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TECHNICAL SERIES

Antimicrobial Susceptibility Testing

Practical Issues

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Foreword

Microxpress ® a part of the Tulip Group of Companies addresses the needs of professional microbiologists across the full spectrum of clinical, analytical, industrial and research laboratories.

The group's commitment in building products to international standards, through indigenous R&D has accorded the company virtual leadership in most product segments domestically. Its state-of-art manufacturing facility conforms to the strictest GMP regulations. In its efforts to build world-class quality products, the group has ISO 9001:2008 certification from TUV and ISO 13485: 2003 – NF EN ISO 13485: 2004 certification from LNE.

Publishing of Technical series is one such initiative to make available to the laboratory professional and clinicians updated knowledge that is vital for them to set trends in their day to day practice.

Background

Antimicrobial Susceptibility Testing (AST) is performed on bacterial isolates in clinical laboratories. The development and improvement of accurate, efficient methods of rapid antimicrobial susceptibility testing is important for public health. Antimicrobial susceptibility information about pathogens may significantly reduce morbidity and mortality, cost of treatment, and duration of hospitalization if this information can be provided to clinicians in a rapid and timely fashion.

Many different clinical laboratories perform Antimicrobial Susceptibility Testing (AST). It is very important that all laboratories use methods that are comparable, for uniform reporting of results and better patient management.

For years, there has been a professional organization, CLSI (Clinical and Laboratory Standards Institute) that was formerly known as NCCLS (National Committee on Clinical Laboratory Standards) that created standards for performance of Antimicrobial Susceptibility Testing (AST). All clinical laboratories performing tests for antimicrobial susceptibility should have access to current CLSI guidelines. This assures that clinicians use appropriate antibiotic therapy for better patient prognosis.

In view of the above we have discussed the following points in our technical series :

1. Features of Mueller Hinton Agar medium used for Antimicrobial Susceptibility Testing.
2. Troubleshooting aspects in Antimicrobial Susceptibility Testing.

Antibiotics : Mechanism of Action

Antibiotics are low molecular weight substances that interfere with specific activities in certain types of organisms. These effects could be cidal (killing) and/or static (inhibitory). If an antibiotic has a widespread effect on Gram-positive and Gram-negative bacteria it is said to be a **broad spectrum antibiotic**. **A narrow spectrum antibiotic** will only act on either Gram-positive or Gram-negative bacterial strains. Antibiotics are found throughout nature and are used by organisms like moulds and soil inhabitants to gain advantages over their competitors. The early antibiotics were isolated from these natural sources, however today many are genetically engineered to be even more effective than their natural counterparts. For an antibiotic to be useful to humans it must have the ability to destroy pathogens while being relatively non-toxic to the host organism. It should be chemically stable and be able to reach the part of the host organism in which the infection persists.

Antibiotics work in a variety of ways, most of which attack Bacterial cell components and their synthesis. Some broad-spectrum antibiotics use several of the following modes of cellular attack:

1. Cell Wall Synthesis Inhibitors
2. Cell Membrane Inhibitors
3. Protein Synthesis Inhibitors
4. Nucleic Acid Effectors
5. Competitive Inhibitors

1. Cell Wall Synthesis Inhibitors : These antibiotics are particularly selective because they typically target the formation of peptidoglycan cell walls which are only found in prokaryotic cells. Cell Wall Synthesis Inhibitors do not impact eukaryotic cells of humans due to the lack of peptidoglycan. These types of antibiotics are referred to as Beta lactam antibiotics. Some common Beta lactam antibiotics are Ampicillin, Cephalothin and Penicillin.

2. Cell Membrane Inhibitors : These are less common than other types of antibiotic inhibiting mechanisms. Cell membrane inhibitors attack integrity of the bacterial membranes. An example of these is Polymyxin, which binds to membrane phospholipids. Once antibiotic has disrupted the membrane, the cell loses its integrity and will die. However a problem arises when there is a similarity between phospholipids in bacterial and eukaryotic cell membranes. These drugs can therefore be very dangerous to the patient due to the lack of selectivity in target cells and thus have only topical application.

