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IN VITRO METHODS TO STUDY ANTIBACTERIAL ACTIVITY

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ABSTRACT

Antimicrobial resistance (AMR) has become a significant global public health concern. Identifying resistant microorganisms is essential for selecting effective treatment options and controlling the transmission of resistant strains. This manuscript summarizes existing and upcoming technologies for detecting microbial resistance and highlights the strengths and limitations of each approach. Microbiological culture and susceptibility testing methods, such as Disk Diffusion, Agar well diffusion, Epsilometer (E test),Tube dilution methods, Chromogenic agar media for rapid detection of AMR pathogens have been employed to identify AMR bacteria. We also examine the non conventional automated methods such as (MALDI-TOF) spectroscopy, VITEK-2 System, Microscan walk away method, Fourier transform infrared spectroscopy (FTIR),BD Phoenix automated microbiology system in antimicrobial resistance, we also summarize the future techniques used in microbial resistance (AMR). By understanding the strengths and limitations of these technologies, stakeholders—including researchers, healthcare professionals, regulatory bodies, health authorities, financial managers, and patients—can make informed decisions that help prevent the emergence and spread of antibiotic-resistant strains, thereby ultimately enhancing patient safety.

Keywords: Antimicrobial Resistance (AMR), AST, Antimicrobial Susceptibility Testing Conventional Methods, Automations, Genotypic Methods.

1. INTRODUCTION

The emergence of antimicrobial resistance (AMR) has become one of the most critical challenges in the management of infectious diseases, jeopardizing major advances achieved within healthcare systems in the new millennium. For successful treatment of infectious diseases, prior knowledge about an organism's susceptibility to relevant antimicrobial agents gained through in-vitro testing is a prerequisite-site. Identification of microbes (ID) and their antimicrobial susceptibility testing (AST) are two key tasks performed by the clinical microbiology laboratories to guide therapeutic choices of antimicrobial agents. Without such knowledge, empirical therapy may result in treatment failure or the emergence of antibiotic-resistant pathogens. Since its introduction in 1929, antimicrobial susceptibility testing (AST) has remained indispensable—not only for selecting appropriate antimicrobial regimens, but also for informing antibiotic policies, supporting infection epidemiology, guiding drug discovery, and monitoring resistance trends. The purposes of AST are manyfolds (Box 1), but it is primarily intended to check the efficacy of an antimicrobial drug or natural product to maximize the best drug dose regimen. The term "antibiotic" has a more limited meaning compared to antimicrobials that act against all microorganisms including bacteria, viruses, parasites and fungi. After the revolutionary "golden era of antibiotics", these agents are being used widely not only in healthcare settings but also in food and animal industries because of their versatile nature.Over time, the improper and excessive use of antibiotics has accelerated the emergence of resistant bacterial pathogens. Currently, the impact of microbial resistance to most available antibiotics, especially emergence of multidrug-resistant (MDR) bacteria has become alarming and threatening the global health It is predicted that death due to AMR related infections could grow from 1 million of current annual approximation to 10 million by 2050 unless rigorous measures are taken to stop the antibiotic abuse and misuse. To combat the situation and to save patient's life, there is no alternative to correct identification of pathogens and to select Appropriate antibiotic through AST (Nathan and Cars, 2014.

In Vitro Methods for Anti-Microbial Testing :

1. Agar Disk diffusion method
2. Agar well diffusion method
3. Broth dilution Method
4. agar Dilution Method
5. Antimicrobial gradient test (E Test)
6. Cross Streak Method
7. Time kill Test
8. ATP bioluminescence method

Automated AST Systems And new technologies :

- VITEK 2 Systems

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- MALDI-TOF MS System
- Microscan WalkAway System
- Selux Next-Generation Phenotyping AST System

1. **Agar disk-diffusion method**:

Principle: The agar disc diffusion method works by placing antimicrobial-impregnated filter paper disks on the surface of an agar plate inoculated with a test microorganism. After incubation, the antimicrobial diffuses into the agar, creating a concentration gradient where the highest concentration is closest to the disk. If the antimicrobial is effective, it will inhibit bacterial growth, forming a clear area called a around the disk. The size of this zone is directly related to the antimicrobial's potency and can be used to determine susceptibility

