EXPLORING ANTIBIOTIC RESISTANT SOIL MICROBES FOR THEIR RESISTANCE MECHANISMS STUDY

A Research Project

Submitted by

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VIVEKANAND COLLEGE, KOLHAPUR

(AN EMPOWERED AUTONOMOUS INSTITUTE)

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This is to certify that Mr. **RAHUL G. MALAVI** studying in M. Sc. part II, Sem-IV at Vivekanand College, Kolhapur (An Empowered Autonomous Institute) has sincerely completed research project work entitled **"EXPLORING ANTIBIOTIC RESISTANT SOIL MICROBES FOR THEIR RESISTANCE MECHANISMS STUDY"** during academic year 2025-26.

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INTRODUCTION

INTRODUCTION

Microorganisms are extremely small living beings that cannot be seen with the naked eye. They include bacteria, fungi, viruses, protozoa, and algae (Hogan, 2010), are found everywhere - in soil, water, air, on plants, and even inside living organisms. According to (Madigan et al. 2023), microorganisms are the foundation of every ecosystem on Earth. They help in recycling nutrients, decomposing organic matter, maintaining soil fertility, and even in producing valuable bioactive compounds (Falkowski et al., 2008).

Microorganisms are classified as beneficial and harmful. Beneficial microorganisms play essential roles such as curd formation (*Lactobacillus*) (Nagaoka, 2018), nitrogen fixation (*Rhizobium*) (Wang et al., 2018; Zahran, 1999), and waste decomposition (Gupta et al., 2016). They are also used in the biotechnology industry for producing vitamins, enzymes, amino acids, and antibiotics (Bharathidasan University, n.d.). For example, *Saccharomyces cerevisiae* (yeast) is used in alcohol and bread production, while *Penicillium chrysogenum* is used to produce penicillin.

On the other hand, harmful microorganisms cause diseases in humans, animals, and plants. *Vibrio cholerae* causes cholera, *Mycobacterium tuberculosis* causes tuberculosis, and *Plasmodium* (a protozoan parasite) causes malaria. Despite these harmful effects, microbes are indispensable to life - without them, nutrient cycling and ecological balance would collapse (Tiwari & Gupta, 2021). Microorganisms are diverse and can be divided into several major groups (Tortora et al., 2022):

1. Bacteria:

   These are single-celled organisms that can survive in almost all environments from glaciers to hot springs. Some are beneficial (*Bacillus subtilis* produces enzymes), while others cause diseases (*E. coli, Staphylococcus aureus*).

2. Fungi:

   These can be unicellular (yeasts) or multicellular (molds). Many fungi are used in fermentation and drug production (*Penicillium, Aspergillus*), while others cause plant diseases.

3. Viruses:

These are acellular (non-living outside a host) particles that infect animals, plants, and even bacteria. Recent pandemics like COVID-19 show how powerful viruses can be.

4. Protozoa:

These are single-celled eukaryotic organisms found mostly in water or soil. Some are harmless, but others like Plasmodium (malaria parasite) are deadly.

5. Algae:

These are photosynthetic organisms found mainly in aquatic environments; they produce oxygen and form the base of many food chains. Microbes exist in every ecological niche - from the deep sea to mountain soils - adapting to different temperatures, salinities, and nutrient conditions. This adaptability makes them powerful producers of unique secondary metabolites, including antibiotics.

Some microorganisms, known as pathogens, are capable of causing diseases. The infection process follows a definite pattern (Tortora et al., 2022):

1. Entry of the microorganism - through air, water, food, skin wounds, or insect bites.
2. Multiplication once inside, the pathogen multiplies in tissues or cells.
3. Toxin Production / Damage - the pathogen releases toxins or enzymes that harm the host.
4. Symptoms Appear - fever, cough, rashes, or weakness occur as a result of immune response.
5. Transmission - pathogens spread from one host to another via contact, droplets, vectors, or contaminated food.

For example, *Vibrio cholerae* spreads through contaminated water, while Plasmodium spreads through mosquito bites.

However, not all microbes are harmful; many non-pathogenic species actually protect humans by competing with pathogens or boosting immunity (Tiwari & Gupta, 2021). In soil ecosystems, microbial interactions are complex. Competition among soil bacteria often leads to production of antibiotic substances that suppress competitors - this natural microbial warfare is what inspired the discovery of antibiotics.

1. Antibiotics

Antibiotics are chemical compounds produced by microorganisms that inhibit the growth of or kill other microbes. The first antibiotic, Penicillin, was discovered accidentally by Alexander Fleming (1928) from the fungus *Penicillium notatum*. Later, Florey and Chain (1940) purified and mass-produced it, marking the beginning of the "Antibiotic Era" (Ventola, 2020).

Antibiotics act through different mechanisms:

1. **Inhibition of cell-wall synthesis:**

These antibiotics prevent bacteria from forming strong peptidoglycan cell walls, causing the cells to burst.

Examples: Penicillin, Cephalosporins, Vancomycin.

2. **Inhibition of protein synthesis:**

They bind to bacterial ribosomes (30S or 504S) and block the production of essential proteins needed for survival.

Examples: Tetracycline, Erythromycin, Chloramphenicol, Aminoglycosides.

3. **Interference with DNA/RNA replication:**

These drugs stop the function of enzymes like DNA gyrase or RNA polymerase, preventing bacterial genetic replication.

Examples: Ciprofloxacin (fluoroquinolone), Rifampicin.

4. **Disruption of cell membranes:**

They damage the bacterial cell membrane integrity, causing leakage of cell contents and death.

Examples: Polymyxin-B, Colistin.

Antibiotics have saved millions of lives by controlling infectious diseases and have also been used in veterinary medicine and agriculture. However, their misuse has created serious global health problems.

According to Berdy (2022), antibiotics can be classified based on spectrum and source:

1. Broad-Spectrum Antibiotics:

   These antibiotics are effective against many bacterial types.

   Examples: Tetracycline, Ampicillin.

2. Narrow-Spectrum Antibiotics:

   These antibiotics are target specific bacteria.

   Examples: Penicillin mainly acts on Gram-positive bacteria.

3. Natural Antibiotics:

   These Antibiotics are produced directly by microorganisms.

   Examples: *Streptomyces, Bacillus.*

4. Semi-synthetic and Synthetic Antibiotics:

   These antibiotics are chemically modified versions designed to improve activity or reduce resistance.

   Examples: Amoxicillin, Ampicillin, Erythromycin.

Antibiotics not only serve as medicines but also as molecular tools in genetics and biotechnology

2. Antibiotic Resistance

Overuse and misuse of antibiotics have resulted in the development of antibiotic resistance - the ability of bacteria to survive even in the presence of drugs meant to kill them.

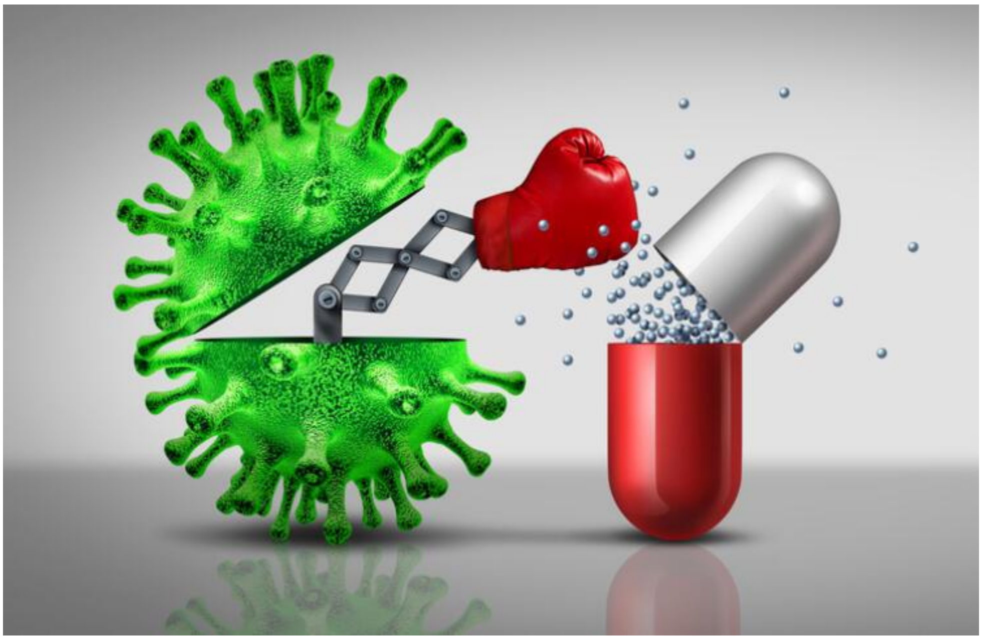


Fig.1.AntibioticResistance

According to the (World Health Organization 2024), antibiotic resistance is now one of the top global health threats. (Laxminarayan et al., 2022) reported that in India, antibiotic resistance is rising rapidly due to unregulated antibiotic use in humans, poultry, and agriculture. Pathogens such as *Staphylococcus aureus* (MRSA) and *Mycobacterium tuberculosis* (MDR-TB) are examples of resistant organisms.

Resistance mechanisms include:

1. **Alteration of drug targets:**

In this mechanism bacteria change the binding site of the antibiotic, so the drug can no longer attach and act effectively.

Examples: MRSA modifies penicillin-binding proteins, reducing penicillin action.

2. **Enzyme production:**

In this mechanism some bacteria produce enzymes that break down or inactive antibiotics before they can work.

Examples: β-lactamase enzyme destroys penicillin and related β-lactam antibiotics.

3. **Efflux pumps (Drug Removal):**

In this mechanism bacteria actively pump antibiotics out of their cells using efflux transport proteins.

Example: Tetracycline resistance due to efflux pump genes (e.g., tetA).

4. **Biofilm formation:**

In this mechanism bacteria form protective biofilms on surfaces which block antibiotics from reaching the cells.

Examples: *Pseudomonas aeruginosa* biofilms in chronic wound or lung infections.

