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Research

Antibacterial Resistance in Soil Bacteria: Comparative Study of Ampicillin, Ciprofloxacin, Levofloxacin Resistance and Its Ecological and Public Implications

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Abstract:
The present study was aimed to conduct a comparative study on the bacterial resistance of soil bacteria isolated from urine contaminated soil samples against three antibiotics: ampicillin, ciprofloxacin and levofloxacin and to evaluate the ecological and public health implications if the findings. Anti-bacterial agents are molecules that kill or stop the growth of bacteria. Antibacterial resistance is a phenomenon where bacteria evolve to survive exposure to antibiotics. Staining techniques and Agar well diffusion microbial assays were conducted to analyze the morphology of bacteria and to estimate the zone of inhibition by treating with three specific antibiotics that are Ampicillin, Ciprofloxacin and Levofloxacin. The study revealed varying levels of bacterial resistance to antibiotics, with Ampicillin showing lower inhibition zones and thus less activity against bacteria from urinated soil compared to Ciprofloxacin and Levofloxacin, which exhibited larger inhibition zones. This suggests soil bacteria are more resistant to ampicillin, likely due to protective mechanisms in Gram-negative bacteria and potential beta-lactamase production or altered PBPs in Gram-positive bacteria. These findings emphasize the need for thorough antibiotic susceptibility testing to select effective treatments and manage resistance. Future studies should explore the genetic basis of resistance, focusing on beta-lactamase genes or target site mutations. The results can guide the development of new antibiotics or adjuvants to overcome identified resistance mechanisms.

Keywords: Antibacterial resistance, urine contaminated soil, Ampicillin, Ciprofloxacin, Levofloxacin.

1. INTRODUCTION

Antibiotics

Antimicrobials – including antibiotics, antivirals, antifungals, and anti-parasitics – are medicines used to prevent and treat infectious diseases in humans, animals and plants. Antibiotics are molecule that kill, or stop the growth of, microorganisms, including both bacteria and fungi. Antibiotics that kill bacteria are called bactericidal. Antibiotics that stop the growth of bacteria are called bacteriostatic. Antibiotics are crucial pharmaceuticals used to treat bacterial infections in humans, animals, and plants. Since their discovery, antibiotics have revolutionized medicine, saving countless lives and enabling complex medical procedures by preventing and treating infections. However, the widespread and often indiscriminate use of antibiotics has led to an alarming rise in antibiotic resistance, rendering many drugs less effective and posing a significant threat to global health.[1]

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enabling complex medical procedures by preventing and treating infections. However, the widespread and often indiscriminate use of antibiotics has led to an alarming rise in antibiotic resistance, rendering many drugs less effective and posing a significant threat to global health.[2]

Antibiotic resistance

Antibiotic resistance is a phenomenon where bacteria evolve to survive exposure to antibiotics that would normally kill them or inhibit their growth. Strains of bacteria that have developed the ability to survive exposure to antibiotics that would normally kill them or inhibit their growth are antibiotic resistant bacteria. This resistance can arise through genetic mutations or the acquisition of resistance genes from other bacteria. The spread of antibiotic-resistant bacteria poses a significant threat to public health, leading to harder-to-treat infections, increased healthcare costs, and higher mortality rates.[4]

Role of antibiotics in public health

Antibiotics play a crucial role in public health by treating bacterial infections, reducing mortality, and improving the quality of life. These medications are effective against a wide range of bacterial diseases, from common infections like strep throat and urinary tract infections to more severe conditions such as pneumonia, sepsis, and meningitis. By eradicating harmful bacteria, antibiotics not only help individuals recover from illnesses but also prevent the spread of infections within communities. This is particularly important in settings such as hospitals, schools, and crowded living conditions, where the risk of bacterial transmission is high.

However, the misuse and overuse of antibiotics pose significant public health risks. When antibiotics are taken unnecessarily or incorrectly, such as for viral infections like the common cold or flu, it contributes to the development of antibiotic-resistant bacteria. These "superbugs" can survive even in the presence of antibiotics, rendering standard treatments ineffective and leading to more severe and prolonged illnesses. This misuse is often fueled by a lack of public understanding about when antibiotics are needed, pressure on healthcare providers to prescribe them, and the availability of antibiotics without prescription in some regions.[4]

To combat antibiotic resistance, public education and stricter regulations on antibiotic use are crucial. Health organizations advocate for responsible antibiotic use, emphasizing that these medications should only be taken when prescribed by a healthcare professional for bacterial infections. Moreover, completing the full course of antibiotics as prescribed, even if symptoms improve, is vital to ensure that all bacteria are eradicated and to prevent the development of resistance. By promoting prudent antibiotic use and improving global awareness, we can help preserve the effectiveness of these life-saving drugs for future generations.[1-4]

