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Review Article

A comprehensive review on in-vitro methods for anti- microbial activity

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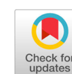

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ABSTRACT

Microbes, also referred to as microscopic creatures because of their minuscule size and invisibility to human sight, are single-celled microorganisms. The first microorganism was discovered between 1665 and 1683 by Robert Hooke and Antoni van Leuwenhoek. There are two categories of techniques for examining microbial activity. methods used in vitro and in vivo. Experiments and tests carried out in a laboratory setting, typically without the presence of a living organism, are referred to as in vitro methods. By creating microbial cultures, cell lines, cell cultures, etc., we may do in vitro experiments. Scientists can study biological processes, assess the effects of medications, and understand systems without having to deal with the complexities of a whole organism by employing in vitro techniques. Growing cells outside of the body in a controlled setting for a range of studies is known as cell culture. Tissues are grown and kept in a lab setting for tissue culture. It is essential to research creating anti-infection therapies. To evaluate a substance's antibacterial activity, in vitro methods such as the brothdilution method and discdiffusion method are commonly used. There are different methods available for the testing of antimicrobial activity and the methods are the agar disk diffusion method, Agar well diffusion method, cross streak method, Bioautography, dilution method, Time kill test, ATP bioluminescence etc. Techniques for determining novel antimicrobial agents and testing for antibiotic susceptibility have been employed. Compared to in-vivo procedures, in vitro methods are less costly. To guarantee an accurate experimental approach, these techniques may be the answer.

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1. Introduction

Microbes can exist as cell colonies or as single-celled organisms. Because of their tiny size and inability to visualise with the unaided eye, germs are also familiar as microscopic organisms. A microscope is a tool that allows us to see them. Microbes play an important function because they can be both beneficial and harmful. They can cause sickness and be poisonous enough to make us sick, yet they can also be helpful in terms of human welfare. Robert Hooke and Antoni van Leuwenhoek made the first microbe discovery between 1665 and 1683. He is considered as microbiology's founder. In 1673, he made the first discovery of microorganisms and conducted the first microbe experiment. On April 23, 1663, during the Royal Society meeting.[1] Two microscopical observations were made by Mr. Hooke. Leeches in vinegar and bluish mould on a piece of leather that has become mouldy are the two microscopical observations that Mr Hooke has made. In his book "Micrographia," he was the first to describe plant cells. Levis Pasteur discovered in the 1850s that food deterioration was caused by microorganisms and developed the theory of spontaneous generation. Robert Koch found

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that microorganisms were the source of anthrax, cholera, diphtheria, and tuberculosis in the 1880s. Both friends and foes have important roles in microorganisms.

Experiments or tests carried out in a controlled laboratory environment, usually without the presence of a living creature, are referred to as in vitro. By using in vitro techniques, scientists can investigate biological processes, evaluate the effects of drugs, and comprehend systems without having to deal with the intricacies of a whole organism. The practice of cultivating cells outside of the body in a regulated environment is known as cell culture. for a variety of investigations. In tissue culture, tissues are grown and maintained in a lab environment.[2] The ability of a chemical to suppress or eradicate microorganisms such as bacteria, viruses, or fungi is known as antimicrobial activity. Research on developing treatments for infections is crucial. In vitro techniques like the broth dilution test and disc diffusion method are frequently employed to assess a substance's antibacterial activity.

2. Useful Micro-organisms

These microbes play an important part in the system. They help in the production of mineral gases (O2 and CO2) and play a role in the medicines and agriculture industry and maintaining a clean environment.[2]

1. **I n Medicines:** Antibiotics serum with antibodies and vaccines obtained from microbes.
2. **Antibiotics: -**like Penicillin streptomycin Erythromycin etc are obtained from bacteria they inhibit the growth of bacteria and are also used to cure diseases like typhoid tuberculosis and other infections.
3. **Serum: -**contains antibodies against particular pathogens. They are used to prevent specific bacterial infections and increase immunity.
4. **Vaccines: -**Vaccines protect against illnesses caused by certain diseases, such as smallpox, cholera, etc. A suspension of pathogens that have been killed makes up a vaccination. Once injected into the body, it triggers the development of immunity and antibodies.

