

12. Dry pellet and suspend in Nuclease free water.

2.7 MIC DETERMINATION

1. DILUTION OF ANTIBIOTICS

- Desired antibiotic concentration should be started from 1000µg/ml.
- Weigh 20mg of antibiotic powder and dissolve in 1ml of autoclaved distilled water and dilute 20 folds.

2. Take a sterilized 96 well plate ELISA titre (u – shaped). Fill 95µl of bacterial culture in each well and add 5µl of each dilution of antibiotic as below. Take bacterial control, Media control, and an empty plate control. Add 95µl bacterial culture + 5µl antibiotic in rest of the wells.

2.8 PCR for Genes Encoding AMEs:

1. DNA of each isolate was exposed to polymerase chain reaction (PCR) to screen all isolates for the presence of the aminoglycoside modifying genes, aac(6), ant(2) and aph(3).

2. PCR amplification was performed using 1 µl of template DNA, 0.5 µl of 10mM dNTPs, 2.5µl of each primer and 0.3 µl of Taq polymerase in a total volume of 12.5µL.

3. PCR amplification was done using Eppendorf thermocycler

4. Thereafter, 5 µl of each PCR product was analyzed on 1 % (w/v) agarose gel supplemented with ethidium bromide. The amplicons were then visualized on a UV transilluminator and photographed.

CHAPTER 3

RESULTS

3.1 Selection of bacteria on differential media:

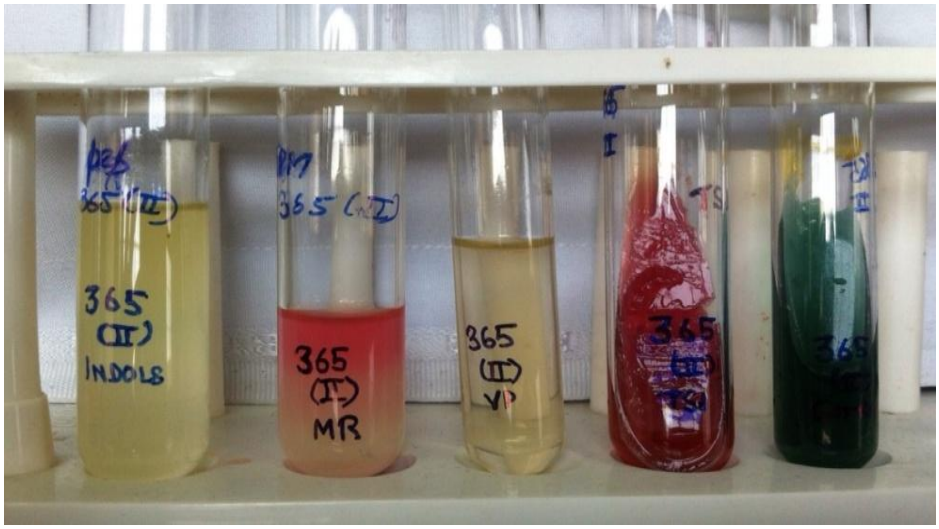
Bacterial Pathogens were isolated by streaking on Nutrient and Mac Conkey plates.

Fig 3.1 showing E. coli isolates in Nutrient agar and MacConkey agar plates

3.2 Biochemical characterization

IMViC and triple sugar iron agar. 1. Indole test, 2. Methyl red test , 3. Voges prauskauer

4. Citrate test 5. Triple sugar iron agar

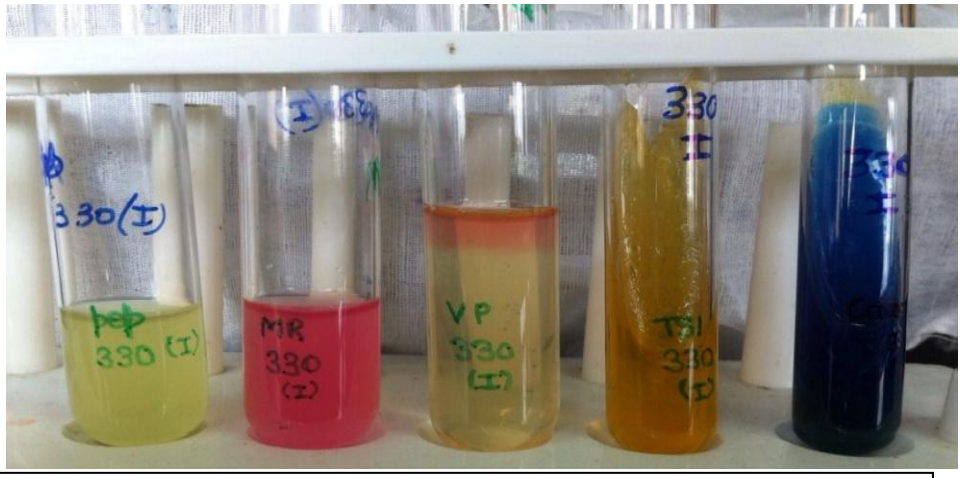


Ind -	MR+	VP -	TSI K/K	Cit -

Fig 3.2 IMViC for Sample No. 365 (II)