Soil

Soil, is a natural, dynamic and complex system, composed of mineral particles, organic matter, water, air, and living organisms. Soil bacteria play a significant role in maintaining soil health and fertility, supporting plant growth, and influencing the overall functioning of terrestrial ecosystems. One critical yet often overlooked reservoir for antibiotic resistance is soil. Soils, particularly those enriched with human and animal waste or impacted by wastewater, can accumulate significant levels of antibiotics and resistant bacteria.[5]

Bacterian content in urinated soil

Human urine, which contains residual antibiotics and nutrients, can alter soil microbial communities and enhance the persistence and spread of antibiotic resistance genes (ARGs). Similarly, wastewater, which often carries a mixture of pharmaceuticals, including antibiotics, contributes to the contamination of soils and water bodies. Urinated soil bacteria, often referred to as soil bacteria exposed to urine or other nitrogen-rich wastes, play a complex role in antibiotic interactions. These bacteria can harbor resistance genes that potentially affect the efficacy of antibiotics. The presence of urine introduces nitrogen and other compounds into the soil, which can stimulate the growth and proliferation of certain bacterial species. This environment might favor bacteria that have developed or acquired resistance mechanisms against antibiotics. Consequently, the presence of these resistant bacteria in the soil can lead to an increased prevalence of antibiotic-resistant strains, making it more challenging to treat bacterial infections effectively.[3]

Understanding antibiotic levels in soils, particularly in urine-enriched and wastewater-affected areas, is crucial for assessing environmental contamination and resistance mechanisms. This study focuses on quantifying antibiotic concentrations in soil bacteria, identifying resistant strains, and assessing the prevalence of antibiotic resistance genes (ARGs).

Through a blend of chemical, microbiological, and molecular techniques, this research aims to illuminate the intricate interactions between antibiotics and soil bacteria, offering insights into their implications for public and environmental health.

These findings will inform strategies to manage and mitigate antibiotic resistance spread, emphasizing integrated approaches like the "One Health" initiative. This initiative underscores the interconnectedness of human, animal, and environmental health in combatting antibiotic resistance, ensuring sustainable practices for safeguarding ecosystems and human wellbeing.[6]

Fig no:1 Structure of bacteria

Bacteria and morphology of bacteria

Bacteria are single-celled microorganisms that lack a nucleus and other membrane-bound organelles, making them prokaryotes. They come in various shapes, such as rods, spheres, and spirals. Bacteria can be found in almost every environment on Earth, including soil, water, and the human body. Some bacteria are beneficial and play essential roles in processes like digestion and nutrient cycling, while others can cause infections and diseases.[7]

Morphology of bacteria

Bacteria is unicellular, free-living, microscopic microorganisms capable of performing all the essential functions of life. They possess both deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA). Bacteria are prokaryotic microorganisms that do not contain chlorophyll. They occur in water, soil, air, food, and all-natural environment. They can survive extremes of temperature, pH, oxygen, and atmospheric pressure.

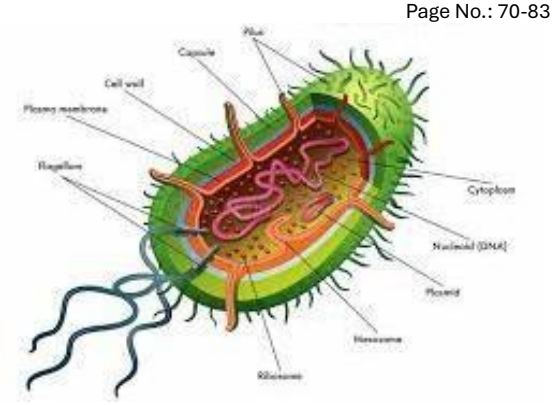

Fig no: 2 Anatomy of bacteria

Size of bacteria

Bacteria are very small microorganisms which are visible under the microscope. They are having the size range in microns. Bacteria are stained by staining reagents and then visualised under high power of magnification (1000X) of compound microscope. An electron microscope is used for clear visualization of internal structure of bacteria.

Shape of bacteria

On the basis of shape bacteria are classified as,

1. Cocci- Cocci are small, spherical or oval cells. In greek 'Kokkos' means berry. Eg: micrococcus.
2. Bacilli- They are rod shaped cells. Eg: Bacillus anthracis. It is derived from Greek word "Bacillus" meaning stick. Eg: Bracella.
3. Vibrios- They are comma shaped curved rods. Eg: Vibrio comma.
4. Spirilla- They are longer rigid rods with several curves or coils. They have a helical shape and rigid body. Eg: Spirillum ruprem.
5. Spirochetes- They are slender and flexuous soiral forms.
6. Actinomycetes- The characteristic shape is due to the presence of rigid cell wall. They are branching filamentous bacteria. Eg: Streptomyces.
7. Mycoplasma- They are cell wall deficient bacteria and hence do not possess stable morphology. They occur as round or oval bodies with interlacing filaments.

