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Essentials of Antimicrobial Stewardship



2nd Edition

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CHAPTER

10

Basics of Antimicrobial Susceptibility Testing

INTRODUCTION

Antimicrobial susceptibility testing (AST) is the most important investigation carried out by a Clinical microbiology laboratory. Bacteria exhibit great strain variations in susceptibility to antimicrobial agents. Therefore, AST plays a vital role to guide the clinician in tailoring the empirical antimicrobial therapy to pathogen-directed targeted therapy.

Indications for Performing AST[1,2]

AST is indicated for any organism isolated in a clinical specimen if:

- The organism isolated is **pathogenic** (at the infection site), present in the **significant count**, contributes to the infectious process, and warrants antimicrobial therapy—which means AST is not performed for normal commensal flora or contaminant grown in culture. For example, *E. coli* isolated from urine specimens in a significant count of $\geq 10^5$ CFU/mL should be subjected to AST, whereas *E. coli* isolated from stool is a commensal; hence, AST is not performed. AST is also not necessary for *E. coli* from urine specimens, if isolated in insignificant count.
- Organism isolated is capable of **exhibiting resistance** to commonly used antimicrobial agents and its susceptibility cannot be reliably predicted from knowledge of the organism's identity—which means AST is not necessary if the organism has predictable susceptibility to an antimicrobial agent that is used for empiric therapy and no resistance is reported; e.g. *S. pyogenes* to penicillin.
- There is an **established method** available to perform and interpret AST results according to the standard guideline.[1,2]
- A sufficient number of **isolated colonies** of the organism is available in the agar plate.
- ASTs are also important in **epidemiological** studies of antimicrobial resistance and studies of **new** antimicrobial agents.

Types of AST Methods

AST methods are classified into phenotypic and genotypic methods. The genotypic methods such as PCR detecting drug-resistant genes are discussed in Chapter 9. The phenotypic methods are performed either manually (conventional) or by an automated AST instrument.

Conventionally performed phenotypic methods are further grouped into:

- *Disk diffusion method:* e.g. Kirby–Bauer's disk diffusion (DD) test, Stokes method.
- *Dilution methods:* such as broth dilution and agar dilution methods.
  - Broth dilution method: broth tube macrodilution and broth microdilution.
  - Agar dilution method including screening agar method
- *Diffusion and dilution (i.e. gradient diffusion) method:* Epsilometer or E-test.

Automated AST systems include VITEK (bioMerieux), Phoenix (Becton Dickinson), and MicroScan (Beckman Coulter) systems: They work on the principle of broth microdilution.

Susceptibility test can also be performed directly from positively-flagged blood culture broth (direct susceptibility test), discussed separately in Chapter 14.

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10.1 Kirby–Bauer's Disk Diffusion Test

INTRODUCTION[1-5]

Kirby–Bauer's disk diffusion (KB-DD) test is the most widely used AST method. They are suitable for rapidly growing pathogenic bacteria; but not for slow-growing bacteria. It is mostly performed from the colony (called colony-DD) or performed directly from the specimens (called direct DD, Chapter 14). The workflow of performing KB-DD is as follows:

- **Workflow:** Selecting colony growth on the appropriate media → preparing the inoculum → inoculation into plates → applying the disks → incubating → taking the reading of the AST result → interpreting (Chapter 11) the AST result **(Fig. 10.1)**.
- **Quality control** of AST tests needs to be performed in parallel (weekly or daily) to ensure accuracy and reproducibility (Chapter 13).

MUELLER-HINTON AGAR (MHA)[3,6]

For performing KB-DD in nonfastidious bacteria, Mueller-Hinton agar (MHA) is considered as the best medium, which can be attributed to various reasons.

- MHA exhibits excellent acceptable batch-to-batch reproducibility for AST.
- Contains the lower amount of inhibitors that can affect the AST results of antimicrobial such as sulfonamide, trimethoprim, and tetracycline.
- It supports satisfactory growth of most nonfastidious aerobic and facultative anaerobic bacteria.
- A large body of data and experience is available depicting the performance of KB-DD on this medium.

