

ANTIBIOTIC SUSCEPTIBILITY TEST

Abstract

Antibiotic Susceptibility Test is measurement of the susceptibility of bacteria to antibiotics. Antibiotic Susceptibility Test may be done if infection does not respond to treatment¹. It helps doctor to recommend the most vital and effective Antibiotic to overcome an infecting micro-organism, pathogenic organisms such as bacteria, fungus etc. that infect our body and lodge infection. Antibiotic Susceptibility Test specifies effective Antibiotic dosage and evaluating Antibiotic Resistance and helps in determining patient's treatment plans. This testing technique is widely used as micro-organisms tend to develop resistant to some antibiotics. It is used to select effective drugs for treatment. It determines ability of the drug to kill bacteria. It is used to check effective antibiotic or a combination of few antibiotics that are most effective in treating the different types of bacteria causing the infection, present in the diagnostic sample². It is used to recognize causative organism and also check if it shows a demonstrable and comparable sensitivity pattern.

Keywords: Antibiotic Susceptibility Test, pathogenic, infection, zone of Inhibition

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Infectious diseases caused by pathogens are burden worldwide. Microbiological laboratory is mainly responsible for patient management and treatment including Isolation and identification of etiological agent and performing Antibiotic Susceptibility Test, thereby guide clinicians for respective therapy. Antimicrobial Susceptibility Test can help doctor to evaluate choice of drug that can be most effective in overcoming the infection². Huge number of Antimicrobial agents is available for treating diseases. These antibiotics are now routine and indispensable part of modern medical practice, so there is need for judgmental use of these antibiotics. Antibiotic Susceptibility Test is measurement of the susceptibility of bacteria to antibiotics. Antibiotic Susceptibility Test may be done if infection does not respond to treatment. It helps doctor to recommend the most vital and effective Antibiotic to overcome an infecting micro-organism, pathogenic organisms such as bacteria, fungus etc. that infect our body and lodge infection¹⁴. The Antimicrobial agents used should aim at destroying the infection causing micro-organisms that can prevent the invasion of infection. It is established that bacteria and other pathogens tend to mutate. Antibiotic that are effective today may not be effective after some years. Antibiotic Susceptibility Test specifies effective Antibiotic dosage and evaluating Antibiotic Resistance and helps in determining patient's treatment plans.

- This testing technique is widely used as micro-organisms tend to develop resistant to some antibiotics.
- Antimicrobial Sensitivity Tests are not only vital in study of the resistance but also in discovery of new antimicrobial drugs and agents.
- Antibiotic Susceptibility Test is used to evaluate the sensitivity of the antibiotics to be used in treatment against disease causing pathogenic bacteria.
- Antibiotic Susceptibility Tests are very helpful for clinicians and therefore play important role in routine procedure in diagnostic bacteriology.

I. USES OF ANTIBIOTIC SUSCEPTIBILITY TEST

1. It is used to select effective drugs for treatment.
2. It determines ability of the drug to kill bacteria.
3. It is used to check effective antibiotic or a combination of few antibiotics that are most effective in treating the different types of bacteria causing the infection, present in the diagnostic sample.
4. It is used to recognize causative organism and also check if it shows a demonstrable and comparable sensitivity pattern.
5. It is **NOT** prescribed if the antibiotic sensitivity pattern of the pathogen detected in the sample cannot be predicted¹.
6. Antibiotic Susceptibility Test is **NOT performed and checked against the commensals and contaminants**⁸.
7. This creates confusion and deludes the physician from prescribing suitable drug and also deceive patient to receive required necessary antimicrobial therapy.
8. Side-effects of such unwanted and unjustified therapy results in major cause of emergence of resistant pathogens against certain drugs.
9. It is used to determine MBC- Minimum Bacterial Concentration and MIC – Minimum Inhibitory Concentration of the all the Antimicrobial Agents used in AST¹⁷.

II. LIMITATIONS

- Antibiotic Susceptibility Test is applied only In-vitro testing and is **Not applicable in In-vivo** drug activity.

