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Theoretical Investigation on Antimicrobial Susceptibility Testing Methods

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Abstract: The resistance to antimicrobial agents has an effect in morbidity and transience from treatment failures and improved health awareness expenses. Although crucial the specific public health risk and estimating the increase in expenses is not an easy responsibility, there is modest uncertainty that growing antibiotic resistance is a severe global problem. Suitable antimicrobial drug use has unquestionable advantage, but physicians and the public frequently use these agents inappropriately. Inappropriate use results from physicians providing antimicrobial drugs to indulge viral infections, using. The simple accessibility of antimicrobial drugs leads to their incorporation into herbal or "folk" remedies that also increase unsuitable use of these agents. In this present investigation the results of in-vitro antibiotic susceptibility testing, guide clinicians in the suitable selection of initial empiric regimens and, drugs used for individual patients in specific situations are explained in detail.

Keywords: Resistance to Antimicrobial Agents, Antimicrobial Drugs, Antibiotic Susceptibility Testing,
Gram Negative Bacilli, Disc Diffusion Method

1. Introduction

AMR [Resistance to antimicrobial agents] has resulted in morbidity and transience from treatment failures and improved health awareness expenses. Suitable antimicrobial drug use has unquestionable advantage, but physicians and the public frequently use these agents inappropriately. Inappropriate use results from physicians providing antimicrobial drugs to indulge viral infections, using insufficient criteria for diagnosis of infections that potentially have a bacterial aetiology, gratuitously prescribing expensive, broad-spectrum agents, and not following recognized recommendations for using chemo prophylaxis. The simple accessibility of antimicrobial drugs leads to their incorporation into herbal or "folk" remedies that also increase unsuitable use of these agents. Antibiotic usage exerts a selective pressure that acts as a driving force in the progress of antibiotic resistance. The connection between increased rates of antimicrobial use and resistance has been predictable for nosocomial infections as well as for resistant area acquired infections. Resistance factors carried on mobile elements, can spread rapidly within human and animal populations. Antibiotic resistance patterns may differ locally and regionally, so observation data needs to be collected from selected sentinel sources. Patterns can change quickly 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. The results of in-vitro antibiotic susceptibility testing, guide clinicians in the suitable selection of initial empiric regimens and, drugs used for individual patients in specific situations.

2. Principle

The principles of determining the affectivity of a noxious agent to a bacterium were well specified by Rideal, Walker and others at the turn of the century, the innovation of antibiotics made these tests (or their variation) too unwieldy for the large numbers of tests needed to be put up as a usual way. 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 at first to assay the

antibiotic enclosed in blood by allowing the antibiotics to diffuse out of reservoirs in the medium in containers placed on the surface.

With the beginning of a variety of antimicrobials it became vital to perform the antimicrobial susceptibility test as a routine. For this, the antimicrobial contained in a reservoir was permitted to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. Even now a variety of antimicrobial containing reservoirs are used but the antimicrobial impregnated absorbent paper disc is by far the commonest type used. The disc diffusion method of AST is the most practical method and is still the method of choice for the average laboratory. Automation may force the method out of the diagnostic laboratory but in this country as well as in the smaller laboratories of even advanced countries, it will certainly be the most commonly carried out microbiological test for many years to come. All techniques involve either diffusion of antimicrobial agent in agar or dilution of antibiotic in agar or broth.

Even automated techniques are variations of the above methods.

3. Factors Influencing Antimicrobial Susceptibility Testing

3.1. PH

The pH of each batch of Müeller-Hinton agar should be checked when the medium is prepared. The precise method used will depend mostly on the type of equipment accessible in the laboratory. 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:
1. Macerate a sufficient amount of agar to submerge the tip of a pH electrode.
2. Allow a small amount of agar to solidify around the tip of a pH electrode in a beaker or cup.
3. Use a properly calibrated surface electrode.

3.2. Moisture

If, just previous to use, surplus 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 surplus surface moisture is lost by evaporation (typically 10 minutes 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.

3.3. Effects of Thymidine or Thymine

Media containing excessive amounts of thymidine or thymine can reverse the inhibitory effect of sulfonamides and trimethoprim, thus yielding lesser and less distinct zones, or

even no zone at all, which may result in false-resistance reports. Müeller-Hinton agar that is as low in thymidine content as possible should be used.

To evaluate a new lot of Müeller-Hinton agar, Enterococcus faecalis ATCC 29212, or instead, 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.

3.4. Effects of Variation in Divalent Cations

Variation in divalent cations, mainly magnesium and calcium, will affect results of aminoglycoside and tetracycline tests with P. aeruginosa strains. Excessive cation content will decrease zone sizes, while low cation content may result in inappropriately huge zones of inhibition. Excess zinc ions may reduce zone sizes of carbapenems. Performance tests with each lot of Müeller-Hinton agar must conform to the control limits.

3.5. Testing Strains That Fail to Grow Satisfactorily

Only aerobic or facultative bacteria that grow well on unsupplemented Müeller-Hinton agar should be tested on that medium. Certain fastidious bacteria such as Haemophilus spp., N. gonorrhoeae, S. pneumoniae, and viridans and ß-haemolytic streptococci do not develop adequately on unsupplemented Müeller-Hinton agar. These organisms require supplements or dissimilar media to develop, and they should be tested on the media described in separate sections.

4. 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	Broth dilution Agar Dilution	-

4.1. Disk Diffusion

4.1.1. Reagents for the Disk Diffusion Test
Müeller-Hinton Agar Medium
Of the many media available, Müeller-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.

Although Müeller-Hinton agar is reliable generally for susceptibility testing, results obtained with some batches may, on occasion, vary significantly. If a batch of medium does not support adequate growth of a test organism, zones obtained in a disk diffusion test will usually be larger than expected and may exceed the acceptable quality control limits. Only Müeller-Hinton medium formulations that have been tested according to, and that meet the acceptance limits described in, NCCLS document M62-A7- Protocols for Evaluating Dehydrated Müeller-Hinton Agar should be used.

Preparation of Müeller-Hinton Agar

Müeller-Hinton agar preparation includes the following steps.

1. Müeller-Hinton agar should be prepared from a commercially available dehydrated base according to the manufacturer's instructions.
2. Immediately after autoclaving, 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. Plates should be used within seven days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimize drying of the agar.
6. 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 antibiotic stock solutions

Antibitiotics may be received as powders or tablets. It is recommended to obtain pure antibiotics from commercial sources, and not use injectable solutions. Powders must be accurately weighed and dissolved in the appropriate diluents (Annexure III) to yield the required concentration, using sterile glassware. Standard strains of stock cultures should be used to evaluate the antibiotic stock solution. If satisfactory, the stock can be aliquoted in 5 ml volumes and frozen at -20ºC or -60ºC. Stock solutions are prepared using the formula (1000/P) X V X C = W, where P+ potency of the anitbiotic base, V = volume in ml required, C = final concentration of solution and W = weight of the antimicrobial to be dissolved in V.