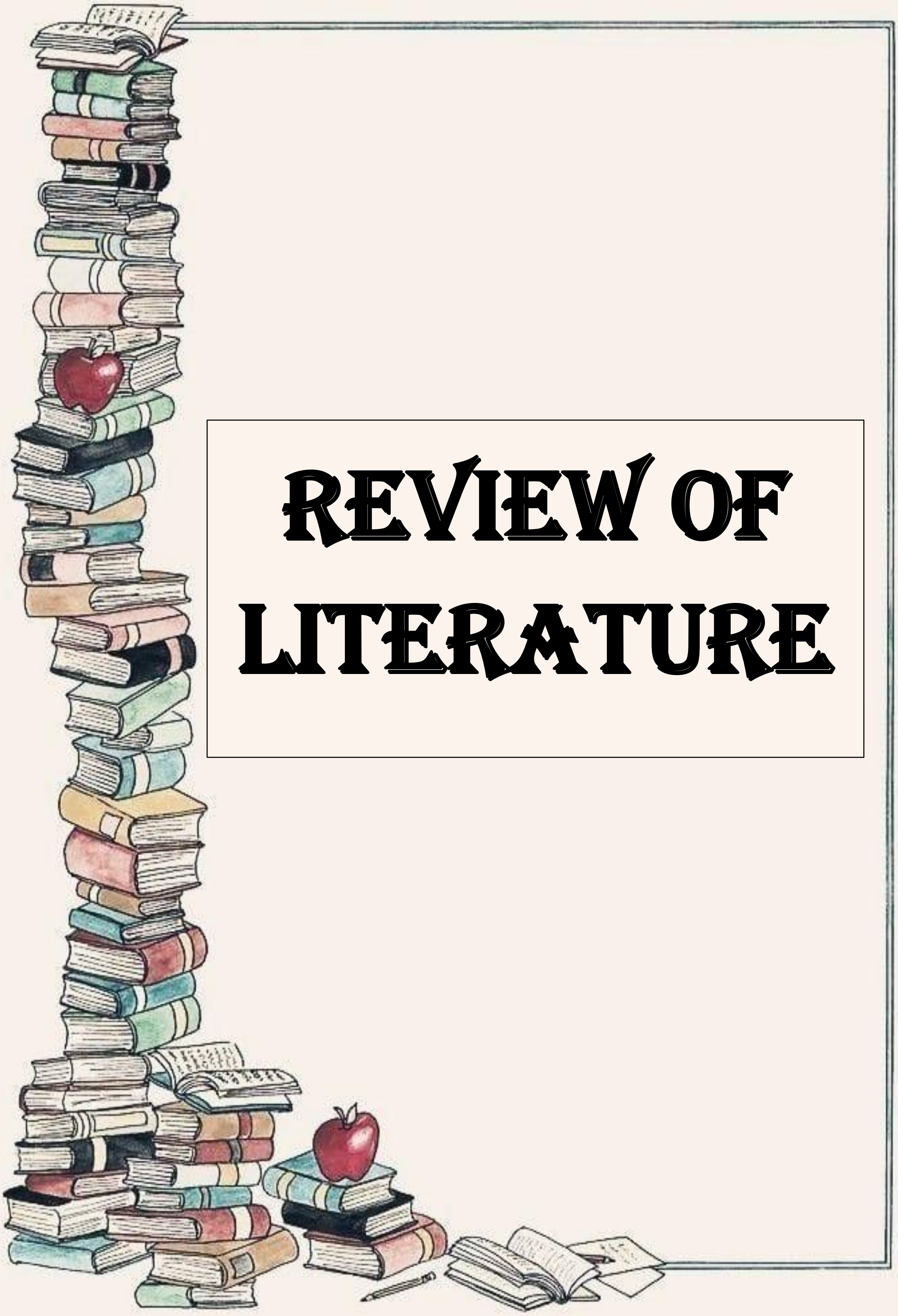
If resistance continues to spread, many modern medical treatments could become ineffective. Hence, scientists are focusing on new antibiotics from natural and unexplored microbial sources (Ventola, 2020; WHO, 2024).

Need for New Antibiotics

Because of the increasing prevalence of antibiotic resistance, there is an urgent need to discover new antimicrobial compounds. The rapid rise of antibiotic-resistant bacteria, particularly in environments exposed to continuous antibiotic use such as hospital surroundings, has made many existing drugs less effective. Hospital-associated environments, including soil, act as important reservoirs of resistant microorganisms due to constant exposure to antibiotics, disinfectants, and biomedical waste.

In the present study, the isolation of bacteria resistant to penicillin, streptomycin, and tetracycline from hospital soil clearly demonstrates the widespread occurrence of resistance in environmental sources. These resistant bacteria possess various mechanisms that allow them to survive in the presence of antibiotics, contributing to the persistence and spread of resistance.

Therefore, the discovery of new antibiotics and the monitoring of environmental sources of resistance are essential to control the spread of resistant infections. Studies like the present work highlight the importance of hospital soil as a potential source of antibiotic-resistant bacteria and emphasize the need for proper antibiotic management and environmental control strategies to safeguard public health.



REVIEW OF LITERATURE

Review of literature

1. Historical Overview of Antibiotic Discovery

Antibiotics are drugs that treat bacterial infections by killing or stopping the growth of bacteria. The history of antibiotics began in the early 20th century with the discovery of penicillin by Alexander Fleming in 1928, which revolutionized modern medicine (Fleming, 1929). However, it was not until the 1940s that penicillin was mass-produced, marking the beginning of the “Golden Age of Antibiotics.” Between 1940 and 1970, numerous antibiotics were discovered from soil-derived microorganisms, particularly from the Actinomycetes group, such as *Streptomyces* species (Waksman & Woodruff, 1942). These discoveries dramatically reduced mortality rates from infectious diseases and laid the foundation for microbial drug discovery.

The soil has always been a crucial source of natural antibiotics due to its diverse microbial ecology. During the mid-20th century, the majority of clinically important antibiotics, including streptomycin, tetracycline, erythromycin, and chloramphenicol, were isolated from soil bacteria and fungi (Berdy, 2012). However, after the 1980s, the rate of new antibiotic discovery sharply declined due to the overexploitation of cultivable microbes and redundancy in isolated compounds (Baltz, 2017). This decline, combined with the rise of antimicrobial resistance (AMR), has shifted research toward exploring uncultured soil microorganisms using molecular and metagenomic approaches.

1.1 Soil Microbial Diversity

Soil represents one of the most complex microbial ecosystems on Earth, harbouring billions of microorganisms per gram, including bacteria, fungi, archaea, and protozoa. These microorganisms play vital roles in biogeochemical cycles, plant growth, and natural product biosynthesis (Torsvik & Ovreas, 2002). Soil microbial diversity is driven by environmental factors such as pH, organic content, moisture, and temperature, which influence microbial population dynamics.

Among soil microbes, Actinomycetes are of particular importance for antibiotic production. The genus *Streptomyces* alone accounts for more than two-thirds of naturally derived antibiotics known today (Barka et al., 2016). Fungal genera such as *Penicillium, Aspergillus*, and *Trichoderma* also produce a wide range of antimicrobial metabolites. Moreover, advances in molecular biology have uncovered that many soil bacteria possess silent or cryptic biosynthetic gene clusters (BGCs) that encode unexpressed antibiotic pathways, offering untapped potential for novel compound discovery (Medema & Fischbach, 2015).

2. Antibiotic-Producing Soil Microbes

Soil is considered one of the richest natural habitats for microbial diversity and is a well-known source of secondary metabolites, including antibiotics, enzymes, and bioactive compounds (Vartoukian et al., 2010). Among soil-inhabiting microbes, members of the phylum *Actinobacteria*, particularly *Streptomyces* species, are the dominant producers of clinically useful antibiotics. These organisms account for nearly 70 % of all antibiotics discovered to date (Barka et al., 2016). Other bacterial taxa-such as *Bacillus, Pseudomonas*, and *Burkholderia* and fungal genera like *Penicillium* and *Aspergillus* also contribute significantly to antibiotic biosynthesis. For instance, *Bacillus subtilis* produces bacitracin and *surfactin*, while *Pseudomonas fluorescens* synthesizes pyocyanin and phenazine derivatives (Newman & Cragg, 2020).

The vast metabolic potential of soil microbes arises from their adaptation to competitive soil environments, where the synthesis of antimicrobial molecules serves as a defence mechanism and a tool for ecological dominance (van Wezel & McDowall, 2011).

2.1 Bacterial antibiotic producers

Most natural antibiotics are produced by Actinomycetes, particularly the genus *Streptomyces*, which inhabit soil and marine environments. Other bacterial genera like *Bacillus*, *Micromonospora* and *Pseudomonas* also contribute significantly. (Table.1)

Table.1 Bacterial antibiotic producers

Sr. No.	Bacterium	Antibiotic produced	Mode of Action
1.	*Streptomyces griseus*	Streptomycin	Binds to the 30S ribosomal subunit, causing misreading of mRNA and inhibition of protein synthesis.
2.	*Streptomyces aureofaciens*	Chlortetracycline	Inhibits protein synthesis by preventing attachment of aminoacyl-tRNA to the 30S ribosomal subunit (broad-spectrum antibiotic).
3.	*Streptomyces venezuelae*	Chloramphenicol	Inhibits protein synthesis by binding to the 50S ribosomal subunit and blocking peptidyl transferase activity.
4.	*Streptomyces nodosus*	Amphotericin B	Binds to ergosterol in fungal membranes, causing cell leakage (antifungal)
5.	*Streptomyces orientalis*	Vancomycin	Inhibits cell wall synthesis in Gram-positive bacteria
6.	*Streptomyces erythraeus*	Erythromycin	Inhibits protein synthesis by binding to the 50S ribosomal subunit

7.	*Streptomyces fradiae*	Neomycin	Inhibits protein synthesis by binding to the 30S ribosomal subunit
8.	*Streptomyces noursei*	Nystatin	Binds to sterols in fungal cell membranes, causing leakage (antifungal)
9.	*Streptomyces clavuligerus*	Clavulanic acid	Inhibits β-lactamase enzymes, enhancing penicillin activity
10.	*Micromonospora purpurea*	Gentamicin	Inhibits protein synthesis by binding to the 30S ribosomal subunit
11.	*Nocardia lactamdurans*	Cephamycin	Inhibits bacterial cell wall synthesis (β-lactam antibiotic)
12.	*Bacillus subtilis*	Bacitracin	Inhibits bacterial cell wall synthesis by preventing dephosphorylation of bactoprenol, a carrier molecule for peptidoglycan precursors.
13.	*Bacillus polymyxa*	Polymyxin	Disrupts the phospholipid structure of bacterial cell membranes, increasing permeability and causing cell death (mainly active against Gram-negative bacteria).

3. Types of Antibiotics

Antibiotics are natural or synthetic compounds that inhibit or kill microorganisms by interfering with vital cellular processes. They are classified based on chemical structure, mode of action, or spectrum of activity (Kohanski et al., 2010).

3.1 Types of antibiotics on the basis of mode of action:

1. β-lactams:

   These antibiotics inhibit bacterial cell wall synthesis.

   Examples: Penicillin, cephalosporins, carbapenems.
2. Aminoglycosides:

   These antibiotic bind to the 30S ribosomal subunit, causing mistranslation.

   Examples: Streptomycin, gentamicin
3. Macrolides:

   These antibiotic bind to the 50S ribosomal subunit to inhibit peptide chain elongation.

   Examples: Erythromycin, Azithromycin.
4. Tetracyclines:

   These antibiotic block attachment of aminoacyl-tRNA to the ribosome.

   Examples: Doxycycline, Minocycline, Tetracycline, Tigecycline.
5. Glycopeptides:

   These antibiotics prevent peptidoglycan synthesis in Gram-positive bacteria.

   Example: Vancomycin.
6. Quinolones:

   These antibiotics inhibit DNA gyrase and topoisomerase IV, disrupting DNA replication.

   Example: Ciprofloxacin.
7. Sulfonamides:

   These antibiotic block folate synthesis by inhibiting dihydropteroate synthase.

   Examples: Sulfadiazine, Sulfisoxazole.

8. Polyketides and non-ribosomal peptides:

   These antibiotics include structurally diverse antibiotics like erythromycin and daptomycin, produced mainly by Actinomycetes (Hopwood, 2007)

   Examples: Erythromycin, Actinorhodin.

   Each class has distinct mechanisms targeting bacterial physiology, making them vital in combating infections of diverse origins.