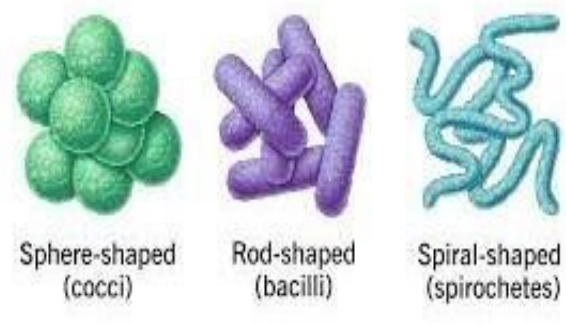

Fig no:3 Shapes of bacteria

• **Arrangements of bacterial cells**

Cocci appears as several characteristics arrangement or grouping.

1.Diplococci- They split in one plane and remains in pair. Eg: diplococcus pneumonia.

2.Streptococci- These cells divide in one plane and remain attached, to form chains. Eg: streptococcus lactis.

3.Tetracocci- They divide in two planes and live in groups of four. Eg: Gaffyka tetragena.

4.Staphylococci- Cocci cells divide in three planes in an irregular pattern. These cells produce bunches of cocci as in grapes. Eg: staphylococcus aureus, staphylococcus albus.

5.Sarcinae- Sarcinae cells divide in three planes in a regular pattern. These cells produce a cuboidal arrangement of group of a eight cells. Eg: Micrococcus tetragena.

Arrangement of grouping formed by bacilli species,

1. Diplobacilli
2. Streptobacilli
3. Trichomes[8]

Gram-positive and gram-negative bacteria

Gram-positive and gram-negative bacteria are distinguished by their cell wall structures and reactions to the Gram stain test. Gram-positive bacteria have a thick peptidoglycan layer with embedded teichoic acids, which retain the crystal violet stain, making them appear purple under a microscope. Common examples include Staphylococcus aureus and Streptococcus pneumoniae. In contrast, gram-negative bacteria have a thin peptidoglycan layer located between an inner membrane and an outer membrane that contains lipopolysaccharides (LPS). This structure does not retain the crystal violet stain, but takes up the counterstain safranin, making these bacteria appear pink or red. Notable examples include Escherichia coli and Pseudomonas aeruginosa.

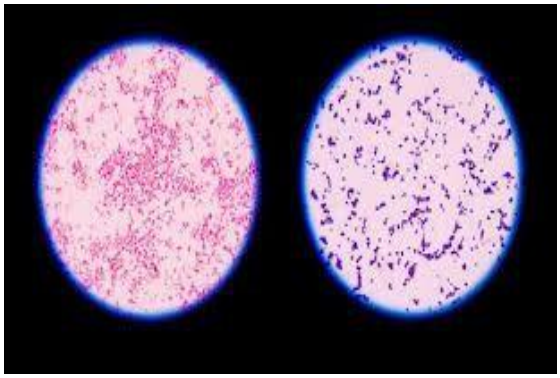

Fig no:4 Gram-positive and Gram-negative bacteria

These structural differences impact their susceptibility to antibiotics and pathogenic mechanisms. The outer membrane of gram-negative bacteria acts as a barrier to certain antibiotics, making them more resistant compared to gram-positive bacteria. Understanding these distinctions is essential for developing effective treatments and managing bacterial infections.[9]

Action of antibiotics on bacteria

Antibiotics are potent medications crucial for treating bacterial infections by targeting specific aspects of bacterial physiology and biochemistry while sparing human cells. They achieve this by inhibiting cell wall synthesis, disrupting cell membrane function, inhibiting protein synthesis, interfering with nucleic acid synthesis, and blocking essential metabolic pathways. By preventing cell wall formation, antibiotics hinder bacterial structure and integrity. Disruption of cell membrane function increases permeability, leading to leakage of essential molecules. Inhibition of protein and nucleic acid synthesis halts bacterial reproduction and essential biochemical processes. Blocking metabolic pathways further cripples bacterial growth. These combined mechanisms effectively eliminate bacterial pathogens, underscoring the vital role of antibiotics in combating infections.[11]

Antibiotic resistance of bacteria

Bacteria develop resistance to antibiotics through multiple mechanisms that enable their survival and proliferation despite treatment. One key strategy involves producing enzymes like β-lactamase, which inactivate antibiotics such as penicillin derivatives. Another approach alters antibiotic target sites, such as ribosomal binding sites, preventing the drug from inhibiting essential processes like protein synthesis. Bacteria can also deploy efflux pumps to expel antibiotics from cells, maintaining non-lethal intracellular drug levels. Horizontal gene transfer plays a critical role by allowing bacteria to acquire resistance genes from other microbes through processes like conjugation, transformation, or transduction, facilitating rapid spread of resistance within populations. Additionally, some bacteria form protective biofilms, hindering antibiotic penetration and effectiveness. These diverse resistance mechanisms underscore the challenge of combating antibiotic resistance, emphasizing the urgent need for new antibiotics and alternative treatment strategies.[10]

Antibiotic resistance of bacteriain soil

Soil is a very complex structure which includes organic particles as well as thousands of living organisms from different taxa including worms, arthropods, fungi, bacteria and some other eukaryotic and prokaryotic organisms. Bacteria are one of the most important living parts of the soil ecosystem. Many of them are decomposers, the other helps to assimilate nitrogen for plants as well as serving as a food for protists. Soil is one of the most favorable settings for acquisition and selection of antimicrobial resistance, due to the abundance of antibiotic-producing microorganisms.

