

ANTIMICROBIAL SUSCEPTIBILITY TEST OF TETRACYCLINE MICROSPHERES

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ABSTRACT

In the treatment and control of infectious diseases, especially when caused by pathogens that are often drug resistant, sensitivity testing is used to select effective antimicrobial drugs. They are not usually indicated if the sensitivity reactions of a pathogen can be predicted. Sensitivity tests must never be performed on commensal organisms or contaminants because this would mislead the clinician and could result in the patient receiving ineffective and unnecessary drug therapy, causing possible side effects and resistance to other potentially pathogenic organisms. The result of antimicrobial sensitivity tests may also help to identify a pathogen. Some bacterial have characteristic antimicrobial sensitivity patterns. Microspheres prepared with tetracycline loaded antibiotic antimicrobial susceptibility test was carried out and compared with the control disc of the tetracycline.

Keywords: Antimicrobial, Microspheres, Tetracycline.

INTRODUCTION

Resistance to antimicrobial agents has resulted in morbidity and mortality from treatment failures and increased health care costs. Although defining the precise public health risk and estimating the increase in costs is not a simple undertaking, there is little doubt that emergent antibiotic resistance is a serious global problem.

Inappropriate use results from physicians providing antimicrobial drugs to treat viral infections, using inadequate criteria for diagnosis of infections that potentially have a bacterial aetiology, unnecessarily prescribing expensive, broad-spectrum agents, and not following established recommendations for using chemoprophylaxis. Widespread antibiotic usages exerts a selective pressure that acts as a driving force in the development of antibiotic resistance. The association between increased rates of antimicrobial use and resistance has been documented for nosocomial infections as well as for resistant communit acquired infections. As resistance develops to "first-line" antibiotics, therapy with new, broader spectrum, more expensive antibiotics increase,

but is followed by development of resistance to the new class of drugs.

Resistance factors, particularly those carried on mobile elements, can spread rapidly within human and animal populations. Multidrug-resistant pathogens travel not only locally but also globally, with newly introduced pathogens spreading rapidly in susceptible hosts. Antibiotic resistance patterns may vary locally and regionally, so surveillance data needs to be collected from selected sentinel sources. Patterns can change rapidly and they need to be monitored closely because of their implications for public health and as an indicator of appropriate or inappropriate antibiotic usage by physicians in that area.

Principle

The principles of determining the effectivity of a noxious agent to a bacterium were well enumerated by Rideal, Walker and others at the turn of the century, the discovery of antibiotics made these tests (or their modification) too cumbersome for the large numbers of tests necessary to be put up as a routine. The ditch plate method of agar diffusion used by

Alexander Fleming was the forerunner of a variety of agar diffusion methods devised by workers in this field. The Oxford group used these methods initially to assay the antibiotic contained in blood by allowing the antibiotics to diffuse out of reservoirs in the medium in containers placed on the surface.

Factors Influencing Antimicrobial Susceptibility Testing

pH

The pH of each batch of Müller-Hinton agar should be checked when the medium is prepared. The agar medium should have a pH between 7.2 and 7.4 at room temperature after gelling. If the pH is too low, certain drugs will appear to lose potency (e.g., aminoglycosides, quinolones, and macrolides), while other agents may appear to have excessive activity (e.g., tetracyclines). If the pH is too high, the opposite effects can be expected. The pH can be checked by one of the following means

- Macerate a sufficient amount of agar to submerge the tip of a pH electrode.
- Allow a small amount of agar to solidify around the tip of a pH electrode in a beaker or cup.
- Use a properly calibrated surface electrode.

Moisture

If, just before use, excess surface moisture is present, the plates should be placed in an incubator (35°C) or a laminar flow hood at room temperature with lids ajar until excess surface moisture is lost by evaporation (usually 10 to 30 minutes). The surface should be moist, but no droplets of moisture should be apparent on the surface of the medium or on the petri dish covers when the plates are inoculated.