3.2 Types of antibiotics based on spectrum of activity:

Antibiotics are also classified based on their spectrum:

1. Broad-spectrum antibiotics:

   These antibiotic act against a wide range of Gram-positive and Gram-negative bacteria. Broad-spectrum agents are useful in empirical therapy but may disrupt normal microbiota and promote resistance (Leekha et al., 2011).

   Examples: Tetracyclines, Chloramphenicol.

2. Narrow-spectrum antibiotics:

   These antibiotics are target specific bacterial groups either Gram positive or Gram negative.

   Examples: Vancomycin, penicillin G.

3.3 Mechanism of Action of Antibiotics

Antibiotics interfere with essential microbial pathways, primarily targeting,

- Cell Wall Synthesis Inhibition
- Protein Synthesis Inhibition
- Nucleic Acid Synthesis Inhibition
- Metabolic Pathway Inhibition
- Membrane Disruption

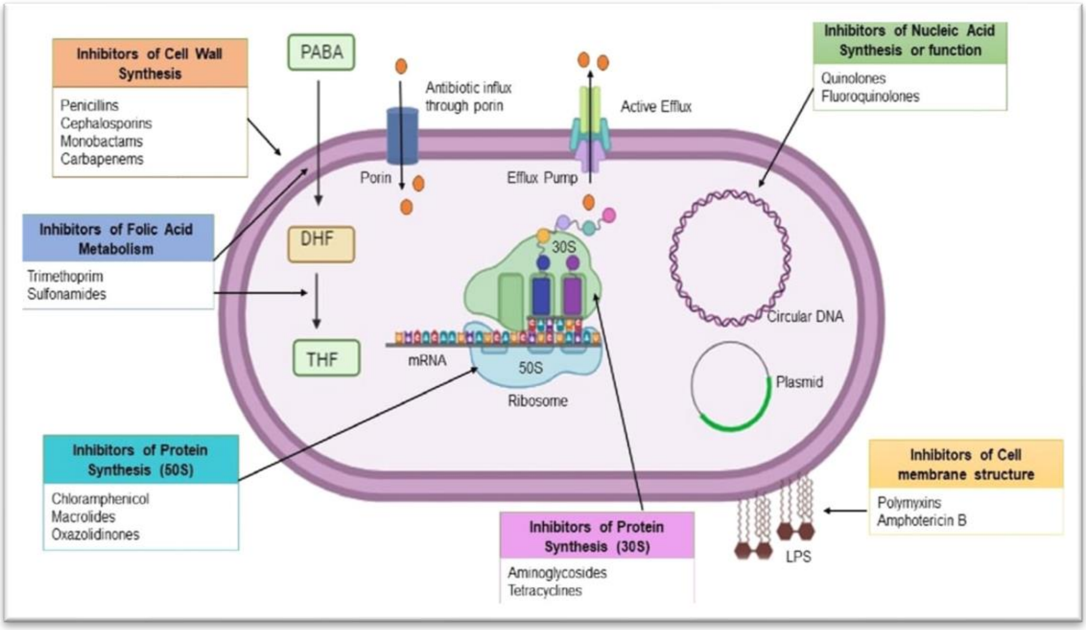


Fig.2. Mechanism of Action of Antibiotics

3.3.1 Cell Wall Synthesis Inhibition

The bacterial cell wall is a critical target since humans lack this structure. β-lactam antibiotics act by inhibiting penicillin-binding proteins (PBPs) that catalyze peptidoglycan cross-linking (Waxman & Strominger, 1983). Glycopeptides, like vancomycin, bind to D-Ala-D-Ala termini of peptidoglycan precursors, preventing polymerization (Reynolds, 1989). Disruption of cell wall synthesis leads to osmotic lysis and bacterial death.

Examples: Penicillin, Cephalosporins, Vancomycin.

Penicillin:

Type: β-lactam antibiotic

Source: *Penicillium notatum* (fungus)

Mechanism of Action:

1. Penicillin targets bacterial cell wall synthesis.
2. It inhibits the enzyme transpeptidase (penicillin-binding protein) responsible for cross-linking peptidoglycan chains in the cell wall.
3. Without cross-linking, the cell wall becomes weak and cannot maintain structural integrity.
4. The bacterial cell eventually bursts (lysis) due to osmotic pressure-hence it is bactericidal.
5. Effective Against: Mainly Gram-positive bacteria such as *Staphylococcus* and *Streptococcus* species.

3.3.2 Protein Synthesis Inhibition

Many antibiotics target bacterial ribosomes, exploiting structural differences between prokaryotic (70S) and eukaryotic (80S) ribosomes. Aminoglycosides cause misreading of mRNA, leading to faulty proteins, while macrolides and chloramphenicol inhibit peptide bond formation (Wilson, 2014). Tetracyclines prevent tRNA binding at the A-site of the ribosome, thereby halting translation.

Example: Streptomycin, Tetracycline, Erythromycin, Chloramphenicol, Aminoglycosides.

Streptomycin:

Type: Aminoglycoside antibiotic

Source: *Streptomyces griseus*

Mechanism of Action:

1. Streptomycin acts by inhibiting protein synthesis in bacteria.
2. It binds specifically to the 30S subunit of the bacterial ribosome.
3. This binding causes misreading of mRNA during translation, leading to the production of nonfunctional or toxic proteins.
4. It also prevents the initiation of protein synthesis in some cases.
5. The result is bactericidal action-the bacteria die because they can't make essential proteins.

6. Effective Against: Mainly Gram-negative bacteria and *Mycobacterium tuberculosis*.

3.3.3 Nucleic Acid Synthesis Inhibition

Fluoroquinolones target bacterial DNA gyrase and topoisomerase IV, enzymes essential for supercoiling and replication (Hooper & Jacoby, 2015). Rifamycins inhibit bacterial RNA polymerase by binding to its β-subunit, blocking transcription initiation (Campbell et al., 2001). These mechanisms make nucleic acid inhibitors highly effective against both Gram-positive and Gram-negative pathogens.

Examples: Ciprofloxacin (fluoroquinolone), Rifampicin.

3.3.4 Metabolic Pathway Inhibition

Sulfonamides and trimethoprim inhibit folic acid biosynthesis, which is necessary for nucleotide formation (Skold, 2000). Since humans acquire folate through diet, these drugs selectively target bacterial metabolism without affecting host cells.

Examples: Sulfonamides, trimethoprim, Isoniazid, Bedaquiline.

3.3.5 Membrane Disruption

Polypeptide antibiotics like polymyxins and daptomycin interact with bacterial membranes, altering permeability and causing leakage of cytoplasmic contents (Falagas & Kasiakou, 2005). Such antibiotics are often used as a last resort against multidrug-resistant Gram-negative bacteria.

Examples: Polymyxins, Daptomycin, Amphotericin B.

4. Antibiotic Resistance

Antibiotic resistance (AR) refers to the ability of microorganisms to survive or proliferate despite the presence of antimicrobial agents that were once effective against them. It has emerged as one of the most pressing global health challenges of the 21st century (Ventola, 2015). Resistance can be **intrinsic**, where microorganisms naturally possess insensitivity to certain antibiotics, or **acquired**, through genetic mutation or horizontal gene transfer (HGT) (Davies & Davies, 2010).

The World Health Organization (WHO) has declared antimicrobial resistance a top global public health threat due to increasing multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains (WHO, 2022). The misuse and overuse of antibiotics in healthcare, agriculture, and animal husbandry accelerate the emergence and spread of resistant strains (Laxminarayan et al., 2013).

4.1 Mechanisms of Antibiotic Resistance

Bacteria employ several biochemical and genetic mechanisms to evade the action of antibiotics. These mechanisms can be broadly classified into four categories:

1. Enzymatic degradation.

2. Alteration of antibiotic targets.

3. Reduced permeability or efflux.

4. Target protection or bypass mechanisms (Blair et al., 2015).

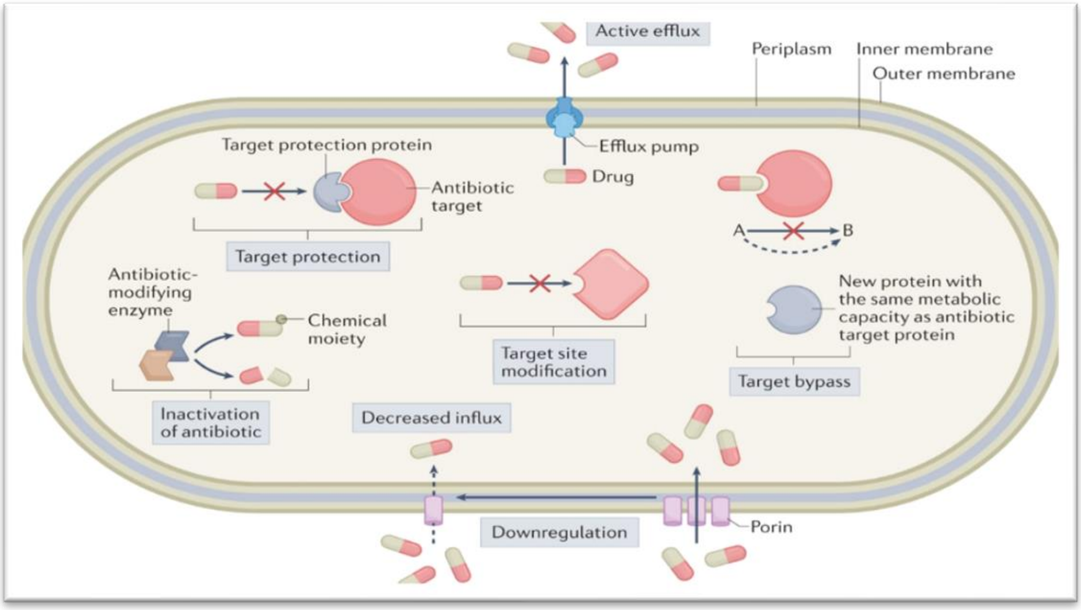


Fig. 3. Mechanisms of Antibiotic Resistance.

4.1.1 Enzymatic Degradation or Modification

One of the most prevalent mechanisms of resistance involves the enzymatic inactivation of antibiotics.

1. **β-lactamases**

   β-lactamase produced by Gram-negative bacteria, hydrolyze the β-lactam ring of penicillins and cephalosporins, rendering them inactive (Bush & Bradford, 2016).

2. **Aminoglycoside-modifying enzymes (AMEs)**

   Aminoglycoside-modifying enzymes (AMEs) such as acetyltransferases, phosphoryl transferases, and nucleotidyl transferases, alter aminoglycoside molecules, preventing them from binding to the ribosome (Ramirez & Tolmasky, 2010).

3. **Chloramphenicol acetyltransferase (CAT)**

   Chloramphenicol acetyltransferase (CAT) provides resistance by acetylating chloramphenicol, thus preventing its binding to ribosomal subunits (Schwarz et al., 2004).

4.1.2 Target Modification

Bacteria can develop mutations in antibiotic target sites, reducing drug binding affinity. For instance:

1. Mutations in the **DNA gyrase (gyrA/gyrB)** and **topoisomerase IV (parC/parE)** genes confer resistance to fluoroquinolones (Hooper & Jacoby, 2015).
2. **Ribosomal mutations** or methylation of rRNA by *erm* genes can result in macrolide resistance (Vester & Douthwaite, 2001).
3. Altered penicillin-binding proteins (PBPs), such as PBP2a in *Methicillin-resistant Staphylococcus aureus* (MRSA), provide resistance to β-lactams (Hackbarth & Chambers, 1993).