Procedure :

1. **Prepare bacterial suspension:**Select 3–5 well-isolated colonies from a fresh culture.Suspend in sterile saline or broth.adjust turbidity to 0.5 McFarland standard (~1.5 × 10^8 CFU/mL).
2. **Inoculate Mueller-Hinton Agar (MHA) plate:** Dip a sterile swab into the standardized suspension.Remove excess fluid by pressing/swiping against the tube wall.Spread evenly across the agar surface in three directions to ensure a confluent lawn.Allow the plate to dry for 3–5 minutes at room temperature.
3. **Place antimicrobial discs:** Use sterile forceps or a disc dispenser.Place discs evenly spaced on the agar surface.Ensure discs are firmly in contact with the agar.
4. **Incubate:**Invert the plates to prevent condensation.Incubate at 37°C for 16–18 hours in ambient air.
5. **Measure zones of inhibition:** After incubation, observe clear zones around discs.Measure diameters in millimeters using a ruler or Vernier caliper.
6. **Interpret results :**Compare measurements with CLSI or EUCAST interpretive charts .

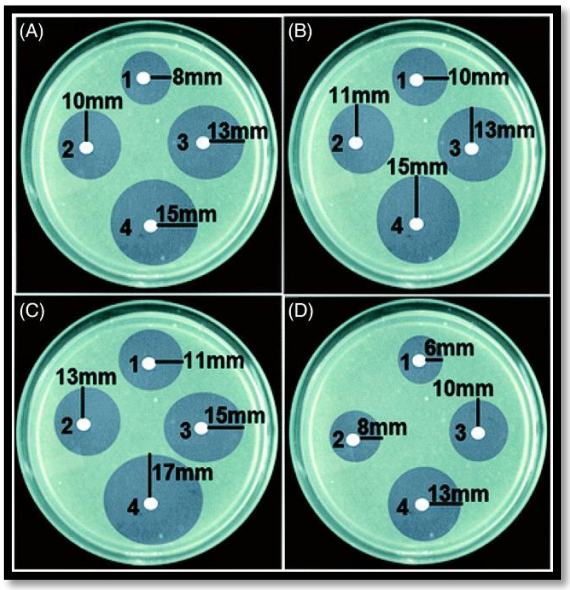

Fig 1: Agar Disk diffusion method

2. **Agar well diffusion method:**

Principle : Agar well diffusion method mainly helps in the research purpose to check the antimicrobial activity of some plant, leaf or the seed extract.The well diffusion assay is preforming in Muller Hinton agar.Similarly to the procedure used in disk-diffusion method, the agar surface plate is inoculated by spreading a volume of the microbial inoculum over the entire agar surface (lawn culture) and make the wells in the MHA with around 6-8mm in diameter.The wells are puncture with a sterile cork borer or with the backside of a sterile blue micropipette

Procedure:

1. **Medium preparation :**Prepare Mueller-Hinton medium and incubate at 37 °C.
2. **Bacterial inoculation ;**Transfer a loopful of test organisms (e.g., Staphylococcus aureus, Escherichia coli) into 2–3 mL of Mueller-Hinton Broth (MHB). Incubate at 37 °C for 24 hours.
3. **Adjusting turbidity :** After incubation, standardize bacterial density using the McFarland solution (barium chloride + sulfuric acid). This ensures a consistent number of bacteria across plates.

4. Inoculation on agar plates : Spread the standardized suspension onto Mueller-Hinton Agar (MHA) plates using the cotton swab method with rotation. Allow plates to dry.

5. **Well formation and sample loading** :Create wells in the agar using a sterile borer.

6. **Add:** Test extract (experimental sample) , Positive control (e.g., Neomycin) , Negative control (e.g., DMSO)

7. **Incubation** : Incubate the inoculated plates at 37 °C for 24 hours.

8. Observation and measurement : After incubation, examine the plates. Measure the zones of inhibition around each well to determine antimicrobial activity.

Positive control: Neomycin, to show the test works when an active antibiotic is present.