Understanding the prevalence and polymorphism of antibiotic resistance genes in soil bacteria and their potential to be transferred horizontally is required to evaluate the likelihood and ecological (and possibly clinical) consequences of the transfer of these genes from transgenic plants to soil bacteria.

Chemicals that are used in conventional farming have potential to induce resistance development. On the other hand, during organic farming manure as a fertilizer is used, therefore antimicrobial resistant bacteria originated from gut of the animals may spread into soil ecosystems and increase resistance.[12]

Antibiotics are medicines that are widely used in livestock production not only for the prevention and treatment of infectious diseases, but also for accelerating the growth of animals. The application of manure for fertilizing agricultural soils leads to the entry into the soil ecosystem not only of the antibiotics themselves, but also the resistance genes to them.

Analysis of antimicrobial resistance in soils demonstrates that microorganisms did not acquire a plethora of genetic determinants encoding resistance mechanisms to the antimicrobials used in human and animal medicine as only a small number and low variety of clinically important genes encoding resistance to those antimicrobials were detected.

Drug resistance in bacteria is often the result of a genetic mutation. Because many soil-dwelling bacteria are exposed to antibiotics produced by surrounding strains in the soil, they have developed a variety of mechanisms to survive the toxic antimicrobial compounds created around them. Often, these mechanisms of resistance resemble those identified in clinical pathogens.

However, the antibiotic resistance of the cultivable agricultural soil bacteria, including clinically relevant species, is largely mediated by the drug efflux mechanisms.[13]

Impact of antibacterial resistance in human health

Antibacterial resistance poses a significant threat to human health. It occurs when bacteria evolve to resist the effects of antibiotics, rendering treatments less effective or ineffective. This leads to longer illnesses, increased healthcare costs, and a higher risk of complications and death. It can also limit treatment options, making it harder to control infections and increasing the spread of resistant bacteria. In severe cases, it can render common infections and medical procedures potentially life-threatening. Combating antibacterial resistance requires global efforts in responsible antibiotic use, infection control, and the development of new treatments.[14]

2. AIM AND OBJECTIVE

Aim: To conduct a comparative study on the antibacterial resistance of soil bacteria isolated from urinated soil samples against three antibiotics: ampicillin, ciprofloxacin, and levofloxacin, and to evaluate the ecological and public health implications of the findings.

Objective:

1. To isolate and identify bacterial strains from urinated soil samples.
2. To evaluate the resistance of these bacterial strains to the antibiotics ampicillin, ciprofloxacin, and levofloxacin using standardized susceptibility testing methods.
3. To compare the resistance patterns among the isolated strains for each antibiotic. To analyze the ecological implications of antibiotic-resistant bacteria in soil, particularly in relation to nutrient cycling and soil health.
4. To assess the potential public health risks associated with the presence and spread of antibiotic-resistant bacteria from soil environments.

3. MATERIALS AND METHODS

3.1. Materials, apparatus and instruments

a. Chemicals

All the chemicals and reagents used in the research work were analytical or synthetic grade.

List of chemicals or reagents used in this project work are listed in the table below:

Table no: 1 List of chemicals used

SERIAL NUMBER	CHEMICALS / REAGENTS	MANUFACTURER
1	Nutrient agar	Sisco research laboratories Pvt.Ltd.
2	Methylene blue	Nice chemicals Pvt.Ltd.
3	Glycerin	Mercl life science Pvt.Ltd.
4	Crystal violet	Linco scientific instruments and chemicals Pvt.Ltd.
5	Iodine solution	Burgoyne burbidges & co(m) laboratory chemical company
6	Ethanol	Chemco
7	Safranin	Chemco
8	Ciprofloxacin	Alkem health science
9	Levofloxacin	Cipla Ltd.
10	Ampicillin	Cadilla Pharmaceuticals Ltd.

b. Apparatus

Petri plates, Glass rod, Conical flask, Burner, Test tubes, Wash bottle, Cotton swab, Twine, Spatula, Cork borer, Glass Slides, Cover slip, Inoculation loop, Tripod stand, pH paper

c. Instruments

Table no: 2 List of Instruments used

S.NO	INSTRUMENTS	MANUFACTURER
1	Autoclave	B & C Industries
2	Bod incubator	Navyug
3	Laminar air flow	B & C Industries
4	Incubator	B & C Industries
5	Micropipette	R V Instruments Pvt.Ltd.
6	Refrigerator	LG
7	Microscope	Unilab
8	Antibiotic zone reader	Optics Technology
9	Electronic balance	Infra Instruments Pvt.Ltd.