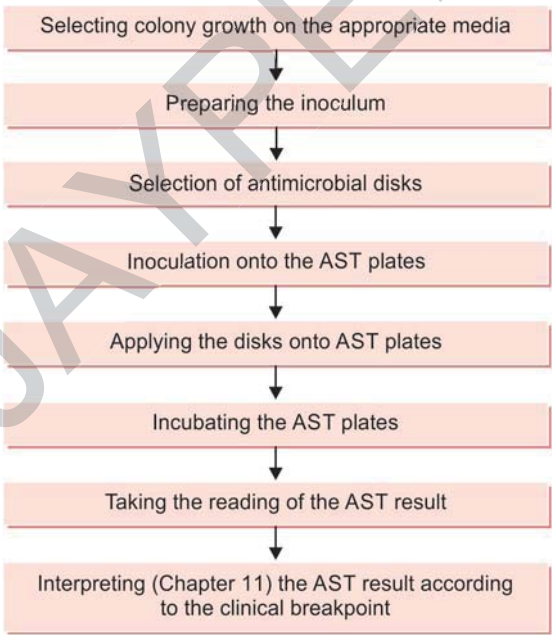

Fig. 10.1: Workflow of performing KB-DD test.

- Does not alter the growth of an organism—i.e., neither promotes, nor inhibits the growth.

Preparation of MHA[3,6]

Although Mueller-Hinton agar is to be prepared according to the manufacturer's instructions, the following general principles need to be followed.

- **Autoclaving:** The medium is autoclaved, allowed to cool to 45–50°C in a water bath, and then poured into Petri plates with an optimum depth of 4 mm. This would correspond to 25–30 mL of medium for 100 mm plates and 60–70 mL for 150 mm plates.
- **pH check**: The pH of each MHA batch is checked after it has cooled and solidified and should have an optimum pH between 7.2 and 7.4.
- **Sterility and pH check:** A representative sample of each batch is incubated at 35°C ± 2°C for 24 h or longer and examined for sterility.
- **Drying of AST plates:** This is important as excess moisture may lead to fuzzy zone edges and/or haze within zones. No drops of water should be visible on the surface of the agar or inside the lid. However, care must be taken not to over-dry the plates.
- **Storage:** The plates can be used on the same day or stored at 2–8°C for up to 7 days but could have a longer shelf life if precautions are taken to prevent drying and QC is in range at the time of testing.

Other Media[1-7]

Unsupplemented MHA medium is ideal for the rapidly growing aerobic pathogens, but is not adequate for AST of fastidious organisms, for which several other media are recommended.

- **5% MH blood agar:** This is the medium of choice for performing KB-DD for *Streptococcus pneumoniae, Streptococcus* species, and *Neisseria meningitidis.* When MHA has cooled to 45–50°C, add 50 mL defibrinated sheep/horse blood to 950 mL of molten MHA with or without β-NAD supplementation (20 mg/L). The remaining steps of preparation are the same as for MHA.
- ***Haemophilus* test medium (HTM):** It is recommended for *H. influenzae* and *H. parainfluenzae.* It is a modification of MHA with addition of yeast extract, hematin and β-NAD.
- **GC agar base medium** + 1% defined growth supplement or *chocolate agar with Columbia agar base*: It is recommended for *Neisseria gonorrhoeae.*

Factors Influencing Zone Diameter in MHA[3-6]

There are several factors that can influence the zone diameter in MHA such as divalent cations, thymine, pH, etc. **(Table 10.1)**.

1. Effects of Divalent Cations[3-6]

Variation in divalent cations, principally magnesium, calcium and zinc affect the zone size of certain antimicrobials.

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Table 10.1: Factors influencing zone diameter of the disk diffusion test.

	Ca^{+2}↑	Ca^{+2}↓	Mg^{+2}↑	Mg^{+2}↓	Zn^{+2}↑	Zn^{+2}↓	pH$^{\$}$↑	pH$^{\$}$↓	Thym↑
Aminoglycoside	↓ (R), *Ps*	↑ (S), *Ps*	↓ (R), *Ps*	↑ (S), *Ps*	–	–	↑ (S)	↓ (R)	–
Tetracycline	↓ (R)	↑ (S)	↓ (R)	↑ (S)	–	–	↓ (R)	↑ (S)	–
Penicillins	–	–	–	–	–	–	↓ (R)	↑ (S)	–
Daptomycin*	↑ (S)	↓ (R)	–	–	–	–	–	–	–
Carbapenem	–	–	–	–	↓ (R)	↑ (S)	–	–	–
Cotrimoxazole	–	–	–	–	–	–	–	–	↓ (R)
Quinolones	–	–	–	–	–	–	↑ (S)	↓ (R)	–
Clindamycin Macrolide	–	–	–	–	–	–	↑ (S) (*S. aureus*)	↓ (R) (*S. aureus*)	–

(Thym-thymine, thymidine; Ps, for *Pseudomonas aeruginosa* only; R, false resistance; S, false susceptible; ↑, increase in zone diameter; ↓, decrease in zone diameter; ↑, increase in content; ↓, decrease in content)

*Disk diffusion is not reliable for daptomycin; $^{\$}$Optimum pH 7.2–7.4.