Preparation of dried filter paper discs

Whatman filter paper no. 1 is used to prepare discs approximately 6 mm in diameter, which are placed in a Petri dish and sterilized in a hot air oven. The loop used for delivering the antibiotics is made of 20 gauge wire and has a diameter of 2 mm. This delivers 0.005 ml of antibiotics to each disc.

Storage of commercial antimicrobial discs

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Discs should be 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. Sealed packages of disks that contain drugs from the ß-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 disks.
* Once a cartridge of discs has been removed from its sealed package, it should be placed in a tightly sealed, desiccated container. The dispenser should be allowed to warm to room temperature before opening. Excessive moisture should be avoided by replacing the desiccant when the indicator changes color.
* When not in use, the dispensing apparatus containing the discs should always be refrigerated.
* 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.

Turbidity standard for inoculum preparation

To standardize the inoculum density for a susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), should be used. A $BaSO_4$ 0.5 McFarland standard may be prepared as follows:

1. A 0.5-ml aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2$. $2H_2O$) is added to 99.5 ml of 0.18 mol/L H_2SO_4 (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. 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.

4.1.2. 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. 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.

Direct Colony Suspension Method

1. As a convenient alternative to the growth method, the inoculum can be prepared by making a direct broth or saline suspension of isolated colonies selected from a 18- to 24-hour agar plate (a nonselective medium, such as blood agar, should be used). The suspension is adjusted to match the 0.5 McFarland turbidity standard, using saline and a vortex mixer.

2. This approach is the recommended method for testing the fastidious organisms, Haemophilus spp., N. gonorrhoeae, and streptococci, and for testing staphylococci for potential methicillin or oxacillin resistance.

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.

2. The dried surface of a Müeller-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.

3. 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.

NOTE: Extremes in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

Application of Discs to Inoculated Agar Plates

1. 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.

2. The plates are inverted and placed in an incubator set to 35°C within 15 minutes after the discs are applied. With the exception of Haemophilus spp., streptococci and N. gonorrhoeae, the plates should not be incubated in an increased CO_2 atmosphere, because the interpretive standards were developed by using ambient air incubation, and CO_2 will significantly alter the size of the inhibitory zones of some agents.

Reading Plates and Interpreting Results

1. After 16 to 18 hours of incubation, each plate is examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated. 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 sliding calipers or 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. If blood was added to the agar base (as with streptococci), the zones are measured from the upper surface of the agar illuminated with reflected light, with the cover removed. If the test organism is a Staphylococcus or Enterococcus spp., 24 hours of incubation are required for vancomycin and oxacillin, but other agents can be read at 16 to 18 hours. Transmitted light (plate held up to light) is used to examine the oxacillin and vancomycin zones for light growth of methicillin- or vancomycin- resistant colonies, respectively, within apparent zones of inhibition. Any discernable growth within zone of inhibition is indicative of methicillin or vancomycin resistance.

2. 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. Strains of Proteus spp. may swarm into areas of inhibited growth around certain antimicrobial agents. With Proteus spp., the thin veil of swarming growth in an otherwise obvious zone of inhibition should be ignored. When using blood-supplemented medium for testing streptococci, the zone of growth inhibition should be measured not zone of inhibition of hemolysis. With trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore, disregard slight growth (20% or less of the lawn of growth), and measure the more obvious margin to determine the zone diameter.

The sizes of the zones of inhibition are interpreted by referring to Tables 2A through 2I (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints) of the NCCLS M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement and the organisms are reported as 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.

4.2. Dilution

Dilution Methods

Dilution susceptibility testing methods are used to determine the minimal concentration of antimicrobial to inhibit or kill the microorganism. This can be achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials are tested in $\log_2$ serial dilutions (two fold).

4.2.1. Minimum Inhibitory Concentration (MIC)

Diffusion tests widely used to determine the susceptibility

of organisms isolated from clinical specimens have their limitations; when equivocal results are obtained or in prolonged serious infection e.g. bacterial endocarditis, the quantitation of antibiotic action vis-a-vis the pathogen needs to be more precise. Also the terms 'Susceptible' and 'Resistant' can have a realistic interpretation. Thus when in doubt, the way to a precise assessment is to determine the MIC of the antibiotic to the organisms concerned.

There are two methods of testing for MIC:
a Broth dilution method and
b Agar dilution method.

i. Broth Dilution Method

The Broth Dilution method is a simple procedure for testing a small number of isolates, even single isolate. It has the added advantage that the same tubes can be taken for MBC tests also:

Materials

Sterile graduated pipettes of 10ml, 5ml, 2ml and 1ml Sterile capped 7.5 x 1.3 cm tubes / small screw-capped bottles, Pasteur pipettes, overnight broth culture of test and control organisms (same as for disc diffusion tests), required antibiotic in powder form (either from the manufacturer or standard laboratory accompanied by a statement of its activity in mg/unit or per ml. Clinical preparations should not be used for reference technique.), required solvent for the antibiotic, sterile Distilled Water - 500ml and suitable nutrient broth medium. Trimethoprim and sulphonamide testing requires thymidine free media or addition of 4% lysed horse blood to the media. A suitable rack hold 22 tubes in two rows i.e. 11 tubes in each row.

Stock solution

Stock solution can be prepared using the formula,

$$\frac{1000}{P} \times V \times C = W,$$

where P = Potency given by the manufacturer in relation to the base, V= Volume in ml required, C=Final concentration of solution (multiples of 1000), W= Weight of the antimicrobial to be dissolved in the volume V.

Example: For making 10 ml solution of the strength 10,000mg/l from powder base whose potency is 980 mg per gram, the quantities of the antimicrobials required is

$$\frac{1000}{980} \times 10 \times 10 = W = 102.04 \, mg,$$

Note: The stock solutions are made in higher concentrations to maintain their keeping qualities and stored in suitable aliquots at -20oC. Once taken out, they should not be refrozen or reused. Suggested dilution ranges of some antimicrobials are shown in Table II.

Method

Prepare stock dilutions of the antibiotic of concentrations 1000 and 100 μg/L as required from original stock solution (10,000mg/L). Arrange two rows of 12 sterile 7.5 x1.3 cm capped tubes in the rack. In a sterile 30ml (universal) screw capped bottle, prepare 8ml of broth containing the concentration of antibiotic required for the first tube in each

row from the appropriate stock solution already made. Mix the contents of the universal bottle using a pipette and transfer 2ml to the first tube in each row. Using a fresh pipette, add 4 ml of broth to the remaining 4 ml in the universal bottle mix and transfer 2ml to the second tube in each row. Continue preparing dilutions in this way but where as many as 10 or more are required the series should be started again half the way down. Place 2ml of antibiotic free broth to the last tube in each row. Inoculate one row with one drop of an overnight broth culture of the test organism diluted approximately to 1 in 1000 in a suitable broth and the second row with the control organism of known sensitivity similarly diluted. The result of the test is significantly affected by the size of the inoculum. The test mixture should contain 106 organism/ml. If the broth culture used has grown poorly, it may be necessary to use this undiluted. Incubate tubes for 18 hours at 37oC. Inoculate a tube containing 2ml broth with the organism and keep at +4oC in a refrigerator overnight to be used as standard for the determination of complete inhibition.