3. Protein Synthesis Inhibitors : These types of antibiotics have numerous ways of attacking protein synthesis in bacterial cells and will usually target

activities occurring at the ribosome. These drugs affect the ribosome and donot bind to any other components of the protein synthesis process. Examples of these are Erythromycin, Streptomycin, Gentamicin and Tetracycline.

4. Nucleic Acid Effectors : Some antibiotics attack the DNA or RNA of a cell. These chemotherapeutic agents affect the synthesis of the DNA (in some case RNA). This serves to block the natural growth of the cell and will lead to death without replication.

One group is called Quinolones, and an example of this type is Nalidixic acid. Nalidixic acid binds itself to the enzyme topoisomerase, which uncoils supercoiled DNA before replication. The drug also inhibits the enzyme DNA gyrase, which returns the DNA to its supercoiled state after replication. This widely utilized nucleic acid effector possesses it's own caveats and has been known to affect animal cells in addition to bacterial DNA. A new class of drugs, called Rifamycins (e.g. rifampin), solves the specificity problem of the quinolones. These only attack the eubacterial RNA polymerase, which is essential to mRNA synthesis. It is inactive towards RNA polymerase found in eukaryotic cells.

5. Noncompetitive and Competitive Inhibitors : A competitive inhibitor blocks an enzyme from performing its normal function by mimicking the substrate and binding to the enzyme's active site. A noncompetitive inhibitor will bind to the enzyme at a different location (the allosteric site), which will change the structure of the enzyme affecting the rate at which it can perform a task.

The Sulfonamides are a class of competitive inhibitors. They inhibit Dihydropteroate Synthase (DHPS) an enzyme present exclusively in bacterial cells. DHPS enzyme is required in a series of reactions to synthesize folic acid. Inhibition of DHPS will results in many damaging effects including failure in biosynthesis of Purine and Thymylidate nucleotides and eventually inhibiting DNA synthesis.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing measure the ability of an antibiotic or other antimicrobial agent to inhibit bacterial growth *in vitro*. This ability may be estimated by either dilution method or diffusion method.

1. The dilution method

For quantitative estimates of antibiotic activity, dilutions of the antibiotic may be incorporated into broth or agar medium, which is then inoculated with the test organism. The lowest concentration that prevents growth after overnight incubation is known as the minimum inhibitory concentration (MIC) of the

agent. The MIC value is then compared with known concentrations of the drug obtainable in the serum and in other body fluids to assess the likely clinical response.

2. The diffusion method

Paper discs impregnated with a defined quantity of antimicrobial agent are placed on agar medium uniformly seeded with the test organism. A concentration gradient of the antibiotic forms by diffusion from the disc and the growth of the test organism is inhibited at a distance from the disc that is related among other factors to the susceptibility of the organism.

The recommended method for intermediate and peripheral laboratories is the modified Kirby-Bauer method. **This method has been recommended by National Committee on Clinical Laboratory Services (NCCLS-USA) Subcommittee on Antimicrobial Susceptibility Testing.** This is the most thoroughly described disc diffusion method for which interpretive standards have been developed and which is supported by laboratory and clinical data.

Kirby-Bauer Disk Diffusion Method

To ascertain antibiotic efficacy, degree of antibiotic resistance or sensitivity to bacterium, is the objective of Kirby-Bauer Test.