Negative control: DMSO, to confirm that the solvent itself does not kill bacteria.

3. **Broth dilution Method :**

Principal:The agar dilution and broth dilution methods are two highly similar techniques that are used for determining the MIC of antimicrobial agents. The MIC is the lowest concentration of an antimicrobial agent that inhibits the visible growth of a particular bacterium The primary distinction between agar dilution and broth dilution methods is the medium used for testing: agar dilution relies on solid agar plates, while broth dilution employs liquid broth media. Both methods involves incorporating different concentrations of the antimicrobial substance into the medium, followed by the application of a standardized number of microbial cells to the medium.The MIC is established by assessing whether microbial growth is present or absent at each antimicrobial concentration. Both agar dilution and broth dilution methods play a critical role in selecting appropriate antimicrobial therapy and in monitoring shifts in antimicrobial susceptibility over time.

Procedure :

1. **Drug Dilution** :Prepare serial dilutions of the antimicrobial agent in Mueller-Hinton broth using test tubes.
2. **Bacterial Inoculation :**Introduce a standardized bacterial suspension into each tube, ideally using a strain known to be sensitive.
3. **Incubation :**Place the tubes at 37 °C for 16–18 hours. For Methicillin MIC testing, incubate at 30 °C instead. PIdentifying MIC fter incubation, check for bacterial growth.
4. **The Minimum Inhibitory Concentration (MIC); is** the lowest drug concentration where growth is completely absent.
5. **Testing for MBC** :From tubes showing no growth, subculture onto nutrient agar plates that do not contain the drug.
6. **Identifying MBC:** The Minimum Bactericidal Concentration (MBC) is the lowest concentration where no colonies appear on the subculture plates.

4. Agar dilution test:

1. Prepare Dilutions :Set up test tubes with sterile distilled water and carry out either a 10-fold or 2-fold serial dilution.
2. Prepare Agar Plates :Pour molten agar into plates, incorporating different concentrations of the antibiotic into each. Inoculate and Incubate Add the test microorganism to the plates and incubate for an appropriate duration.
3. Identify MIC :After incubation, determine the Minimum Inhibitory Concentration (MIC) as the lowest antibiotic concentration that prevents visible growth.
4. This method, which allows testing of multiple strains at once, was first introduced in the 1920s and later refined in the 1940s.

5. Antimicrobial gradient method (E-test):

This technique, known as the exponential gradient method, is used to measure antibiotic resistance. It combines two principles: Dilution of the antibiotic to create varying concentrations.Diffusion of the antibiotic into the medium to establish a gradient. The Etest is an enhanced version of the agar diffusion method and is carried out in a way similar to the disc diffusion technique. Instead of paper discs, a plastic strip containing a predefined exponential gradient of an antibiotic is placed on the agar surface, allowing the drug to diffuse into the medium. After 24 hours of incubation with the bacterial inoculum, the Minimum Inhibitory Concentration (MIC) can be directly read from the strip at the point where bacterial growth is halted. Another method with similar functionality is the spiral gradient endpoint technique, in which a spiral plater distributes a stock solution of the antibiotic across the agar plate to create a gradient. The distance from the center of the plate to the point where growth stops is used to determine the MIC. This approach has proven effective for detecting heterogeneous vancomycin-non-susceptible Staphylococcus aureus (hVISA) and vancomycin-intermediate Staphylococcus aureus (VISA), as previously reviewed by Jorgensen and Ferraro.