3.2 Procedure

Isolation of bacteria from soil

There are over a million species of microorganisms widely distributed in nature. Less than 1% of the World's microorganisms have been studied. In fact, only a few species are important for industrial use. The good source for the isolation of microorganisms are soils,lakes and river muds. It is estimated that a gram of soil contains 10^6-10^8 bacteria, 10^4-10^6 actinomycete spores and 10^2-10^4 fungal spores.

The common techniques employed for the isolation of bacteria are given below:

1. Direct sponge of the soil
2. Soil dilution
3. Gradient plate method (pour plate and streak plate technique)
4. Aerosal dilution
5. Flotation
6. Centrifugation.

The actual technique for the isolation of bacteria depends on the source and the physiological properties of bacteria. The general scheme adopted for isolating bacteria from soil is given below;

- Collection of soil sample
- Preparation of dilution series
- Plating dilutions
- Incubation
- Colonies identification
- Sub-culturing
- Characterisation
- Preservation of pure strains.[24]

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1. Collection of soil sample

Fig no:5 Urine contaminated soil areas

Collect soil from the desired locations ie., urinated soil from malls, hospital and bus stand areas using sterile techniques to avoid contamination by using sterile spoon or shovel. Place the soil in sterile containers or bags and transport it to the laboratory.

Fig no:6 Soil samples collected

2. Preparation of soil suspension

Add a small amount of soil to a sterile tube containing 9ml of sterile water. Shake the mixture vigorously for several minutes to create a homogeneous suspension.

3. Preparation of dilution series

Perform serial dilutions of the soil suspension to reduce the concentration of bacteria. Take 1ml of the soil suspension and add it to a tube containing 9ml of sterile water. Repeat this process to obtain several dilutions.

Fig no:7 Dilution series

4. Preparation of agar plates

The 1.68g of nutrient agar was measured which was introduced into a conical flask and is dissolved in water. The mixture was heated to facilitate the dissolution of agar. Upon complete dissolution of the powder, the total volume was made upto 60ml. The pH of the solution was assessed utilizing pH paper, aimed for a value of approximately

7. The flask was sealed with a cotton plug, encased in paper, secured with a thread. Sterilization of the test tube was achieved by subjecting it into autoclaving at 121℃ for 20 minutes. Following autoclaving, the test tube was allowed to cool down to room temperature. The liquefied agar was transferred into sterile petri dishes, which was left undisturbed until the agar solidified.

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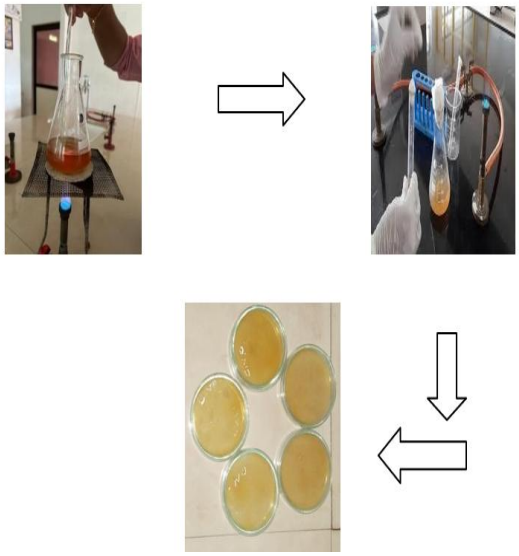

Fig no:8 Preparation of Agar plates

Fig no:9 Autoclave

5. Plating dilutions

Using a sterile micropipette, transfer 0.1ml of each dilution onto separate nutrient agar plates. By using a sterile inoculating loop streak the liquid evenly across the surface of the agar plate. The plates are properly labelled to identify the dilution.

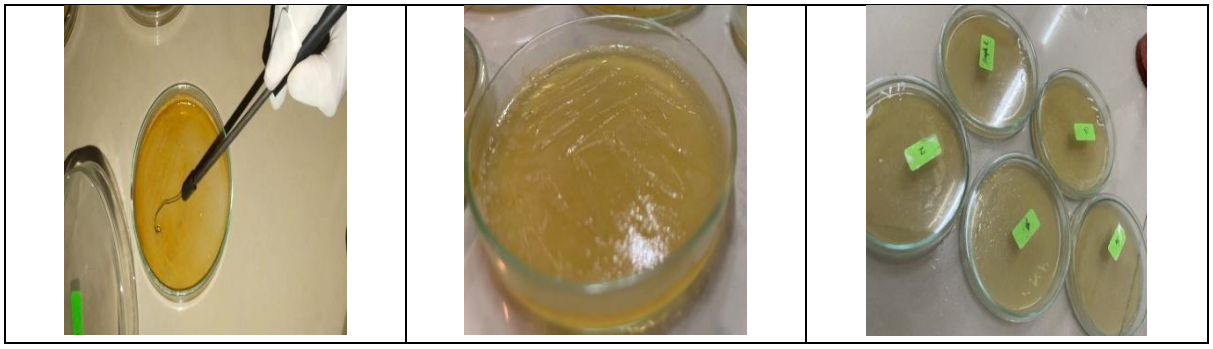

Fig no:10 Plating dilutions

6. Incubation

Invert the petri dishes to prevent condensation from dripping on to the agar surface. Incubate the plates at 30℃ for 24-48 hours. After 48 hours observe the plates for bacterial colonies.