Calculations for the preparation of the original dilution

This often presents problems to those unaccustomed to performing these tests. The following method advocated by Pamela M Waterworth is presented. Calculate the total volume required for the first dilution. Two sets of dilution are being prepared (one for the test and one for the control), each in 2ml volumes i.e. a total of 4 ml for each concentration as 4ml is required to make the second dilution, the total requirement is 8ml. Now calculate the total amount of the antibiotic required for 8ml. For 64 g/l concentration, $8 \times 64mg/l = 512\mu g$ in 8 ml. Place a decimal point after the first figure (5.12) and take this volume in ml (i.e. 5.12 ml) of the dilution below 512mg/l and make up to 8ml with broth. In this example given above, the series has to be started again mid way at 2 mg/l which would be obtained in the same way:

8ml of 2mg/l=16µg in 8ml.

16ml of 10 mg/ l + 6.4 ml of broth.

Reading of result

MIC is expressed as the lowest dilution, which inhibited growth judged by lack of turbidity in the tube. Because very faint turbidity may be given by the inoculum itself, the inoculated tube kept in the refrigerator overnight may be used as the standard for the determination of complete inhibition.

Standard strain of known MIC value run with the test is used as the control to check the reagents and conditions.

Minimum Bactericidal Concentrations (MBC)

The main advantage of the 'Broth dilution' method for the MIC determination lies in the fact that it can readily be converted to determine the MBC as well.

Method

Dilutions and inoculations are prepared in the same manner as described for the determination of MIC. The control tube containing no antibiotic is immediately subcultured (Before incubation) by spreading a loopful evenly over a quarter of the plate on a medium suitable for the growth of the test organism and incubated at 37oC

overnight. The tubes are also incubated overnight at 37oC. Read the MIC of the control organism to check that the drug concentrations are correct. Note the lowest concentration inhibiting growth of the organisms and record this as the MIC. Subculture all tubes not showing visible growth in the same manner as the control tube described above and incubate at 37oC overnight. Compare the amount of growth from the control tube before incubation, which represents the original inoculum. The test must include a second set of the same dilutions inoculated with an organism of known sensitivity. These tubes are not subcultured; the purpose of the control is to confirm by its MIC that the drug level is correct, whether or not this organism is killed is immaterial.

Reading of result

These subcultures may show

Similar number of colonies- indicating bacteriostasis only.

A reduced number of colonies-indicating a partial or slow bactericidal activity.

No growth- if the whole inoculum has been killed.

The highest dilution showing at least 99% inhibition is taken as MBC.

Micro-broth dilution test

This test uses double-strength Müeller-Hinton broth, 4X strength antibiotic solutions prepared as serial two-fold dilutions and the test organism at a concentration of 2×10^{6}/ml. In a 96 well plate, 100 µl of double-strength MHB, 50 µl each of the antibiotic dilutions and the organism suspension are mixed and incubated at 35°C for 18-24 hours. The lowest concentration showing inhibition of growth will be considered the MIC of the organism.

Reading of result

MIC is expressed as the highest dilution which inhibited growth judged by lack of turbidity in the tube. Because very faint turbidity may be given by the inoculum itself, the inoculated tube kept in the refrigerator overnight may be used as the standard for the determination of complete inhibition. Standard strain of known MIC, run with the test is used as the control to check the reagents and conditions.

ii. The Agar Dilution Method

Agar dilutions are most often prepared in petri dishes and have advantage that it is possible to test several organisms on each plate. If only one organism is to be tested e.g M. tuberculosis, the dilutions can be prepared in agar slopes but it will then be necessary to prepare a second identical set to be inoculated with the control organism. The dilutions are made in a small volume of water and added to agar which has been melted and cooled to not more than 60°C. Blood may be added and if 'chocolate agar' is required, the medium must be heated before the antibiotic is added. It would be convenient to use 90 mm diameter petri dishes and add one ml of desired drug dilutions to 19 ml of broth. The factor of agar dilution must be allowed for in the first calculation as follows.

Final volume of medium in plate = 20 ml

Top antibiotic concentrations = 64 mg/l

Total amount of drug = 1280 µg to be added to 1 ml of water

2ml of 1280 µg /ml will be required to start the dilution = 2560 µg in 2 ml = 1.28 ml of 2000 µg /ml ± 0.72 ml of water

1ml of this will be added to 19 ml agar. (Note: stock dilution of 2000µg /ml is required for the range of MIC)

The quickest way to prepare a range of dilutions in agar is as follows:

Label a sterile petri dish on the base for each concentration required. Prepare the dilutions in water placing 1 ml of each in the appropriate dish. One ml water is added to a control plate. Pipette 19 ml melted agar, cooled to 55oC to each plate and mix thoroughly. Adequate mixing is essential and if sufficient technical expertise is not available for the skilled manipulation, it is strongly recommended that the agar is first measured into stoppered tubes or universal containers and the drug dilution added to these and mixed by inversion before pouring into petri dishes. After the plates have set they should be well dried at 37oC with their lids tipped for 20 to 30 minutes in an incubator. They are then inoculated either with a multiple inoculator as spots or with a wire loop or a platinum loop calibrated to deliver 0.001ml spread over a small area. In either case the culture should be diluted to contain 105 to 106 organisms per ml. With ordinary fast growing organisms, this can be obtained approximately by adding 5 µl of an overnight broth culture to 5ml broth or peptone water.

It is possible to test spreading organism such as P. mirabilis by this method either by cutting ditches in the agar between the inocula, or by confining each with small glass or porcelain cylinders pressed into the agar. Although swarming of P. mirabilis can be prevented by the use of higher concentration of agar in the medium, this is not recommended for determination of MIC because of the difficulty of ensuring adequate mixing of the drug with this very viscous medium. Selective media should not be used and electrolyte deficient media will give false results because of the effect of variation in the salt content on the action of many antibiotics.

Reading of results

The antibiotic concentration of the first plate showing ≥ 99% inhibition is taken as the MIC for the organism.

4.3. Dilution & Diffusion

E test also known as the epsilometer test is an 'exponential gradient' testing methodology where 'E' in E test refers to the Greek symbol epsilon (ε). The E test(AB Biodisk) which is a quantitative method for antimicrobial susceptibility testing applies both the dilution of antibiotic and diffusion of antibiotic into the medium.. A predefined stable antimicrobial gradient is present on a thin inert carrier strip. When this E test strip is applied onto an inoculated agar plate, there is an immediate release of the drug. Following incubation, a symmetrical inhibition ellipse is produced. The intersection of the inhibitory zone edge and the calibrated carrier strip indicates the MIC value over a wide concentration range (>10 dilutions) with inherent precision and accuracy.