- **Magnesium and calcium** affect the AST results of aminoglycoside and tetracycline for *P. aeruginosa*. Excess cation content (Ca^{2+}/Mg^{2+}) reduces zone sizes (false-resistance), whereas low cation content may result in large zones of inhibition (false-susceptibility). Supplemental Ca^{2+} or Mg^{2+} should not be added to MHA.
- **Variation in calcium** levels also affects daptomycin test results. For daptomycin, insufficient calcium content reduces zone sizes, whereas high calcium content may increase zone sizes; therefore, disk diffusion testing is not reliable for daptomycin.
- **Excess zinc ions** may reduce carbapenem zone sizes.

2. Effects of Thymidine or Thymine[3-6]

MHA should have as low thymidine content as possible.

- **Reason:** MHA containing excessive amounts of thymidine or thymine can reverse the inhibitory effect of sulfonamides, cotrimoxazole, and trimethoprim, making these drugs ineffective; thus, yielding smaller and less distinct zones, or no zone at all, which may result in false-resistance reports. Therefore, MHA should have as low thymidine content as possible.
- **Evaluating MHA:** The thymidine content of a MHA should be evaluated—(i) routinely for every batch of MHA before use, (ii) if the weekly QC test fails for cotrimoxazole. It is performed by testing *Enterococcus faecalis* ATCC 29212 with cotrimoxazole disks.
  - For a *satisfactory MHA medium*, a clear distinct inhibition zone of ≥21 mm is obtained.
  - If *MHA medium is unsatisfactory* (i.e., too high in thymidine content), it produces no zone of inhibition, growth within the zone, or a zone of ≤20 mm.

3. Effect of pH of the Medium[3-6]

The MHA medium should have a pH between 7.2 and 7.4 at room temperature. Variation of pH can affect the zone sizes of certain antimicrobials **(Table 10.1)**.

- **Increase in pH** is commonly observed when plates are incubated in a capnophilic environment.
  - It can lead to an increase in zone diameter of aminoglycoside (*P. aeruginosa*), quinolones, clindamycin (*S. aureus*), and macrolide (*S. aureus*), giving rise to false susceptible reports.
  - Whereas it reduces the zone diameter of penicillins and tetracycline, leading to false resistant results.
- **Decrease in pH:** The opposite occurs when there occurs a decrease in pH of the medium.

ANTIMICROBIAL DISKS[3]

Antimicrobial disks should be procured only from a reliable commercial vendor, who can provide a certificate of analysis stating the content of the disks, lot number, expiration date, and assurance that they were tested and performed according to quality control recommendations.

Storage of Disks[3]

The commercial antimicrobial disk cartridges are generally packaged in such a way (e.g. along with desiccant) to ensure appropriate anhydrous conditions. The steps for storing and handling disks are as follows.

- **Temperature:** For most antimicrobial agents (crucial for β lactams), the **stock disks** (sealed cartridges) should be stored in a freezer (≤–14°C).
  - The **working disks** (opened cartridges) can be kept in refrigerator (2–8°C) for a maximum of 1 week.
  - Certain labile disks (e.g. imipenem, cefaclor, clavulanate combinations) need to be frozen (≤–14°C) till the day of use to retain greater stability.
- **Removal:** The working disks cartridge or box with or without the disk dispenser apparatus should be removed from the refrigerator at least 1–2 h before use, so as to equilibrate to room temperature before opening. This will help to minimize the amount of condensation that occurs on the disk.
- **Desiccant:** Once opened, the cartridges with or without the disk dispenser apparatus should be stored in a tightly-sealed desiccated container at 2–8°C after use. Desiccant such as silica gel is hygroscopic in nature, help to absorb water/moisture. Desiccant should be replaced when its indicator changes color.
- Expiry of the disks needs to be checked regularly and the expired disks should always be discarded.

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PREPARING INOCULUM[3-5]

The preparation of bacterial inoculum is one of the most crucial steps of performing AST. Care should be taken to prepare the inoculum by correct method and appropriate density (0.5 McF, discussed subsequently). In general, there are two methods of preparing bacterial inoculum.

Colony Suspension Method[3-5]

It is the most preferred and convenient method for inoculum preparation.