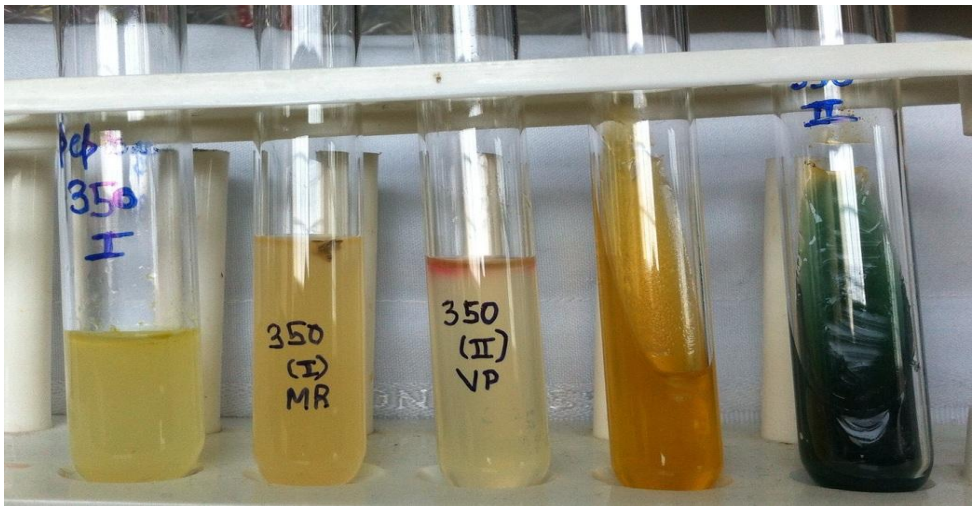
Ind +	MR+	VP-	TSI K/A	Cit +

Fig 3.3. IMViC for Sample No. 351 (II)



Ind-	MR+	VP+	TSI A/A	Cit+

Fig 3.4. IMViC for Sample No. 330 (I)



Ind-	MR-	VP+	TSI A/A	Cit-

Fig 3.5 IMViC for Sample no. 350 (II)

S.No.	Region	Samples	Sample type	Organism
1	Shimla	49sL	Urine	*E. coli*
2	Shimla	68s	Urine	*E. coli*
3	Shimla	48s	Urine	*E. coli*
4	Shimla	58s	Urine	*E. coli*
5	Shimla	54	Urine	*E. coli*
6	Shimla	49sS	Urine	*E. coli*
7	Kasaulli	CRI KII	Pus	*E. coli*
8	Shimla	75LeL	Urine	*E. coli*
9	Shimla	75LeS	Urine	*E. coli*
10	Shimla	50sSF	Urine	*Klebsiella spp.*
11	Kasaulli	CRI KIII	Urine	*Klebsiella spp.*
12	Kasaulli	Pus ND2S	Pus	*Shigella spp.*
13	Kasaulli	Pus ND1L	Pus	*Shigella spp.*
14	Shimla	50SFl	Urine	*Klebsiella spp.*
15	Shimla	U(3)	Urine	*E. coli*
16	Shimla	U(10)	Urine	*E. coli*
17	Shimla	U(24)	Urine	*E. coli*
18	Shimla	U(27)	Urine	*E. coli*
19	Shimla	3F lab (U)	Urine	*E. coli*
20	Shimla	1 Lab (U)	Urine	*E. coli*
21	Shimla	4M	Urine	*E. coli*
22	Kasaulli	Anil CRI (U)	Urine	*E. coli*
23	Shimla	6 Fu	Urine	*E. coli*
24	Kasaulli	8 Fu CRI	Urine	*E. coli*
25	Shimla	2F	Urine	*E. coli*
26	Shimla	8	Urine	*E. coli*
27	Shimla	Neelam U(9)	Urine	*E. coli*
28	Shimla	11	Urine	*E. coli*
29	Shimla	5M	Urine	*E. coli*
30	Kasaulli	F(v) CRI	Urine	*E. coli*