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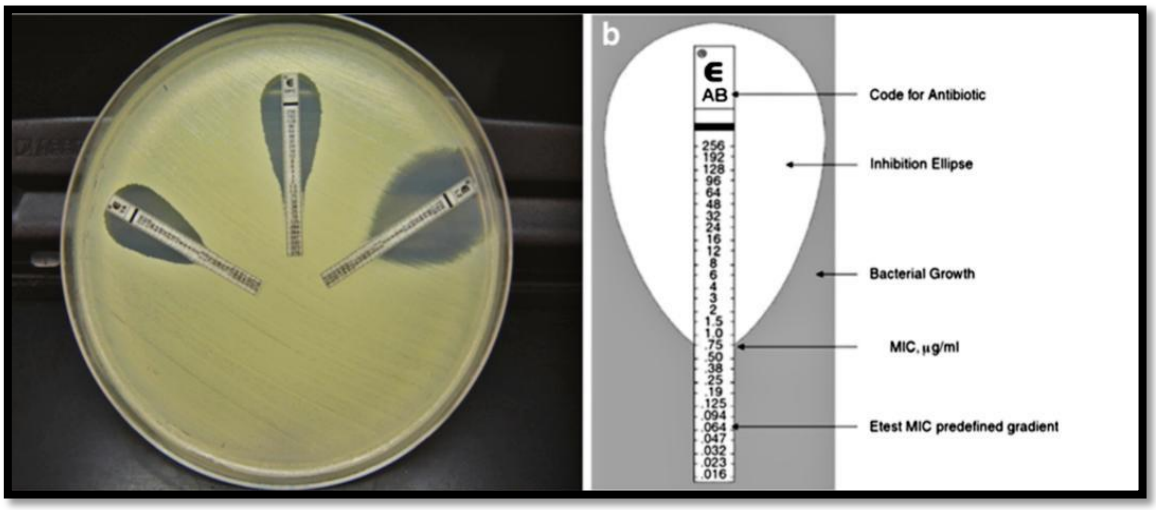

Fig 2: Antimicrobial gradient method (E Test)

6. Cross streak method:

A pure culture of a single microbial species is obtained through inoculation. Using a bacteriological loop, millions of cells are spread across the surface of a solid medium, allowing individual cells to be separated from the larger population. These isolated cells grow and multiply, eventually forming distinct colonies. A pure culture can then be derived from one or more colonies that remain clearly separated from the res**t.**

Procedure;

1. Check the prepared streak plates and locate well-isolated colonies. Transfer a portion of each colony onto a fresh agar plate.
2. Flame-sterilize the inoculating loop for 3–5 seconds, then use it to pick up a colony.
3. Choose a nutrient agar (NA) plate and place the microorganisms from the selected colony onto its surface.
4. Hold the loop in your right hand and carefully remove the plate cover. Gently sweep the loop across one edge of the agar to deposit the sample.
5. Streak the agar using the same tri-streak technique applied during the initial isolation. After re-sterilizing the loop, repeat the process on another plate with a different colony.
7. **Time Kill Test :**

The time-kill assay is used to study the pharmacodynamics of antibiotics by tracking bacterial survival over a set period. This method demonstrates both concentration-dependent and time-dependent bactericidal effects, showing how the drug reduces bacterial viability as exposure increases.

1. Preparation of Stock Solution and MIC Assay ;Stock Solution Setup :Prepare test tubes containing 0.5 ml of sterile distilled water.
2. Serial Dilution Perform a two-fold dilution by transferring 0.5 ml of the stock solution into 0.5 ml of sterile water. Continue the process by transferring 0.5 ml into the next tube until the desired Minimum Inhibitory Concentration (MIC) range is achieved.
3. Addition of Growth Medium Add 0.5 ml of Mueller-Hinton broth (MHB) to each tube to support bacterial growth. Arrange concentrations in descending order: 4×MIC, 2×MIC, MIC, 0.5×MIC, and 0.25×MIC.
4. Microtiter Plate Assay Add 100 µl of antibiotic solution to wells already containing 100 µl of the 0.5×MIC stock solution.
5. Fill subsequent rows with 200 µl of stock solution, decreasing the concentration stepwise as outlined above.
6. Controls and Inoculation Positive control (affirmative): 200 µl of MHB plus bacterial suspension.Negative control (dissentious): 200 µl of MHB only. Add 20 µl of the prepared bacterial suspension to each well except the negative control. Incubate the plate at 37 °C.

