Chapter 9

Determination of AMR by phenotypic method – Disk Diffusion assay

G. K. Sivaraman and U. Prinetha

Over 70 years have passed since the disc diffusion testing principle was first applied in microbiology labs. Drs. Bauer, Kirby, Sherris, and Turck meticulously developed by considering all parameters including the media, temperature, and depth of agar and were published in the year 1966. The essential operational stages from the Bauer paper's disc diffusion reference approach were adopted by CLSI. The CLSI is approved by FDA-USA and recommended by WHO.

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 = ~1.5×10^8 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 hrs) 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 3.0g

Peptone 5.0g

Agar 12g to 18g

Water 1000 mL

Adjust pH to ~7.0 after sterilisation,

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^{o}C for 20 min

Procedure

1st Step: Select colonies

Check the bacteria and the quality control strains are pure and well isolated colonies on the grown agar plates and free of any visible contamination. Colonies cannot be more than 18 to 24 hours old when using the direct colony suspension method. Except for staphylococci, most quickly growing organisms are studied using the log phase approach.

2nd Step: Prepare inoculum suspension:

Pick up at least 4 to 5 well isolated colonies with a sterile loop or swab and transfer to the tube of saline and emulsify the inoculum on the inside of the tube to avoid clumping of the cells. Make sure that the microorganism suspension is thoroughly mixed, and vortex it.

3rd Step: Standardize inoculum suspension:

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 accordingly.

4th Step: Inoculate plate:

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. The adjusted suspensions should be used within 15 minutes. 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.

5th Step: Add antimicrobial disks

Apply the disks containing the antimicrobial agents within 15 minutes of inoculating the MHA plate. Dispense the antibiotic disks on the agar surface with a dispenser or sterile forceps (5 disks on a 100 mm plate). To ensure complete, level contact with the agar, firmly press each disc into the surface. Once a disc was placed on the agar, it should not be repositioned. Select the FDA approved products and antibiotic disks with the specified contents as listed in the CLSI standards (Table 1 and 2).

6th Step: Incubate plate

Incubate the plate at 35±2°C for 18-24hrs within 15 minutes of standardizing the inoculum suspension. For nonfastidious bacteria, incubate in ambient air at 35°C for 16–18 hours (refer as per CLSI recommendations conditions).

7th Step Measure inhibition zones

Check for the growth is even and confluent and the zone of inhibition is of very clear. Measure the diameter of the clear inhibition zones margin. Measure zone of inhibition with respect to each antibiotic using reflected light from the back of the plate for the *Enterobacteriaceae*, *Staphylococci*, and *Enterococci* (except for oxacillin and vancomycin). Use transmitted light for *Staphylococci* with oxacillin and *Enterococci* with vancomycin. Measure the zone of inhibition where there is a clear distinction of growth and no growth. Even if the swarm type (*Proteus mirabilis)* are tested, only measure the obvious zone. It may be challenging to interpret zones with trimethoprim-sulfamethoxazole and sulfonamides and trimethoprim alone since these antibiotics may not prevent bacterial growth until the bacteria have undergone several generations of growth. Therefore, measure the zone at the point when there is an 80% reduction in growth within the zone. If

there is no zone of inhibition, the disk's diameter needs to be recorded as 6mm.

8th Step: Interpret and report of the results:

Refer the CLSI Guideline M100-S22, S-20: Performance Standards for Antimicrobial Susceptibility Testing, Table 2A- 2I (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints) and report as sensitive (S), intermediate (I) or resistant.

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.

NOTE: The situation with respect to susceptibility testing of bacteria isolated from aquatic animal is essentially different. In general there are not a wide number of testing protocols that have been developed by different national agencies. Only one international agency, CLSI, has started to develop standardized testing protocols suitable for bacteria isolated from aquatic animals that require incubation at temperatures <35°C or longer than 16-20hrs. Although the CLSI protocols do not yet cover all the diverse species encountered in aquatic animals, the progress they have made is substantial.

Table 1: List of antibiotics for susceptibility testing of *Staphylococci*

Antibiotic	**Disc content**	**Zone diameter interpretive criteria nearest whole mm**		
		S	**I**	**R**
Penicillin	10 units	≥ 29	-	≤ 28
Cefoxitin	30 µg	≥ 22 ≥ 25 (for CONS)	- -	≤ 21 ≤ 24 (for CONS)

Gentamicin	10 µg	≥ 15	13-14	≤ 12
Tetracycline	30 µg	≥ 19	15-18	≤ 14
Ciprofloxacin	5 µg	≥ 21	16-20	≤ 15
Trimethoprim-Sulfamethoxazole	1.25/23.75 µg	≥ 16	11-15	≤ 10
Chloramphenicol	30 µg	≥ 18	13-17	≤ 12
Erythromycin	15 µg	≥ 23	-	≤ 13
Linezolid	30 µg	≥ 21	-	-

Table 2: List of antibiotics for susceptibility testing of *E. coli*

Antibiotic	**Disc content**	**Zone diameter interpretive criteria nearest whole mm**		
		S	**I**	**R**
Ampicillin	10 µg	≥ 17	14-16	≤ 13
Amoxicillin-clavulanic acid	20/10 µg	≥ 18	14-17	≤ 13
Ceftriaxone	30 µg	≥ 26	23-25	≤ 22
Cefpodoxime	10 µg	≥ 21	18-20	≤ 17
Ceftazidime	30 µg	≥ 21	18-20	≤ 17
Aztreonam	30 µg	≥ 21	18-20	≤ 17
Cefotaxime	30 µg	≥ 26	23-25	≤ 22
Cefoxitin	30 µg	≥ 18	15-17	≤ 14
Imipenem	10 µg	≥ 23	20-22	≤ 19
Amikacin	30 µg	≥ 17	15-16	≤ 14
Tetracycine	30 µg	≥ 15	12-14	≤ 11
Ciprofloxacin	5 µg	≥ 21	16-20	≤ 15
Nalidixic acid	30 µg	≥ 19	14-18	≤ 13
Chloramphenicol	30 µg	≥ 18	13-17	≤ 12
Colistin	10 µg	≥ 11	-	≤ 10

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