4.1.3 Efflux Pumps and Reduced Permeability

1. Efflux pumps actively expel antibiotics from bacterial cells, decreasing intracellular concentrations.
2. **Multidrug efflux pumps**, such as those belonging to the **RND (Resistance-Nodulation-Division)** family in Gram-negative bacteria, expel a broad range of drugs (Li & Nikaido, 2009).
3. Gram-negative bacteria also possess outer membrane porins that control drug influx; loss or alteration of these porins contributes to resistance by reducing permeability (Delcour, 2009).

4.1.4 Target Protection and Bypass Mechanisms

Some bacteria develop resistance by protecting their target sites or bypassing inhibited pathways.

For Examples:

1. **Tet(M)** and **Tet(O)** proteins protect ribosomes from tetracycline binding (Connell et al., 2003).
2. In *Enterococcus faecalis*, **VanA** and **VanB** gene clusters modify peptidoglycan precursors, preventing vancomycin binding (Courvalin, 2006).

3. The production of **alternative enzymes**, such as dihydrofolate reductase variants, confers resistance to trimethoprim (Huovinen et al., 1995).

4.2 Horizontal Gene Transfer and Resistance Gene Dissemination

Horizontal gene transfer (HGT) is a key driver in the dissemination of resistance genes among microbial populations. The primary modes include **transformation, transduction, and conjugation** (von Wintersdorff et al., 2016).

- **Transformation** involves uptake of naked DNA fragments from the environment.
- **Transduction** occurs through bacteriophages transferring genetic material between bacteria.
- **Conjugation** is mediated by plasmids and transposons that facilitate direct DNA transfer between cells.

Soil environments act as natural reservoirs of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs), collectively referred to as the **"resistome"** (Wright, 2010). Molecular techniques such as **qPCR, metagenomics,** and **shotgun sequencing** have been instrumental in identifying and quantifying these resistance genes in soil microbiota (Forsberg et al., 2014).

4.3 Global Impact of Antibiotic Resistance

The spread of resistant pathogens threatens the effectiveness of modern medicine. Common bacteria such as *E. coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* have developed resistance to last-resort antibiotics like carbapenems and colistin (Tacconelli et al., 2018). The economic burden of antimicrobial resistance is enormous, with global estimates predicting up to 10 million deaths annually by 2050 if current trends continue (O'Neill, 2016).

Therefore, molecular exploration of soil microorganisms plays a dual role-not only in the discovery of new antibiotics but also in understanding the natural origins and evolution of resistance mechanisms.

Research Gap and Rationale:

Nowadays, many infectious diseases are increasing rapidly, and microorganisms are becoming resistant to commonly used antibiotics. This problem arises mainly due to the overuse and misuse of antibiotics in clinical and environmental settings. As a result, bacteria get more opportunities to adapt, multiply rapidly, and develop resistance through genetic mutations and other mechanisms that help them survive even in the presence of antibiotics.

Although extensive research has been carried out on antibiotic resistance in clinical isolates, limited attention has been given to environmental sources, particularly hospital-associated soil, which is continuously exposed to antibiotics, disinfectants, and biomedical waste. Such environments create strong selective pressure that promotes the development and persistence of antibiotic-resistant bacteria. However, there is still a lack of sufficient data on the distribution, characteristics, and metabolic activity of these resistant microorganisms in hospital soil.

In the present study, an attempt has been made to address this gap by isolating bacteria resistant to penicillin, streptomycin, and tetracycline from hospital soil. These antibiotics represent different mechanisms of action, enabling a broader understanding of resistance patterns. Furthermore, the study includes morphological and biochemical characterization, along with protein estimation using the Biuret method, to evaluate the metabolic activity of the isolates.

While the development of new antibiotics is a time-consuming and expensive process, understanding environmental reservoirs of resistance is equally important. The identification of aerobic, non-fermentative, and metabolically active bacteria in hospital soil highlights the potential role of such environments in the spread of resistance.

Therefore, this study provides valuable insights into the presence and behavior of antibiotic-resistant bacteria in hospital soil, helping to bridge the gap between environmental microbiology and clinical concerns. It also emphasizes the importance of monitoring environmental sources and implementing proper antibiotic management strategies to control the spread of resistance.


OBJECTIVE

Research Objectives

1. To collect soil samples from hospital surroundings as a potential source of antibiotic-resistant bacteria.
2. To isolate antibiotic-resistant bacteria using media containing: Penicillin, Streptomycin and Tetracycline.
3. To study the morphological, Biochemical characters of selected isolates
4. To study protein expression pattern of isolate in presence and absence of antibiotics.

MATERIALS AND METHODS

Materials and Methods

1. Study Area and Sample Collection

Soil samples were collected from different locations using sterile spatulas and sterile polyethylene bags. Approximately 10-20 g of soil was collected from a depth of 5-10 cm below the surface to avoid surface contaminants. The samples were labelled properly and transported to the laboratory. All samples were stored at room temperature until further processing

2. Isolation of resistant organism

A. Penicillin
B. Streptomycin
C. Tetracycline

A. PENICILLIN

3. Isolation and characterisation of penicillin resistant microorganisms

Penicillin is a β-lactam antibiotic that inhibits bacterial cell wall synthesis by interfering with peptidoglycan formation. Bacteria that possess resistance mechanisms such as β-lactamase enzyme production, altered penicillin-binding proteins, or reduced membrane permeability can survive in the presence of penicillin (Stat Pearls. 2025, February 20) Therefore, nutrient agar containing penicillin can be used for selective isolation of penicillin-resistant bacteria from hospital soil.

3.1 Preparation of Soil Suspension

Soil samples used in the present study were collected from a hospital environment, which is considered a high-risk zone for the presence of antibiotic-resistant microorganisms due to continuous exposure to antibiotics, disinfectants, and biomedical waste. The collected soil was analysed based on its physical appearance and texture (Suresh, G., K., S., & V., S. 2026).

1 g of soil sample was added to 9 mL sterile distilled water in a test tube to prepare the stock soil suspension (10^{-1} dilution). The tube was vortexed thoroughly to mix the soil particles evenly.

3.2 Serial Dilution

Serial dilutions were prepared by transferring 1 mL of the suspension into 9 mL sterile distilled water to obtain dilutions up to 10^{-10}.

3.3 Preparation of Antibiotic-Containing Nutrient Agar

Nutrient agar medium (Table.2) was prepared according to standard microbiological procedures and sterilized in an autoclave at 121°C for 15 minutes (Cappuccino, J. G., & Sherman, N. 2014). After cooling the medium to 45–50°C, sterile penicillin solution was added aseptically to obtain a final concentration of 100 µg/ml. The medium was mixed thoroughly and poured into sterile Petri plates. Plates were allowed to solidify under aseptic conditions.

Table.2 Composition of nutrient agar

1.	Peptone	0.5gm
2.	Beef extract	0.3gm
3.	Sodium chloride	0.5gm
4.	Agar	1.5
5.	Distilled water	100 ml
6.	pH	7.0
7.	Antibiotic	100 µg/ml

3.4 Isolation of antibiotic-resistant Bacteria

From each serial dilution, 0.1 mL of suspension was spread on nutrient agar plates containing antibiotics using a sterile spreader. The plates were incubated at 37°C for 24-72 hours. After incubation, colonies showing growth in the presence of antibiotics were considered antibiotic-resistant isolates (Rabiu and Falodun 2017).

3.5 Purification and Maintenance of Isolates

Distinct colonies were picked using a sterile inoculating loop and streaked onto fresh nutrient agar plates to obtain pure cultures. Pure colonies were then transferred onto nutrient agar slants and incubated at 37 °C for 48 hours. The cultures were stored at 4°C for further analysis.

3.6 Morphological Characterization

3.6.1 Colony Morphology

Colony characteristics such as size, shape, colour, margin, surface, elevation, consistency, opacity of penicillin resistant organism were recorded (Smith burn, 1935; Petroff & Steenken, 1930).

3.6.2 Gram Staining

Gram staining was performed to determine the Gram reaction and cellular morphology of bacterial isolates (Gram, H.C. 1884).

Procedure:

1. A smear was prepared on a clean slide and heat-fixed.
2. The smear was stained with crystal violet (1 minute).
3. Rinse in distilled water for 2 seconds.
4. Gram's iodine was added for 1 minute.
5. Decolorization was done using 95% ethanol for a few seconds.
6. Rinse in distilled water.
7. The slide was counterstained with basic fuchsin for 1 minute.
8. Rinse in distilled water and keep it for air dry.
9. Slides were observed under a microscope using oil immersion (100×).

3.7 Biochemical Characterization

3.7.1 Sugar Fermentation Test

Sugar fermentation tests are carried out to determine the ability of bacteria to utilize carbohydrates and to differentiate between fermentative and non-fermentative organisms. Sugar fermentation tests were performed using 1% specific sugar (glucose, lactose, sucrose, mannitol) added in peptone water containing Andrade's indicator (R.K. Verma et al.2022) (Table 3).

Table.3 Peptone water composition

1.	Peptone	1.0 gm
2.	Sodium chloride	0.5 gm
3.	Distilled water	100 ml
4.	pH	7.2

Procedure:

1. The peptone water plus specific sugar tubes were inoculated with bacterial isolates.
2. Inoculated tubes were incubated at 37 °C for 48 hours.
3. Colour change from pink to yellow indicated fermentation of the sugar.

3.7.2 Catalase Test

A small amount of bacterial culture was placed on a clean glass slide. A drop of 3% hydrogen peroxide (H_2O_2) was added. Formation of bubbles indicated a positive catalase reaction.

3.7.3 Oxidase Test

A piece of filter paper was soaked with oxidase reagent (1% tetramethyl-p-phenylenediamine). A colony was rubbed onto the paper using a sterile loop. Development of a dark purple colour within 10 seconds indicated a positive oxidase test.

4. Protein Estimation

Protein estimation is done to measure the protein content of a sample and to assess the metabolic activity of microorganisms. For comparative analysis of protein concentration, bacterial isolates were cultured in two different broth conditions-one serving as control (without antibiotic) and the other containing the respective antibiotic. After incubation, protein estimation was performed for both samples to evaluate the effect of antibiotic stress on protein production.

4.1 Preparation of Bacterial Broth Culture

A loopful of each bacterial isolate was inoculated into nutrient broth (100 ml) in sterile conical flasks (Table 4).

Table. 4 Composition of Nutrient broth

1.	Peptone	0.5 gm
2.	Beef extract	0.3 gm
3.	Sodium chloride	0.5 gm
4.	Distilled water	100 ml
5.	pH	7.0

The broth was sterilized at 121°C for 15 minutes and inoculated under aseptic conditions.

Protein estimation by the Biuret method is based on the formation of a violet complex between proteins and copper ions in alkaline medium, where colour intensity is proportional to protein concentration.

4.1.1 Incubation

The inoculated broth cultures were incubated at 37°C for 72 hours under shaking conditions to obtain sufficient bacterial growth.

4.1.2 Centrifugation

Before centrifugation, the centrifuge was pre-cooled to 4°C. The broth cultures were transferred into sterile centrifuge tubes and centrifuged at 5000 rpm for 10-15 minutes 4°C

After centrifugation:

Supernatant → extracellular proteins

Pellet → bacterial cells

4.1.3 Cell Lysis Procedure (for Intracellular Protein)

The bacterial pellet obtained after centrifugation was used for cell lysis.