8. ATP bioluminescence method:

Antimicrobial susceptibility tests have been accomplishedswiftly with ATP bioluminescence- based approaches.The four steps of the eager ATP bioluminescence-basedapproach are as follows: 1. Preparation of bacterial culture. 2. Reduce extracellular ATP in the broth. 3. intrinsic ATP in bacteria can be released withthe 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

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Procedure:

1. **Sample Collection** : Collect samples from the surface or area to be tested for microbial contamination using swabs or other suitable methods.
2. **ATP Extraction**: Use a specialized reagent to release ATP (adenosine triphosphate) from the collected sample. ATP, present in all living cells, serves as an indicator of microbial activity. The reagent contains the enzyme luciferase.
3. **Luciferin-Luciferase Reaction** :Add the luciferin-luciferase reagent to the extracted ATP. This reaction generates bioluminescence (light) in direct proportion to the amount of ATP present, as ATP is converted into ADP with energy release.
4. .**Light Measurement** : The emitted light is measured using a luminometer, which records the intensity of bioluminescence. The measurement reflects the level of microbial contamination or activity in the sample.
5. **Comparison with Controls:**Compare the recorded light intensity against appropriate controls or standards to determine the degree of microbial contamination or the effectiveness of antimicrobial treatment.
6. **Application :**ATP bioluminescence testing provides a rapid, quantitative assessment of microbial contamination. It is widely applied in hygiene monitoring, food safety, and environmental testing.

Automated AST Systems And new technologies :

1. VITEK 2 SYSTEM :

The VITEK®2 Compact and VITEK®2 systems, developed by bioMérieux in the 1970s, are fully automated platforms based on the broth microdilution technique for bacterial identification and antimicrobial susceptibility testing (AST).These systems utilizeSpecialized "AST cards," each containing 64 microwells preloaded with dehydrated culture medium and antibiotics at varying concentrations. One of the wells serves as a positive control, containing only the culture medium without any antibiotic. After isolating the target organism, it is emulsified in 0.45% saline to match the turbidity of a 0.5 McFarland standard. This standardized inoculum is then placed into a VITEK®2 Cassette at the SMART CARRIER STATION™, which virtually links the sample to the AST card Once loaded, the system performs all identification and susceptibility testing steps automatically, eliminating the need for manual intervention. The AST card features fluidic connections that allow simultaneous filling of multiple wells with the prepared sample. Bacterial growth is detected through light attenuation, measured by an integrated optical scanner that combines a multi-channel fluorimeter and photometer to record fluorescence, turbidity, and colorimetric changes. The original VITEK system could process up to 120 test cards at once, while the VITEK®2 system can handle up to 240 cards simultaneously. Using susceptibility cards, results for rapidly growing gram-positive and gram-negative aerobic bacteria can be obtained within 4 to 8 hours.