3. In the Industry

1. **Curd preparation:** Milk is fermented and curdled using Lactobacillus bacteria to produce curd. Lactic acid is produced from lactose, the milk sugar.[2]
2. **Production of Vinegar (acetic acid):** Fermentation of sugar solution is accomplished by acetic acid bacteria.
3. **T ea and tobacco leaf ripening and curing:** Bacteria aid in the development of flavour and aroma. Production of Alcohol and Acetone: Acetic acid is converted to alcohol and acetone by Clostridium aceto-butylicum.
4. **Creation of Biogas:** Excreta from humans and animals, such as cows and buffaloes, break down to produce biogas. Gas is used in cooking and is combustible. The Gobar gas plants have two functions: they provide rich manure and fuel.[3]
5. **Regarding Agriculture:** Nitrogen Fixation: Legumes' root nodules contain Rhizobium bacteria. They improve soil fertility by fixing free nitrogen from the atmosphere into nitrates. Nitrogen fixation is the process of transforming the free atmospheric nitrogen in the soil into forms that plants can use, such as nitrates. We refer to it as nitrification.
6. **Nitrification:** The process by which Nitrobacter bacteria transform ammonia from the soil into nitrates is known as nitrification.
7. **Denitrification:** The course of change of nitrates from squanders and dead bodies into nitrogen gas is called denitrification. Like Pseudomonas.

4. Harmfulmicro-organisms: -Disease:- Bacteria are also Responsible to Cause Diseases Like

Disease	Caused by
1. Cholera	Vibrio cholera
2. Diphtheria	Corynebacterium diphtheriae
3. Dysentery	Bacillus dysenteries
4. Pneumonia	Streptococcus pneumoniae
5. Tetanus	Clostridium tetani
6. Tuberculosis	Bacillus tuberculosis
7. Typhoid	Bacillus typhosus

Bioweapons: Some pathogenic bacteria are used as bioweapons to create *Bioterrorism. Bacillus anthracis* which can cause anthrax. Bacteria causing cholera *botulism and poliomyelitis* can also be used as bioweapons.[3]

5. *In Vitro* Methods for Anti-Microbial Testing

5.1. Diffusion method

1. (a) Agardisk diffusion
   (b) Antimicrobial gradient assessment (E-test)
   (c) Agarwells diffusion
   (d) Cross streak method

5.2. Thin layer chromatography (TLC)

1. (a) Agar diffusion
   (b) Bio-autography

5.3. Dilution method

1. (a) Broth dilutions
   (b) Agar dilution method
   (c) TimeKill (TimeKill Curve)
   (d) ATP bioluminescence Assay

(e) Flow Cytofluorometric technique

5.4. Diffusion method

5.4.1. Agardisk diffusion

Since its invention in 1940, the agreed tech-nique for conducting routine anti-microbial suscepti-bility in many clinical micro-biology labs is agardisk-diffusion testing. The Clinical and Laboratory Standards Institute (CLSI) currently issues recognized and certified standards for bacterial and yeast testing. These standards include specific guidelines for using designated culture mediums, diverse incubation conditions, and interpretation criteria for inhibition zones.[4] While this method may not apply to all fastidious bacteria, it has been standardized to assess a range of prevalent fastidious bacterial pathogens. These include streptococci, Haemophilus influenzae, Haemophilus parainfluenzae, Neisseria gonorrhoeae, and Neisseria meningitidis.

Principle: Risk of antibiotic impregnation. diffusion of an antibiotic absorbed in water with the aid of agar media. A defined quantity of antibiotic is utilized to produce a clear one in agar near the disc. Mueller-Hinton Agar is the material used in the agar diffusion method (MHA).[4] Because it helps develop the majority of bacterial pathogens and has low concentrations of sulfonamide trimethoprim and tetracycline inhibitors, it is the best material for the test.

5.4.2. Preparation of medium

1. scheme MHA (using deionized or refined water). To completely break up the medium, heat with successive disturbances and bubbles.
2. Autoclave for 15 minutes at 121°C to sanitize. Check the pH after sterilization; it should range from 7.2 to 7.4 at room temperature.
3. After bringing the agar medium down to 40–50°C, let it solidify.
4. before using dry plates in a hatchery at 30-37oC for no more than 30 minutes.[4]
5. Inoculum Preparation: - (i) Take 4-5 colonies with a wire loop from isolated culture of bacteria. Place colonies in 5 millilitres of either 0.9% saline or Trypticase soy broth.
6. Incubate the broth at 30°C to achieve the cloudiness or 0.5 Macfarland standard (Combine 0.5 millilitres of 0.048 M BaCl2 with 99.5 millilitres of 0.36 NH2SO4.)
7. Assess the turbidity of the bacterial suspension against a white background with a contrasting black line, comparing it to the turbidity of 0.5 MacFarland under light.
8. Introduce sterile saline or broth to diminish the turbidity.