Step-by-step Procedure:

1. The pellet was resuspended in phosphate buffer (pH 7.0)
2. Cells were disrupted using the Vortexing vigorously method
3. The suspension was incubated for 10-15 minutes to ensure complete lysis
4. The lysed sample was centrifuged again at 5000 rpm for 15 minutes
5. The clear supernatant obtained was collected as cell lysate (protein extract)

4.1.4 Biuret Assay Procedure

1. 1 mL of each standard solution and sample was taken in separate test tubes.
2. 1 mL of Biuret reagent was added to each tube.
3. The tubes were mixed gently and incubated at room temperature for 20–30 minutes.
4. The violet colour developed was measured using a spectrophotometer at 540 nm against a blank.

4.1.5 Calculation

A standard curve was plotted with protein concentration on the X-axis and absorbance on the Y-axis. The protein concentration of the unknown sample was determined from the standard curve and multiplied by the dilution factor.

B. STREPTOMYCIN

5. Isolation and characterisation of Streptomycin resistant soil microorganisms

Streptomycin is a broad-spectrum antibiotic that inhibits protein synthesis in bacteria. Only those microorganisms that possess resistance mechanisms-such as modification of ribosomal binding sites, enzymatic inactivation, or efflux systems-are able to grow in its presence. Therefore, incorporation of streptomycin into culture media allows selective isolation of streptomycin-resistant bacteria (Luzzatto, L., Apirion, D., & Schlessinger, D. 1968).

5.1 Preparation of Soil Suspension

Soil samples used in the present study were collected from a hospital environment, which is considered a high-risk zone for the presence of antibiotic-resistant microorganisms due to continuous exposure to antibiotics, disinfectants, and biomedical waste. The collected soil was analysed based on its physical appearance and texture (Suresh, G., K., S., & V., S. 2026).

1 g of soil sample was added to 9 mL sterile distilled water in a test tube to prepare the stock soil suspension (10^{-1} dilution). The tube was vortexed thoroughly to mix the soil particles evenly.

5.2 Serial Dilution

Serial dilutions were prepared by transferring 1 mL of the suspension into 9 mL sterile distilled water to obtain dilutions up to 10^{-5} or 10^{-9}.

5.3 Preparation of Antibiotic-Containing Nutrient Agar

Nutrient agar medium (Table.5) was prepared and sterilized by autoclaving at 121°C for 15 minutes (Cappuccino, J. G., & Sherman, N. 2014). After cooling to about 45–50°C, sterile streptomycin solution was added to obtain the desired final concentration (100 µg/ml). The medium was mixed thoroughly and poured into sterile Petri plates under aseptic conditions and allowed to solidify.

Table.5 Composition of nutrient agar

1.	Peptone	0.5gm
2.	Beef extract	0.3gm
3.	Sodium chloride	0.5gm
4.	Agar	1.5
5.	Distilled water	100 ml
6.	pH	7.0
7.	Antibiotic	100 µg/ml

5.4. Isolation of antibiotic-resistant Bacteria

From each serial dilution, 0.1 mL of suspension was spread on nutrient agar plates containing antibiotics using a sterile spreader. The plates were incubated at 37 °C for 24-72 hours. After incubation, colonies showing growth in the presence of antibiotics were considered antibiotic-resistant isolates (Rabiu and Falodun 2017).

5.5 Purification and Maintenance of Isolates

Distinct colonies were picked using a sterile inoculating loop and streaked onto fresh nutrient agar plates to obtain pure cultures. Pure colonies were then transferred onto nutrient agar slants and incubated at 37 °C for 48 hours. The cultures were stored at 4°C for further analysis.

5.6. Morphological Characterization

5.6.1 Colony Morphology

Colony characteristics such as size, shape, colour, margin, surface, elevation, consistency, opacity of penicillin resistant organism were recorded (Smith burn, 1935; Petroff & Steenken, 1930).

5.6.2 Gram Staining

Gram staining was performed to determine the Gram reaction and cellular morphology of bacterial isolates (Gram, H.C. 1884).

Procedure:

1. A smear was prepared on a clean slide and heat-fixed.
2. The smear was stained with crystal violet (1 minute).
3. Rinse in distilled water for 2 seconds.
4. Gram's iodine was added for 1 minute.
5. Decolorization was done using 95% ethanol for a few seconds.
6. Rinse in distilled water.

7. The slide was counterstained with basic fuchsin for 1 minute.
8. Rinse in distilled water and keep it for air dry.
9. Slides were observed under a microscope using oil immersion (100×).

5.7 Biochemical Characterization

5.7.1 Sugar Fermentation Tests

Sugar fermentation tests are carried out to determine the ability of bacteria to utilize carbohydrates and to differentiate between fermentative and non-fermentative organisms (R.K. Verma et al.2022). Sugar fermentation tests were performed using 1% specific sugar (glucose, lactose, sucrose, mannitol) was added in peptone water containing Andrade's indicator. (Table.6)

Table. 6 Peptone water composition

1.	Peptone	1.0 gm
2.	Sodium chloride	0.5 gm
3.	Distilled water	100 ml
4.	pH	7.2

Procedure:

5. The broth tubes were inoculated with bacterial isolates.
6. Tubes were incubated at 37 °C for 48 hours.
7. Colour change from pink to yellow indicated fermentation of the sugar.

5.7.2 Catalase Test

A small amount of bacterial culture was placed on a clean glass slide. A drop of 3% hydrogen peroxide (H_2O_2) was added. Formation of bubbles indicated a positive catalase reaction.

5.7.3 Oxidase Test

A piece of filter paper was soaked with oxidase reagent (1% tetramethyl-p-phenylenediamine). A colony was rubbed onto the paper using a sterile loop. Development of a dark purple colour within 10 seconds indicated a positive oxidase test.

6. Protein Estimation

Protein estimation is done to measure the protein content of a sample and to assess the metabolic activity of microorganisms. For comparative analysis of protein concentration, bacterial isolates were cultured in two different broth conditions-one serving as control (without antibiotic) and the other containing the respective antibiotic. After incubation, protein estimation was performed for both samples to evaluate the effect of antibiotic stress on protein production.

6.1 Preparation of Bacterial Broth Culture

A loopful of each bacterial isolate was inoculated into nutrient broth (50 ml) in sterile conical flasks. (Table.7)

Table. 7 Composition of Nutrient broth

1.	Peptone	0.5 gm
2.	Beef extract	0.3 gm
3.	Sodium chloride	0.5 gm
4.	Distilled water	100 ml
5.	pH	7.0

The broth was sterilized at 121°C for 15 minutes and inoculated under aseptic conditions.

Protein estimation by the Biuret method is based on the formation of a violet complex between proteins and copper ions in alkaline medium, where colour intensity is proportional to protein concentration.

6.1.1 Incubation

The inoculated broth cultures were incubated at 37°C for 24 hours under shaking conditions to obtain sufficient bacterial growth.

6.1.2 Centrifugation

The broth cultures were transferred into sterile centrifuge tubes and centrifuged at 5000 rpm for 10-15 minutes 4°C

After centrifugation:

Supernatant → extracellular proteins

Pellet → bacterial cells

6.2 Cell Lysis Procedure (for Intracellular Protein)

The bacterial pellet obtained after centrifugation was used for cell lysis.

Step-by-step Procedure:

1. The pellet was resuspended in phosphate buffer (pH 7.0)
2. Cells were disrupted using the Vortexing vigorously method
3. The suspension was incubated for 10-15 minutes to ensure complete lysis
4. The lysed sample was centrifuged again at 5000 rpm for 15 minutes
5. The clear supernatant obtained was collected as cell lysate (protein extract)

6.3 Biuret Assay Procedure

1. 1 mL of each standard solution and sample was taken in separate test tubes.
2. 1 mL of Biuret reagent was added to each tube.
3. The tubes were mixed gently and incubated at room temperature for 20–30 minutes.
4. The violet colour developed was measured using a spectrophotometer at 540 nm against a blank.

6.4 Calculation

A standard curve was plotted with protein concentration on the X-axis and absorbance on the Y-axis. The protein concentration of the unknown sample was determined from the standard curve and multiplied by the dilution factor.

C. TETRACYCLINE

7. Isolation and characterisation of tetracycline resistant soil microorganisms

Tetracycline is a broad-spectrum antibiotic that inhibits protein synthesis by binding to the 30S ribosomal subunit. Bacteria that grow in its presence possess resistance mechanisms such as:

- Efflux pumps (removal of antibiotic from cell)
- Ribosomal protection proteins
- Enzymatic inactivation

Thus, tetracycline-containing medium acts as a selective medium to isolate resistant bacteria (Grossman, T. H. 2016).

7.1 Preparation of Soil Suspension

Soil samples used in the present study were collected from a hospital environment, which is considered a high-risk zone for the presence of antibiotic-resistant microorganisms due to continuous exposure to antibiotics, disinfectants, and biomedical waste. The collected soil was analysed based on its physical appearance and texture (Suresh, G., K., S., & V., S. 2026).

1 g of soil sample was added to 9 mL sterile distilled water in a test tube to prepare the stock soil suspension (10^{-1} dilution). The tube was vortexed thoroughly to mix the soil particles evenly.

7.2 Serial Dilution

Serial dilutions were prepared by transferring 1 mL of the suspension into 9 mL sterile distilled water to obtain dilutions up to 10^{-5} or 10^{-9}.

7.3 Preparation of Antibiotic-Containing Nutrient Agar

Nutrient agar medium (Table.8) was prepared according to standard microbiological procedures and sterilized in an autoclave at 121°C for 15 minutes (Cappuccino, J. G., & Sherman, N. 2014). After cooling the medium to 45–50°C, sterile penicillin solution was added aseptically to obtain a final concentration of 100 μg/ml. The medium was mixed thoroughly and poured into sterile Petri plates. Plates were allowed to solidify under aseptic conditions.

Table.8 Composition of nutrient agar

1.	Peptone	0.5gm
2.	Beef extract	0.3gm
3.	Sodium chloride	0.5gm
4.	Agar	1.5
5.	Distilled water	100 ml
6.	pH	7.0
7.	Antibiotic	100 µg/ml

7.4 Isolation of Bacterial Colonies

From each serial dilution, 0.1 mL of suspension was spread on nutrient agar plates containing antibiotics using a sterile spreader. The plates were incubated at 37 °C for 24-72 hours. After incubation, colonies showing growth in the presence of antibiotics were considered antibiotic-resistant isolates (Rabiu and Falodun 2017).

7.5 Purification and Maintenance of Isolates

Distinct colonies were picked using a sterile inoculating loop and streaked onto fresh nutrient agar plates to obtain pure cultures. Pure colonies were then transferred onto nutrient agar slants and incubated at 37 °C for 48 hours. The cultures were stored at 4°C for further analysis.

7.6 Morphological Characterization

7.6.1 Colony Morphology

Colony characteristics such as size, shape, colour, margin, surface, elevation, consistency, opacity of penicillin resistant organism were recorded (Smith burn, 1935; Petroff & Steenken, 1930).

7.6.2 Gram Staining

Gram staining was performed to determine the Gram reaction and cellular morphology of bacterial isolates (Gram, H.C. 1884).