31	Shimla	64	Urine	*E. coli*
32	Shimla	57	Urine	*E. coli*
33	Shimla	69	Urine	*E. coli*
34	Shimla	65	Urine	*E. coli*
35	Shimla	105	Urine	*E. coli*
36	Shimla	61	Urine	*E. coli*
37	Shimla	330 (I)	Stool	*E. coli*
38	Shimla	285 (I)	Stool	*Vibrio spp.*
39	Shimla	350 (I)	Stool	*Vibrio spp.*
40.	Shimla	330 (II)	Stool	*Salmonella spp.*
41	Shimla	350 (II)	Stool	*Salmonella spp*
42	Shimla	365 (II)	Stool	*Shigella spp.*
43	Shimla	351 (III)	Stool	*Salmonella spp.*
44	Shimla	351 (II)	Stool	*E. coli*
45	Shimla	351 (I)	Stool	*Vibrio spp.*
46	Shimla	350 (III)	Stool	*Shigella spp.*
47	Shimla	365 (I)	Stool	*E. coli*
48	Shimla	317 (I)	Stool	*Vibrio spp.*
49	Shimla	317 (II)	Stool	*E. coli*
50	Shimla	316	Stool	*E. coli*
51	Shimla	358 (I)	Stool	*Shigella spp.*
52	Shimla	358 (II)	Stool	*E. coli*
53	Shimla	383 (II)	Stool	*E. coli*
54	Shimla	345 (I)	Stool	*Shigella spp.*
55	Shimla	278 (I)	Stool	*E. coli*
56	Shimla	333 (II)	Stool	*Salmonella spp.*
57	Shimla	383 (I)	Stool	*Shigella spp.*
58	Shimla	345 (II)	Stool	*E. coli*
59	Shimla	333 (III)	Stool	*E. coli*
60	Shimla	333 (I)	Stool	*Vibrio spp.*
61	Shimla	278 (II)	Stool	*Shigella spp.*

62	Shimla	357 (I)	Stool	*E. coli*
63	Shimla	378 (I)	Stool	*E. coli*
64	Shimla	357 (I)	Stool	*Shigella spp.*

Table 3.1. shows Biochemically characterized isolates found in clinical specimens.

Percentage prevalence of Microorganisms in Clinical isolates

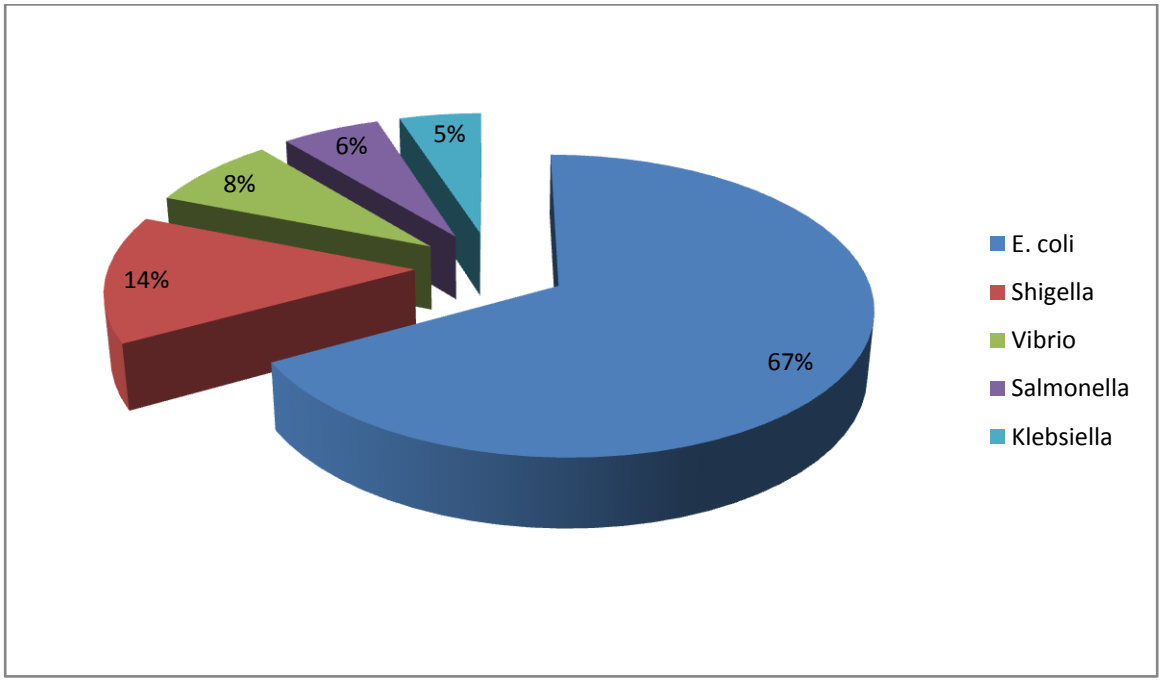


Fig 3.6. shows higher percentage of E. coli followed by *Shigella , Vibrio, Salmonella and Klebsiella spp.*