To carry out Kirby-Bauer Test following components are used :

1. Mueller Hinton Agar - Culture Media
2. Antimicrobial Susceptibility Discs
3. Standard Strains for Quality Control
4. Turbidity Standard (McFarland standards)
5. Swabs

Mueller-Hinton Agar : Mueller-Hinton Agar is considered the best medium to use for routine susceptibility testing of nonfastidious bacteria for the following reasons:

- It shows acceptable batch-to-batch reproducibility for susceptibility testing
- It is low in sulfonamide, trimethoprim, and tetracycline inhibitors
- It supports satisfactory growth of most nonfastidious pathogens
- A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

Please note that the use of media other than Mueller-Hinton Agar may result in erroneous results. Aerobic or facultative bacteria grow well on unsupplemented Mueller-Hinton Agar. Fastidious organisms require Mueller-Hinton Agar to be supplemented with additional nutrients.

1. Mueller-Hinton Agar (AM1071/AM5071) should be prepared from a dehydrated base or purchased as Mueller Hinton Agar ready prepared plates. Follow the manufacturer's recommendation for storage of prepared plates. Be sure to prepare the media according to the manufacturer's directions. The medium should be such that control zone sizes within the standard limits are produced. It is important not to overheat the medium.
2. If you prepare Mueller-Hinton Agar plates from dehydrated media, **the plates must be poured to a depth of 4 mm**. Plates that are too shallow will produce false susceptible results as the antimicrobial compound will diffuse further, creating larger zones of inhibition. Conversely, plates poured to a depth more than 4 mm will result in false resistant results.
3. While pouring media into plates medium temperature should be in between 45-50ºC.
4. Dry the plates at 35-37ºC in the incubator in an upright position for immediate use.
5. Any unused plates may be stored in a plastic bag, which should be sealed and placed in the refrigerator. Plates stored this way can be kept for 2 weeks.
6. pH of the Mueller-Hinton Agar should fall between 7.2 and 7.4 at room temperature after solidification and should be tested when the media is first prepared. If the pH is less than 7.2 certain drugs will appear to lose potency (aminoglycosides, quinolones, macrolides), while other agents may appear to have excessive activity (tetracycline). If the pH is more than 7.4, the opposite results may occur.
7. Excessive thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim resulting in smaller and less distinct zones of inhibition, or no zones at all.
8. The incorrect concentration of divalent cations (calcium and magnesium) will affect the results of aminoglycoside and tetracycline tests against *Pseudomonas aeruginosa*. Excess cation concentration will result in reduced zone sizes and low concentration will increase zone sizes. Excess calcium will increase the zone size of *P. aeruginosa* against daptomycin. Excess zinc ions may reduce the zone size of carbapenems against *P. aeruginosa.*
9. Mueller-Hinton Agar should be tested with known strains of organism at least weekly in order to verify that the media and discs are working as expected.

Mueller-Hinton Agar Physical Parameters

PARAMETERS	SPECIFICATIONS
Dehydrated powder appearance	Yellow coloured, homogenous, free flowing powder
Final pH at 25ºC	7.3 ± 0.1
Gelling temperature	Gel is formed at 32ºC
Colour and clarity of prepared media	Light amber coloured, clear gel

Cultural Characteristics Observed with Mueller-Hinton Agar after incubation for 18 to 24 hours at 30 - 35ºC

ORGANISM	TYPE CULTURE	GROWTH
Escherichia coli	ATCC (8739)	Luxuriant
Streptococcus faecalis	ATCC (11420)	Luxuriant
Neisseria gonorrhoeae	ATCC (49226)	Luxuriant
Pseudomonas aeruginosa	ATCC (9027)	Luxuriant
Staphylococcus aureus	ATCC (6538)	Luxuriant
Escherichia coli	ATCC (25922)	Luxuriant
Staphylococcus aureus	ATCC (25923)	Luxuriant
Pseudomonas aeruginosa	ATCC (27853)	Luxuriant