6. Inoculating Plates Procedure

1. Immerse a sterile cotton swab in a standardized microorganism suspension. Remove excess inoculum by gently tapping the swab against the tube's wall.
2. Apply the microorganism to the agar, rotate the plate by 60 degrees, and repeat the rubbing process (repeat twice).
3. Permit the medium's surface to air-dry for a duration of three to five minutes, ensuring not to exceed fifteen minutes, to facilitate absorption of any additional moisture.[5]

7. Procedure

7.1. Placing the antibiotic disk on the plate

1. Employ sterile forceps or a disk dispenser to place the antibiotic disk onto the surface of the plate. Ensure gentle pressure to ensure full contact between the disk and the agar surface.
2. Apply gentle pressure to guarantee that the antibiotic disk and the agar surface are in complete contact.
3. In a 9-cm Petri dish, position a maximum of six disks, and for 150-mm plates, arrange up to twelve disks.
4. Ensure a minimum centre distance of 24 mm between disks, and they should not be positioned closer than 10 to 15 mm from each other.
5. After 16 to 18 hours, check for inhibition by keeping the plates inverted and observing them at 30°C.[5]

7.2. Antimicrobial gradient method (E-test)

The method for determining antibiotic resistance is called an exponential gradient method. Both antibiotic dilution and antibiotic diffusion into the medium are used in this quantitative approach.[6]

8. Equipment and Materials

1. Mueller-Hinton agar (MHA) media
2. Etest strips
3. McFarland standard 0.5
4. Forceps and Sterile cotton swabs
5. Sterile saline, 4ml volume tubes
6. Bacterial strain for testing (Non-Fastidious cultures plated out for single colonies)
7. Quality control strain: E.coli ATCC 25922.

9. Preparation of inoculum

1. Retrieve the Etest strips from the freezer (-20°C) at least 30 minutes before use.
2. Emulsify three or four distinct test strain colonies and transfer them to a saline tube.
3. Adjust the turbidity of the inoculum to meet the 0.5 McFarland standard by visual comparison.[5]

10. Inoculation in Muller Hinton Agar

1. Gently select a sterile cotton swab, dip it into the inoculum, and rotate swabs inside tubes to remove excess liquid.
2. Turn the agar plate 60 degrees and keep the lid on for five to fifteen minutes.
3. Spread the swab over the entire agar surface.[6]

11. E-test Application

1. Open the Etest package and use forceps to attach strips to the agar surface.
2. Position the strips with the E end near the plate's edge and visible.
3. Avoid touching other areas of the strips; use formats to place 4 to 6 strips on a 150mm plate or one strip on a 90mm plate.
4. Do not remove or replace a strip once it has touched the agar.
5. Repeat the entire process for the quality control strain.
6. Incubate the plate at 37°C for 18 - 24 hrs.

11.1. AgarWell diffusion method

1. This method is utilized in antimicrobial tests to assess the inhibitory capacity of plant extracts and secondary metabolites against bacteria.
2. Similar to the disk diffusion strategy, the surface of the agar plate is inoculated with a measured microbial inoculum distributed across the entire agar surface.[7]

12. Experimental Procedure

12.1. Medium preparation

Prepare the medium and incubate at 37 degrees Celsius.

12.2. Inoculation of bacteria

Introduce a loop containing tested bacteria (e.g., S. aureus and E. coli) into 2-3 millilitres of Mueller-Hinton Broth (MHB).

Incubate the bacteria at 37 degrees Celsius for 24 hours.

12.3. Bacterial turbidity adjustment

After 24 hours, remove the bacteria and add McFarland solution (barium chloride + sulfuric acid) to match turbidity, ensuring an approximate number of bacteria on each plate.[7]

12.4. Inoculation on mueller-hinton agar (MHA) plates

Transfer bacteria onto MHA plates with continuous rotation at 60 degrees Celsius using the cotton bud method.

Allow the plates to dry.[7]

12.5. Well creation and sample application

Use a Borer to create a well on the dried MHA plate.