- **Organisms:** This method can be used for most organisms and is the only recommended method for fastidious organisms such as *H. influenzae*, *N. gonorrhoeae, N. meningitidis*, and streptococci as well as for testing methicillin (oxacillin) resistance in *Staphylococcus*.
- **Method**: About 3–5 fresh colonies from a nonselective medium such as blood agar are touched to make a direct suspension in sterile normal saline. Turbidity is adjusted to 0.5 McF standard.
- **Fresh colony:** This method is appropriate only when the fresh colony (16–24 h overnight incubated plates) are available, but not suitable for refrigerated plates or older colony growth.
- **Single morphotype:** Care should be taken to pick colonies of a single morphotype, avoiding mixing with dissimilar colony morphotypes.
- **Resistant mutants:** The purpose of touching 3–5 colonies is to represent the possible resistant variants/mutants present.

Broth Culture Method[3-5]

Also called as growth method, it is the preferred method for organisms in which colonies are difficult to suspend in saline/broth and therefore smooth suspension cannot be made by colony suspension method (e.g. molds, *Nocardia*). It can also be used for inoculum preparation for colonies of nonfastidious organisms (except *Staphylococcus*) from refrigerated or >24 h old plates (i.e. when fresh colonies are not available).

- **Method**: About 3–5 well-isolated colonies of the same morphotype are touched on the top with a loop/sterile swab and inoculated into a tube containing 4–5 mL of a suitable broth medium, such as BHI, tryptic soy broth. The broth is incubated at 35°C ± 2°C until the turbidity of the 0.5 McF standard (usually 2–6 h) is achieved.
- **Not suitable:** This method is not suitable for fastidious organisms such as *H. influenzae*, *N. gonorrhoeae, N. meningitidis*, and streptococci and testing methicillin (oxacillin) resistance in staphylococci.

The following inoculum should never be used for performing AST: inoculum density other than 0.5 McF, undiluted overnight broth cultures, or any other unstandardized inocula.

McFarland Turbidity Standard for Inoculum Preparation[3-5]

McFarland turbidity standards (MFTS) are used as the reference for measuring the turbidity of the bacterial inoculum, which in turn ensures the presence of an approximate number of bacteria in the suspension, i.e. approximate quantification **(Table 10.2)**. MFTS is prepared in a varying concentration ranging from 0.5 to 4. The concentration used for AST is 0.5 McF—which means the inoculum would contain an organism load of 1.5 (range, 1–2) × 10^8 CFU/mL. The following methods of MFTS preparation are available.

Table 10.2: McFarland turbidity standard by barium sulfate method.

McFarland standard	$BaCl_2$ 1% (mL)	H_2SO_4 1% (mL)	Approximate CFU/mL
0.5	0.05	9.95	1.5×10^8
1.0	0.1	9.9	3.0×10^8
2.0	0.2	9.8	6.0×10^8
3.0	0.3	9.7	9.0×10^8
4.0	0.4	9.6	12.0×10^8

(CFU, colony-forming units of bacteria)

Barium Sulfate Method

The most common method to prepare MFTS is by making a chemical solution of barium chloride and sulfuric acid.

- **0.5 MFTS** is prepared by adding 0.5 mL of the 1% $BaCl_2$ solution to 99.5 mL of the 1% H_2SO_4 solution (i.e. 0.5%) to produce fine crystals of barium sulfate.
- Similarly, **1, 2 and 4 MFTS** can be prepared by adding 1 mL, 2 mL and 4 mL of 1% $BaCl_2$ to 99 mL, 98 mL and 96 mL of 1% H_2SO_4 solution respectively.
- **Stirring:** The solution is stirred constantly, which helps to maintain a suspension.
- **Verify turbidity:** The density of the turbidity standard is verified correctly by measuring absorbance using a spectrophotometer with a 1-cm light path and matched cuvettes. The absorbance at 625 nm should be 0.08–0.13 for the 0.5 McF standard.
- **Aliquot:** The $BaSO_4$ suspension is transferred to screw-cap tubes in 4- to 6-mL aliquots. The tubes of the same size and thickness should be used as those used for routine preparation of the bacterial inoculum during AST. The tubes are tightly sealed and stored in the dark at room temperature.
- **Vortex before use:** The $BaSO_4$ tubes should be vortexed vigorously before each use. The tubes must be visually inspected regularly to look for a uniformly turbid appearance; and need to be replaced if large particles are seen.
- **Measuring turbidity:** The turbidity of the bacterial suspension is visually compared with that of the corresponding MFTS tube (e.g. 0.5) by holding both the tubes up against a card with a white background and contrasting black lines (Wickerham card, **Fig. 10.2**) in presence of adequate light.
- **Adjustment:** In case of heavy turbidity of bacterial suspension, it is adjusted by adding more volume of broth or saline via a sterile pipette. If the bacterial suspension is too light, additional colonies are inoculated or the tube is incubated further until the turbidity matches that of the standard.