Antimicrobial Susceptibility Discs : Antimicrobial susceptibility discs with proper diameter and potency should be used. Discs should be made of an absorbent material, usually paper, which has no interfering effect either on bacterial growth or on the action of the antibiotic. It must be capable of absorbing moisture rapidly and the antibiotic should be evenly distributed in it. Sealed cartridges containing commercially prepared paper discs should be stored at either 8°C or frozen at -14°C in a non-self-defrosting freezer. A small working supply of discs can be kept in the refrigerator for one week. On removal from the refrigerator, the containers should be left at room temperature for about 1 hour to allow the temperature to equilibrate. **Once opened, store the cartridges in a storage container containing desiccant for not more than 1 week.**

Standard Strains for quality control : The quality control should use standard reference strains of bacteria that are tested in parallel with the clinical culture. They should preferably be run every week (Fig 11.1), or with every fifth batch of tests, and in addition, every time that a new batch of Mueller Hinton agar or a new batch of discs is used.

Standard Strains :

Staphylococcus aureus (ATCC 25923)
Escherichia coli (ATCC 25922)
Pseudomonas aeruginosa (ATCC 27853)

Culture for day-to-day use should be grown on slants of Nutrient Agar or Tryptic Soya Agar and stored in the refrigerator. These should be subcultured onto fresh slants after every 2 weeks.

Turbidity Standard (McFarland standard) : McFarland standard may be prepared in-house as described below :

1. Add a 0.5-ml aliquot of a 0.048 mol/liter $BaCl_2$ (1.175% wt/vol $BaCl_2 \cdot 2H_20$) to 99.5 ml of 0.18 mol/liter H_2SO_4 (1% vol/vol) with constant stirring to maintain a suspension.
2. Verify the correct density of the turbidity standard by measuring absorbance using a spectrophotometer with a 1-cm light path and matched cuvette. The absorbance at 625nm should be 0.08 to 0.13 for the 0.5 McFarland standard.
3. Transfer the barium sulfate suspension in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in standardizing the bacterial inoculums.
4. Tightly seal the tubes and store in the dark at room temperature.

Use of McFarland standard in the Kirby-Bauer procedure :

1. Prior to use, vigorously agitate the barium sulfate standard on a mechanical vortex mixer and inspect for a uniformly turbid appearance. Replace the standard if large particles appear. If using a standard composed of latex particles, mix by inverting gently, not on a vortex mixer.
2. While adding bacterial colonies to saline in the "preparation of the inoculum" step of the procedure, compare the resulting suspension to the McFarland standard. This is done by holding both the standard and the inoculum tube side by side and no more than 1 inch from the face of the Wickerham card (with adequate light present) and comparing the appearance of the lines through both suspensions. If the bacterial suspension appears lighter than the 0.5 McFarland standard, more organisms should be added to the tube from the culture plate. If the suspension appears denser than the 0.5 McFarland standard, additional saline should be added to the inoculum tube in order to dilute the suspension to the appropriate density. In some cases it may be easier to start over rather than to continue to dilute a bacterial suspension that is too dense for use.

Swabs : Sterilized cotton swab stick can used or cotton swabs sticks can be sterilized in tins, culture tubes, or on paper, either in the autoclave or by dry heat.

Procedure to Perform Kirby-Bauer Disc Diffusion Test

- To prepare the inoculum from a primary culture plate, touch with a loop the top of each of 3-5 colonies of similar appearance of the organism to be tested (Fig.1).

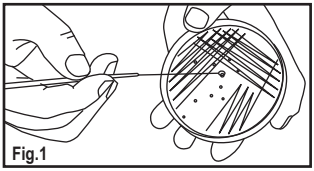
Fig.1

- When the inoculum has to be made from a pure culture, a loopful of the confluent growth is similarly suspended in saline (Fig. 2).

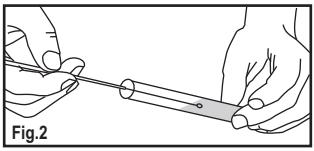
Fig.2

- Compare the tube with turbidity standard and adjust the density of test suspension to that of the standard by adding more bacteria or more sterile saline. Proper adjustment to the turbidity of the inoculum is essential to ensure that the resulting lawn of growth is confluent or almost confluent. Use this suspension within 15 minutes of preparation (Fig.3).