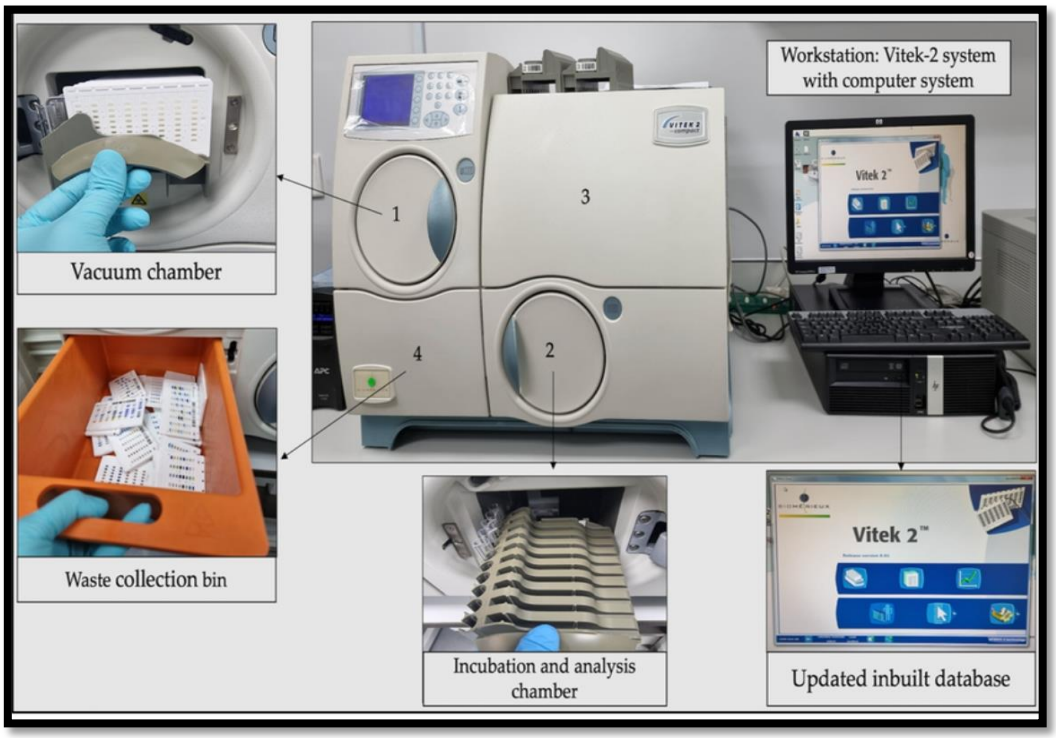

Fig 3: Illustration of the workflow pattern using the VITEK 2 automated compact system for microbial identification and antimicrobial susceptibility test (AST)

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2. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS):

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS)Mass spectrometry, introduced in the 1980s for proteomics, has evolved into a powerful tool in clinical microbiology through the development of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Since 2000, this technique Has been widely applied for bacterial identification and antimicrobial susceptibility testing (AST). MALDI-TOF MS operates by rapidly ionizing abundant ribosomal proteins directly from bacterial isolates or cell pellets using a laser pulse and an energy-absorbent matrix. The resulting ionized proteins are detected as peptide mass fingerprints (PMFs) by a time- of-flight (TOF) analyzer, which measures the mass-to-charge (m/z) ratio of the ions. These PMFs are then matched against extensive databases to identify microorganisms down to the genus, species, or even strain level. Sample preparation involves mixing or coating the specimen with a dried matrix solution—typically a derivative of benzoic or cinnamic acid— which crystallizes and facilitates co-crystallization of the sample. Upon laser irradiation, the sample is ionized to generate single protonated ions in an automated process. The TOF analyser then separates and measures these charged analytes based on their m/z ratios.MALDI-TOF MS has proven effective in identifying antibiotic resistance genes such as vanA and mecA. Although the instrument itself is expensive, it is cost-efficient for laboratories processing large volumes of samples due to minimal consumable costs.Despite its advantages, routine application of MALDI-TOF MS for rapid AST in clinical settings still requires further validation, including standardized protocols, test kits, and software. A recent advancement in this area is the direct-on-target micro droplet growth assay (DOT- MGA), which uses microdroplets incubated with and without antibiotics enable rapid, phenotypic AST. This method, combined with automated sample processing and enhanced software analysis, allows for concurrent testing of multiple antibiotics, offering a streamlined workflow and increased speed in clinical diagnostics.