Effects of Thymidine or Thymine

Media containing excessive amounts of thymidine or thymine can reverse the inhibitory effect of sulfonamides and trimethoprim, thus yielding smaller and less distinct zones, or even no zone at all, which may result in false-resistance reports. Müller-Hinton agar that is as low in thymidine content as possible should be used. To evaluate a new lot of Müller-Hinton agar, *Enterococcus faecalis* ATCC 29212, or alternatively, *E. faecalis* ATCC 33186, should be tested with trimethoprim/sulfamethoxazole disks. Satisfactory media will provide essentially clear,

distinct zones of inhibition 20 mm or greater in diameter. Unsatisfactory media will produce no zone of inhibition, growth within the zone, or a zone of less than 20 mm.

Effects of Variation in Divalent Cations

Variation in divalent cations, principally magnesium and calcium, will affect results of aminoglycoside and tetracycline tests with *P. aeruginosa* strains. Excessive cation content will reduce zone sizes, whereas low cation content may result in unacceptably large zones of inhibition. Excess zinc ions may reduce zone sizes of carbapenems. Performance tests with each lot of Müller-Hinton agar must conform to the control limits.

Testing strains that fail to grow satisfactorily Only aerobic or facultative bacteria that grow well on unsupplemented Müller-Hinton agar should be tested on that medium. Certain fastidious bacteria such as *Haemophilus* spp.,

N. gonorrhoeae, *S. pneumoniae*, and viridans and β -haemolytic streptococci do not grow sufficiently on unsupplemented Müller-Hinton agar. These organisms require supplements or different media to grow, and they should be tested on the media described in separate sections.

Preparation of Tetracycline encapsulated chitosan microspheres by spray drying technique

Encapsulation of tetracycline in chitosan microspheres was carried out by spray drying method. Required volume (200 ml) of chitosan solution was prepared using glacial acetic acid solution (1% W/V) by overnight swelling. The required volume of tetracycline was dissolved in 20 ml of methanol. The tetracycline solution was then added to aqueous chitosan solution and homogenized at 400 rpm for 30 min using universal motors Mumbai stirrer. The different drug polymer ratios used. Spray -drying was then performed using a Lu-222 spray drier (Labultima-mumbai) with a standard nozzle with a peristaltic pump, atomization occurred by the force of the compressed air, disrupting the liquid in to small droplets. The droplets together with hot air, were blown in to a drying chamber. The products was then collected in the collector. In standard conditions, the atomization pressure 2 kg cm⁻² and vacume 120 mm respectively.

Table: Batch specifications of the Tetracycline encapsulated chitosan microspheres

Batch code	Drug : polymer	Inlet Temperature	Outlet Temperature	Pump Speed	Nozzle Size
A1	1:0.25	110°C	80°C	2 rpm	1.5 mm
A2	1:0.5	110°C	80°C	2 rpm	1.5 mm
A3	1:0.75	110°C	80°C	2 rpm	1.5 mm
A4	1:1	110°C	80°C	2 rpm	1.5 mm

Methods of Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system. They include

Diffusion	Dilution	Diffusion & Dilution
Stokes method	Minimum Inhibitory Concentration	E-Test method
Kirby-Bauer method	i) Broth dilution ii) Agar Dilution	

MATERIALS AND METHODS

- 1) Plan antibiotic disc of size 6 mm purchased from Himedia
- 2) Tetracycline containing antibiotic disc size 6 mm purchased from Himedia
- 3) Mueller-Hinton Agar Medium
- 4) Sterile distilled water
- 5) Wire loop of 2mm size
- 6) Sterile cotton swab
- 7) UV-visible Spectrophotometer
- 8) Fourier Transformation Infrared Spectrophotometer
- 9) Particle size analyzer
- 10) Analytical weighing balance
- 11) Dissolution test apparatus

METHOD

Disk Diffusion

Reagents for the Disk Diffusion Test

1. Müller-Hinton Agar Medium

Of the many media available, Müller-Hinton agar is considered to be the best 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 sulphonamide, trimethoprim, and tetracycline inhibitors.
- It gives satisfactory growth of most nonfastidious pathogens.
- A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

Preparation of Müller-Hinton Agar

Müller-Hinton agar preparation includes the following steps.