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- **Density check:** $BaSO_4$ standards should be replaced or their densities verified monthly.

Other Methods of Preparing MFTS

Other methods to prepare MFTS include:

- **Latex method:** McFarland standards made from latex particle suspension are commercially available. When used, they should be mixed by inverting gently (not on a vortex mixer) immediately before use.
- **Photometric devices** are commercially available such as DensiCHECK of VITEK AST instrument **(Fig. 10.3)**, however, the calibration of the device needs to be ensured daily **(Table 10.3)**. If the turbidity of calibration standards is out of acceptable range, then it has to be recalibrated using the same standards.



Fig. 10.2: McFarland turbidity standards and Wickerham card.



Fig. 10.3: DensiCHECK of VITEK AST instrument.
Source: Department of Microbiology, JIPMER.

Table 10.3: Calibration of DensiCHECK.

Calibration standards	Acceptable Range	
	Lower limit	Upper limit
0.0 McF	0.00	0.00
0.5 McF	0.44	0.56
2.0 McF	1.85	2.15
3.0 McF	2.79	3.21

INOCULATING THE TEST PLATES[3-5]

A sterile cotton swab is dipped in the inoculum broth, rotated several times, and pressed firmly on the inside wall of the tube above the fluid level to remove excess fluid.

- **Lawn culture:** Swab is inoculated on the entire plate three times (with 60° rotation), finally the rim to ensure an even distribution of inoculum **(Fig. 10.4)**.
- **Lid ajar:** To absorb any excess surface moisture, the lid is left ajar for 3–5 minutes (ideally), but no more than 15 minutes before applying the disks.

15–15–15 rule

Inoculum preparation (i.e. turbidity adjusted)
↓ within 15 minutes
Lawn culture of the inoculum on to MHA plate
↓ within 15 minutes
Applying the disks on to MHA plate
↓ within 15 minutes
Incubating the MHA plates

APPLYING THE DISKS ONTO MHA PLATE[1,3-5]

Within 15 minutes of lawn culture, a set of antimicrobial disks are placed onto the surface of the inoculated MHA plate either manually with sterile forceps or by using a disk dispenser **(Fig. 10.5)**.

- **Pressed:** After being placed, each disk is pressed down with forceps to ensure complete contact with the agar surface.
- **Small zone disk next to the large zone:** The disks expected to produce larger zones (e.g., erythromycin, cephalosporins, penicillins) should be placed next to those that produce small zones (e.g., gentamicin, amikacin, vancomycin) so as to avoid overlapping zones.

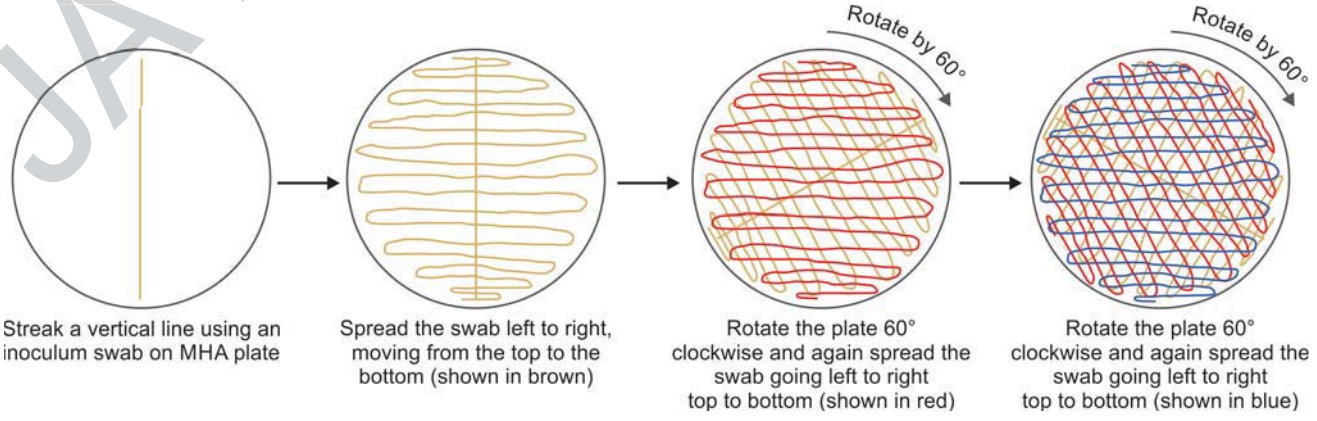

Fig. 10.4: Technique of inoculating the swab onto the MHA plates.