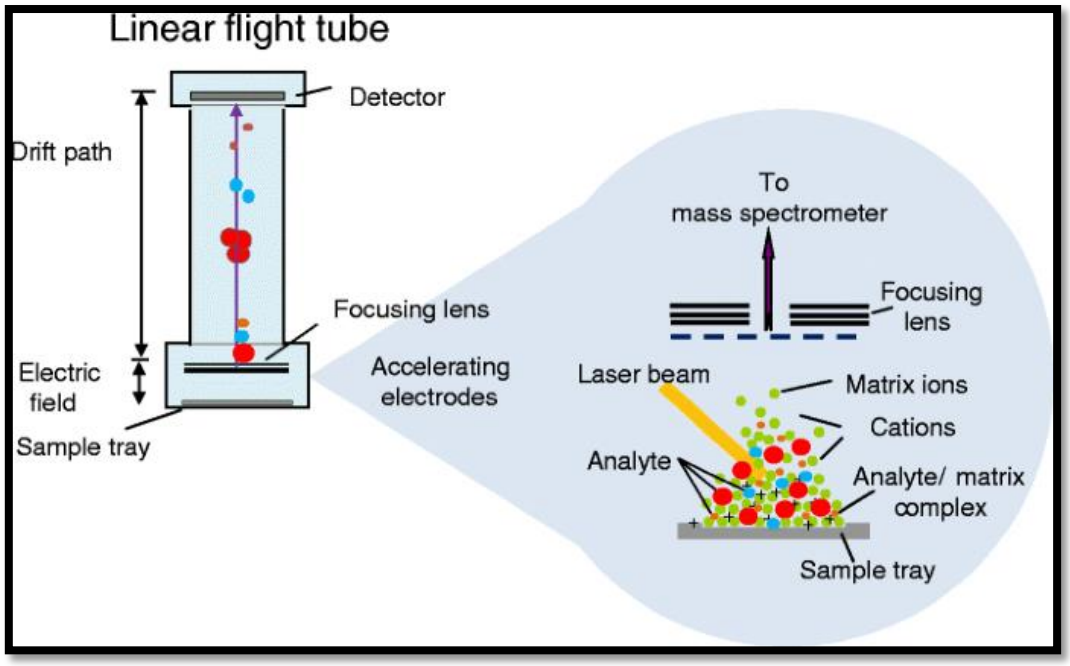

Fig 4: Schematic representation of MALDI-TOF MS System

3. Microscan Walkaway system :

The Microscan WalkAway system, developed by Beckman-Coulter Diagnostics, is an intelligent and automated microbiology platform designed for the identification of bacteria and antimicrobial susceptibility testing (ID/AST). It is based on the broth microdilution technique and is particularly suited for mid- to high-volume laboratories. The system uses microdilution trays containing 40 to 96 wells, which are first hydrated and then manually inoculated with bacterial samples. These trays are placed into a large, self-contained incubator/reader unit that analyzes the samples.This system offers gold-standard minimum inhibitory concentration (MIC) accuracy and is recognized for its ability to detect emerging resistance in pathogens. It employs optical detection methods to monitor bacterial growth in the wells and interprets biochemical reactions using a photometric or fluorogenic reader. The results are compiled into computerized reports that can be integrated with hospital information systems for streamlined data management.For rapidly growing bacteria, the MicroScan WalkAway system can generate AST profiles within 4.5 to 7 hours, thanks to its high bacterial concentration threshold of (2 \times 10^7) CFU/mL. In contrast, testing for slow-growing organisms

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may require up to 18 hours. This combination of automation, accuracy, and integration makes the MicroScan WalkAway system a valuable tool in clinical microbiology.

4. Selux Next-Generation Phenotyping AST System:

Bacterial isolates were handled strictly following the manufacturer's protocol. In summary, cultures were first sub-cultured onto Remel 5% sheep blood agar (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 18–24 hours. After incubation, colonies were suspended in 2.5 mL of saline to reach a turbidity equivalent to a 0.4–0.6 McFarland standard. These standardized suspensions were then assigned to the appropriate antimicrobial susceptibility testing (AST) panels: the Gram-positive (GP) panel included 15 antibiotics, while the Gram-negative (GN) panel contained 24 antibiotics. The suspensions were automatically diluted in cation-adjusted Mueller-Hinton broth and inoculated into the AST panels using the Selux DX Inoculator (Selux Diagnostics Inc., Charlestown, MA, USA). The inoculated panels were placed into the Selux DX Analyzer, which performed incubation and real-time monitoring. Once the bacterial identification was entered into the system software, the analyzer calculated minimum inhibitory concentrations (MICs) for each antibiotic and applied interpretive criteria according to CLSI or FDA breakpoints. The turnaround time (TTR)—defined as the interval between panel setup and final result—was recorded directly from the Selux DX sys