Add the tested extract, positive control (e.g., Neomycin, depending on the protocol), and negative control (e.g., DMSO) to the well.[7]

12.6. Incubation

Incubate the plates for 24 hours at 37 degrees Celsius.

12.7. Observation

After 24 hours, observe the plates.

Measure the zone of inhibition, which indicates the extent to which the extract inhibits bacterial growth.[8]

1. ***Controls:*** Neomycin is used as a positive control, DMSO as a negative control, and the tested extract is the experimental condition.
2. ***Result:*** This process allows for the assessment of the antimicrobial activity of the extract by observing the inhibition zones around the well on the agar plates after incubation.

12.8. Cross streak method

A pure strain of a single species of microorganism is inoculated using it. To disperse millions of cells across a solid medium's surface, an inoculating loop is employed. to separate some particular cells from the rest of the population. Developing into an isolated colony, these cells multiply and thrive. Pure culture will be sourced from one or more colonies that are kept well apart from the rest.[9]

Material Required:- Streak plate, Nutrient agar plates(NA), Bunsen burner, Bacteriological loop.

13. Procedure

1. Inspect the streak plates that have been prepped and recognize well-isolated colonies. Transfer a segment of each colony to an individual agar plate.
2. Using an inoculating loop, pick a colony. Conclude the loop's movement with the isolated cluster after sterilising it in the burner flame for three to five seconds.
3. Selecting the NA plate and removing the microorganisms from the colony.[10]
4. The right hand's loop and the NA plate are injected. Remove the plate's cover, then apply a sweeping motion to one edge of the plate to position the inoculating loop.
5. Inoculate the agar using the identical tristreak method employed during the initial isolation process. Inoculate a second plate with a different colony after you've flamed the loop..
6. rovide the plates with an incubator set at 37°C.

13.1. TLC bioautography

Compounds which are having micro-organisms and produce antibiotics are called Bioautography by some chromatography techniques such as TLC. The major applications of bioautography are the fast screening of large numbers of samples for bioactivity like antimicrobial, antioxidant enzyme inhibitions etc.

There are two methods of TLC bioautography

14. Agar diffusion or Contact Bioautography

14.1. Antimicrobial application

Apply the antimicrobial sample onto a developed TLC plate or paper.

14.2. Chromatogram placement

Position the chromatogram face down onto an inoculated agar plate.

Allow a specific duration for the antimicrobial substance to diffuse into the agar.

14.3. Chromatogram lifting

Lift the chromatogram after the designated diffusion period.

14.4. Inoculation of new agar layer

Inoculate a new agar layer with the lifted chromatogram.

14.5. Observation of zone of inhibition

Observe the zone of inhibition on the agar surface, corresponding to the spot in the plate This zone indicates the presence of antimicrobial substances.

Incubation: Incubate the inoculated agar plate for microbial growth.

The incubation period typically spans between 16 and 24 hours, although it can be shortened to 5-6 hours through spraying with 2,6-dichlorophenol-indophenol.

15. Result

This Agar Diffusion or Contact Bioautography method provides a visual representation of antimicrobial activity by detecting zones of inhibition on the agar surface, indicating the presence of bioactive compounds. The process facilitates a direct correlation between chromatographic spots and antimicrobial effects.

15.1. Direct bioautography

15.2. Preparation of TLC plates

Prepare TLC plates for the bioautography process.

16. Application of Microbial Suspension

Spray or dip a bacterial or fungal suspension onto the prepped TLC plates. Use a test fungus or bacteria suspension for this process.

16.1. Incubation

Place the bioautogram in a humid environment at 25 degrees Celsius for 48 hours to allow microbial growth.[14]

16.2. Visualization using tetrazolium salts

Utilize tetrazolium salts to visualize the microbial growth.

Dehydrogenases from living organisms transform these salts into vibrantly coloured formazan.

16.3. Detection of antibacterial activity

Observe the TLC plates for distinct white zones against a purple background.

The antibacterial activity of the sample is indicated by these white zones, suggesting inhibition of microbial growth.[14]

Result: This direct TLC bioautography method serves as a valuable tool for assessing antibacterial activity in samples, and the visualization using tetrazolium salts helps identify areas of microbial growth inhibition on the TLC plates.