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Fig. 10.5: Automated disk dispenser.

Table 10.4: Maximum number of disks per plate.[1,3]

Organisms	150 mm plate	100 mm plate
Nonfastidious organisms • Enterobacterales • Nonfermenters • *Staphylococcus* • *Enterococcus*	12	6
Fastidious organisms • *S. pneumoniae* • βH streptococci • Viridans streptococci • *N. gonorrhoeae* • *Haemophilus*	9	4
N. meningitidis	5	2

- The **minimum distance** between two adjacent disks should be 24 mm from center to center. Disks should not be placed very close to the edge of the plate, else the zones of some disks may not be fully round.
- The **maximum number of disks** per plate should be ≤6 disks on a 100-mm plate and ≤12 disks on a 150-mm plate for most nonfastidious organisms; whereas for fastidious organisms it should be ≤4 and ≤9 disks respectively **(Table 10.4)**.
- **Place a new, rather than relocating:** The disk should not be relocated after being placed, as some of the drugs diffuse almost instantaneously. If needed, a new disk can be placed in another location on the MHA pate.

INCUBATING THE MHA PLATES[1,3-5]

Within 15 minutes after the disks are placed, the MHA plates are inverted (with lid down) and placed inside the aerobic incubator.

- **Temperature:** The MHA plates should be incubated at 35°C ± 2°C. When testing for oxacillin, temperature above 35°C may not reliably detect methicillin-resistant *S. aureus* (MRSA).
- **Capnophilic environment:** For fastidious organisms such as *Haemophilus, Neisseria,* and streptococci for which MHBA is used, the plates may be incubated in CO_2 jar. Otherwise, the routine MHA plates should not be incubated in capnophilic environment as the increase in CO_2 can significantly alter the zone size of some agents.
- **Duration:** For colony KB-DD, an incubation of 16–18 h is sufficient for most organisms before examining the plates.
  - However, for certain drug/organisms an extended duration of incubation for 20–24 h and full 24 h is warranted **(Table 10.5)**.
  - Conversely, the direct KB-DD performed from the blood culture broth can be read after a short incubation (8 h as per CLSI; 4 h/6 h/8 h as per EUCAST), but may need to be reconfirmed by colony KB-DD subsequently (Chapter 14).

Table 10.5: Antimicrobial agent/organism for which the AST results are read after an extended incubation.[1,3-5]

Full 24 h incubation	20–24 h incubation
• Cefoxitin disk diffusion[a] for CoNS • Vancomycin screen agar and MIC for *S. aureus, Enterococcus* • Vancomycin disk diffusion for *Enterococcus* • Oxacillin MIC for *S. aureus* • Mupirocin for *S. aureus* • High level gentamicin/ streptomycin MIC[b] • *Pseudomonas aeruginosa*—for cystic fibrosis cases	Disk diffusion or MIC for: • *Acinetobacter* species • *Burkholderia cepacia* complex • *Stenotrophomonas maltophilia* • Fastidious organisms such as ▪ *Streptococcus* including *S. pneumoniae* ▪ *Neisseria* spp. ▪ *Haemophilus* spp. (MIC only)[c]

[a]Cefoxitin for *S. aureus, S. lugdunensis, S. pseudintermedius,* and *S. schleiferi*—16–18 h, Cefoxitin for CoNS: may be reported after 18 h, if resistant.
[b]For HLG/HLS disk diffusion—16–18 h is needed.
[c]*Haemophilus* disk diffusion—16–18 h is needed.

READING PLATES AND INTERPRETING RESULTS[1,3-5]

The MHA plate should be examined only after the recommended duration of incubation **(Table 10.5)**.

- If the inoculum concentration used is correct, and streaking is done satisfactorily, there will be a confluent lawn of growth produced and the resulting zones of inhibition will be uniformly circular **(Figs. 10.6A and B)**.
- If individual colonies are apparent, instead of a confluent lawn of growth, the inoculum concentration was too light and the test must be repeated **(Figs. 10.6C and D)**.

The following points are to be kept in mind while measuring the zone diameter of inhibition.