III. SELECTION OF SUITABLE ANTIMICROBIAL DEPENDS ON

For therapeutic use in patients, an Antimicrobial agent or the drug of choice must show Selective Toxicity. This refers that; it must show greater and effective sensitivity and toxicity to the infecting pathogens than to the Human host^{18, 19}. A drug which is capable of producing harmful effects or if that drug kills patients, then that drug should not be used in treating infectious diseases, besides the fact that, it is lethal on micro-organisms especially pathogens.

Antibiotics are known to be the major class of antimicrobial agent. Antibiotics are the biochemical agents produced by the micro-organisms that paralyse, inhibit growth of the pathogen, also, it may kill the micro-organisms²⁰. The discovery, use and application of these Antibiotics have revolutionised and transformed the entire medical field. But now a day, organic chemists are able to synthesize the biochemical structures of many naturally occurring Antibiotics. Many antibiotics that are routinely used in current medical practice are nothing but are modified chemically forms of microbial biosynthetic products^{3, 21}.

Selection of suitable Antimicrobial drug depends on:-

- Patient's clinical condition and signs and symptoms shown.
- Type and site of infection in the host.
- History of the patient against drug hypersensitivity.
- Activity of Drug like absorption, diffusion in tissues, metabolism, excretion, toxicity, effect on patient's normal flora **CANNOT** be predicted by antimicrobial susceptibility testing^{5,6,7}.

IV. ANTIBIOTIC SUSCEPTIBILITY TECHNIQUES

Antibiotic Susceptibility Technique has become concern worldwide as there is increase in Antibiotic Resistant forms due to indiscriminate use of Antibiotics in both man and animal. Antibiotic Susceptibility Test is useful and effective technique that helps to quickly determine if the bacteria are resistant to any particular drug^{4,14}. There are two methods of Antibiotic Susceptibility Techniques:-

- Diffusion Technique
- Dilution Technique

1. Requirements for Antibiotic Susceptibility Techniques^{22-25,2936}:

- Mueller - Hinton Agar is used and considered best because Mueller Hinton Media is acceptable solid media for majority of micro-organism, batch reproducibility, for susceptibility testing. It is having low concentration of sulphonamides, trimethoprim. It also produces ample growth of non-fastidious pathogens. In addition, it also contains low concentration of Thymidine or Thymine.
- Other media that can be used are according to the nutritional requirement of test bacteria for example Lowenstein Jenson and Loefflers Serum Slope.
- Depth of medium should be 4mm (25 ml) in a sterilized petri plate.

- Pour sterilized media on plates on a level surface using Pour plate method in a sterilized petri plate.
- For spreading Inoculum Pour Plate or Swab Method is used.
- Too thin & too thick media if plated on the sterilized petri plate may show flawed inhibition zones.
- Culture Media Plates should be kept in sealed plastic bags and store at 2 - 8°C for up to 2 weeks and can be used later after bringing it to Room Temperature before Inoculating.

2. Diffusion Sensitivity Technique

- In laboratory, it is used in routine sensitivity testing.
- The Inoculum if applied properly gives semi-confluent growth.
- Filter paper discs cut into the size of 6mm in diameter are dipped in required concentration of drugs so that it gets charged with the drug and then is stored in dry and cold place.
- This disc of filter paper dipped and impregnated with the known volume and concentration of a certain antimicrobial agent of choice is placed on an agar media that is pre-inoculated with a test organism as explained above.

Kirby - Bauer Technique

- Control organisms are also seeded in inoculated plate for effective comparison.
- Charged Discs are placed on the sterilized agar plate and kept for 20 – 30 minutes, so that Drug diffuses into the medium.
- The plates having discs are subjected for overnight incubation,
- The plates are then taken out of Incubator and examined for areas of no growth or the zones of clearance (inhibition zones) surrounding the antibiotic discs.
- The bacteria that are Sensitive are inhibited at a visible particular distance from disc showing larger zone of clearance.
- The Resistant bacteria are seen to grow up to the edge of the disc.
- According to the Diameter of the Zone of Inhibition, the Antibiotics are then classified as Sensitive, Intermediate and Resistant.