Fig no:11 Incubation

7. Identification

Once pure cultures are obtained, perform further tests like gram staining to identify bacterial species. 8.PRESERVATION The obtained pure cultures are stored by refrigeration or freezing at -4℃.

8. Preservation

The obtained pure cultures are stored by refrigeration or freezing at -4℃.

Identification & morphology of bacteria

Bacteria are microscopic organism they are also colourless for the most part. Improves visibility by greater contrast between the organism and the background, differentiate various morphological types (by shape, size, arrangement, etc.), determine the staining characteristic of organism and, at times, direct diagnosis of disease, and demonstrate the purity of culture. observe certain structures (flagella, capsules, endospores, etc.[7]

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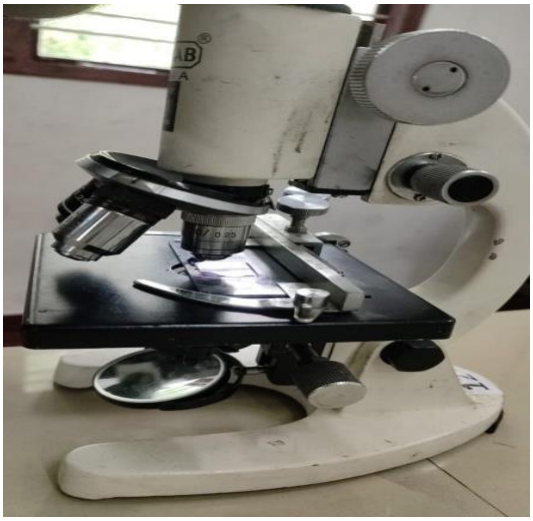

Fig no:12 Microscope

Simple staining:

Simple staining requirements

- Glass slide and coverslip
- Inoculation loop
- Dropper
- Staining rack
- Compound microscope
- Methylene blue
- Glycerin
- Bunsen burner

Simple staining- Procedure

1. Select oil/ grease free slide. Do it by washing with detergent and wiping the excess water and then dry the slide by passed through flame.
2. The slide is allowed to dry and smear of sample is applied.
3. After air drying, the slide is rapidly passed through a flame for three to four times for heat fixation.
4. After the heat fixation the slide is flooded with the stain methylene blue and is allowed to react for three minutes.
5. Further the slide is washed under running water.
6. The slide is air dried and watched under oil immersion microscope.

Gram staining:

Gram staining -Requirements

Gram-staining is a four-part procedure.

The specimen is mounted and heat fixed on a slide before proceeding to stain it.

The reagents required are:

- Crystal Violet (the Primary Stain)
- Iodine Solution (the Mordant)
- ethanol (Decolorizer)
- Safranin (the Counter stain)
- Water (preferably in a squirt bottle)

Gram staining-Procedure

1. The bacteria are first stained with the basic dye crystal violet (primary stain). Both gram-positive and gram-negative bacteria become directly stained and appear purple after this step.

2. The bacteria are then treated with gram's iodine solution (mordant). This allows the stain to be retained better by forming an insoluble crystal violet-iodine complex, called as 'iodine lake'. Both gram-positive and gram-negative bacteria remain purple after this step.

3. Gram's decolorizer, a mixture of ethyl alcohol and acetone, is then added. This is the differential step. Gram-positive bacteria retain the crystal violet-iodine complex while gram-negative are decolorized.

4. Finally, the counter stain safranin (also a basic dye) is applied. Since the gram positive bacteria are already stained purple, they are not affected by the counter stain. Gram-negative bacteria, that are now colorless, become directly stained by the safranin. Thus, gram-positive appear purple, and gram-negative appear pink.

Gram-positive bacteria show blue or purple after gram-staining in a laboratory test. They have thick cell walls. Gram-negative bacteria show pink or red on staining and have thin walls. They release different toxins and affect the body in different ways.[9]

Antibiotic susceptibility testing

Agar well diffusion assay:

1. Preparation of antibiotic solutions

The desired concentration of antibiotic solution is determined based on the assay requirements and it should be appropriate for the expected sensitivity of the bacterial strain being tested. Ampicillin is obtained in powder form and therefore stock solution is prepared by dissolving appropriate amount of AMP in a small volume of sterile distilled water and ensured the AMP powder is completely dissolved by gentle swirling and sterile it.

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Fig no:13 Antibacterial samples collected

2. Preparation of wells in agar plates

Three separate Nutrient agar plates are taken for AMP, CIP and LFX and wells are created in the solidified agar plates using a sterile cork borer. The wells should not be too close to the edge of the plate to prevent zone of inhibition.