- The zone edge should be read at the *point of complete inhibition*, as judged by the *unaided eye* (not by magnified lens) **(Fig. 10.7A)**.
- Reading includes the *diameter of the disk* (6 mm) **(Fig. 10.7B)**.
- Measurement is taken preferably by a *sliding caliper* (e.g. Vernier caliper, **Fig. 10.8**) or a *ruler*.
- Measurement is taken to the *nearest* whole millimeter: e.g. any zones between 23.0 and 23.5 mm should be taken as 23 mm; whereas the zones between 23.6 and 23.9 mm should be read as 24 mm.
- If an automated zone reader is used, it must be *calibrated* to manual reading.
- **Holding the plate:** The plate is held *about 30 cm* from the eye and against a *black,* nonreflecting background. Holding

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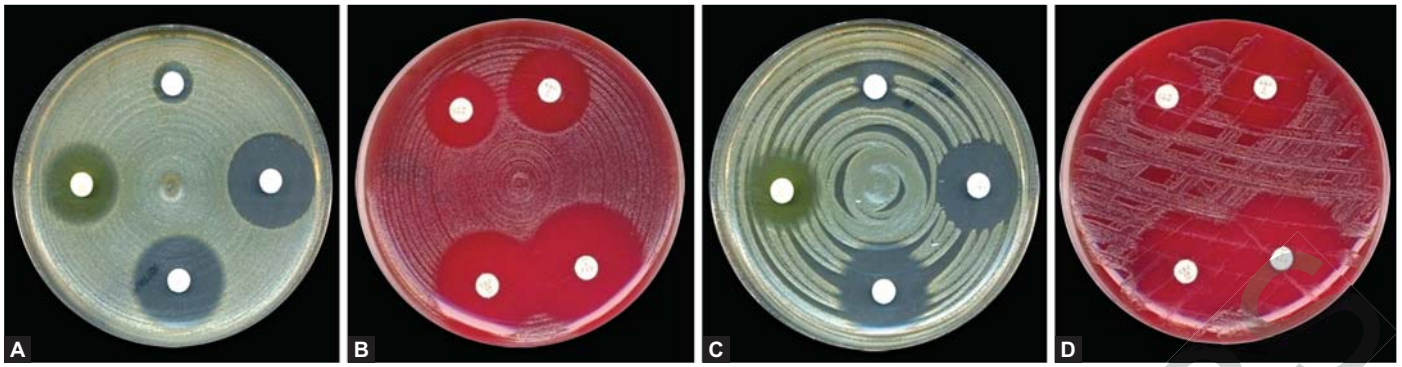
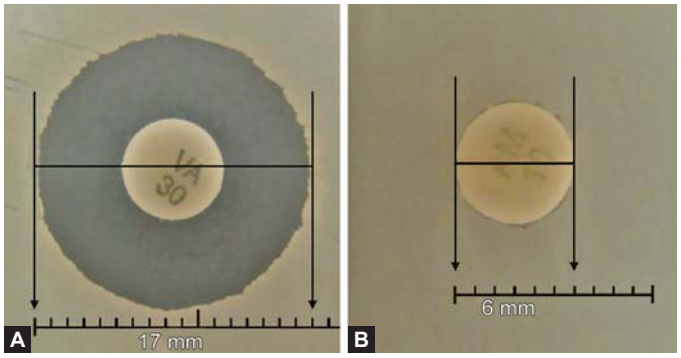


Figs. 10.6A to D: Growth in AST plates: **A and B.** Acceptable growth, confluent lawn of growth with zones of inhibition will be uniformly circular; **C and D.** Unacceptable growth, as individual colonies are apparent and therefore the test must be repeated.



Figs. 10.7A and B: Zones of complete inhibition include the diameter of the disk and show no obvious, visible growth as judged by the unaided eye: **A.** Zone is measured as 17 mm; **B.** Measure growth with no zone of inhibition as 6 mm.

Table 10.6: Recommendations for reading of the AST plate in reflected light from back and front side.[1–5]

Reading from back of the plate (Fig. 10.8)	Reading from front of the plate with lid open (Fig. 10.10)
Usually done on Mueller-Hinton agar • Enterobacterales • Nonfermenters • *Staphylococcus* • *Enterococcus* • *Bacillus* • *Aeromonas* • *Vibrio*	Usually done on Mueller-Hinton blood agar • *S. pneumoniae* • βH streptococci • Viridans streptococci • *Neisseria* • *Haemophilus* and HACEK • *Moraxella* • *Listeria* • *Campylobacter* • *Helicobacter* • *Pasteurella* • *Corynebacterium* • Anaerobes



Fig. 10.8: Measuring zones of inhibition using reflected light from back of the plate.