1. Müller-Hinton agar should be prepared from a commercially available dehydrated base according to the manufacturer's instructions.
2. Immediately after autoclaving, It was allow it to cool in a 45 to 50°C water bath.
3. Pour the freshly prepared and cooled medium into glass or plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30 ml for plates with a diameter of 100 mm.
4. The agar medium should be allowed to cool to room temperature and, unless the plate is used the same day, stored in a refrigerator (2 to 8°C).
5. A representative sample of each batch of plates should be examined for sterility by incubating at 30 to 35°C for 24 hours or longer.

Preparation of Tetracycline Microsphere antibiotic stock solutions

Stock solutions are prepared using the formula $(1000/P) \times V \times C = W$, where P=potency of the antibiotic base, V=volume in ml required, C=final concentration of solution and W=weight of the antimicrobial to be dissolved in V.

Storage of commercial antimicrobial discs

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Discs was stored as follows

- Refrigerate the containers at 8°C or below, or freeze at -14°C or below, in a nonfrost-free freezer until needed.
- The unopened disc containers was 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 disks.
- Once a cartridge of discs has been removed from its sealed package, it was placed in a tightly sealed, desiccated

container. When using a disc-dispensing apparatus, it is fitted with a tight cover and supplied with an adequate desiccant. The dispenser should be allowed to warm to room temperature before opening. Excessive moisture was avoided by replacing the desiccant when the indicator changes color.

- When not in use, the dispensing apparatus containing the discs should always be refrigerated.
- Turbidity standard for inoculum preparation

To standardize the inoculum density for a susceptibility test, a BaSO₄ turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), should be used. A BaSO₄ 0.5 McFarland standard may be prepared as follows

1. A 0.5-ml aliquot of 0.048 mol/L BaCl₂ (1.175% w/v BaCl₂ · 2H₂O) is added to 99.5 ml of 0.18 mol/L H₂SO₄ (1% v/v) with constant stirring to maintain a suspension.
2. The correct density of the turbidity standard should be verified by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.008 to 0.10 for the 0.5 McFarland standard.
3. The Barium Sulfate suspension should be transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum.
4. These tubes should be tightly sealed and stored in the dark at room temperature.
5. The barium sulfate turbidity standard should be vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance. If large particles appear, the standard should be replaced. Latex particle suspensions should be mixed by inverting gently, not on a vortex mixer
6. The barium sulfate standards should be replaced or their densities verified monthly.

Disc diffusion methods

The Kirby-Bauer and Stokes' methods are usually used for antimicrobial susceptibility testing, with the Kirby-Bauer method being recommended by the NCCLS. The accuracy and reproducibility of this test are dependent on maintaining a standard set of procedures as described here.

NCCLS is an international, interdisciplinary, non-profit, non-governmental organization composed of medical professionals, government, industry, healthcare providers, educators etc. It promotes accurate antimicrobial susceptibility testing (AST) and appropriate reporting by developing standard reference methods, interpretative criteria for the results of standard AST methods, establishing quality control parameters for standard test methods, provides testing and reporting strategies that are clinically relevant and cost-effective.

Interpretative criteria of NCCLS are developed based on international collaborative studies and well correlated with MIC's and the results have corroborated with clinical data. Based on study results NCCLS interpretative criteria are revised frequently. NCCLS is approved by FDA-USA and recommended by WHO.

Procedure for Performing the Disc Diffusion Test

Inoculum Preparation

Growth Method

The growth method is performed as follows

1. At least three to five well-isolated colonies of the same morphological type are selected from an agar plate culture. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of a suitable broth medium, such as tryptic soy broth.
2. The broth culture is incubated at 35°C until it achieves or exceeds the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours)
3. The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain a turbidity optically comparable to that of the 0.5 McFarland standard. This results in a suspension containing approximately 1 to 2 x 10⁸ CFU/ml for *E.coli* ATCC 25922. To perform this step properly, either a photometric device can be used or, if done visually, adequate light is needed to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.