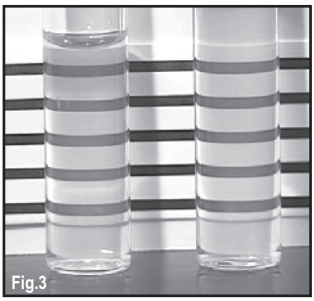
Fig.3

- Inoculate the plates by dipping a sterile swab into the inoculum. Remove excess inoculum by pressing and rotating the swabs firmly against the side of the tube above the level of the liquid (Fig. 4).

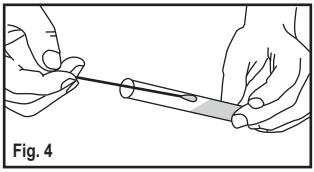
Fig. 4

- Streak the swab all over the surface of the medium three times, rotating the plate through an angle of 60º after each application. Finally, pass the swab round the edge of the agar surface. Leave the inoculum to dry for a few minutes at room temperature with the lid closed (Fig. 5).

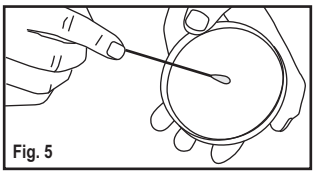
Fig. 5

- The antibiotic discs may be placed on the inoculated plates using a pair of sterile forceps or a sterile needle tip. Alternatively, an antibiotic disc dispenser can be used to apply the discs to the inoculated plate (Fig. 6).

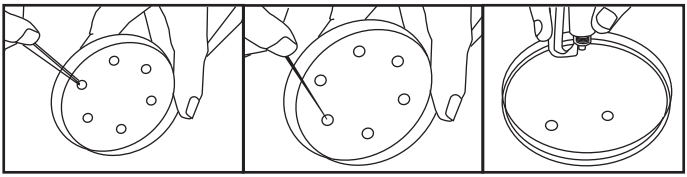

Fig. 6

- A maximum of 4-5 discs can be placed in a 90-100 mm diameter petridish.
- The plates should be placed in an incubator at 35-37ºC within 30 minutes of preparation.
- Incubate fastidious organism in an atmosphere of carbon dioxide.
- After overnight incubation, the diameter of each zone (including the diameter of the disc) should be measured and recorded in mm. The results should then be interpreted according to the critical diameters by comparing them with standard tables (Fig. 7).

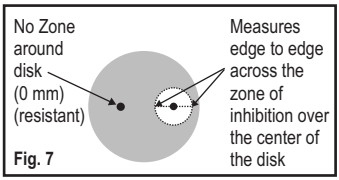

Fig. 7

- The measurements can be made with a ruler on the under surface of the plate without opening the lid (Fig. 8).

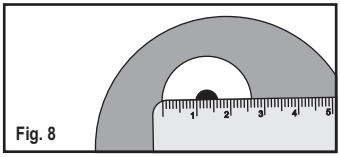

Fig. 8

- The endpoint of inhibition is judged by the naked eye at the edge where growth starts, but there are three exceptions,
  - With sulfonamides and co-trimoxazole, slight growth occurs within the inhibition zone; such growth should be ignored.
  - When β-lactamase producing staphylococci are tested against penicillin, zones of inhibition are produced with a heaped-up, clearly defined edge; these are readily recognizable when compared with the sensitive control and regardless of the size of the zone of inhibition, they should be reported as resistant.
  - Certain *Proteus* species may swarm into the area of inhibition around some antibiotics, but the zone of inhibition is usually clearly outlined and the thin layer of swarming growth should be ignored.