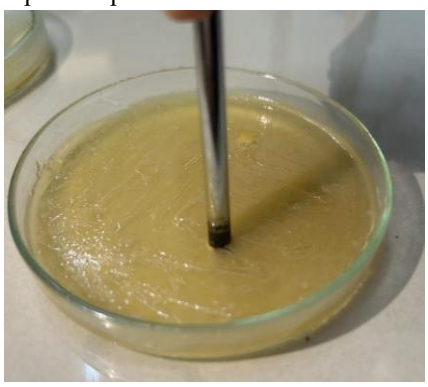

Fig no:14 Preparation of wells in Agar plates

3. Add antibiotics to the wells in agar plates

Fill each well with specific antibiotic solution of AMP, CIP and LFX using a sterile micropipette. Use the same volume volume of consistency for the three antibiotics. After adding antibiotics, kept it in refrigeration for 1 hour for diffusion.[17]

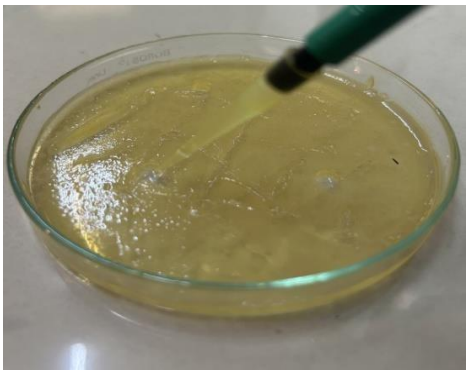

Fig no:15 Adding antibacterial samples using micropipete

4. Incubation

Take it out of refrigeration and let it come to room temperature. Incubate the plates at an appropriate temperature of 37℃ for the bacterial stain for 24 hours in a BOD incubator.

Fig no:16 B.O.D Incubator

5. Observation and measurement

After, incubation measure the diameter of the zone of inhibition around each well and noted down on a tabular column. This clear zone indicates where the antibiotic has inhibited bacterial growth.

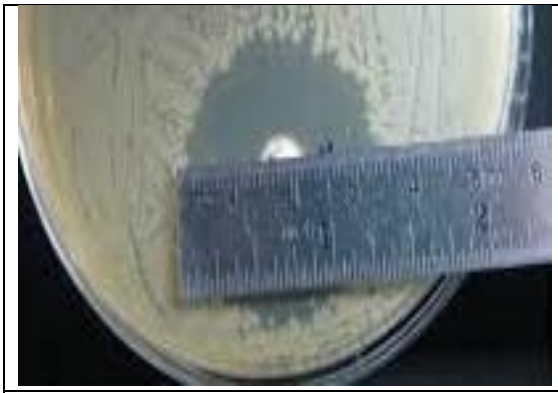

Fig no:17 Measuring diameter of Zone of inhibition

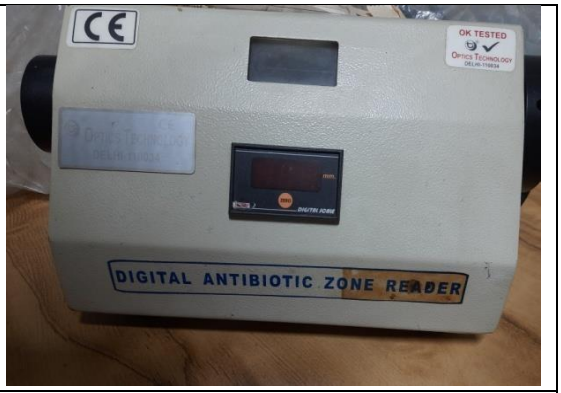

Fig no:18 Antibiotic Zone Reader

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Interpretation

The size of the zone of inhibition correlates with the sensitivity of the bacteria to the antibiotic. Larger zones indicate higher sensitivity, while smaller or non-existent zones suggest resistant. By comparing the zone of inhibition, we can determine the relative effectiveness of each antibiotic against the soil bacterial culture.

4. RESULT AND DISCUSSION

RESULTS

Antibiotic resistance in urinated soil is a growing concern as human and animal urine can introduce antibiotic residues into the environment, promoting the development of resistant bacteria. This poses a significant public health threat by potentially transferring resistant genes to pathogens, complicating infections and treatment. This resistance can spread through water, food, and direct contact with soil. Consequently, it undermines the effectiveness of antibiotics, leading to more severe and harder-to-treat infections. Factors affecting antibiotic resistance in urinated soil bacteria include the concentration of antibiotics in urine, soil microbial composition, and environmental conditions like pH, temperature, and moisture. Overuse and misuse of antibiotics, genetic mutations in bacteria, and horizontal gene transfer also play significant roles. Inadequate infection control measures further contribute to the spread of resistance. The agar well diffusion method is used to detect antibiotic resistance in soil bacteria by observing growth inhibition around antibiotic wells. Staining techniques are then performed to study bacterial morphology. These combined methods help assess resistance and characterize bacterial structure. The result of our study are presented in tables below, including identification of bacteria and analysis of antibiotic using agar well diffusion method of antibiotics.