3.3 ANTIBIOTIC SENSTIVITY TESTING

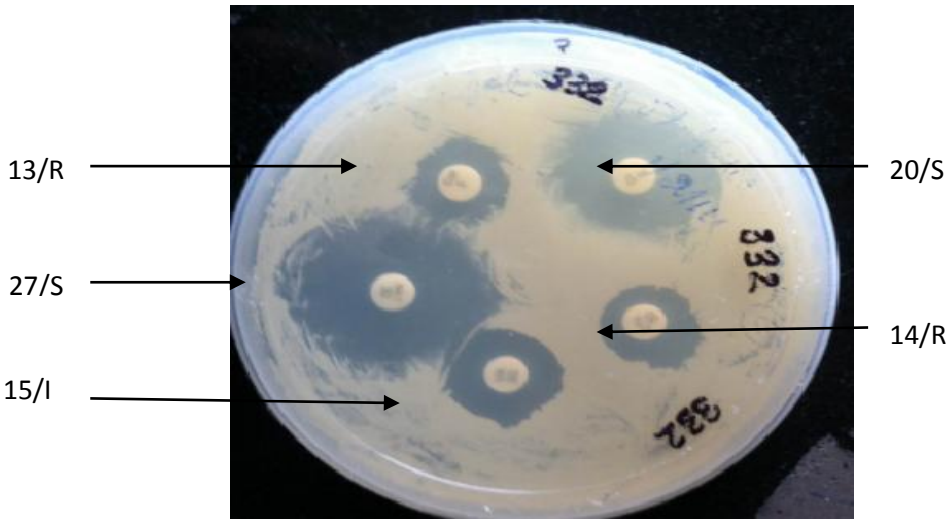


Fig 3.7. shows antibiotic sensitivity tests for E. coli isolates against antibiotics

S. No.	Sample code	Amikacin zone	Streptomycin zone	Gentamicin zone	Kanamycin zone
1.	49sL	17mm/S	No zone/R	10mm/R	No zone/R
2.	68s	18mm /S	No zone/R	8mm/R	15mm/I
3.	48s	20mm /S	8mm/R	8mm/R	No zone/R
4.	58s	15mm /S	10mm/R	9mm/R	No zone/R
5.	54	23mm /S	No zone/R	15mm/S	No zone/R
6	49sS	18mm /S	12mm/R	11mm/R	8mm/R
7	CRI KII	24mm /S	No zone/R	10mm/R	No zone/R
8	$75Le_L$	24mm /S	No zone/R	13mm/I	No zone/R
9	$75Le_S$	22mm/S	No zone/R	13mm/I	No zone/R
10	50_SSF	24mm/S	22mm/S	11mm/R	12mm/R
11	CRI KIII	26mm/S	No zone/R	10mm/R	No zone/R
12	Pus ND_2S	40mm/S	No zone/R	21mm/S	No zone/R
13	Pus ND_1L	28mm/S	No zone/R	11mm/R	No zone/R
14	$50SF_L$	25mm/S	22mm/S	12mm/R	No zone/R
15	U(3)	21mm/S	No zone/R	12mm/R	No zone/R

16	U(10)	23mm/S	No zone/R	12mm/R	No zone/R
17	U(24)	25mm/S	No zone/R	16mm/S	No zone/R
18	U(27)	27mm/S	No zone/R	17mm/S	No zone/R

Table 3.2 shows AST results obtained by measurement of zones of inhibition (mm) for antibiotics Amikacin, Streptomycin, Gentamicin, Kanamycin. R and S indicates Resistant or Susceptible respectively.

S.No	**Samples**	**AK 10**	**NX 10**	**CIP 5**	**GEN 10**	I	II	III	IV
19	3Flab(U)	14mm/R	10mm/R	12mm/R	14mm/I	32mm	30mm	34mm	18mm
20	1Lab(U)	24mm/S	9mm/R	8mm/R	8mm/R	30mm	24mm	20mm	10mm
21	4M	22mm/S	10mm/R	6mm/R	10mm/R	32mm	30mm	24mm	12mm
22	ACRI (U)	21mm/S	8mm/R	10mm/R	14mm/I	18mm	26mm	26mm	18mm
23	6 Fu	16mm/I	14mm/I	20mm/I	8mm/R	28mm	30mm	34mm	30mm
24	8Fu CRI	14mm/R	10mm/R	12mm/R	10mm/R	34mm	39mm	42mm	32mm
25	2F	26mm/S	16mm/I	16mm/I	9mm/R	16mm	32mm	24mm	18mm
26	8	22mm/S	8mm/R	14mm/R	10mm/R	18mm	26mm	26mm	12mm
27	NU(9)	12mm/R	10mm/R	12mm/R	10mm/R	18mm	26mm	28mm	8mm
28	11	16mm/I	20mm/S	26mm/S	14mm/I	24mm	22mm	22mm	20mm
29	5M	24mm/S	10mm/R	14mm/R	12mm/R	20mm	36mm	18mm	20mm
30	F(v) CRI	14mm/R	20mm/S	24mm/S	12mm/R	34mm	36mm	34mm	32mm
31	64	15mm/I	22mm/S	22mm/S	0mm/R	12mm	11mm	13mm	16mm
32	57	20mm/S	0mm/R	26mm/S	11mm/R	26mm	22mm	20mm	27mm
33	69	25mm/S	31mm/S	29mm/S	19mm/S	23mm	22mm	24mm	0mm