Procedure:

1. A smear was prepared on a clean slide and heat-fixed.
2. The smear was stained with crystal violet (1 minute).
3. Rinse in distilled water for 2 seconds.
4. Gram’s iodine was added for 1 minute.
5. Decolorization was done using 95% ethanol for a few seconds.
6. Rinse in distilled water.

7. The slide was counterstained with basic fuchsin for 1 minute.
8. Rinse in distilled water and keep it for air dry.
9. Slides were observed under a microscope using oil immersion (100×).

7.7 Biochemical Characterization

7.7.1 Sugar Fermentation Tests

Sugar fermentation tests are carried out to determine the ability of bacteria to utilize carbohydrates and to differentiate between fermentative and non-fermentative organisms (R.K. Verma et al.2022). Sugar fermentation tests were performed using 1% specific sugar (glucose, lactose, sucrose, mannitol) was added in peptone water containing Andrade's indicator. (Table.9)

Table.9 Petone water composition

1.	Peptone	1.0 gm
2.	Sodium chloride	0.5 gm
3.	Distilled water	100 ml
4.	pH	7.2

Procedure:

1. The broth tubes were inoculated with bacterial isolates.
2. Tubes were incubated at 37 °C for 48 hours.
3. Colour change from pink to yellow indicated fermentation of the sugar.

7.7.2 Catalase Test

A small amount of bacterial culture was placed on a clean glass slide. A drop of 3% hydrogen peroxide (H_2O_2) was added. Formation of bubbles indicated a positive catalase reaction.

7.7.3 Oxidase Test

A piece of filter paper was soaked with oxidase reagent (1% tetramethyl-p-phenylenediamine). A colony was rubbed onto the paper using a sterile loop. Development of a dark purple colour within 10 seconds indicated a positive oxidase test.

8. Protein Estimation by Biuret Method

Protein estimation is done to measure the protein content of a sample and to assess the metabolic activity of microorganisms. For comparative analysis of protein concentration, bacterial isolates were cultured in two different broth conditions-one serving as control (without antibiotic) and the other containing the respective antibiotic. After incubation, protein estimation was performed for both samples to evaluate the effect of antibiotic stress on protein production.

8.1 Preparation of Bacterial Broth Culture

A loopful of each bacterial isolate was inoculated into nutrient broth (50 ml) in sterile conical flasks (Table.10).

Table. 10 Composition of Nutrient broth

1.	Peptone	0.5 gm
2.	Beef extract	0.3 gm
3.	Sodium chloride	0.5 gm
4.	Distilled water	100 ml
5.	pH	7.0

The broth was sterilized at 121°C for 15 minutes and inoculated under aseptic conditions.

Protein estimation by the Biuret method is based on the formation of a violet complex between proteins and copper ions in alkaline medium, where colour intensity is proportional to protein concentration.

8.1.1 Incubation

The inoculated broth cultures were incubated at 37°C for 24 hours under shaking conditions to obtain sufficient bacterial growth.

8.1.2 Centrifugation

The broth cultures were transferred into sterile centrifuge tubes and centrifuged at 5000 rpm for 10-15 minutes 4°C

After centrifugation:

Supernatant → extracellular proteins

Pellet → bacterial cells

8.2 Cell Lysis Procedure (for Intracellular Protein)

The bacterial pellet obtained after centrifugation was used for cell lysis.

Step-by-step Procedure:

1. The pellet was resuspended in phosphate buffer (pH 7.0)
2. Cells were disrupted using the Vortexing vigorously method
3. The suspension was incubated for 10-15 minutes to ensure complete lysis
4. The lysed sample was centrifuged again at 5000 rpm for 15 minutes
5. The clear supernatant obtained was collected as cell lysate (protein extract)

8.3 Biuret Assay Procedure

1. 1 mL of each standard solution and sample was taken in separate test tubes.
2. 1 mL of Biuret reagent was added to each tube.
3. The tubes were mixed gently and incubated at room temperature for 20-30 minutes.
4. The violet colour developed was measured using a spectrophotometer at 540 nm against a blank.

8.4 Calculation

A standard curve was plotted with protein concentration on the X-axis and absorbance on the Y-axis. The protein concentration of the unknown sample was determined from the standard curve and multiplied by the dilution factor.


RESULTS

RESULTS

1. Isolation of Antibiotic-Resistant Bacteria

Soil samples were processed and plated on nutrient agar plate supplemented with three antibiotics separately: Streptomycin, Penicillin, tetracycline. After incubation at 37°C for 24-48 hours, bacterial colonies were observed on all antibiotic-containing plates.

The growth of colonies in the presence of these antibiotics indicates that the isolates possess resistance against one or more antibiotics (Table.11).

1.1 Antibiotic wise growth observations

Table.11 Different antibiotics showed different growth patterns

Antibiotic	Observation	Interpretation
Penicillin	Heavy growth	High resistance
Streptomycin	Moderate growth	Moderate resistance
Tetracycline	Low growth	Lower resistance

2. Penicillin-resistant isolates

Penicillin-resistant isolates are bacteria capable of surviving in the presence of penicillin due to specific resistance mechanisms. These may include the production of β-lactamase enzymes or modification of penicillin-binding proteins.

Fig.1 Before incubation

Fig.2 After incubation

Isolation of penicillin- resistant organism

2.1 Colony Characters

Colony characters of well isolated colony of organism isolated from soil sample grown on nutrient agar plate with penicillin antibiotic after incubation at 37°C for 48 hrs.

Isolates 1	**Size**	**Shape**	**Colour**	**Margin**
	2mm	circular	Pale yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 2	**Size**	**Shape**	**Colour**	**Margin**
	2mm	circular	Pale yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 3	**Size**	**Shape**	**Colour**	**Margin**
	2mm	circular	Pale yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 4	**Size**	**Shape**	**Colour**	**Margin**
	2mm	circular	Pale yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 5	**Size**	**Shape**	**Colour**	**Margin**
	2mm	circular	Pale yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 6	Size	Shape	Colour	Margin
	2mm	circular	Pale yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

2.2 Morphological Characters:

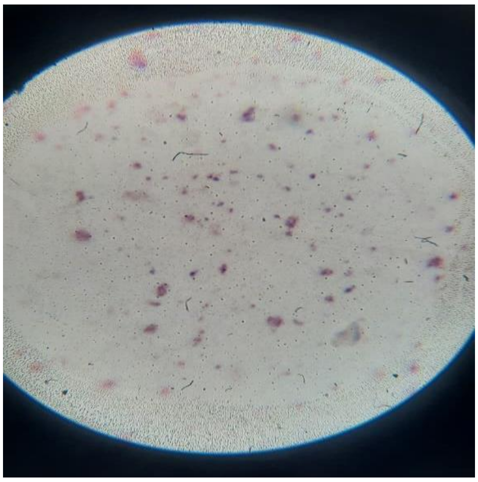


Fig.3 Gram Staining

Table.12 Gram staining

Sr. No.	Isolates	Gram nature	Shape and arrangement
1.	Isolate 1	Gram positive	Rod shaped arranged singly
2.	Isolate 2	Gram positive	Rod shaped arranged singly
3.	Isolate 3	Gram positive	Rod shaped arranged singly
4.	Isolate 4	Gram positive	Rod shaped arranged singly
5.	Isolate 5	Gram positive	Rod shaped arranged singly
6.	Isolate 6	Gram positive	Rod shaped arranged singly

Table.13 Biochemical Tests

Sr. No.	Isolates	Glucose	Lactose	Sucrose	Mannitol	Oxidase	Catalase
1.	Isolate 1	+	+	-	-	+	+
2.	Isolate 2	+	+	-	-	+	+
3.	Isolate 3	+	+	-	-	+	+
4.	Isolate 4	+	+	-	-	+	+
5.	Isolate 5	-	-	-	-	+	+
6.	Isolate 6	-	-	-	-	+	+

2.3 Protein Estimation of Antibiotic-Resistant Isolates (Biuret Method)

Protein concentration was determined using a casein standard curve at 540 nm. (Table.14).

Table.14 Standard Curve

Sr. No.	Concentration (µg/ml)	Absorbance (OD at 540 nm)
1.	20	0.12
2.	40	0.25
3.	60	0.38
4.	80	0.52
5.	100	0.65

A linear increase in absorbance with concentration was observed.

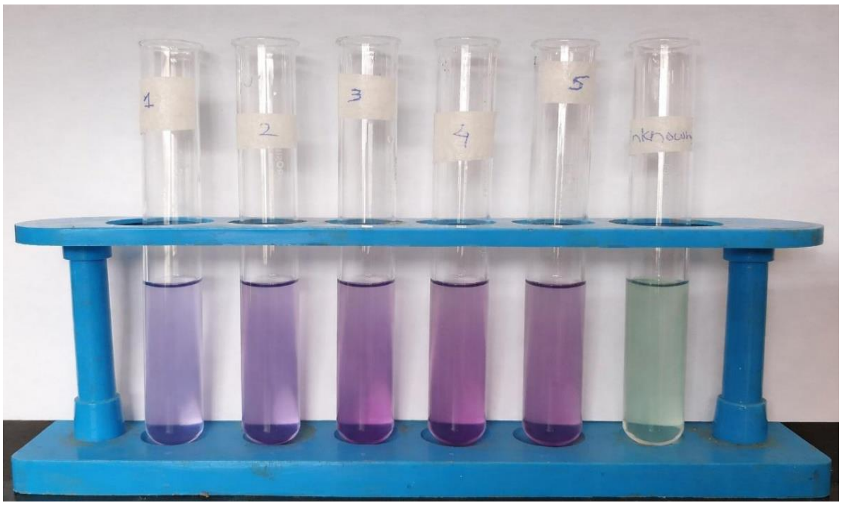


Fig.4 Protein estimation

A total of 6 isolates were analysed (Table.15).

Table.15 Protein Estimation of Penicillin-Resistant Isolates

Sr. No.	Isolates No.	OD at 540 nm	Protein conc. (µg/ml)
1.	Isolate 1	0.26	40
2.	Isolate 2	0.30	47
3.	Isolate 3	0.32	50
4.	Isolate 4	0.35	50
5.	Isolate 5	0.29	45
6.	Isolate 6	0.31	49

3. Streptomycin-resistant isolates

Streptomycin-resistant bacterial isolates were obtained from hospital soil using selective media supplemented with streptomycin. The principle of this method is based on the inhibition of protein synthesis by streptomycin, allowing only those bacteria with resistance mechanisms to survive and grow.

Fig.5 Before incubation

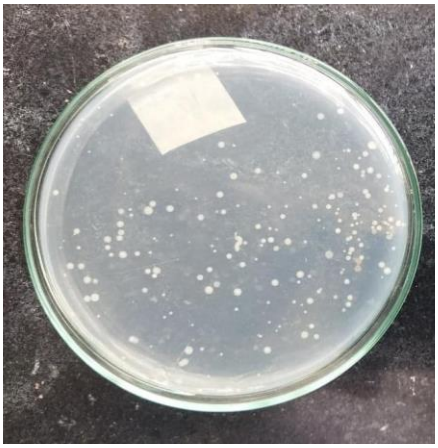


Fig.6 After incubation

Isolation of streptomycin- resistant organism

3.1 Colony Characters

Colony characters of well isolated colony of organism isolated from soil sample grown on nutrient agar plate with streptomycin antibiotic after incubation at 37°C for 48 hrs.