16.4. Dilution methods

The determination of Minimum Inhibitory Concentration (MIC) values, crucial in assessing antimicrobial effectiveness, involves dilution tests. These tests are performed in either agar (agar dilution) or broth medium (Macrodilution or microdilution). Both methods allow for quantitative analysis of in vitro antimicrobial activity.[11]

16.5. Broth dilution method

1. Dilute the drug and add it to Mueller-Hinton broth in tubes.
2. Inoculate the tubes with a standardized suspension of test bacteria, ideally a known-sensitive organism.
3. Incubate the tubes at 37°C for 16-18 hours (or at 30°C for Methicillin MIC determination).
4. Determine MIC by identifying the lowest drug concentration with no visible growth.
5. To find Minimum Bactericidal Concentration (MBC), subculture from tubes with no growth onto nutrient agar plates without antimicrobial agents.
6. Identify the lowest drug concentration that fails to show growth in subculture as the MBC.

Table 1: The invitro methods for antimicrobial testing

Methods	Growth medium	Description	Incubation temp.&	time	Advantages	Disadvantages	References
Disk diffusion method	Bacteria- MHA Yeast- MHA+GMB Molds- non-supplemented MHA	Measured is the diameter of the inhibitory hole in the disk or wells holding the extracts. The activity is better the larger the diameter.	Temp. 37°C Time- 24 hrs	35- 16-	Screening of more than 6 extracts per plate is feasible. Cost-effective.	Not applicable to non-polar samples. -Challenging to implement in High Throughput Screening (HTS).	4,5
Broth microdilution test	-RPMI1640 growth medium with glucose. -Suborned dextrose broth	Incubate with varying concentrations of the extract, plate the samples, and count the colonies on agar. A reduced number of colonies indicates a more potent activity.	Temp. 37°C Time bacteria 20hrs yeasts 50 hrs	35- for and 46-	Benchmark Minimum Inhibitory Concentrati on (MIC) can be determined	Insoluble samples that might disrupt readings. Adjustments may be required in the presence of fungal growth.	11,12
Time-kill test	MHA stands for Mueller Hinton Agar, while MHB stands for Mueller Hinton Broth.	Agar plates are spread out to calculate the survivor colony count (CFU/ml) of the control and antimicrobial agent sample, which is cultured at intervals of 0, 4, 8, 10, 12, and 24 hours. A higher level of activity is indicated by fewer colonies.	Temp. 37°C Time- 24 hrs.	35- 16-	It is possible to determine MICs and identify synergistic and antagonistic interactions.	In the presence of fungal growth, adjustments may be necessary. The process can be labor- intensive.	12,13
TLC bioautograph y	Maltagar, Filamentous fungi & yeast	Inoculate fungal cells into melted agar. Apply the mixture to a TLC plate along with plate extract. Incubate and stain to visualize inhibition bands.	Temp. - higher than room temp. Two to three days	This is consistent with the production of spores using fungi. Inexpensive and simple.	Difference in obtaining complete contact b/w agar and TLC plate.	10,14	

16.6. Agar dilution test

1. Prepare test tubes with sterile distilled water and perform a 10-fold or 2-fold dilution series.
2. Prepare plates with melted agar and varying concentrations of antibiotics.
3. Inoculate plates with the test organism and incubate for a suitable period.
4. Determine MIC by identifying the lowest antibiotic concentration inhibiting visible growth.
5. This method is convenient for testing multiple strains simultaneously, developed in the 1920s and refined in the 1940s.

16.7. Time kill test

The time-kill assay assesses antibiotic pharmacodynamics by monitoring bacterial viability over time, revealing concentration-dependent and time-dependent bactericidal activities.

17. Requirements

1. In a microtiter plate, one row (Row A) contains the antibiotic alone, while another row (Row B) holds a combination of two agents.
2. Determine the antibiotic's Minimum Inhibitory Concentration (MIC) for the tested strain.
3. Dilute the antibiotic stock solution to four times the MIC (4MIC).
4. Perform serial double dilutions by adding 0.5 ml Mueller-Hinton broth and 0.5 ml of the previous concentration, resulting in concentrations double that of MIC (2MIC).
5. In each well of one row, add decreasing concentrations of antibiotic solutions (4MIC, 2MIC, and MIC).
6. In another row, add 100 μl of antibiotic concentration (4MIC, 2MIC, and MIC) in each well.
7. Perform bacterial viability quantification at different time intervals, typically in 96-well microtiter plates, presenting results graphically on a semi-log plot.
8. In certain cases, additional concentrations such as half of the MIC and a $\frac{1}{4}$ of the MIC (0.5MIC and 0.25MIC) can also be included in the assay.