34	65	25mm/S	28mm/S	26mm/S	18mm/S	0mm	0mm	0mm	0mm
35	105	22mm/S	33mm/S	14mm/R	12mm/R	27mm	29mm	28mm	22mm
36	61	0mm/R	0mm/R	0mm/R	0mm/R	14mm	24mm	25mm	17mm

Table 3.3 shows AST results obtained by measurement of zones of inhibition (mm) for antibiotics Amikacin, Norfloxacin, Ciprofloxacin, Gentamicin. Here, I signifies combination of cefepime hydrochloride and amikacin sulphate referred to as Potentox, II - Supime comprising Cefepime, Sulbactam, III - Elores comprising Ceftriaxone and sulbactam, IV - Vancoplus comprising Ceftriaxone sodium and Vancomycin hydrochloride (Venus Remedies Limited, Baddi, India). R and S indicates Resistant or Susceptible respectively.

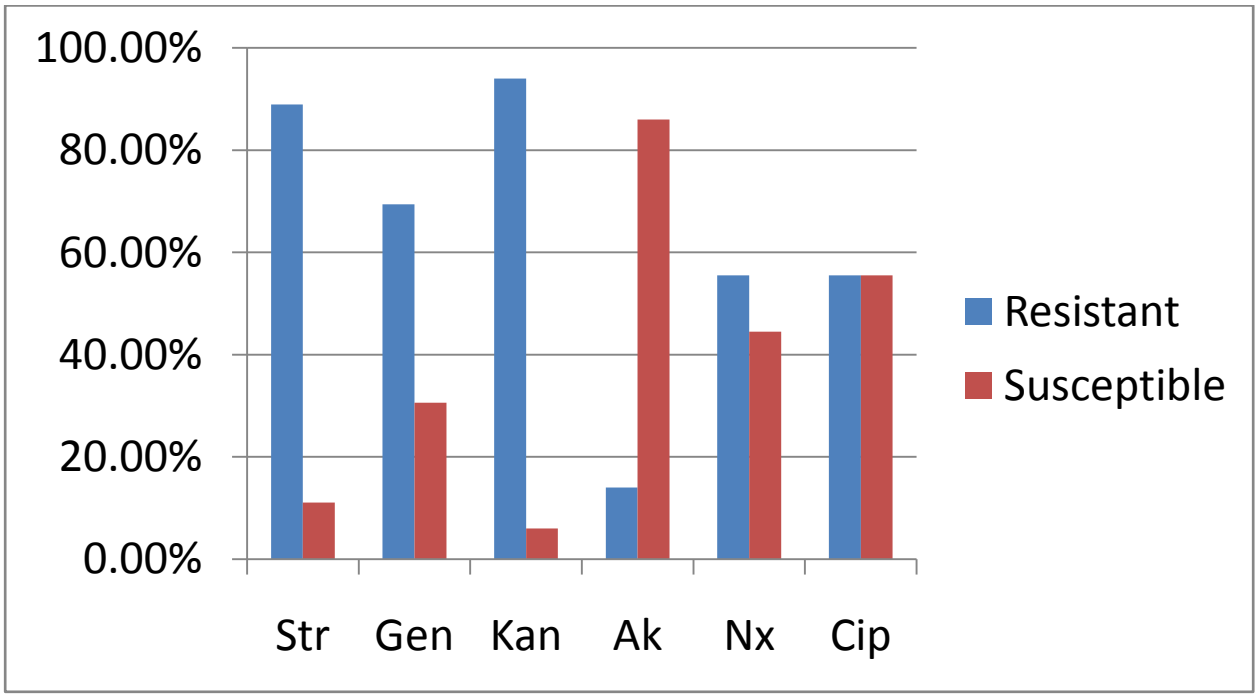


Fig 3.8. shows higher antibiotic resistance against various antibiotics (Streptomycin, Gentamicin, Kanamycin, Amikacin, Norfloxacin, Ciprofloxacin.