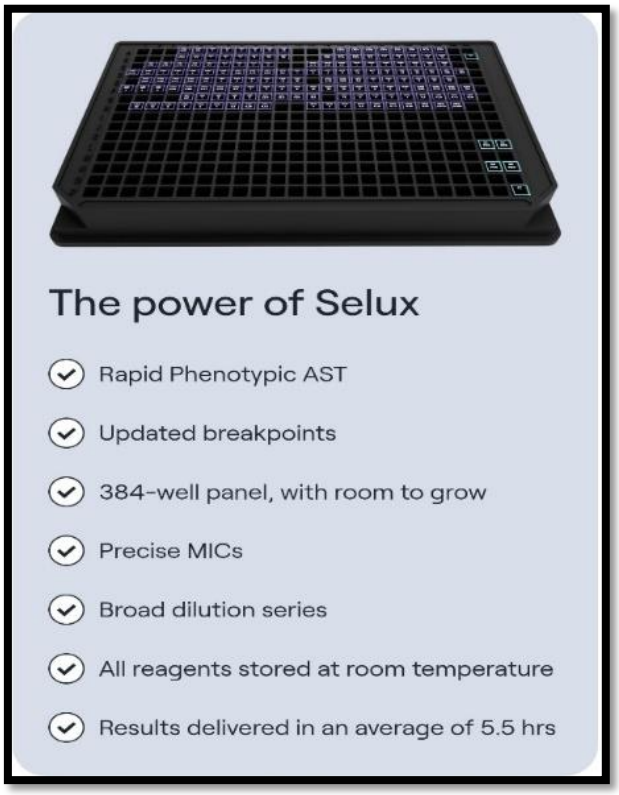

Figure 5: Selux Next Generation Phenotyping AST Systems

2. DATA ANALYSIS

Minimum inhibitory concentration (MIC) values and their interpretive categories generated by the Selux DX system were compared against results from the SOC MicroScan system (Beckman Coulter Inc., Franklin Lakes, NJ, USA) for clinical isolates, as well as against established susceptibility profiles for AR Bank reference strains. Concordance analysis (CA)—defined as agreement in susceptibility interpretation (susceptible, intermediate, susceptible-dose-dependent [SDD], or resistant) regardless of the exact MIC—was performed. CA percentages were calculated for each antibiotic–organism pairing. Both MicroScan and Selux DX system applied the most recent CLSI M100 or FDA breakpoints valid during the isolate collection period (March–May2024).

Error classifications followed CLSI guidelines. A very major error (VME) was noted when an isolate deemed resistant by the MicroScan system or AR Bank reference data was interpreted as susceptible by Selux DX. A major error (ME) occurred when an isolate categorized as susceptible by the reference method was reported as resistant by Selux DX. A minor error (mE) was defined as a discrepancy where one method reported intermediate or SDD, while the other reported either susceptible or resistant. All discrepancies were documented for each antibiotic–organism combination.

Error rates were calculated as follows:

Very Major Error Rate (%) = (Number of VMEs ÷ Number of resistant isolates by reference method) × 100

Major Error Rate (%) = (Number of MEs ÷ Number of susceptible isolates by reference method) × 100

Minor Error Rate (%) = (Number of mEs ÷ Total number of isolates tested) × 100 .

3. CONCLUSION

Antimicrobial research is experiencing significant growth as scientists intensify efforts to discover new and potent agents to combat infectious diseases. This surge is driven by the urgent global need to address antimicrobial resistance, which poses a serious threat to public health. To meet this challenge, researchers are continuously developing and refining methods to identify and evaluate potential antimicrobial compounds. However, no single method can be universally considered the best, as the choice depends on factors such as equipment availability, sample volume, and the nature of the agents being tested.

Increasingly, scientists are favoring approaches that are less labor-intensive, more automated, faster, and user-friendly to streamline the screening process. While advanced technologies like high-throughput sequencing and sophisticated microscopy offer promising insights into the mechanisms of action and detailed properties of antimicrobial agents, their widespread application in routine assays is still evolving. To enhance accuracy and reliability, researchers are exploring the integration of multiple methods or the supplementation of existing ones. Continuous improvements to both manual and automated systems are essential to ensure consistent and robust results. By tailoring method selection to specific research needs and optimizing techniques accordingly, scientists can strike a balance between simplicity and sensitivity. Ultimately, the adoption and refinement of these testing methods hold great promise for the discovery and development of effective antimicrobial agents, contributing to improved management of infectious diseases and reinforcing the global fight against antimicrobial resistance.

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