Determination of AMR in bacteria

G. K. Sivaraman* and T. Muthulakshmi
Microbiology, Fermentation & Biotechnology Division
ICAR- Central Institute of Fisheries Technology, Cochin
*gk.sivaraman@icar.gov.in/gkshivraman@gmail.com

Introduction

Presently, different methods of detection of antibiotic susceptibility are being available for both phenotypic and genotypic characterization of antimicrobial resistance (AMR) in different bacterial isolates. Some are regularly used in diagnostic laboratories, while others are still employed by academicians and professionals as research tools. The routine/ conventional testing of AMR involves plating of samples (fish, water, sediment & feed etc.) for the isolation and identification of a bacterial species of interest with pre-enrichment with selective and differential media. Disk diffusion, broth dilution, and gradient strip with respect to type of bacteria (Gram positive/ Gram negative) a panel of antibiotics is used to determine the AST as per minimum inhibitory concentration (MIC), and the breakpoint values set by the Clinical Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines and is known as phenotypic susceptibility tests. Recently, World Health Organization (WHO) has developed the software viz. WHONET for the analysis of antibiotic sensitive test (AST) to derive multiple interpretations with world unified protocol to support clear and error-free concept. The phenotypic method can be tested by conventional method (Diffusion and Dilution) or advanced method (Automated systems and Mass spectrometry). Bauer and Kirby's invented the disc diffusion method in 1956 and it is now the most used for determining the results of the phenotypic antibiotic sensitive test (AST). This conventional method is the gold standard and is very informative, but labor intensive and time consuming, sometime need several days to complete the AST with different classes of antibiotics. Recently, Automated bacterial characterization systems such as VIDAS, Vitek, BD Phoenix, MicroScan WalkAway, Micronaut and and Sensititre ARIS 2X based on turbidimetric, colorimetric, fluorometer or photometer or its combination are in used in large multispecialty hospitals, diagnostic centers and research institutions for the high throughput screening of more samples in a shorter time but is costlier, technical skill is required and it does not provide the mechanisms of resistance. However, PC based detection of antimicrobial resistance genes (ARGs) could able to detect the mechanisms of resistance. The possibility of quickly determining the ARGs in bacteria by the introduction of next-generation sequencing methods (Whole Genome Sequence). Although there are more and more phenotypic and genotypic characterization techniques for AMR detection now accessible, each of these techniques has some drawbacks. The adaptability of the bacterial genome should not be undervalued, either, given the potential future development of hitherto unimaginable new and unique resistance mechanisms. So, do both phenotypic and genotypic screening of AMR pathogens if possible as per the situations. Moreover, monitoring of AMR bacteria is a continuous process not only in clinical setting but also in healthy humans, animals and environment for proper understanding and to make effective combat strategies.

Disk diffusion assay: One phenotypic technique that can be used to assess the antibiotic resistance is disc diffusion testing i.e. in vitro susceptibility testing of antimicrobial resistance

(antibiogram). A standard inoculum of the bacteria (McFarland Standard 0.5 = ~1.5×108 CFU/mL) is used to inoculate agar plates, and then an antimicrobial disc is placed on the inoculated agar plate. Following the recommendations of the Clinical and Laboratory Standards Institute (CLSI), the plate is incubated under controlled circumstances. When in contact with the surface of the agar, the antimicrobial agent (set concentration, as per CLSI) contained in the discs used for a disc diffusion experiment diffuses into the agar. A "zone of inhibition" forms around the disc as a result of the antimicrobial drug diffusing into the agar during incubation and preventing bacterial growth. The diameter of this zone is measured and the findings are classified as resistant, moderate, or susceptible (CLSI M7, M31 and M100) and the inhibition zone's size reveals the level of resistance. This disk diffusion assay is extremely sensitive to changes in the following factors: bacterial concentration, media composition, pH, agar depth, diffusion rate of the antibiotics, growth rate of the bacteria, and incubation time. Internal quality control testing must be carried out on a regular basis as advised by CLSI (CLSI M2) to ensure the accuracy and repeatability of antimicrobial susceptibility test results.

Practical

Sample Preparation: The purified, single, and young culture (18-24 h) grown on non-selective agar must be used.

Media required

- Sterile saline solution (0.85 %) 3-4 mL each tube
- Mueller-Hinton agar plates (4 mm)
- Antimicrobial Disks (stored in -10 °C to -20 °C)
- Nutrient agar plates/ non-selective agar
- Quality control Strain

Equipment

- McFarland standard 0.5/ nephelometer
- Vortex
- Disk dispenser/ forceps
- Micropipette & tips (100 µl)
- Bunsen burner
- Small sterile cotton swabs/ spreader
- Ruler or caliper

Composition and preparation of culture media and reagents

- **Mueller Hinton Agar:** Mueller-Hinton Agar may be prepared from a commercially available base. Ensure that the Mueller-Hinton agar formulations have met the quality standards prescribed by CLSI document M6 *Protocols for Evaluating Dehydrated Mueller-Hinton Agar*.
- **Nutrient agar** (ISO 6579:2002)

  Meat extract 3.0 g
  Peptone 5.0 g
  Agar 12 g to 18 g
  Water 1000 mL

  Adjust pH to ~7.0 after sterilization
  Autoclave at 121 ^{O}C for 20 min.

- **Saline solution**

  Sodium chloride 8.5g

  Water 1000 mL

  Adjust pH to 7.0.

  Autoclave at 121 ºC for 20 min

Procedure

- Check the bacteria and the quality control strains are pure and well isolated colonies on the grown agar plates and free of visible contamination
- Pick up at least 4 to 5 well isolated colonies with a loop or sterile swab and transfer to the tube of saline and emulsify the inoculum on the inside of the tube to avoid clumping of the cells.
- Prepare the inoculum standard to a 0.5 McFarland by compare turbidity to that in the 0.5 McFarland standards using a paper with black lines or nephelometer and adjust it.
- Dip a sterile cotton swab into the inoculum, rotate the swab several times and press firmly on the inside wall of the tube above the fluid level to remove excess inoculum.
- Streak the swab over the entire surface of the Mueller Hinton agar plate
- Keep the plates 3-5 minutes to allow the excess moisture to be absorbed
- Dispense the antibiotic disks on the agar surface with dispenser or sterile forceps (5 disks on a 10 cm plate)
- Incubate at 35+2ºC for 18-24hrs

Results: Measure the diameter of inhibition zones and measure the more obvious margin of the zone diameter. If no inhibition is present, the diameter of the disk should be recorded (6mm).

Interpretation and reporting of the results: Refer the CLSI Guideline M100 and report as sensitive (S), intermediate (I) or resistant ®.

Reference

- Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 19th Informational Supplement. M100-S19. CLSI, Wayne, Pennsylvania, 2009.