18. Procedure

1. To prepare the stock solution, arrange tubes containing 0.5 ml of sterile water.
2. Utilize a double dilution technique by adding 0.5 ml of the stock solution to 0.5 ml of sterile water. Repeat the technique by transferring 0.5 ml to the next tube to achieve the desired Minimum Inhibitory Concn. (MIC).
3. To support bacterial growth, add 0.5 ml of Mueller-Hinton broth (MHB) to each tube. The appropriate concentrations should be arranged in dwindle order: equal to four times the MIC (4MIC), double MIC (2MIC), MIC concn. $\frac{1}{2}$ the MIC (0.5MIC), and $\frac{1}{4}$ MIC (0.25MIC).
4. For the microtiter plate assay, follow the protocol by adding 100 μl of the antibiotic solution to wells filled with 100 μl of the 0.5MIC stock solution. Subsequent rows should be filled with200μl of stock solution, gradually decreasing the concn. as primed above.[12]
5. The affirmative control should contain 200 μl of MHB and the bacterial solution, while the dissentious control should consist of 200 μl of MHB. Add 20 μl of the prepared bacterial solution to single well, excluding the dissentious control, and incubate at 37°C.

18.1. Colony counting

During the incubation period from 0 to 24 hours, the first 10 μl of solution was ingested at multiple times from every well. These aspirated samples were then dil.to 1:10 in PBS. Subsequently, the A blood agar plate had been daubed with diluted samples, and the plates were incubated for 24 hours in an incubator set at 37°C. After the incubation period, the colonies on the agar plates were counted, and the data should be recorded in an Excel sheet. To generate a timekill curve, a semilog plot would be created. In this plot, the colony counts would be represented as the logarithm base 10 (log10) of Colony Forming Units per millilitre (CFU/ml).

18.2. ATP bioluminescence method

Antimicrobial susceptibility tests have been accomplished swiftly with ATP bioluminescence- based approaches. The four steps of the eager ATP bioluminescence-based approach are as follows: 1. Preparation of bacterial culture. 2. Reduce extracellular ATP in the broth. 3. After incubation, intrinsic ATP in bacteria can be released with the use of an ATP-releasing reagent, which also turns off the ATP-eliminating reagent.4. ATP levels of this solution utilizing ATP bioluminescence as a gauge.[13]

19. Procedure

1. Collect the sample from the surface or area you want to test for microbial contamination, it can be done by using swabs or other suitable methods.
2. Use a specialized reagent to extract ATP (adenosine triphosphate) from the collected samples. ATP is a molecule found in all living cells and is an indicator of microbial activity. The Reactant that was used in the test contains an enzyme called Luciferase.
3. Add a Luciferin-Luciferase reagent to the extracted ATP. This reaction produces light (bioluminescence) in proportion to the amount of ATP present. ATP converts into ADP and energy is released.

4. It emits light as a by-product, it is measured to determine the level of ATP. Use a luminometer to measure the intensity of light emitted during ATP – luciferase reaction. This measurement reflects the level of microbial contamination or activity in the sample.
5. Compare the measured light intensity to the tested samples with suitable controls or standards. This helps in the determination of the level of microbial contamination or effectiveness of antimicrobial contamination.[13,15]
6. ATP bioluminescence testing provides a rapid and quantitative assessment of microbial contamination. It is commonly used in hygiene monitoring, food safety and environmental testing.

19.1. Flow Cytometry antimicrobial test

It was used in World War II by the U.S. Army in an experiment for the detection of bacteria and spores. The instrument could be used with biological samples (bacteria). FCM is used as a tool for studying mammalian cells. The detector used was a then recently developed device called a Photomultiplier tube. It allows rapid measurement of light scattered and fluorescence emission produced by suitably illuminated cells. In an antimicrobial test using flow cytometry, the goal is to assess the effectiveness of antimicrobial agents against microorganisms. This technique allows researchers to measure changes in cell viability and determine the impact of antimicrobial treatments. It's a valuable tool for studying the effects of different antimicrobial strategies.[16]