E test can be used to determine MIC for fastidious organisms like S. pneumoniae, ß-hemolytic streptococci, N. gonorrhoeae, Haemophilus sp. and anaerobes. It can also be used for Nonfermenting Gram Negative bacilli (NFGNB) for eg-Pseudomonas sp. and Burkholderia pseudomallei.

Resistance of major consequence may be detected for e.g., the test is very useful in detecting glycopeptide resistant Enterococci (GRE) and glycopeptide intermediate S. aureus (GISA) and slow growing pathogens such as Mycobacterium tuberculosis. Further it can be used for detection of extended spectrum beta lactamases (ESBL). In conclusion E test is a simple, accurate and reliable method to determine the MIC for a wide spectrum of infectious agents.

5. Susceptibility Testing of Fastidious Bacteria

5.1. Disc Diffusion for Fastidious Organisms

5.1.1. Antibiotic Susceptibility Testing of S. Pneumoniae

i. Media for Disc Diffusion

Müeller -Hinton Sheep blood agar
Standardization of inoculum.

The inocula for seeding the susceptibility media with S. pneumoniae is prepared from fresh pure cultures (grown overnight on Chocolate agar). Cell suspensions of the bacteria to be tested are prepared in sterile saline or Müeller-Hinton broth. The cell suspension is prepared by transferring a portion of the fresh growth with a swab or inoculating loop to the suspending medium, using caution when mixing the cells with the suspending medium so as not to form bubbles. The suspension is then compared to the McFarland standard by holding the suspension and McFarland standard in front of a light against a white background with contrasting black lines and comparing the turbidity. If the turbidity is too heavy, the suspension should be diluted with additional suspending medium. If the turbidity is too light additional cells should be added to the suspension. For S. pneumoniae – Direct colony suspension is made in normal saline and turbidity adjusted to 0.5 McFarland standard. Within 15 minutes after adjusting the turbidity of the suspension the plate should be inoculated.

ii. Inoculation of the Susceptibility Test Media

After proper turbidity is achieved, a new sterile swab (cotton or dacron) is submerged in the suspension, lifted out of the broth, and the excess fluid is removed by pressing and rotating the swab against the wall of the tube. The swab is then used to inoculate the entire surface of the supplemented Müeller Hinton agar plate three times, rotating the plate 60 degrees between each inoculation. The inoculum is allowed to dry (usually taking only a few minutes but no longer than 15 minutes) before the discs are placed on the plates. The discs should be placed on the agar with sterile forceps and tapped gently to ensure the adherence to the agar. The plates containing the disks are incubated at 35°C for 16 to 18 h in an inverted position in a 5% CO_2 incubator. A candle

extinction jar may be used if a CO_2 incubator is not available.

iii. Estimating the Susceptibility of the Strains

After overnight incubation, the diameter of each zone of inhibition is measured with a ruler or calipers. The zones of inhibition on the media containing blood are measured from the top surface of the plate with the top removed. It is convenient to use a ruler with a handle attached for these measurements, holding the ruler over the surface of the disk when measuring the inhibition zone. Care should be taken not to touch the disk or surface of the agar. Sterilize the ruler occasionally to prevent transmission of bacteria. In all measurements, the zones of inhibition are measured from the edges of the last visible colony-forming growth. The ruler should be positioned across the center of the disc to make these measurements. The results are recorded in millimeters (mm) and interpretation of susceptibility is obtained by comparing the results to the standard zone sizes. For S. pneumoniae the zone measurement is from top of plate with the lid removed. Faint growth of tiny colonies that may appear to fade from the more obvious zone should be ignored in the measurement.

iv. Interpretation

Each zone size is interpreted by reference to the Table 2G (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints for S. pneumoniae) of the NCCLS M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement as susceptible, intermediate and resistant.

5.1.2. Antibiotic Susceptibility of Haemophilus Species

The medium of choice for disc diffusion testing of Haemophilus sp. is Haemophilus Test Medium (HTM). Müeller-Hinton chocolate agar is not recommended for routine testing of Haemophilus spp. In its agar form, Haemophilus Test medium consists of the following ingredients.
* Müeller-Hinton agar,
* 15 µg/ml ß-NAD,
* 15 µg/ml bovine hematin, and
* 5-mg/ml yeast extract.

To make HTM, first a fresh hematin stock solution is prepared by dissolving 50 mg of bovine hematin powder in 100 ml of 0.01 mol/L NaOH with heat and stirring until the powder is thoroughly dissolved. Thirty ml of the hematin stock solution are added to 1 L of MHA with 5 g of yeast extract. After autoclaving and cooling to 45°C to 50°C, 3 ml of an NAD stock solution (50 mg of NAD dissolved in 10 ml of distilled water and filter sterilized) are also aseptically added. The pH should be 7.2 to 7.4.

i. Test Procedure

1. The direct colony suspension procedure should be used when testing Haemophilus sp. Using colonies taken directly from an overnight (preferably 20 to 24 hour) chocolate agar culture plate, a suspension of the test organism is prepared in Müeller-Hinton broth or 0.9% saline. The suspension should be adjusted to a turbidity equivalent to a 0.5 McFarland standard using a photometric device. This suspension will contain approximately 1 to 4 x 108 CFU/ml. Care must be exercised in preparing this suspension, because higher inoculum concentrations may lead to false-resistant results with some ß-lactam antibiotics, particularly when ß-lactamase producing strains of H. influenzae are tested. Within 15 minutes after adjusting the turbidity of the inoculum suspension, it should be used for plate inoculation.

2. The procedure for the disc test should be followed as described for nonfastidious bacteria, except that, in general, no more than 9 discs should be applied to the surface of a 150-mm plate or no more than 4 discs on a 100-mm plate.

3. Plates are incubated at 35°C in an atmosphere of 5% CO_2 for 16 to 18 hours before measuring the zones of inhibition.

4. The zone margin should be considered as the area showing no obvious growth visible with the unaided eye. Faint growth of tiny colonies that may appear to fade from the more obvious zone, it should be ignored in the measurement.

ii. Zone Diameter Interpretive Criteria

The antimicrobial agents suggested for routine testing of Haemophilus sp. are indicated in Annexure I. Each zone size is interpreted by reference to the Table 2E (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints for Haemophilus sp.) of the NCCLS M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement as susceptible, intermediate and resistant. Disc diffusion testing of Haemophilus spp. with other agents is not recommended.