Isolates 1	**Size**	**Shape**	**Colour**	**Margin**
	1mm	Circular	White	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 2	**Size**	**Shape**	**Colour**	**Margin**
	1mm	Circular	White	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 3	**Size**	**Shape**	**Colour**	**Margin**
	1mm	Circular	White	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 4	**Size**	**Shape**	**Colour**	**Margin**
	1mm	Circular	White	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 5	**Size**	**Shape**	**Colour**	**Margin**
	1mm	Circular	White	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 6	**Size**	**Shape**	**Colour**	**Margin**
	1mm	Circular	White	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 7	**Size**	**Shape**	**Colour**	**Margin**
	1mm	Circular	White	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

Isolates 8	**Size**	**Shape**	**Colour**	**Margin**
	1mm	Circular	White	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Smooth	Convex	Moist	Opaque

3.2 Morphological Characters:

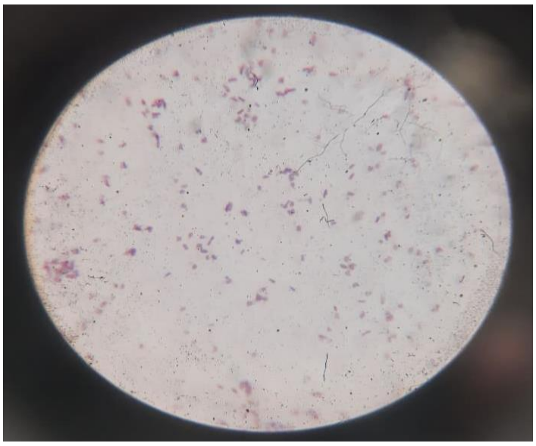


Fig.7 Gram Staining

Table.16 Gram staining

Sr. No.	Isolates	Gram nature	Shape and Arrangement
1.	Isolate 1	Gram positive	Cocci shaped arranged in cluster
2.	Isolate 2	Gram positive	Cocci shaped arranged in cluster
3.	Isolate 3	Gram positive	Cocci shaped arranged in cluster
4.	Isolate 4	Gram positive	Cocci shaped arranged in cluster
5.	Isolate 5	Gram positive	Cocci shaped arranged in cluster
6.	Isolate 6	Gram positive	Cocci shaped arranged in cluster
7.	Isolate 7	Gram positive	Cocci shaped arranged in cluster
8.	Isolate 8	Gram positive	Cocci shaped arranged in cluster

Table.17 Biochemical Tests

Sr. No.	Isolates	Glucose	Lactose	Sucrose	Mannitol	Oxidase	Catalase
1.	Isolate 1	+	+	-	-	+	+
2.	Isolate 2	+	+	-	-	+	+
3.	Isolate 3	+	+	-	-	+	+
4.	Isolate 4	+	+	-	-	+	+
5.	Isolate 5	+	+	-	-	+	+
6.	Isolate 6	+	+	-	-	+	+
7.	Isolate 7	+	+	-	-	+	+
8.	Isolate 8	+	+	-	-	+	+

3.3 Protein Estimation of Antibiotic-Resistant Isolates (Biuret Method)

Protein concentration was determined using a casein standard curve at 540 nm. (Table.18)

Table.18 Standard Curve

Sr. No.	Concentration (µg/ml)	Absorbance (OD 540 nm)
1.	20	0.12
2.	40	0.25
3.	60	0.38
4.	80	0.52
5.	100	0.65

A linear increase in absorbance with concentration was observed.

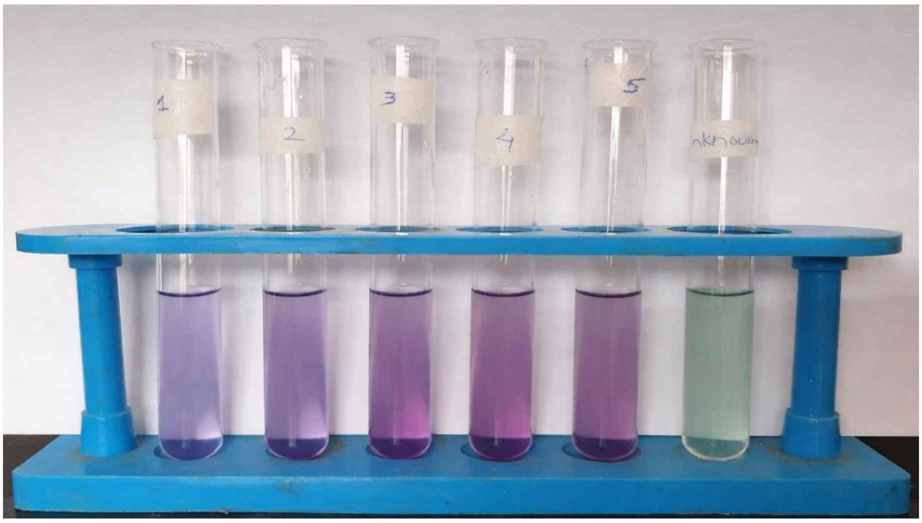


Fig.8 Protein estimation

A total of 8 isolates were analysed (Table.19)

Table.19 Protein Estimation of Streptomycin-Resistant Isolates.

Sr. No.	Isolates No.	OD at 540 nm	Protein conc. (µg/ml)
1.	Isolate 1	0.30	0.47
2.	Isolate 2	0.34	0.50
3.	Isolate 3	0.36	0.50
4.	Isolate 4	0.37	0.50
5.	Isolate 5	0.33	0.50
6.	Isolate 6	0.35	0.50
7.	Isolate 7	0.34	0.50
8.	Isolate 8	0.35	0.50

4. Tetracycline-resistant isolates

Isolation of tetracycline-resistant bacteria was performed using antibiotic-containing media, where tetracycline inhibits bacterial protein synthesis. Only those organisms possessing resistance mechanisms such as efflux pumps or ribosomal protection were able to grow.

Fig.9 Before incubation

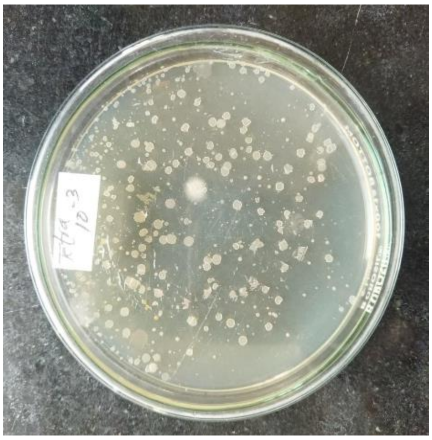


Fig.10 After incubation

Isolation of tetracycline-resistant organism

4.1 Colony Characters

Colony characters of well isolated colony of organism isolated from soil sample grown on nutrient agar plate with tetracycline antibiotic after incubation at 37°C for 48 hrs.

	Size	**Shape**	**Colour**	**Margin**
Isolate 1	3mm	Irregular	Yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Dry	Convex	Sticky	Opaque

	Size	**Shape**	**Colour**	**Margin**
Isolate 2	3mm	Irregular	Yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Dry	Convex	Sticky	Opaque

Isolate 3	**Size**	**Shape**	**Colour**	**Margin**
	3mm	Irregular	Yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Dry	Convex	Sticky	Opaque

Isolate 4	**Size**	**Shape**	**Colour**	**Margin**
	3mm	Irregular	Yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Dry	Convex	Sticky	Opaque

Isolate 5	**Size**	**Shape**	**Colour**	**Margin**
	3mm	Irregular	Yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Dry	Convex	Sticky	Opaque

Isolate 6	**Size**	**Shape**	**Colour**	**Margin**
	3mm	Irregular	Yellow	Entire
	Surface	**Elevation**	**Consistency**	**Opacity**
	Dry	Convex	Sticky	Opaque

4.2 Morphological Characters:

Fig.10 Gram Staining

Table.20 Gram Staining

Sr. No.	Isolates	Gram nature	Shape and Arrangement
1.	Isolate 1	Gram positive	Filamentous arranged in branched
2.	Isolate 2	Gram positive	Filamentous arranged in branched
3.	Isolate 3	Gram positive	Filamentous arranged in branched
4.	Isolate 4	Gram positive	Filamentous arranged in branched
5.	Isolate 5	Gram positive	Filamentous arranged in branched
6.	Isolate 6	Gram positive	Filamentous arranged in branched

Table.21 Biochemical Tests

Sr. No.	Isolates	Glucose	Lactose	Sucrose	Mannitol	Oxidase	Catalase
1.	Isolate 1	+	+	-	-	+	+
2.	Isolate 2	+	+	-	-	+	+
3.	Isolate 3	+	+	-	-	+	+
4.	Isolate 4	+	+	-	-	+	+
5.	Isolate 5	+	+	-	-	+	+
6.	Isolate 6	+	+	-	-	+	+

4.3 Protein Estimation of Antibiotic-Resistant Isolates (Biuret Method) (Table.22)

Protein concentration was determined using a casein standard curve at 540 nm.

Table.22 Standard Curve

Sr. No.	Concentration (µg/ml)	Absorbance (OD 540 nm)
1.	20	0.12
2.	40	0.25
3.	60	0.38
4.	80	0.52
5.	100	0.65

A linear increase in absorbance with concentration was observed.

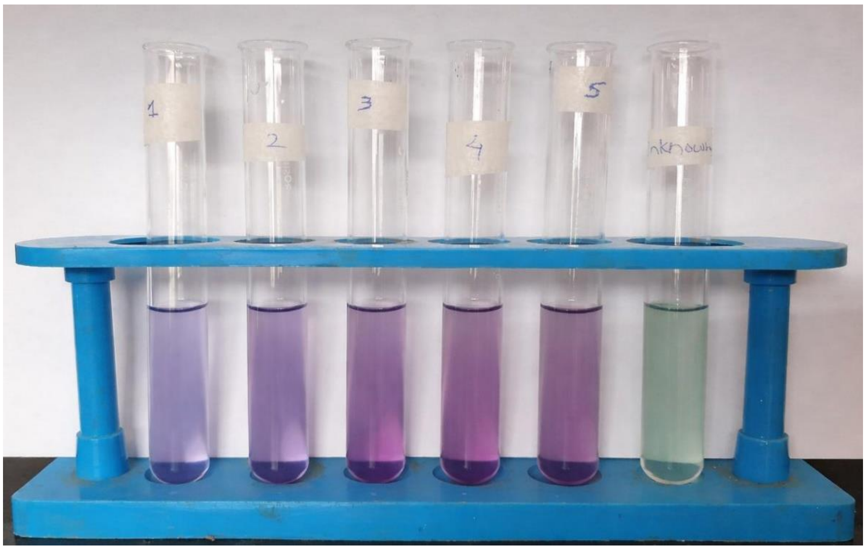


Fig.11 Protein estimation

A total of 6 isolates were analysed (Table.23).