Stokes Technique:

- A disc made from filter paper is dipped and impregnated with a known volume & concentration of an antimicrobial drug.
- This filter paper disc once charged with antimicrobial drug is taken and placed on the sterilized Mueller Hinton Agar medium inoculated with a test organism.
- In this technique Both the test organism and control organisms are inoculated on same culture plate.
- The Disc is placed on the surface of the media so that the Drug diffuses easily into medium.
- The Inhibition zone formed after incubation is compared directly with that of control.

V. PLATING FOR ANTIBIOTIC SUSCEPTIBILITY TEST

- By the help of a sterile cotton swab, apply the suspension of the test organism to the entire sensitivity plate having sterilized media.

- It is advisable that, Do not use the same sterile swab for both application and spreading the inoculum on the surface of the media.
- For making the Inoculum - Check the turbidity of the inoculum using Mac Farland turbidity standard using BaCl₂ and H₂SO₄ and not by naked eye.
- Similarly, for Stoke's method of antimicrobial test, we should inoculate the broth culture of control strain either on the upper or the lower half of the plate.
- The Control suspension to be used for the test must be standardized against the standard.
- Allow the inoculum to dry for few minutes, so that the bacteria firmly adhere to the surface of the media. Keep the Petri dish closed to avoid contamination.
- Place the antibiotic discs (after warming to room temp.), to be used for the test between test and control inoculum junction line.
- Press the antibiotic disc a little with the help of back side of inoculating loop and do not move or shift once it is placed.
- Then after 30 minutes, keep the plates for incubation having discs at 37°C for overnight or 24 hours. (For methicillin, incubate at 35°C)
- The test is read and interpreted when: Bacterial growth of both test strain and control strains is not too heavy or too light.
- The Inhibition zones of Control Strain should measure between 8-15 mm.
- Measurement of the Inhibition Zone is from edge of the antibiotic disc to edge of inhibition zone.
- If the bacterial growth of test & control strains does not show semi confluent growth, the sensitivity test must be repeated.

VI. TURBIDITY (OPACITY) STANDARD

- It is also called as Mac Farland Standard, which is said to give rise to 1×10^8 CFU/ml, if taken loopful.
- Mac Farland Standard is basically a suspension of Barium Chloride in Sulphuric Acid and is used for matching turbidity of test & control strains inoculum.
- For making Mac Farland Standard, to make 1% H₂SO₄, Add 1 ml of H₂SO₄ to 99 ml of distilled water.
- Then dissolve 2.35g Barium Chloride in 200 ml distilled water.
- Then mix 0.5 ml Barium Chloride solution to 99.5 ml H₂SO₄ solution, from the above made solutions.
- Transfer the turbid solution made by mixture of both BaCl₂ and H₂SO₄ to screw-cap bottle of same type as that to be used to prepare test & control strain suspension.
- This MacFarland Turbidity standard can be easily stored in sealed container and kept in dark, at room temperature for 6 months.
- The Turbidity of standard is equivalent to the turbidity shown by overnight broth culture having test organism.

VII. ANTIMICROBIAL DISCS¹⁰⁻¹⁶ –

- To select antimicrobial agents for sensitivity, one should consult physicians and clinicians to correlate the drug of choice with patients sign and symptoms and also health status of that patient.

- The list of the Drugs to be used for the sensitivity test must be restricted, limited & should be reviewed at regular intervals.
- If resistance is seen against certain drug, then one member of each drug group should be selected.
- We should select Antimicrobial Discs according to :
- Control strains should be used in the test.
- Selection of the drug should be according to the Site of infection in patient.
- The Concentration of the Drug to be used at the site should be considered.
- The Control Strain should respond to treatment with normal doses of the drug chosen.
- Control Strain should grow at the same rate as the test organism – when bacterial growth is too much effluent and heavy – the inhibition Zones formed are smaller and if growth is very less and scanty the inhibition zone will be larger.
- A difference in molecular structure of drugs also influence the inhibition zone size – larger inhibition zones are obtained when antimicrobial agents diffuses rapidly in the medium.