S.No	Code	CIP50	S 10	VA 30	Net 30	CTX30	CAZ30	TOB 10
37	330(II)	22/S	18/S	-/R	12/R	20/S	21/S	17//S
38	350 (II)	22/S	20/S	-/R	19/S	18/R	21/S	17/S
39	351 (III)	27/S	20/S	-/R	23/S	18/R	14/R	18/S
40	351 (III)	7/R	16/S	-/R	19/S	7/R	-/R	17/S
41	365 (II)	22/S	13/I	-/R	11/R	22/R	13/R	8/R
42	350 (I)	14/R	9/R	-/R	11/R	13/R	22/S	29/S
43	365 (I)	-/R	19/S	-/R	12/R	-/R	-/R	15/I
44	332	13/R	20/S	15/I	27/S	14/R	11/R	7/R
45	285 (I)	20/R	14/I	20/S	27/S	29/S	13/R	21/S
46	330 (I)	21/I	16/S	-/R	16/S	17/R	12/R	21/S
47	351 (I)	9/S	16/S	-/R	10/R	19/R	22/S	28/S
48	350 (III)	-/R	14/I	-/R	15/S	-/R	-/R	16/S
49	317 (I)	14/R	16/S	7/R	16/S	20/R	23/S	18/S
50	317 (II)	13/R	18/S	18/S	17/S	18/R	24/S	20/S
51	316	22/S	22/S	-/R	10/R	12/R	15/R	23/S
52	358 (I)	17/I	13/I	-/R	9/R	29/S	24/S	9/R
53	358 (II)	10/R	10/R	15/I	11/R	24/I	25/S	18/R
54	383 (II)	9/R	9/R	-/R	22/S	26/S	14/R	14/I
55	345 (I)	8/R	17/S	-/R	23/S	16/R	19/I	22/S
56	278 (I)	21/S	18/S	-/R	10/R	12/R	12/R	23/S
57	333 (II)	19/I	16/S	5/R	9/R	22/R	12/R	25/S
58	383 (I)	24/S	14/I	14/R	10/R	12/R	13/R	20/S
59	345 (II)	6/R	10/R	12/R	11/R	26/S	10/R	19/S
60	333 (III)	14/R	16/S	-/R	13/I	21/R	22/S	20/S

61	333 (I)	13/R	20/S	7/R	24/S	20/R	23/S	16/S
62	278 (II)	13/R	21/S	-/R	15/S	18/R	17/R	7/R
63	357 (I)	18/I	16/S	10/R	12/R	19/R	23/S	5/R
64	378 (I)	17/I	14/I	9/R	11/R	20/R	26/S	17/S

Table 3.4. shows AST results obtained by measurement of zones of inhibition (mm) for antibiotics Ciprofloxacin, Streptomycin, vancomycin, Netillin, Cefotaxime, Ceftazidime, Tobramycin. R and S indicates Resistant or Susceptible.

S.No	**Code**	**AK 10**	**LE 5**	**NX 10**	**CAT (30/10)**	**NA 30**	**CPM 30**	**CTR 30**	**OF 5**
37	330(II)	19/S	22/S	20/S	9	11/R	-/R	12/R	29/S
38	350 (II)	16/I	14/I	24/S	21	14/I	26/S	28/S	26/S
39	351 (III)	18/S	15/I	17/S	17	12/R	23/S	25/S	23/S
40	351 (III)	18/S	6/R	-/R	11	-/R	12/R	-/R	5/R
41	365 (II)	20/S	25/S	22/S	16	16/I	22/S	25/S	25/S
42	350 (I)	26/S	28/S	28/S	-	-/R	-/R	-/R	19/S
43	365 (I)	15/I	10/R	-/R	11	-/R	16/I	8/R	-/R
44	332	15/I	16/I	7/R	10	-/R	19/S	17/R	15/I
45	285 (I)	21/S	30/S	27/S	-	9/R	-/R	-/R	12/R
46	330 (I)	16/I	16/I	14/I	20	10/R	21/S	24/S	24/S
47	351 (I)	26/S	26/S	28/S	19	10/R	22/S	22/S	13/I
48	350 (III)	15/I	9/R	-/R	12	-/R	14/R	-/R	6/R
49	317 (I)	22/S	18/S	20/S	20	12/R	20/S	24/S	28/S
50	317 (II)	23/S	15/I	11/R	16	-/R	18/S	18/R	26/S
51	316	28/S	21/S	18/S	18	10/R	22/S	22/I	22/S
52	358 (I)	19/S	19/S	19/S	9	8/R	10/R	26/S	6/R
53	358 (II)	16/I	15/I	25/S	18	15/I	12/R	28/S	24/S
54	383 (II)	17/S	10/R	10/R	10	8/R	18/S	8/R	20/S

55	345 (I)	20/S	22/S	14/I	18	6/R	20/I	17/R	-/R
56	278 (I)	22/S	7/R	8/R	-	17/I	12/R	-/R	15/I
57	333 (II)	22/S	28/S	18/S	10	6/R	10/R	16/R	12/R
58	383 (I)	25/S	9/R	24/S	22	10/R	18/S	14/R	26/S
59	345 (II)	26/S	20/S	28/S	18	12/R	22/S	24/S	13/I
60	333 (III)	16/I	15/I	10/R	16	12/R	21/S	26/S	6/R
61	333 (I)	15/I	8/R	8/R	24	8/R	12/R	21/I	10/R
62	278 (II)	21/S	7/R	16/I	20	-/R	10/R	24/S	12/R
63	357 (I)	22/S	23/S	19/S	16	-/R	8/R	22/S	16/S
64	378 (I)	20/S	24/S	20/S	14	6/R	8/R	20/I	22/S

Table 3.5. shows AST results obtained by measurement of zones of inhibition (mm) for antibiotics Amikacin, Levofloxacin, Norfloxacin, Nalidixicacid, Cefepime, Ceftriaxone, Ofloxacin. R and S indicates Resistant or Susceptible.