Clinical Definition of Terms Resistant and Susceptible

The result of the susceptibility test, as reported to the clinician, is the classification of the microorganism in one of two or more categories of susceptibility. The simplest system comprises only two categories, susceptible and resistant. This classification, although offering many advantages for statistical and epidemiological purposes, is too inflexible for the clinician to use. Therefore, a **three-category classification** is often adopted. The Kirby-Bauer method recognizes three categories of susceptibility and it is important that both the clinician and the laboritarian understand the exact definitions and the clinical significance of these categories.

Susceptible : An organism is called "susceptible" to a drug when the infection caused by it is likely to respond to treatment with this drug at the recommended dosage.

Intermediate susceptibility : This term covers two situations. It is applicable to strains that are "moderately susceptible" to an antibiotic that can be used for treatment at a higher dosage because of its low toxicity or because the antibiotic is concentrated in the focus of infection (e.g. urine). The term also applies to those strains that are susceptible to a more toxic antibiotic that cannot be used at a higher dosage. In this situation this category serves as a buffer zone between susceptible and resistant.

Resistant : This term implies that the organism is expected not to respond to a given drug, irrespective of the dosage and of the location of the infection.

Quality Assurance in Antimicrobial Susceptibility Discs

Antimicrobial Susceptibility Testing has become a very essential step for properly treating infectious diseases and monitoring antimicrobial resistance in various pathogens. The choice of antimicrobial needs to be made taking into consideration the susceptibility profile of the pathogen, pharmacology of the antimicrobial, the need for antimicrobial therapy, and its cost effectiveness.

Factors Influencing Zone Size and Common Problems Encountered in Performing Susceptibility Testing

FACTORS	IMPACT ON ZONE SIZE
Inoculum density	Larger zones with light inoculum and smaller zone with heavy inoculum
Timing of disc application	If after application of disc, the plate is kept for longer time at room temperature, small zones may form
Temperature of incubation	Larger zones are seen with temperatures < 35ºC
Incubation time	Ideal 16-18 hours; less time does not give reliable results
Depth of the agar medium	Thin media results excessively large inhibition zones and very thick media results small inhibition zone.
Proper spacing of the discs	Avoids overlapping of zones
Potency of antibiotic discs	Deterioration in contents leads to reduced size
Composition of medium	Affects rate of growth, diffusion of antibiotics and activity of antibiotics
Addition of thymidine to medium	Decreases activity of trimethoprim
Acidic pH of medium	Tetracycline, novobiocin, methicillin zones are larger
Alkaline pH of medium	Aminoglycosides, erythromycin zones are larger
Incubation in the presence of CO_2	Increases zone size of tetracycline and methicillin
Reading of zones	Subjective errors in determining the clear edge
Chelating agents such as calcium, magnesium and iron	Decreases diffusion of tetracycline, gentamicin
On chocolate agar	Decreased activity of Sulfonamides, trimethoprim, aminoglycosides
Addition of defibrinated blood	Decreases activity of sulfonamides

Troubleshooting Guide for Disc Diffusion Test in Antimicrobial Susceptibility Testing

ERRONEOUS RESULT	PROBABLE CAUSE	CORRECTIVE ACTION
Tetracycline zone too large and clindamycin zone too small with *E. coli* or *S. aureus* control strains.	pH of medium too low.	Adjust pH 7.2 to 7.4 before pouring media. Commercial media should not have pH problems. Report to manufacturer.
Tetracycline zone too small and clindamycin zone too large with *S. aureus* or *E. coli* control strain.	pH of medium too high.	Get a new lot. (Incubation in CO_2 may alter agar surface pH.)
Aminoglycoside zone too small with *P. aeruginosa,* Acinetobacter control strain.	Calcium ion and/or Magnesium ion too high in medium.	Acquire a new lot of agar medium that will meet QC criteria.
Aminoglycoside zone too large with *P. aeruginosa* control strain.	Calcium ion and/or Magnesium ion too low in medium.	Acquire a new lot of agar medium that will meet QC criteria.
Zones universally too large on control plates.	Inoculum too light.	Adjust inoculum to a McFarland 0.5 turbidity standard.
	Nutritionally poor medium.	Use only Mueller Hinton Agar medium.
	Slow-growing organism. (not seen with controls)	Use minimum inhibtory concentration (MIC) procedure only.
	Improper medium depth. (too thin)	Use 4-5mm depth.
Zones universally too small on control plates.	Inoculum too heavy.	Adjust inoculum to a McFarland 0.5 turbidity standard.
	Agar depth too thick. (minor)	Use 4-5mm depth.
Methicillin zone decreasing over days or weeks with control organisms.	Methicillin degrading during refrigerator storage	Change methicillin discs or use oxacillin or nafcillin as the routine disc.