5.1.3. Antibiotic Susceptibility Testing for Neisseria Gonorrhoeae

The recommended medium for testing N. gonorrhoeae consists of GC agar to which a 1% defined growth supplement is added after autoclaving. Cysteine-free growth supplement is not required for disc testing. Enriched chocolate agar is not recommended for susceptibility testing of N. gonorrhoeae.

i. Test Procedure

1. The direct colony suspension procedure should be used when testing N. gonorrhoeae. Using colonies taken directly from an overnight chocolate agar culture plate, a suspension equivalent to that of the 0.5 McFarland standards is prepared in either Müeller-Hinton broth or 0.9% saline. Within 15 minutes after adjusting the turbidity of the inoculum suspension, it should be used for plate inoculation.

2. The disc diffusion test procedure steps, as described for nonfastidious bacteria, should be followed. No more than 9 antimicrobial discs should be placed onto the agar surface of a 150-mm agar plate not more than 4 discs onto a 100-mm plate. However, when testing

some agents (e.g., quinolones) which produce extremely large zones, fewer discs may need to be tested per plate.
3. The plates are incubated at 35°C in an atmosphere of 5% CO2 for 20 to 24 hours before measuring the zones of inhibition.

ii. Zone Diameter Interpretive Criteria

The antimicrobial agents suggested for routine testing of N. gonorrhoeae are indicated in Annexure I. Each zone size is interpreted by reference to the Table 2F (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints for N. gonorrhoeae) of the NCCLS M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement as susceptible, intermediate and resistant. Disc diffusion testing of N. gonorrhoeae with other agents is not recommended.

[Note: Organisms with 10-μg penicillin disc zone diameters of < 19 mm generally produce ß-lactamase.]

However, ß-lactamase tests are faster and are therefore preferred for recognition of this plasmid-mediated resistance. Organisms with plasmid-mediated resistance to tetracycline also have zones of inhibition (30-μg tetracycline discs) of < 19 mm.

Chromosomal mechanisms of resistance to penicillin and tetracycline produce larger zone diameters, which can be accurately recognized using the interpretive criteria indicated in Table 2F (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints for N. gonorrhoeae) of the NCCLS M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement.

5.2. Determination of MIC for Fastidious Organisms

5.2.1. The Agar Dilution Method

i. Standardization of Inoculum

The inoculum should be an actively growing culture diluted in saline to 104 to 105 microorganism per ml.

For S. pneumoniae – Direct colony suspension from a 12-15 hour culture from TSBA medium is to be used. The colonies are suspended in 0.5ml of normal saline and the opacity adjusted to McFarland 0.5. A 1/10 dilution of this suspension is made and within 15 minutes of making the diluted suspension the test plates should be inoculated with either a platinum loop calibrated to deliver 0.001ml or multipoint inoculator. For N. gonorrhoeae and H. influenzae-Similar to the procedure described above for S. pneumoniae

ii. Inoculation of Test Plate

In general the inoculum should be applied as a spot that covers a circle about 5-8mm in diameter. A platinum loop calibrated to deliver 0.001ml of the inoculum is used to spot inoculate the cultures. Appropriate ATCC quality control organism(s) should be included alongwith each test. Inoculated plates are left undisturbed until the spots of inoculum have dried.

iii. Incubation

After the spots of inoculum have dried, the plates are incubated at 35°C for 16 to 18 h in an inverted position in a 5% CO_2 incubator. A candle extinction jar may be used if a CO_2 incubator is not available.

iv. Reading

The control plate should show the growth of the QC test organism. The MIC of the quality control strain should be in the expected quality control range. The end point is the lowest concentration of antibiotic that completely inhibits growth. A barely visible haziness or single colony should be disregarded. Results are reported as the MIC in micrograms or units/ml. Interpretation is made in accordance to the guidelines laid down in the NCCLS M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement (MIC Interpretative Standards) as susceptible, intermediate and resistant.

6. Errors in Interpretation and Reporting Results

In the interpretation of test results there are possibilities for errors to occur. Based on impact of errors in treatment of patient they are classified as minor errors, major errors and very major errors. This is achieved by comparing disk diffusion, which is widely used to report with the MIC, which is reference method.

Figure 1. The errors in reporting.

The above flow chart shows the errors in reporting. (Adopted from Manual of Clinical Microbiology, 7th edition)

7. 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)

Staphylococccus aureus ATCC 25923 (beta-lactmase negative, oxacillin susceptible)

Staphylococccus aureus ATCC 38591 (beta-lactmase 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)

Neissseria gonorrheae 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.

VIII Standard Methods for the Detection of Antibacterial Resistance

7.1. Detection of Oxacillin/Methicillin-Resistant Staphylococcus Aureus (MRSA)

Strains that are oxacillin and methicillin resistant, historically termed methicillin-resistant S. aureus (MRSA), are resistant to all beta-lactam agents, including cephalosporins and carbapenems. MRSA isolates often are multiply resistant to commonly used antimicrobial agents, including erythromycin, clindamycin, and tetracycline. Since 1996, reports of MRSA strains with decreased susceptibility to vancomycin (minimum inhibitory concentration [MIC], >8 µg/ml) have been published.

Glycopeptides, vancomycin and Teicoplanin are the only drug of choice for treatment of severe MRSA infections, although some strains remain susceptible to fluoroquinolones, trimethoprim/sulfamethoxazole, gentamicin, or rifampin. Because of the rapid emergence of rifampin resistance, this drug should never be used as a single agent to treat MRSA infections. The National Committee for Clinical Laboratory Standards (NCCLS) has recommended "Screening Test for Oxacillin-resistant S. aureus" and uses an agar plate containing 6 microg/ml of oxacillin and Müeller-Hinton agar supplemented with NaCl (4% w/v; 0.68 mol/L). These plates can be stored refrigerated for up to 2 weeks.

The inoculum is prepared by matching a 0.5 McFarland tube. Two methods can be followed for inoculation:

1. Dilute the supension 1: 100, and inoculate 10 microL on the plate, to get an inoculum of 104 CFU.
2. Dip a swab in the suspension and express excess fluid by pressing swab against the wall of the tube. Streak swab over a 1-1.5 inch area.

In both methods, any growth after 24 hours incubation at 35°C denotes oxacillin resistance, if controls are satisfactory. Accurate detection of oxacillin/methicillin resistance can be difficult due to the presence of two subpopulations (one susceptible and the other resistant) that may coexist within a culture. All cells in a culture may carry the genetic information for resistance but only a small number can express the resistance in vitro. This phenomenon is termed heteroresistance and occurs in staphylococci resistant to penicillinase-stable penicillins, such as oxacillin. Heteroresistance is a problem for clinical laboratory personnel because cells expressing resistance may grow more slowly than the susceptible population. This is why isolates being tested against oxacillin, methicillin, or nafcillin should be incubated at 35° C for a full 24 hours before reading. The breakpoints for S. aureus are different from those for coagulase-negative staphylococci (CoNS), have shown in below tables.