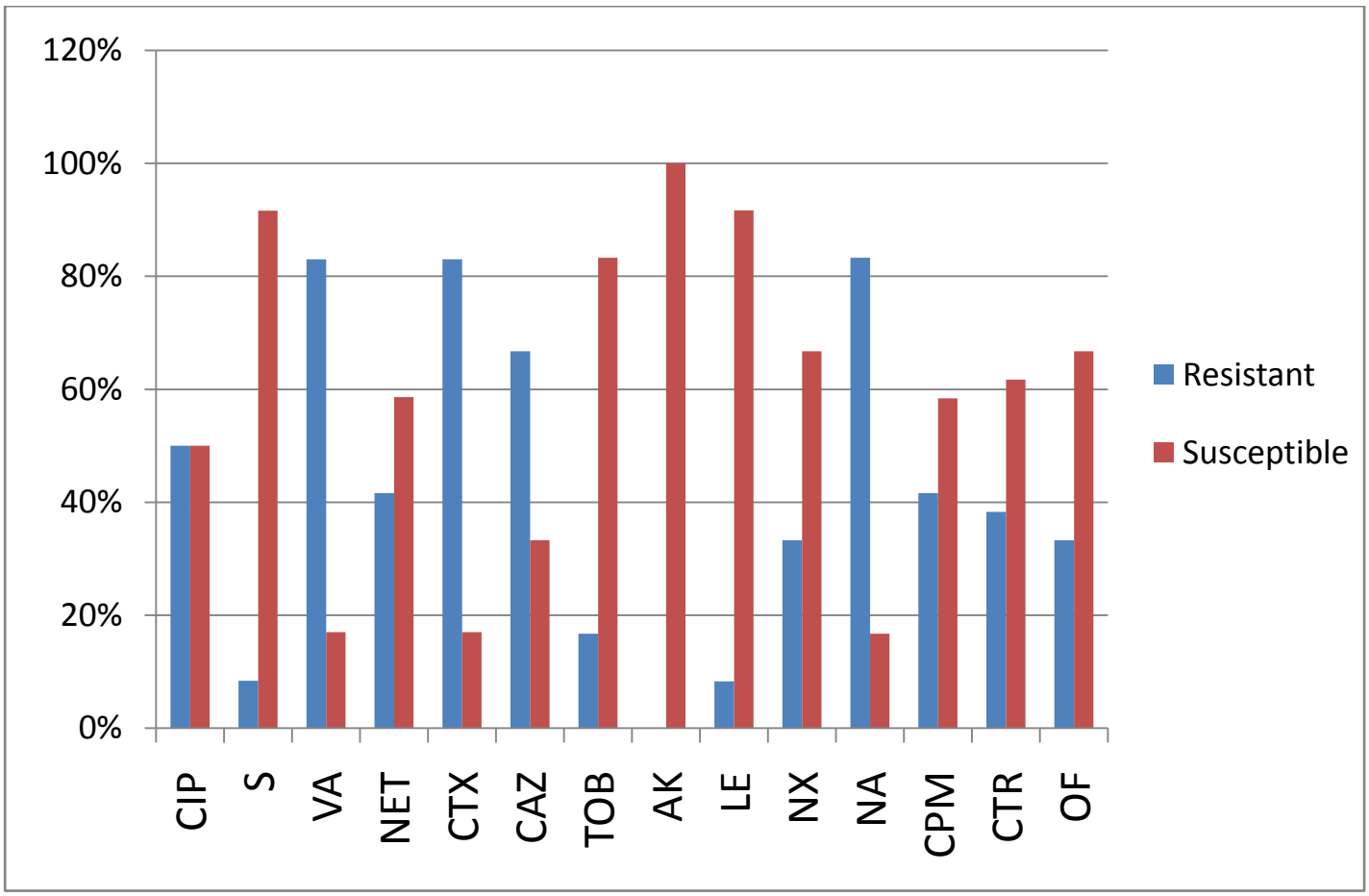


Figure 3.9. shows higher antibiotic resistance against various antibiotics Ciprofloxacin, Streptomycin, vancomycin, Netillin, Cefotaxime, Ceftazidime, Tobramycin, Amikacin, Levofloxacin, Norfloxacin, Nalidixic acid, Cefepime, Ceftriaxone, Ofloxacin in the order.