1. Recommended Control Strains -

- For Staphylococcus aureus – Oxford strain is used with code - NCTC 6571
- This strain is used for all tests except polymyxins & for all the pathogens of all specimens except that of urine.
- For E. coli – NCTC 10418 - is used for all drugs against pathogens from urine.
- P. aeruginosa – NCTC 13921- is used for controlling all drugs against Pseudomonas.

2. Precautions for Using Control Strains are:

- Control Strain should be cultured on sterilized Basal Agar i.e. Nutrient Agar slopes.
- The Strains must be stored in cool and dark place at room temperature i.e. (20-28°C).
- The strain should be subculture in specific media in quarterly or half – yearly interval.
- Weekly, a nutrient broth or agar culture is made and the strains are grown into it and stored at low temperature of 2 – 8°C and from this stored culture; suspensions and inoculums are prepared for daily use for Antibiotic Sensitivity Test.
- In Kirby-Bauer technique: the Inhibition zone formed in the sensitivity test is measured & compared against a previously prepared Antibiotic Zone Reader scale that can be correlated with the Inhibition zone size with MIC.
- In Stokes technique: The Inhibition zone formed in the sensitivity test is compared directly with that of control in the same test plate.

VIII. INTERPRETATION OF RESULTS^{9-16, 19-20, 28, 30- 37} _

- Antibiotic Susceptibility Test is reported according to the size of the Inhibition Zone formed as: Sensitive Zone, Intermediate Zone & Resistant.
- Sensitive Zone: If the Sensitivity Zone of Test is wider as compared to the control zone or equal to the control zone or the inhibition zone should not be less than 3 mm or be smaller than that of control zone.
- Intermediate Zone: If the Inhibition Zone of Test is more than 3 mm or smaller to that of control zone but not less than 3 mm in diameter.
- Resistant: If the Inhibition Zone of Test is 2 mm or less or if No zone of sensitivity is seen.

- Drugs falling in Intermediate zone should be recommended and prescribed in high doses to cure infection or when drug is concentrated at site of infection, e.g. Urinary Tract Infection.
- In sensitivity test with Sulphonamides & Trimethoprim, slight growth of bacteria is seen within the inhibition zone. (This is because of the presence of inhibitors (thymidine) and it must be ignored)
- The Strains are considered resistant if:
- Growth is too much heaped-up upto the inhibition zone edge without gradual fading up towards disc (penicillin-resistant Staphylococcus)
- If large and heavy colonies of bacteria are seen growing within inhibition zone.
- With the use of Colistin & Polymyxin, smaller zones of inhibition are observed because of their large molecular size. (control zone must be at least 3-4mm)
- It is advisable to check the discs and its inhibition zone, daily for noticing any decrease in inhibition zone size resulting from drug deterioration.

IX. INHIBITION ZONES

- Inhibition zones vary in size due to:
- Difference in molecular structures of drugs.
- When bacterial growth is heavy.
- Factors affecting the medium used in the sensitivity test are – volume or the quantity of the media used, moisture present in the media that may influence the diffusion of the drug, pH of the media & the constituents present in the media.
- Factors affecting the disc used in the sensitivity test are - concentration of drug, storage property & application on the site.

X. DILUTION SENSITIVITY TECHNIQUE

- Performed under conditions such as::
- When the Patient is not responding to the routine therapy.
- When the Patient is seen to be immunosuppressed.
- METHODS : 2 methods
- Agar Dilution
- Tube Dilution

XI. USES

- It evaluates the Minimum Bactericidal concentration (MBC), the concentration of the drug that is capable of killing the bacteria.
- It measures the Minimum Inhibitory Concentration (MIC), the concentration of the drug that is capable of inhibiting the bacteria.
- It is useful in checking and measuring adequate Drug concentration in blood and body fluids.
- It also protects the patients against excessive concentrations and levels of drugs in blood that could be toxic.

1. Technique:

- Variable and different Dilutions of drug to be checked are added to the sterilized medium.