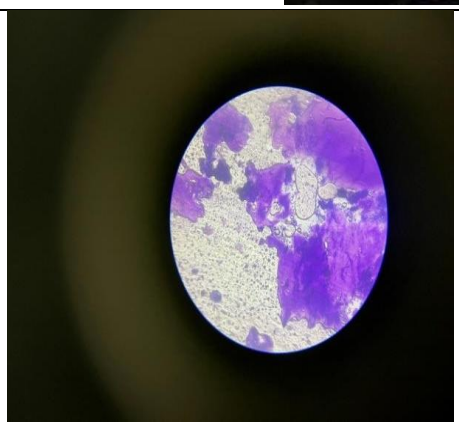

Fig no:19 Simple staining result

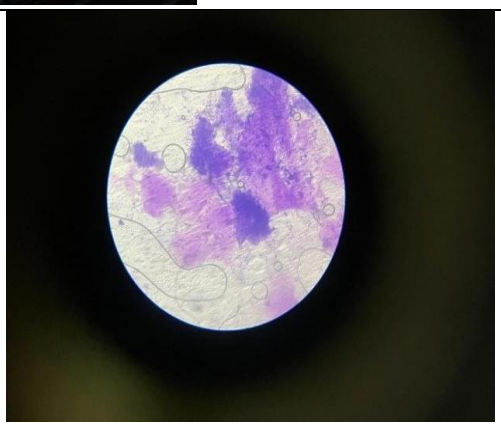

Fig no:20 Gram staining result

STAINING METHOD	RESULT	INTERPRETATION
SIMPLE STAINING	ROD SHAPED, BLUE COLOURED CELLS	BACILLI
	ROUND SHAPED, BLUE COLOURED CELLS	COCCI

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GRAM STAINING	PINK ROD SHAPE	GRAM NEGATIVE BACTERIA
	PURPLE COLOURED COCCI	GRAM POSITIVE BACTERIA

Table No. 3 Simple and gram staining results

Fig no:21 Zone of inhibition of Sample 1

Fig no:22 Zone of inhibition of Sample 2

Fig no:23 Zone of inhibition of Sample 3

The results of agar well diffusion assay are as follows:

Sample no:	Antibiotic	Zone of inhibition for standard (mm)	Concentration of Standard (mg/ml)	Volume applied for soil sample (ml)	Zone of inhibition (mm)
1	AMPICILLIN	16mm	10	0.1ml	12mm
2	CIPROFLOXACIN	28mm	10	0.1ml	25mm
3	LEVOFLOXACIN	26mm	10	0.1ml	23mm

Table No. 4 The results of the agar well diffusion assay

Ampicillin

- Zone of inhibition is 12mm.
- When compared to Standard Ampicillin the zone of inhibition of Sample no:1 shows lesser activity, suggesting the presence of ampicillin-resistant bacteria. This could be due to mechanisms like beta-lactamase production or reduced permeability of the bacterial cell wall, which is common in various bacterial species.

Ciprofloxacin

- Zone of inhibition is 25mm
- When compared to standard Ciprofloxacin the zone of inhibition of Sample no: 2 shows lesser activity, indicating that most bacteria present are susceptible to this antibiotic. Ciprofloxacin, a fluoroquinolone, effectively inhibits DNA gyrase, preventing bacterial DNA replication.

Levofloxacin

- Zone of inhibition is 23mm
- When compared to standard Levofloxacin the zone of inhibition of Sample no:3 shows lesser activity, with a slightly smaller zone of inhibition. This indicates that levofloxacin is also effective against the bacterial population, likely due to its similar mechanism of action in inhibiting bacterial DNA processes.

5. CONCLUSION

An in vitro comparative study on antibacterial resistance in soil bacteria isolated from urinated soil was conducted. Microbial assays and staining techniques were utilized to estimate the zones of inhibition and analyze bacterial morphology when treated with three specific antibiotics: ampicillin, levofloxacin, and ciprofloxacin. The study revealed differences in the diameters of the zones of inhibition, indicating varying levels of resistance in bacterial communities to each antibiotic. The assays showed that the zone of inhibition was lower for Ampicillin (12mm) which exhibits that AMP shows lesser activity towards bacteria, while having more diameter of zone of inhibition of ciprofloxacin(25mm) and levofloxacin(23mm), when compared to AMP. This leading to the conclusion that ampicillin showing lesser activity towards urinated soil bacteria than Ciprofloxacin and Levofoxacin. When comparing the zone of

inhibition, Ampicillin, Ciprofloxacin and Levofloxacin show less activity against bacteria isolated from urinated soil than standard antibacterial drugs. This suggests that the Gram-negative bacteria likely possess protective mechanisms such as outer membrane and beta-lactamase enzymes, whereas Gram- positive bacteria might have altered Penicillin Binding Proteins or produce beta-lactamases. These findings highlight the importance of conducting thorough antibiotic susceptibility testing to determine effective treatment options and manage resistance. Selecting the right antibiotic based on these tests is crucial for treating infections, as different bacteria may require different therapeutic strategies.

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