3.4 DNA ISOLATION

Nanodrop readings

Sample code	DNA conc. (ng/µl)	OD 260/280 ratio	OD 260/230 ratio
49sL	737	2.16	2.05
68s	963	1.07	1.68
48s	386	2.17	2.15
58s	642	1.65	1.12
54	878	2.03	1.36
49sS	348	1.71	1.76
CRI KII	765	1.06	1.82
$75Le_L$	662	2.08	1.04
$75Le_S$	598	1.69	1.68
50_SSF	959	1.54	1.12
CRI KIII	288	1.69	1.39
Pus ND_2S	692	1.93	1.78
Pus ND_1L	788	2.13	1.86
$50SF_L$	396	1.97	1.47
U(3)	248	2.24	2.39
U(10)	195	1.53	2.97
U(24)	457	1.41	2.55
U(27)	168	1.0	1.626
3Flab(U)	783	1.03	1.02
1Lab(U)	289	0.982	1.8
4M	322	0.893	2.222
ACRI (U)	416	0.25	2.364
6 Fu	697	0.848	2.667
8Fu CRI	180	1.03	2.63
2F	63	1.06	2.102
8	563	0.937	2.542
NU(9)	263	1.13	2.121

11	542	1.17	1.704
5M	42	2.23	1.947
F(v) CRI	192	1.87	1.749
64	367	1.44	2.04
57	544	0.947	2.26
69	278	0.893	1.825
65	424	1.87	1.45
105	259	1.16	2.20

Table 3.6. indicates DNA concentration (ng/µl) and DNA quality using the NanoDrop. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA . A ratio of ~1.8 is generally accepted as "pure" for DNA. 260/230 ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2.

CONCLUSION

After 50 years of use, aminoglycosides continue to provide a mainstay in the therapy of serious Gram- negative infections. Recently, new therapeutic approaches have emerged that exploit aminoglycoside-induced read- through of premature stop codons in the treatment of certain human genetic disorders. Thus, aminoglycosides, while being "old" drugs, will continue to have impact on modern medicine as powerful antibiotics, experimental therapeutics and invaluable tool compounds for drug discovery.

In this study, a total of 64 samples were bacterial culture positive and thus were processed further. From the isolated samples, majority of the pathogen found was E. coli (67%) followed by *Shigella spp*.(14%), *Vibrio spp*.(8%), *Salmonella spp*.(6%), *Klebsiella spp*. (5%) through biochemical characterization. Among the tested drugs, traditionally used antibiotics showed the maximum resistance and the newly introduced ones showed excellent in vitro antibacterial activity. Amikacin which is a newly introduced aminoglycoside was shown to exhibit maximum antibacterial activity upto 86 % of all the isolates, whereas the tradionally used aminoglycoside such as Streptomycin, Kanamycin and Gentamicin were shown to exhibit quite a high level of resistance (89%, 94%, 70% respectively). Among other tested antibiotics, many were shown to exhibit high level antimicrobial resistance but the ones which were showing low level of resistance were Tobramycin (17%) which is an aminoglycoside, Levofloxacin (8%) which is a broad-spectrum antibiotic of the fluoroquinolone drug class. We suggest that that Amikacin which has been introduced recently into clinical settings would allow clinicians to overcome the aminoglycoside resistance acquired by some bacterial strains.

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APPENDIX

- **Nutrient Broth Medium (HiMedia Laboratories)**
  Composition :

Ingredients	**gms/Litre**
Peptone	10.000
Beef extract	10.000
Sodium chloride	5.000
pH after sterilization	7.3±0.1

- **Nutrient Agar (HiMedia Laboratories)**
  Composition :

Ingredients	**gms/Litre**
Peptic digest of animal tissue	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

- **MacConkey Agar(HiMedia Laboratories)**
  Composition :

Ingredients	**gms/Litre**
Peptones (meat and casein)	3.000
Pancreatic digest of gelatin	17.000
Lactose monohydrate	10.000
Bile salts	1.500
Sodium chloride	5.000
Crystal violet	0.001
Neutral red	0.030
Agar	13.500
pH after sterilization (at 25°C)	7.1±0.2

- **Xylose–Lysine–Deoxycholate Agar (HiMedia Laboratories)**
  Composition :

Ingredients	**gms/Litre**
Xylose	3.500
L-Lysine	5.000
Lactose monohydrate	7.500

Sucrose	7.500
Sodium chloride	5.000
Yeast extract	3.000
Phenol red	0.080
Sodium deoxycholate	2.500
Sodium thiosulphate	6.800
Ferric ammonium citrate	0.800
Agar	13.500
pHafter heating (at 25°C)	7.4±0.2

- **TCBS Agar (HiMedia Laboratories)**

Composition :

Ingredients	**gms/Litre**
Proteose peptone	10.000
Yeast extract	5.000
Sodium thiosulphate	10.000
Sodium citrate	10.000
Oxgall	8.000
Sucrose	20.000
Sodium chloride	10.000
Ferric citrate	1.000
Bromothymol blue	0.040
Thymol blue	0.040
Agar	15.000
Final pH (at 25°C)	8.6±0.2