- A standard size of inoculum of organism is added on to the surface of the sterilized media for the test.
- After overnight incubation or after 24 hours of incubation MIC is reported.
- The Clinical response of the patient is evaluated by comparing MIC recorded with already known concentrations of the drug.
- MBC can be determined by sub-culturing last dilution tube in the dilution series to check for the viable and visible growth.
- Other tubes should show no growth on subculture.

2. Dilution Techniques Require:

- A perfect standardization of the process, strains used, drugs and concentration of drugs must be done before performing the sensitivity test.
- A perfect control of: inoculum, medium, drugs, incubation time, diluting techniques, reading of results must be done to get accurate result.

3. MIC may be determined by automated machines.

- Before use, dry the plates with lids slightly open for half an hour at 37°C and let it come at room temperature.
- 5% blood is added to Mueller - Hinton Agar to test for fastidious organisms (Neisseria, Haemophilus, Streptococcus)

XII. FACTORS AFFECTING ANTIBIOTIC SUSCEPTIBILITY TEST -

- Media containing substances having inhibiting action of Aminoglycosides, Tetracyclines, Trimethoprim, e.g: the substance Thymidine, may affect the inhibition zone formed.
- pH of media: False large zones are formed if the medium is acidic (Tetracycline), or false small zones are formed if medium is alkaline (Aminoglycosides).
- Fermentable sugars are not added to medium to avoid production of acid that can change the pH of the media.

1. Other Factors Include

- Read and follow the pack insert having instructions from the manufacturer regarding discs: store temperature, expiry date, etc. and follow accordingly.
- Before performing the sensitivity test bring antibiotic discs to room temperature.
- Strictly do not keep or expose or store antibiotic discs to sunlight.
- The Quality control of the antibiotic discs is mandatory before subjecting the disc in the sensitivity test.
- Strictly avoid dryness and heat that can decrease the control zone size.

2. Sensitivity Testing

- Direct (Primary) test: Inoculum is a specimen.
- Indirect (Secondary) test: Inoculum is a pure culture.

3. Methods of Indirect Testing

- Stokes technique for Antibiotic Sensitivity should be applied as follows: Firstly, Emulsify colonies of the organism to be tested in the sterilized Muller-Hinton broth media.

- Match and compare turbidity developed against standard turbidity given by MacFarland.
- In this technique - No incubation period is required.

4. Direct Sensitivity Testing

- It is performed when the Gram stain of the bacterial smear show large number of organism, showing increased load of bacteria.
- It is performed to get a presumptive result for serious cases.
- This technique is usually adopted for urine, pus and blood cultures.
- It is done if it is difficult to isolate and identify a pathogen or to detect a resistant strain.

5. An Antibiotic Sensitivity Plate Should Not Replace Routine Culture Plate When -

- Blood is added to Muller Hinton agar to be used for direct sensitivity testing.
- The procedure for direct sensitivity is same as for indirect sensitivity.
- The result of direct sensitivity must be confirmed by indirect sensitivity.

6. Do Not Report Direct Sensitivity Result if -

- The growth of the bacteria is too heavy and heaped up or if it is too light and scanty.
- If the Inhibition Zone size is too smaller than that of the control.

XIII. COMMON ANTIBIOTICS IN USE

1. Active Against Gram Positive

- Penicillin (G & V)
- Methicillin
- Cloxacillin
- Erythromycin
- Novobiocin
- Vancomycin
- Bacitracin
- Fucidin

2. Active Against Gram Negative

- Polymyxin
- Aminoglycoside

3. Active Against both Gram Positive & Gram Negative

- Tetracycline
- Chloramphenicol
- Ampicillin
- Cephalosporins

4. Active Against Fungi –

- Greseofulvin
- Iodides
- Nystatin

- Amphotericin B

XIV. DRAWBACKS AND LIMITATIONS OF ANTIBIOTIC SUSCEPTIBILITY TESTING

1. The Antibiotic Sensitivity Test requires bulk amount of reagents.
2. This test needs large space to carry the complete procedure.
3. It is tedious in performing the procedure.
4. The procedure is basically manual and so is time taking.
5. Since it is manual procedure, so chances of error is there.
6. In Disc Diffusion process, MIC and MBC cannot be evaluated.