Table.23 Protein Estimation of Penicillin-Resistant Isolates

Sr. No.	Isolates No.	OD at 540 nm	Protein conc. (µg/ml)
1.	Isolate 1	0.27	0.42
2.	Isolate 2	0.30	0.47
3.	Isolate 3	0.31	0.49
4.	Isolate 4	0.34	0.50
5.	Isolate 5	0.28	0.45
6.	Isolate 6	0.30	0.48


DISCUSSION

Discussion

The present study was undertaken to isolate and characterize antibiotic-resistant bacteria from soil samples and to evaluate their morphological, biochemical, and protein profiles. Soil is widely recognized as a complex and dynamic ecosystem that harbours an immense diversity of microorganisms (Rabiu, A. S., & Falodun, A. 2017). These microorganisms are constantly exposed to naturally occurring antibiotics produced by other microbes, as well as anthropogenic antibiotic contamination, which contributes to the development and spread of antibiotic resistance.

In the current investigation, bacterial isolates were successfully obtained on nutrient agar plates supplemented with penicillin, streptomycin, and tetracycline. The ability of these organisms to grow in the presence of antibiotics clearly indicates that they possess resistance mechanisms (B Kowalska-Krochmal 2021). These mechanisms may include enzymatic degradation of antibiotics (e.g., β-lactamase production), modification of antibiotic target sites, reduced permeability, or active efflux systems. The presence of such resistant organisms in soil highlights the ecological importance of soil as a natural reservoir of antibiotic resistance genes (Kummer, S., et al. 2020).

The colony morphology observed in this study exhibited considerable diversity. Colonies varied in terms of colour (white, cream, yellow), size, margin (smooth, undulate, rough), elevation (flat, raised, convex), and shape (circular and irregular). Such variation in colony characteristics suggests that the isolates belong to different bacterial genera and species (Madigan, M. T., Martinko, J. M., Bender, K. S., Buckley, D. H., & Stahl, D. A. 2021). The diversity of colony morphology is a reflection of the heterogeneity of soil microenvironments, where different microorganisms adapt to specific niches based on nutrient availability, moisture content, and environmental stress factors.

Microscopic examination through Gram staining revealed the presence of both Gram-positive cocci and Gram-negative rods. This indicates that the soil sample contained a mixed population of bacteria with different structural and physiological characteristics. Gram-positive bacteria possess a thick peptidoglycan layer, which may contribute to resistance against certain antibiotics, particularly β-lactam antibiotics like penicillin. On the other hand, Gram-negative bacteria have an outer membrane that can act as a barrier to antibiotic entry, thereby contributing to intrinsic resistance (N Tripathi2025).

One of the most significant findings of this study was the biochemical characterization of the isolates, particularly the sugar fermentation tests. All isolates were found to be negative for glucose, lactose, sucrose, and mannitol fermentation. This result indicates that the isolates are non-fermentative bacteria, which do not utilize carbohydrates through fermentation pathways to produce energy. Instead, these organisms rely on oxidative metabolic pathways, utilizing oxygen as the final electron acceptor in the electron transport chain (Muthukumar, A., et al. 2021).

Non-fermentative bacteria are commonly found in soil and water environments and are known for their ability to survive under nutrient-limited and stressful conditions. Their metabolic flexibility allows them to utilize a wide range of organic compounds other than simple sugars. The absence of carbohydrate fermentation in all isolates suggests that the microbial population in the studied soil sample is adapted to an environment where alternative carbon sources are utilized more efficiently than simple sugars (Li, Y., et al. 2024).

In contrast to the negative sugar fermentation results, all isolates showed positive catalase and oxidase activity, which provides important insights into their metabolic nature. The catalase test detects the presence of the enzyme catalase, which breaks down hydrogen peroxide into water and oxygen. The positive catalase reaction observed in all isolates indicates that these bacteria are capable of protecting themselves against oxidative stress caused by reactive oxygen species. This is particularly important for aerobic organisms that generate hydrogen peroxide as a byproduct of metabolism (A Nandi 2019).

Similarly, the oxidase test detects the presence of cytochrome c oxidase, an important enzyme in the electron transport chain. The positive oxidase reaction in all isolates suggests that they possess a functional aerobic respiratory system. This confirms that the isolates are predominantly aerobic organisms that depend on oxygen for energy production (Vet Bact. 2017, December 20). The combination of oxidase and catalase positivity strongly supports the conclusion that the isolates are strictly aerobic, non-fermentative bacteria.

Based on these biochemical characteristics, it is likely that some of the isolates belong to genera such as *Pseudomonas, Alcaligenes*, or other aerobic soil bacteria.

These genera are well known for their non-fermentative metabolism, oxidase positivity, and high intrinsic resistance to antibiotics. *Pseudomonas* species, in particular, are widely distributed in soil and are recognized for their metabolic versatility and ability to resist multiple antibiotics through various mechanisms (Quintieri, L., Fanelli, F., & Caputo, L. 2019).

Protein estimation using the Biuret method further supported the biochemical findings of the study. The development of a violet colour in the Biuret reaction confirmed the presence of proteins in all bacterial samples. The variation in absorbance values observed among isolates indicates differences in protein concentration, which may be associated with variations in metabolic activity, enzyme production, or cell density. Higher protein content in some isolates may reflect increased synthesis of enzymes involved in antibiotic resistance or metabolic adaptation further study is required to confirm the same (Gornall, A. G., Bardawill, C. J., & David, M. M. 1949).

The distribution of isolates among different antibiotics revealed that streptomycin-resistant isolates were the most abundant, followed by penicillin and tetracycline-resistant isolates. This pattern may be explained by the widespread use of streptomycin in agriculture and medicine, which creates selective pressure for the survival of resistant strains. Additionally, the natural production of antibiotic-like compounds in soil environments may contribute to the development of resistance even in the absence of direct human intervention (Popowska, M., Miernik, A., Rzeczycka, M., & Lopaciuk, A. 2010).

The presence of antibiotic-resistant bacteria in soil has important environmental and public health implications. Soil acts as a reservoir of resistance genes, which can be transferred to pathogenic bacteria through horizontal gene transfer mechanisms such as conjugation, transformation, and transduction. This can lead to the emergence of multidrug-resistant pathogens, posing a significant challenge to modern medicine (Martinez, J. L., et al. 2007).

Overall, the findings of this study highlight the diverse, aerobic, non-fermentative nature of antibiotic-resistant bacteria in soil. The combination of morphological diversity, negative sugar fermentation, and positive oxidase-catalase reactions indicates that these organisms are well adapted to survive in challenging environmental conditions.

Conclusion

Conclusion

Antibiotic resistance has become a major global health concern, affecting both clinical and environmental systems. The continuous and excessive use of antibiotics has accelerated the development of resistant microorganisms, making many conventional treatments less effective. Environmental sources, particularly hospital-associated soils, play a significant role in the emergence and spread of antibiotic-resistant bacteria due to constant exposure to antimicrobial agents and biomedical waste.

In the present study, bacterial isolates were successfully obtained from hospital soil using selective media containing penicillin, streptomycin, and tetracycline, confirming the presence of resistant microorganisms. A total of 20 antibiotic-resistant isolates were identified, indicating the widespread occurrence of resistance in hospital surroundings.

Morphological, biochemical, and protein analysis revealed that the isolates exhibited diverse colony characteristics, were non-fermentative, catalase-positive, and oxidase-positive, and showed comparatively higher protein concentration under antibiotic stress conditions, indicating their aerobic, metabolically active, and adaptive nature.

The findings of this study highlight that hospital soil acts as a reservoir of antibiotic-resistant bacteria, which can contribute to the spread of resistance in the environment. Such resistant microorganisms may transfer their resistance traits to other bacteria, posing a serious threat to public health.

Therefore, this study emphasizes the urgent need for continuous monitoring of environmental sources, proper management of antibiotic usage, and effective disposal of biomedical waste to control the spread of antibiotic resistance.

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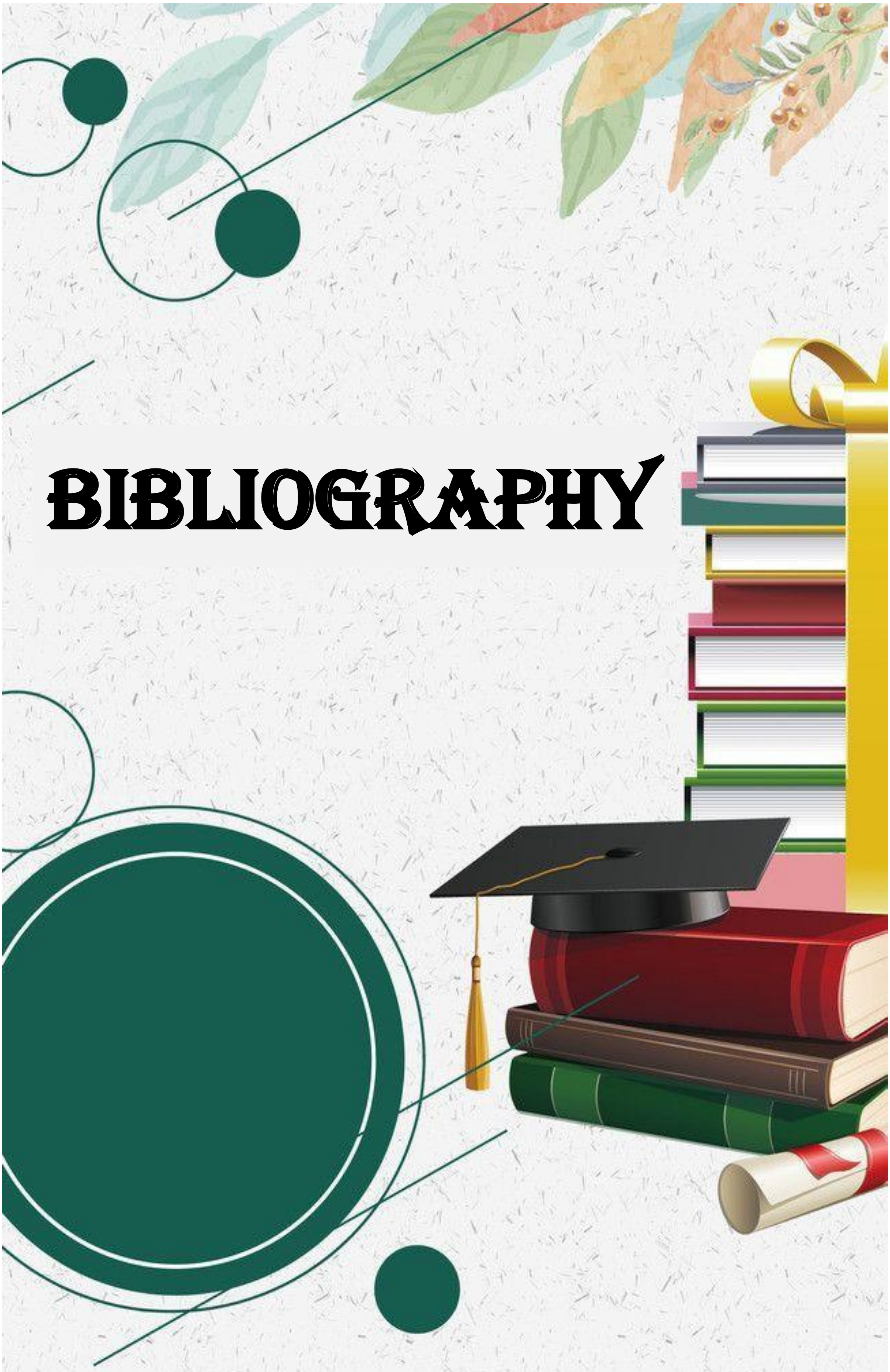

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