MICs	Oxacillin Susceptible	Oxacillin Intermediate	Oxacillin Resistant
S. aureus	<2 µg/ml	no intermediate MIC	MIC>4 µg /ml
CoNS	<0.25µg /ml	no intermediate MIC	MIC>0.5 µg /ml

Zone sizes	Oxacillin Susceptible	Oxacillin Intermediate	Oxacillin Resistant
S. aureus	>13 mm	11-12 mm	<10 mm
CoNS	>18 mm	no intermediate zone	<17 mm

When used correctly, broth-based and agar-based tests usually can detect MRSA. Oxacillin screen plates can be used in addition to routine susceptibility test methods or as a back-up method. Amplification tests like those based on the polymerase chain reaction (PCR) detect the mecA gene. These tests confirm oxacillin/methicillin resistance caused by mecA in Staphylococcus species.

7.2. Detection of Oxacillin-Resistant Coagulase-Negative Staphylococcus Sp

Although there are about 20 CoNS species, they often are considered to be a single group. Some species are more resistant to commonly used antimicrobial agents than others. Identification to species level can aid in the recognition of outbreaks and in tracking resistance trends. S. epidermidis is the most common CoNS isolated in

clinical laboratories. Usually, S. epidermidis, S. haemolyticus and S. hominis are more likely to be multiply resistant to antimicrobial agents than are other CoNS species. However, resistance patterns of CoNS may differ between hospitals and wards. Oxacillin-resistant CoNS isolates are resistant to all beta-lactam agents, including penicillins, cephalosporins, and carbapenems. In addition, oxacillin-resistant CoNS isolates are often resistant to other commonly used antimicrobial agents, so vancomycin is frequently the drug of choice for treatment of clinically significant infections. Accurate detection of oxacillin resistance can be difficult. Colony sizes of CoNS are often smaller than those of S. aureus, making growth more difficult to read. In addition, like S. aureus, two subpopulations (one susceptible and the other resistant) may coexist within a culture. When studies were performed to evaluate oxacillin breakpoints for CoNS, the current breakpoints for S. aureus failed to detect many CoNS that contained the mecA gene. In general, the new breakpoints for CoNS correlate better with mecA production for CoNS.

7.3. Detection of Extended-Spectrum Beta-Lactamases (ESBLs)

ESBLs are enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (e.g., ceftazidime, cefotaxime, and ceftriaxone) and monobactams (e.g., aztreonam) but do not affect cephamycins (e.g., cefoxitin and cefotetan) or carbapenems (e.g., meropenem or imipenem). ESBLs can be difficult to detect because they have different levels of activity against various cephalosporins. Thus, the choice of which antimicrobial agents to test is critical. For example, one enzyme may actively hydrolyze ceftazidime, resulting in ceftazidime minimum inhibitory concentrations (MICs) of 256 microg/ml, but have poor activity on cefotaxime, producing MICs of only 4 microg/ml. If an ESBL is detected, all penicillins, cephalosporins, and aztreonam should be reported as resistant, even if in vitro test results indicate susceptibility. There are standard broth microdilution and disc diffusion screening tests using selected antimicrobial agents. Each K. pneumoniae, K. oxytoca, or Escherichia coli isolate should be considered a potential ESBL-producer if the test results are as follows:

Disk diffusion	MICs
cefpodoxime < 22 mm	cefpodoxime > 2 μg /ml
ceftazidime < 22 mm	Ceftazidime > 2 μg /ml
aztreonam < 27 mm	aztreonam > 2 μg /ml
cefotaxime < 27 mm	cefotaxime > 2 μg /ml
ceftriaxone < 25 mm	ceftriaxone > 2 μg /ml

The sensitivity of screening for ESBLs in enteric organisms can vary depending on which antimicrobial agents are tested. The use of more than one of the five antimicrobial agents suggested for screening will improve the sensitivity of detection. Cefpodoxime and ceftazidime show the highest sensitivity for ESBL detection. Phenotypic confirmation of potential ESBL-producing isolates of K. pneumoniae, K. oxytoca, or E. coli can be done by testing both cefotaxime

and ceftazidime, alone and in combination with clavulanic acid. Testing can be performed by the broth microdilution method or by disk diffusion. For MIC testing, a decrease of > 3 doubling dilutions in an MIC for either cefotaxime or ceftazidime tested in combination with 4 μg /ml clavulanic acid, versus its MIC when tested alone, confirms an ESBL-producing organism. For disc diffusion testing, a > 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL-producing organism.

Discs can be made by adding 10 μl of a 1000 μg /ml stock solution of clavulanic acid to cefotaxime and ceftazidime disks each day of testing. In future, commercial manufacturers of antimicrobial discs may produce discs containing cefotaxime and ceftazidime with clavulanic acid. Until commercial discs are available, SmithKline Beecham can provide clinical laboratories with clavulanic acid powder for routine use.

K. pneumoniae ATCC 700603 (positive control) and E. coli ATCC 25922 (negative control) should be used for quality control of ESBL tests.

Some organisms with ESBLs contain other beta-lactamases that can mask ESBL production in the phenotypic test, resulting in a false-negative test. These beta-lactamases include AmpCs and inhibitor-resistant TEMs (IRTs). Hyper-production of TEM and/or SHV beta-lactamases in organisms with ESBLs also may cause false-negative phenotypic confirmatory test results. Currently, detection of organisms with multiple beta-lactamases that may interfere with the phenotypic confirmatory test can only be accomplished using isoelectric focusing and DNA sequencing, methods that are not usually available in clinical laboratories. If an isolate is confirmed as an ESBL-producer by the NCCLS-recommended phenotypic confirmatory test procedure, all penicillins, cephalosporins, and aztreonam should be reported as resistant. This list does not include the cephamycins (cefotetan and cefoxitin), which should be reported according to their routine test results. If an isolate is not confirmed as an ESBL-producer, current recommendations suggest reporting results as for routine testing. Do not change interpretations of penicillins, cephalosporins, and aztreonam for isolates not confirmed as ESBLs. Other isolates of Enterobacteriaceae, such as Salmonella species and Proteus mirabilis, and isolates of Pseudomonas aeruginosa also produce ESBLs. However, now the methods for screening and phenotypic confirmatory testing of these isolates have not been determined.

7.4. Detection of Imipenem or Meropenem Resistance in Gram-Negative Organisms

Within a health care setting, increases in species-specific carbapenem resistance should be monitored and sudden increases investigated to rule out an outbreak of resistant organisms or spurious test results. Published reports indicate some resistance in a variety of clinical Gram-negative organisms, including Pseudomonas aeruginosa, Burkholderia cepacia, Acinetobacter species, Proteus species, Serratia

marcescens, Enterobacter species, and K. pneumoniae. Stenotrophomonas maltophilia isolates are intrinsically resistant to imipenem. Organisms can produce more than one hydrolyzing enzyme and may show modifications in more than one porin, producing high-level resistance to the carbapenems (minimum inhibitory concentration [MIC] >16 µg/ml). Organisms with decreased susceptibility produced by porin changes alone often have lower MICs (2-8 microg/ml). Organisms with MICs near interpretation breakpoints have greater potential for reporting errors. For example, isolates of P. aeruginosa often have MICs that are at or near the carbapenem intermediate (8 µg/ml) and resistant (>16 µg/ml) breakpoints. Some species, such as P. mirabilis, P. vulgaris, and Morganella morganii, often have MICs (1-4 µg/ml) just below the carbapenem intermediate breakpoint of 8 mg/ml. Most other species of Enterobacteriaceae are very susceptible (<0.5 µg/ml).