ERRONEOUS RESULT	PROBABLE CAUSE	CORRECTIVE ACTION
Methicillin zone indeterminate in disc test.	Methicillin being degraded by strong beta-lactamase producing staphylococci.	Change methicillin discs or use oxacillin or nafcillin as the routine disc.
Carbenicillin zone disappears with *Pseudomonas* control.	Resistant mutant has been selected for testing.	Change *Pseudomonas* control strain every two weeks and whenever

ERRONEOUS RESULT	PROBABLE CAUSE	CORRECTIVE ACTION
The methicillin disc test shows "resistant" but an MIC shows "sensitive" for *S.aureus.*	Mueller Hinton Broth is inadequate in this case. A modified broth used in some commercial MIC systems frequently eliminates this problem.	No action necessary with disc test. To be expected if Mueller Hinton Broth is used in MIC test. Use broth with 2% NaCl if MIC testing is necessary.
	Low methicillin content in disc.	Use new discs
Zones overlap.	Discs too close together.	Use no more than 12 discs on a 150mm plate and 4 to 5 discs on a 100mm plate. Place discs no closer than 15mm from the edge of the plate.
"Zone within a zone"	Swarming / movement of *Proteus* spp.	Read the wide distinct zone and disregard the growth that swarmed over.
	Feather edges of zones around penicillin or ampicillin discs usually occur with beta-lactamase-negative strains of *S. aureus.*	Take half the distance from the inner zone to outermost zone as measure mark.
	Sulfonamides	Disregard growth from disc margin to the major inner zone.
	Beta-lactamase-positive *Haemophilus influenzae* with penicillin or ampicillin.	Use inside zone.

Salient Features of Quality Control in Antimicrobial Susceptibility Testing are Summarized below

- Use antibiotic discs of 6 mm diameter.
- Use correct content of antimicrobial agent per disc.
- Store supply of antimicrobial discs at -20ºC.
- Refrigerate the containers at 8ºC or below, or freeze at -14ºC or below, in a nonfrost-free freezer until needed.
- Sealed packages of discs that contain drugs from the ß(Beta)-lactam class should be stored frozen, except for a small working supply, which may be refrigerated for at most one week.
- Some labile agents (e.g., imipenem, cefaclor, and clavulanic acid combinations) may retain greater stability if stored frozen until the day of use.
- The unopened disc containers should be removed from the refrigerator or freezer one to two hours before use, so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold discs.
- **Once a cartridge of discs has been removed from its sealed package, it should be placed in a tightly sealed, desiccated container.**
- Use Mueller-Hinton medium for antimicrobial sensitivity determination.
- Use appropriate control cultures.
- Use standard methodology for the test.
- Use coded strains from time to time for internal quality control.
- Incubate the sensitivity plates for 16-18 hours before reporting.
- Incubate the sensitivity plates at 35-37ºC.
- Space the antibiotic discs properly to avoid overlapping of inhibition zone.
- Use inoculum size that produces 'near confluent' growth.
- Ensure even contact of the antibiotic disc with the inoculated medium.
- Measure zone sizes precisely.
- Interpret zone sizes by referring to standard charts.
- Only those discs that have not reached the manufacturer's expiration date stated on the label may be used. Discs should be discarded on the expiration date.

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