20. Procedure

1. Get your example ready: Prepare your microbiological sample (such as yeast or bacteria) first. The microorganisms might need to be cultivated and grown in an appropriate medium.
2. Setting up the treatment: Assemble diverse treatment settings, incorporating varied antimicrobial agent doses for your investigation. For comparison, it's critical to include control samples that have not received any treatment.
3. Labeling: Apply a fluorescent dye or other marker that shows the vitality of the microbial cells to their labels. This can be achieved by incorporating the dye into the specimen and permitting it to engage with the cells.
4. Analyze the labelled material using flow cytometry: A flow cytometer is a specialized device that uses a laser beam to examine individual cells. Detectors measure the fluorescence that is released when the fluorescent dye is excited by a laser.
5. Collecting data: Gather data on the light emitted by each cell. The flow cytometer measures the fluorescence intensity, which allows you to determine the cell viability.
6. Analyzing the collected data will allow you to ascertain the percentage of live cells in each treatment condition. Compare the treated samples' viability to that of the control samples to determine the antimicrobial agent's effectiveness.

21. Conclusion

Due to the widespread prevalence of microbial resistance in contemporary antimicrobial drugs, microbial infections have emerged as a major global health challenge. Various techniques for identifying new antimicrobial agents and assessing antibiotic susceptibility have been implemented and continue to evolve. In vitro methods, being more cost-effective than in- vivo procedures, have gained prominence. Several standardised methods by organisations like CLSI and EUCAST provide a foundation for accurate experimental approaches, offering potential solutions to address the ongoing issue of microbial resistance.

22. Source of Funding

None.

23. Conflict of Interest

None.

References

1. Fuerst JA. Microorganisms—A Journal and a Unifying Concept for the Science of Microbiology. *Microorganisms*. 2014;2(4):140–6.
2. Pitt TL, Barer MR. Classification, identification and typing of micro-organisms. *Med Microbiol*. 2012;p. 24–38.
3. Doley K, Terkar A, Borde M. Applications of microorganisms in Agriculture. In: Microbial Technology for Health and Environment Microorganisms for sustainability. vol. 22. Singapore: Springer; 2020. p. 410.
4. Balouiri M, Sadiki M. Methods for in vitro evaluating antimicrobial activity: A review. *J Pharm Anal*. 2015;6(2):3–9.
5. CLSI document M02-A11. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500; 2012. Available from: https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf.
6. Pastor RG, Carrera-Pacheco SE. Current Landscape of Methods to Evaluate Antimicrobial activity of natural extracts. *Molecules*. 2023;28(3):1068.
7. Ansari J, Prakash K, S. Agar well diffusion : A prominent method for In vitro Screening of antimicrobials. *Int J Botany Sci*. 2021;6(2):836–9.
8. Pereira SV, Kamat NM. Antimicrobial screening of Actinobacteria using a Modified Cross-Streak Method. *J Pharm Sci*. 2011;73(2):223–8.
9. Dubale S, Kebebe D. Phytochemical Screening and Antimicrobial activity Evaluation of selected medicinal plant in Ethiopia. *J Exp Pharm*. 2023;15:51–62.
10. Weinstein M. Methods for Dilution Antimicrobial Susceptibility test for Bacteria. *J Antimic Agents Chemother*. 2018;7(3):21–50.
11. ngroff AE, Chaturvedi V, Fothergill A. Optimal testing conditions for determining MICs and minimum fungicidal concentrations of new and established antifungal agents for uncommon molds: NCCLS collaborative study. *J Clin Microbiol*. 2002;40(10):3776–81.
12. Methods for Antimicrobial Dilution and Disk Susceptibility of Infrequently Isolated or Fastidious Bacteria, Approved Guideline, 2nd;

2010. Available from: https://clsi.org/media/1450/m45ed3_sample.pdf.

13. Beckers B, Lang HR, Schimke D. Evaluation of a bioluminescence assay for rapid antimicrobial susceptibility testing of mycobacteria. *Eur J Clin Microbiol*. 1985;4:5561.
14. Pfaller MA, Sheehan DJ, Rex JH. Determination of fungicidal activities against yeasts and molds: lessons learned from bactericidal testing and the need for standardization. *Clin Microbiol Rev*. 2004;17(2):268–80.
15. Green L, Petersen B, Steimel L. Rapid determination of antifungal activity by flow cytometry. *J Clin Microbiol*. 1994;32(4):1088–91.
16. Ramani R, Ramani A, Wong SJ. Rapid flow cytometric susceptibility testing of Candida albicans. *J Clin Microbiol*. 1997;35:2320–4.

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