Broth microdilution methods usually detect carbapenem resistance when the tests are performed properly. However, studies have shown false resistance to imipenem in commercially prepared test panels due to degradation of the drug or to a manufacturing problem where concentrations of imipenem were too low. When performed properly, disc diffusion and agar gradient diffusion also are acceptable methods for carbapenem testing. Imipenem degrades easily. Studies suggest that meropenem may be more stable than imipenem. However, for either antimicrobial agent, storage conditions of susceptibility panels, cards, and discs must be monitored carefully and quality control results checked frequently. If possible, store supplies containing carbapenems at the coldest temperature range stated in the manufacturer's directions. An additional test method [agar gradient diffusion (E test)], can be used to verify intermediate or resistant results.

Quinolones and resistance

The number and location of mutations affecting critical sites determine the level of resistance. Organisms may have alterations in more than one enzyme target site and, in gram-negative organisms, may contain more than one porin change. Many resistant organisms have multiple enzyme target site, porin, and efflux mutations, producing high-level resistance to quinolones. In contrast, organisms with decreased susceptibility produced only by porin changes usually have lower minimum inhibitory concentrations (MICs).

The fluoroquinolone susceptibility profile for each clinical isolate is determined by the number and location of mutational changes in specific enzyme target sites, porin proteins, and efflux mechanisms. The effect of each mutation in an isolate is not equivalent for all fluoroquinolones, due to variations of the chemical structures among this class of agents. Therefore, an organism with one or more mutations may have resistant MICs/zone sizes to one quinolone but have intermediate or susceptible MICs/zone sizes to another quinolone. During therapy, the potential exists for an organism with a single mutation to acquire a second mutation, leading to high-level resistance. After multiple mutations occur, an organism is generally highly resistant to all quinolones. Resistance to quinolones has been reported in a variety of important bacterial pathogens, including E. coli,

K. pneumoniae and other enteric organisms; P. aeruginosa; Chlamydia trachomatis, Mycoplasma pneumoniae; Campylobacter jejuni, B. cepacia; S. maltophilia, N. gonorrhoeae, S. aureus (especially oxacillin-resistant strains), Enterococcus faecium and S. pneumoniae.

7.5. Detection of Resistant Enterococci

Penicillin/Ampicillin Resistance

Enterococci may be resistant to penicillin and ampicillin because of production of low-affinity, penicillin-binding proteins (PBPs) or, less commonly, because of the production of ß-lactamase. The disc diffusion test can accurately detect isolates with altered PBPs, but it will not reliably detect ß-lactamase producing strains. The rare ß-lactamase- producing strains are detected best by using a direct, nitrocefin-based, ß-lactamase test. Certain penicillin- ampicillin- resistant enterococci may possess high-level resistance (i.e., penicillin MICs > 128 µg/ml or ampicillin MICs > 64 µg/ml). The disc test will not differentiate those with normal resistance from this high-level resistance. For enterococci recovered from blood and CSF, the laboratory should consider determining the actual MIC for penicillin or ampicillin since E. faecium strains with normal lower level resistance (penicillin MICs < 64 µg/ml and ampicillin < 32 µg/ml) should be considered potentially susceptible to synergy with an aminoglycoside (in the absence of high-level aminoglycoside resistance) whereas strains with higher level resistance may be resistant to such synergy.

Vancomycin Resistance

Accurate detection of vancomycin-resistant enterococci by the disc diffusion test requires that plates be incubated for a full 24 hours (rather than 16 to 18 hours), that any zone surrounding the vancomycin disc be examined with transmitted light for evidence of small colonies/a light film growing within the zone. An intermediate category result by the disc diffusion test should be verified by determining vancomycin MIC.

High-level Aminoglycoside Resistance

High-level resistance to aminoglycosides is an indication that an enterococcal isolate will not be affected synergistically by a combination of penicillin or glycopeptide plus an aminoglycoside. Special, high-content gentamicin (120 µg) and streptomycin (300 µg) discs can be used to screen for this type of resistance. No zone of inhibition indicates resistance, and zones of > 10 mm indicate a lack of high-level resistance. Strains that yield zones of 7 to 9 mm should be examined using a dilution screen test.

Aminoglycoside Resistance in Enterobacteriaceae and Pseudomonas aeruginosa

Resistance to one aminoglycoside may not predict resistance to the others. In general, resistance is relatively common in P. aeruginosa but less common in Enterobacteriaceae. Enterobacteriaceae resistant to gentamicin and tobramycin can be susceptible to amikacin or netilmicin because these drugs are not affected by many of the aminoglycoside modifying enzymes (AMEs). Therefore, the prevalence of amikacin resistance can be lower than the prevalence of resistance to gentamicin and tobramycin,

depending upon the resistance mechanisms present at a healthcare facility.

8. Application of Computers in Antibacterial Susceptibility Testing

Antimicrobial resistance is a global problem.

Emergence of multidrug resistance has limited the therapeutic options, hence monitoring resistance is of paramount importance.

Antimicrobial resistance monitoring will help to review the current status of antimicrobial resistance locally, nationally and globally and helpful in minimizing the consequence of drug resistance, limit the emergence and spread of drug resistant pathogens.

Thousands of laboratories are distributed world-wide and need to be linked to integrate data on most clinically relevant organism on a daily basis to obtain an accurate picture of "real resistance". For this purpose software was developed for the management of routine laboratory results by WHO called 'WHONET'. This software has focused on data analysis particularly on the results of AST.

WHONET 5.1 is a data base software compatible with, Windows 95,

Windows 98, Windows NT and later versions of Windows. Installation capacity of the software is 9.34 mb, this software is available in website http://www.who.int/emc/WHONET/Instructions.html.

This programme is useful in supplying current guidelines, protocols to local laboratories, in identifying the clusters of resistant isolates and emerging outbreaks. Use of WHONET software may aid the local laboratory in networking efficiently with the national reference laboratory, in developing guidelines for use of antimicrobial agents and review the same periodically, in studying the effect of antimicrobial control programmes on resistant bacteria, to network efficiently with international bodies such as WHO, NCCLS etc., used for research studies.