Inoculation of Test Plates

1. Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the

- tube above the fluid level. This will remove excess inoculum from the swab.
- The dried surface of a Müller-Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed.
 - The lid may be left ajar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

Application of Discs to Inoculated Agar Plates

- The predetermined battery of antimicrobial discs is dispensed onto the surface of the inoculated agar plate. Each disc must be pressed down to ensure complete contact with the agar surface. Whether the discs are placed individually or with a dispensing apparatus, they must be distributed evenly so that they are no closer than 24 mm from center to center. Ordinarily, no more than 12 discs should be placed on one 150 mm plate or more than 5 discs on a 100 mm plate. Because some of the drug diffuses almost instantaneously, a disc should not be relocated once it has come into contact with the agar surface. Instead, place a new disc in another location on the agar.
- The plates are inverted and placed in an incubator set to 35°C within 15 minutes after the discs are applied.

RESULT AND DISCUSSION

Reading Plates and Interpreting Results

Interpretation of results

Report the reaction of the test organism to each antibiotic as 'sensitive', 'intermediate' or 'resistant'; as follows

A. Sensitive (S)

Zone radius is wider is than, equal to, or not more than 3 mm smaller than the control.

A pathogen reported as sensitive suggested that the infection it has caused is likely to respond to treatment if the drug to which it is susceptible is used in normal recommended doses.

B. Intermediate (I)

Zone radius is more than 3 mm smaller than control but not less than 3 mm.

A pathogen reported as being intermediately sensitive suggests that the infection it has caused is likely to respond to treatment if the to which it is susceptible is used in larger dose than normal or when the drug is concentrated at the site of infection, for example in the urinary tract.

C. Resistant (R)

No zone of inhibition or zone radius measure 2 mm or less.

A pathogen reported as resistant implies that the infection it has caused will not respond to treatment with the drug to which it is resistant irrespective of dose or site of infection.

- After 16 to 18 hours of incubation, each plate is examined. The resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the disc. Zones are measured to the nearest whole millimeter, using a ruler, which is held on the back of the inverted petri plate. The petri plate is held a few inches above a black, nonreflecting background and illuminated with reflected light.
- The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, is ignored. However, discrete colonies growing within a clear zone of inhibition should be subcultured, re-identified, and retested.
- The sizes of the zones of inhibition are interpreted by referring to (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints) the NCCLS M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement, and the organisms are reported as either susceptible, intermediate, or resistant to the agents that have been tested. Some agents may only be reported as susceptible, since only susceptible breakpoints are given.

**Zone Size Interpretative Chart
(Based on Results obtained using Mueller Hinton Agar)**

Product Code	Antimicrobial Agent	Symbol	Disc Content	Interpretative Criteria		
				Sensitive mm or more	Intermediate mm	Resistant mm or less
SD037	Tetracycline Enterobacteriaceae. Acientobacter, staphylococcus, Enterococcus, N.meningitidis, H. influenzae, N.gonorhoease, Streptococcus app. Beta haemolytic group & Streptococcus spp	TE	30 mcg	15 19 29 23	12-14 15-18 26-28 19-22	11 14 25 18

Reading Plates and Interpreting Results

Product Code	Antimicrobial Agent	Disc content	Control disc mm	Test disc mm
ATCC 25922	Shigella flexneri	30 mcg	25	23
	Salmonella typhi	30 mcg	30	28
	Streptococcus spp.	30 mcg	41	38
	E. coli	30 mcg	20	19



Fig. 1: Stokes disc diffusion technique showing inhibition zones of control and test organism against shigella flexneri organism



Fig. 3: Stokes disc diffusion technique showing inhibition zones control and test organism against Streptococcus spp. Organism



Fig. 2: Stokes disc diffusion technique showing inhibition zones of control and test organism against salmonella typhi organism



Fig.4: Stokes disc diffusion technique showing inhibition zones control and test organism against.E.coli. organism

Quality Control in Antibiotic Susceptibility Testing

QC is performed to check the quality of medium, the potency of the antibiotic, to check manual errors. Quality control strains should be included daily with the test. Not more than 1 in 20 results should be outside accuracy limits. No zone should be more than 4 standard deviations away from midpoint between the stated limits.

If, for reasons of expense or manpower constraints, it is not possible to include all strains on a daily basis, then the following guidelines should be followed.