XV. LATEST TECHNIQUES: EMERGING TECHNIQUES ^{1, 3,7-11,19,20,33,}

Newer Antibiotic Sensitivity Test techniques, which are currently and actively being pursued by commercial entities for clinical translation, are considered as emerging technologies. With due increase of clinical demands for rapid Antibiotic Sensitivity Test, these techniques have been formulated. These techniques detect growth using cell lengths and numbers, forward light scattering, measuring vibrational amplitude changes of magnetic beads etc. The resonators use nanoscale fluctuations. In these techniques Bio-markers or Bio-chemical markers use 16s rRNA, ATP and Luciferase.

Now a day many commercially available systems use automatic inoculating devices with multichannel pipettors. Broth micro dilutions results can be determined visually through automated instruments. Automation provides more precise, reliable and quantitative Antibiotic Sensitivity Test. These automated Antibiotic Sensitivity Test instruments require bacterial isolates obtained through routine culture from patients samples. Some of these instruments use photometer to detect turbidity over 4.5 to 18 hours to reveal Antibiotic Sensitivity Test results. However, in some Turbidometer has been replaced by colorimeter. Some instruments use Fluorescent intensity monitored over 18-24 hours post incubation. Each of these instruments consists of the following-

- A single used AST cassette – micro-dilution tray – containing varied Antibiotics at different concentration
- An Antibiotic Sensitivity Test instrument which reads multiple cassettes over a period of time (usually overnight) to give Antibiotic Sensitivity Test results.

1. Latest Instruments -

- MicroShcan Walk Away – In this technique the report is ready in 3.5 – 7 hours.
- Vitek 2 – Turbidimetric detection – In this technique the report is ready in 4 – 15 hours.
- BD Phoenix automated System.
- Sensiture system.

2. Emerging Techniques -

- Optical Imaging
- Micro-Channel Resonators
- Biosensors
- Quantitating molecular or Biochemical markers

3. Imaging – Based AST:

- Multiplexed Automated Digital Microscopy (MADM) – This technique uses Gel filters, electrokinetic loading and Fluorescent in-situ hybridized (FISH) probes – It identifies bacterial cell in 1 hour, followed by AST.
- Single Cell Morphological Analysis (SCMA) – uses Bright Field microscopy.
- oCelloscope

4. Non - Imaging – based AST:

- Forward Laser Light Scattering – (FLIS)

XVI. MOLECULAR TECHNIQUES

With the use of this technique, Detection of Gene coding for resistance to one or several drugs by technique such as PCR and DNA hybridization can be done easily. These approaches can significantly improve the current commercial Antibiotic Sensitivity Test technology but still rely on culturing, sample preparations, etc., and have some limitation.

1. Limitations:

- Its mechanism is limited to number of Antibiotics.
- It has limited concentrations of the drug to be tested.
- It is limited to analyzing Polymicrobial samples.

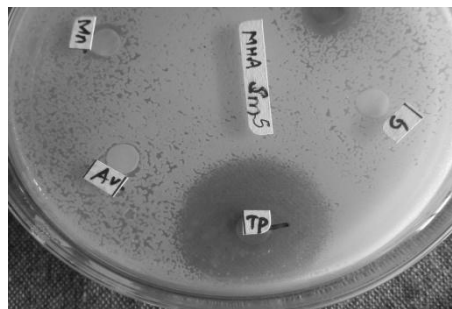


Figure 1: Antibiotic Sensitivity Test Using Herbal Extract (Triphala) Against Streptococcus Mutans - Zone of Inhibition Can be Noted



Figure 2: Antibiotic Sensitivity Test Using Commercially Available Antibiotic Discs Against Actinomycetes - Zones of Inhibition Can Be Noted



Figure 3: Antibiotic Sensitivity Test Using Commercially Available Antibiotic Discs Against Streptococcus Mutans - Zones of Inhibition can be Noted



Figure 4: Antibiotic Sensitivity Test Using Herbal Extract Against Fusobacterium - Zone Of Inhibition can be Noted



Figure 5: Antibiotic Sensitivity Plates

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