Guidelines for Antimicrobial Susceptibility Testing

Table 1. *Suggested Battery of Antibiotics for Susceptibility Testing.*

Staphylococcus sp.	Gram negative bacilli	Streptococcus (Enterococcus, Pneumococcus)	Haemophilus sp	N. gonorrhoeae
Pencillin	Ampicillin	Pencillin	Ampicillin	Pencillin
Oxacillin	Piperacillin	Oxacillin	Amoxycillin/ Clavulanic acid	Cefazolin
Cephalothin	Cephalothin	Ampicillin	Cefuroxime	Ceftriaxone
Gentamicin	Cefotaxime	Cefotaxime	Cefotaxime	Chloramphenicol
Netilmicin	Ceftazidime	Erythromycin	Tetracycline	Ciprofloxacin
Amikacin	Gentamicin	Chloramphenicol	Erythromycin	
Chloramphenicol	Netilmicin	Tetracycline	Chloramphenicol	
Tetracycline	Amikacin	Vancomycin		
Erythromycin	Chloramphenicol			
Co-trimoxazole	Tetracycline			
Clindamycin	Co-trimoxazole			
Ofloxacin	Nalidixic Acid			
Rifampicin	Ciprofloxacin			
Vancomycin	Ofloxacin			
Teicoplanin	Nitrofurantoin			
	Imipenem			
	Meropenem			

Note: The choice of antibiotic depends on the pattern exhibited locally and selection of antibiotics varies based on specimen and the isolates.

Table 2. *Suggested Antibiotic Dilution Ranges for MIC Testing.*

ANTIMICROBIAL AGENT	CONCENTRATION RANGE(µg/ml)
Penicillin G, Ampicillin(Gram +ve)	0.015-32
Methicillin, Naficillin/Oxacillin	0.003-64
Ampicillin(Gram-ve), Cephalosporin I generation (Gram -ve)	0.13-256
Cephalosporin(Gram +ve)	0.015-32
Cephalosporin II&III generation (Gram-ve), Trimethoprim	0.03-64
Cephalosporin III generation (Pseudomonas sp.)	0.13-256
Vancomycin	0.015-16
Amikacin	0.06-128
Gentamicin/Tobramycin	0.03-64
Erythromycin, Clindamycin, Rifampicin	0.015-32
Chloramphenicol, Tetracycline	0.06-256
Sulphamethoxazole	0.6-1216
Trimethoprim(UTI, Gram - ve)	0.5-64

Source-A guide to senstivity testing: Report of working party on antibiotic sensitivity testing of British society for Antimicrobial Chemotherapy.

Table 3. *Solvents and Diluents for Antibiotics.*

Antimicrobial Agents[a]	Solvent[d]	Diluent[d]
Amoxicillin, Clavulanic acid and icarcillin	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Ampicillin	Phosphate buffer, pH 8.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Azithromycin	95 % ethanol or glacial acetic acid[g]	Broth media
Aztreonam	Saturated solution sodium bicarbonate	Water
Cefepime	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Cefotetan	Dimethyl sulfoxide[f]	Water
Cefpodoxime	(0.10 %; 11.9 mmol/L) aqueous sodium bicarbonate	Water
Ceftazidime	Sodium bicarbonate[e]	Water
Cephalothin	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Chloramphenicol	95 % ethanol	Water
Cinoxacin and Nalidixic acid	½ volume of water, then add NaOH 1 mol/L, drop wise to dissolve	Water
Clarithromycin	Methanol[f] or glacial acetic acid[g]	Phosphate buffer, pH 6.5, 0.1 mol/L
Enoxacin, Fleroxacin, Norfloxacin&Ofloxacin	½ volume of water, then 0.1 mol/L NaOH drop wise to dissolve	Water
Erythromycin	95 % ethanol or glacial acetic acid[g]	Water
Imipenem	Phosphate buffer, pH 7.2, 0.01 mol/L	Phosphate buffer, pH 7.2, 0.01 mol/L
Moxalactam (diammonium salt)[b]	0.04 mol/L HCl (let sit for 1.5 to 2h)	Phosphate buffer, pH 6.0, 0.1 mol/L
Rifampin	Methanol[f]	Water (with stirring)
Sulfonamides	½ volume of hot water and minimal amount of 2.5 mol/L NaOH to dissolve	Water
Telithromycin	½ volume of water, then add glacial acetic acid drop wise until dissolved not to exceed 2.5 µL/mL	Water
Trimethoprim	0.05 mol/L lactic[f] or HCl acid, 10% vol.	Water (may require heat)

a. Cephalosporins and cephems not listed above or in footnote d are solublized (unless the manufacturer indicates otherwise) in phosphate buffer, pH 6.0, 0.1 mol/L, and further diluted in sterile distilled water.

b. The diammonium salt of moxalactam is very stable, but it is almost pure R isomer. Maxolactam for clinical use is a 1: 1 mixture of R and S isomers. Therefore, the salt is dissolved in 0.04 mol/L HCL and allowed to react for 1.5 to 2.0 hours to convert it to equal parts of both isomers.

c. Alternatively, nitrofurantoin is dissolved in dimethyl sulfoxide.

d. These solvents and diluents are for making stock solutions of antimicrobial agents that require solvents other than water. They should be diluted further, as necessary, in water or broth. The products known to be suitable for water solvents and diluents are amikacin, azlocillin, carbencillin, cefaclor, cefemandole, cefonicid, cefotaxime, cefoperazone, cefoxitin, ceftizoxime, ceftriaxzone, ciprofloxacin, clindamycin, gatifloxacin (with stirring), gemifloxacin, gentamicin, kanamycin, linezolid, mecillinam, meropenem, methicillin, metronidazole, mezlocillin, minocyclin, moxifloxacin, nafcillin, netilmycin, oxacillin, penicillin, piperacillin, quinupristin-dalfopristin, sparfloxacin, sulbactam, tazobactam, teicoplanin, tetracyclines, tobramycin, trimethoprim (if lactate), trospectomycin and vancomycin.

e. Anhydrous sodium carbonate is used at a weight of exactly 10% of the ceftazidme to be used. The sodium carbonate is dissolved in solution in most of the required water. The antibiotic is dissolved in this sodium carbonate solution and water is added to desired volume. The solution is to be used as soon as possible, but can be stored up to six hours at not more than 25°C.

f. These compounds are potentially toxic. Consult the material safety datasheets (MSDS) available from the product manufacturer before using any of these materials.

g. Use ½ volume of water then add glacial acetic acid drop wise until dissolved not to exceed 2.5µl/ml.

h. Table 3: Reproduced with permission, from NCCLS publication M100-S12 Performance Standards for Antimicrobial Testing: Twelfth Informational Supplement (ISBN 1-56238- 454 -6). Copies of the current edition may be obtained from NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

9. Conclusions

The present search deals the results of in-vitro antibiotic susceptibility testing, guide clinicians in the suitable selection of initial empiric regimens and, drugs used for individual patients in specific situations are elucidated in detail. The resistance to antimicrobial agents has an effect in morbidity and transience from treatment failures and improved health awareness expenses. These require supplements to develop, and they should be tested on the media described in separate sections. For that the different methods of Antimicrobial Susceptibility Testing are analyzed in this investigation. We hope these theoretical search will be useful to nanoresearchers.

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