The frequency can be decreased to once weekly if proficiency has been demonstrated by

- 1) Performing QC daily for 30 days with less than 10% inaccuracy for each drug
- 2) Proficiency testing is repeated for each new drug included in the testing
- 3) All documentation is maintained indefinitely.
- 4) Proficiency testing is repeated for each new batch of media or reagents

All tests must be within accuracy limits if QC is done once weekly.

Reference strains for quality control

Escherichia coli ATCC 25922 (beta-lactamase negative)

Escherichia coli ATCC 35218 (beta-lactamase positive)

Staphylococcus aureus ATCC 25923 (beta-lactamase negative, oxacillin susceptible)

Staphylococcus aureus ATCC 38591 (beta-lactamase positive)

Pseudomonas aeruginosa ATCC 27853 (for aminoglycosides)

Enterococcus faecalis ATCC 29212 (for checking of thymidine or thymine level of MHA)

Haemophilus influenzae ATCC 49766 (for cephalosporins)

Haemophilus influenzae ATCC 10211 (for medium control)

Neisseria gonorrhoeae ATCC 49226

Stock cultures should be kept at -70°C in Brucella broth with 10% glycerol for up to 3 years. Before use as a QC strain, the strain should be subcultured at least twice and retested for characteristic features. Working cultures are maintained on TSA slants at 2-8°C for up to 2 weeks.

CONCLUSION

The microspheres were able to entrap the drug at high levels and sustain its release over a prolonged time. Microspheres prepared with tetracycline loaded antibiotic antimicrobial susceptibility test was carried out and compared with the control disc of the tetracycline. The

diameter of the zones of complete inhibition are measured, including the diameter of the disc. Zones are measured to the nearest whole millimeter, using sliding caliper disc prepared with diffusion method shows positive result.

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REFERENCES

1. Doern GV, Murray PR, Baron EJ, Pfaller MA, Tover FC and Tenover R, Susceptibility tests of fastidious bacteria. Manual of Clinical Microbiology, 6th edition, American Society for Microbiology, Washington DC. 1995; 1342-1349.
2. Ira R. Bacteriology, Standard Operative procedure manual for microbiology laboratories, National Institute of Biologicals. 1995;73-97.
3. John DT and James H, Murray PR, Baron EJ, Pfaller MA, Tenover FC and Tenover R. J Antimicrobial Susceptibility testing: General Considerations. Manual of Clinical Microbiology 7th edition. American Society for Microbiology, Washington DC. 1999;1469-1473.
4. National Committee for Clinical Laboratory Standards. Performance Standards for antimicrobial susceptibility testing. 8th Informational Supplement. M100 S12. National Committee for Clinical Laboratory Standards, 2002; Villanova, Pa.
5. Thrupp LD. Susceptibility Testing of Antibiotic in Liquid Media. Antibiotics In Laboratory Medicine 2nd Edition, Victor Lorian, Williams and Wilkins, Baltimore. 1986;93-150.
6. Water worth PM, Reeves DS, Philips I, Williams JD and Wise R. Quantitative methods for bacterial sensitivity testing. Laboratory methods in antimicrobial chemotherapy. Churchill Livingstone, Baltimore. 1978;31-41.
7. Kramer J and Kirshbaum A. Effect of paper on the performance assay in the control of antibiotic sensitivity discs. Appl Microbiol. 1961; 9:334-336.
8. Rippere RA. Effects of paper on the performance of antibiotic-impregnated discs. J Pharma Sc.i 1978;67:367-371.

9. Lalitha MK, Manayani DJ, Priya L, Jesudason MV, Thomas K and Steinhoff MC. E test as an alternative to conventional MIC determination for surveillance of drug resistant *S.pneumoniae*. *Indian J Med Res.* 1997; 106:500-503.
10. Morley DC. A simple method for testing the sensitivity of wound bacteria to penicillin and sulphathiazole by use of impregnated blotting paper disc. *J Pathol Bacteriol.* 1945: 57;379-382.
11. A guide to sensitivity testing: Report of working party on antibiotic sensitivity testing of the British Society for Antimicrobial Chemotherapy. *J Antimicrob Chemotherap.* 1991;27: Supplement D,1-50.