Source: Department of Microbiology, JIPMER.

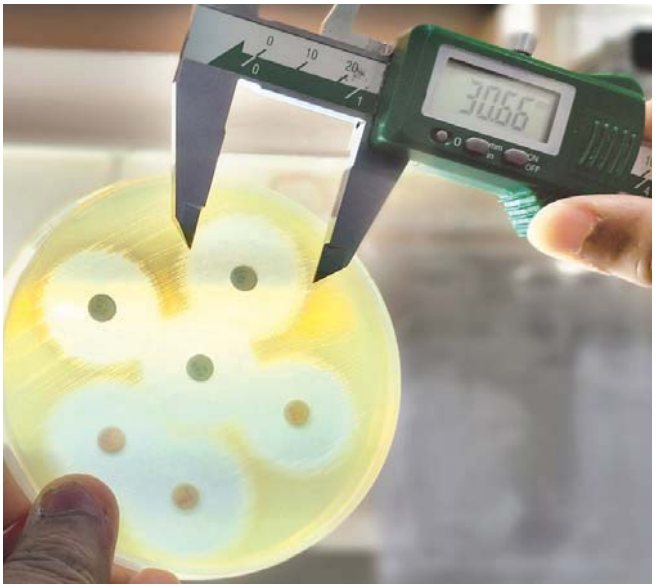


Fig. 10.9: Measuring zones of inhibition using transmitted light (linezolid against *Staphylococcus*).

Source: Department of Microbiology, JIPMER.

the plate at a *45° angle* to the work bench may facilitate reading when zone edges are difficult to define. The sliding calliper is held on the *back of the inverted* Petri plate, with lid closed **(Fig. 10.8, Table 10.6)**.

- **Reflected light:** Reading of MHA plate is mostly taken under *reflected light* **(Fig. 10.8)**. However, there are few drug/organisms for which *transmitted light* **(Fig. 10.9)** is recommended to take the reading, not reflected light; which include:
  - Penicillin/*S. aureus* (Zone-edge test).
  - Mupirocin/*S. aureus*.
  - Vancomycin screen agar/*S. aureus*.

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- Vancomycin/*Enterococcus.*
- Colistin agar test
- Tedizolid disc/*Enterococcus faecalis* ATCC 29212 only for QC testing
- Oxacillin salt screen agar/*S. aureus*

General Rule While Reading KB-DD Plate[3-5]

Only the inhibition zone with no obvious visible growth is measured as judged by unaided eye. If faint growth of tiny colonies are seen by magnifying lens, but not by unaided eye, then it should be ignored.

- **Colonies within zone**: If discrete visible colony is seen by naked eyes within a clear zone of inhibition, then the colony is subjected to identification to check for the purity **(Fig. 10.11)**.
  - If identification is different, then the outer zone should be measured.
  - If identification is found to be same, the test should be repeated with a pure culture.
  - If repeat AST yields similar discrete colonies within the zone of inhibition, then the colony-free inner zone should be measured.

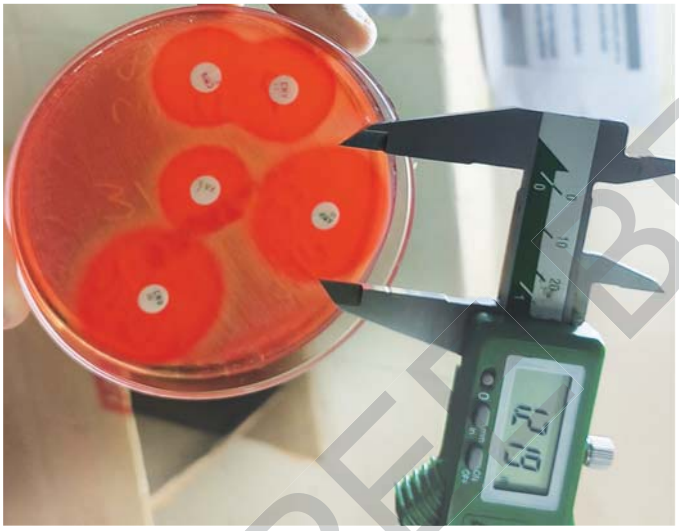


Fig. 10.10: Mueller-Hinton blood agar (MHBA) plate are read from the front against white background with the lid removed and illuminated with reflected light.

Source: Department of Microbiology, JIPMER.



Fig. 10.11: Reading of colonies within zone (if purity is confirmed, measure the colony-free inner zone).

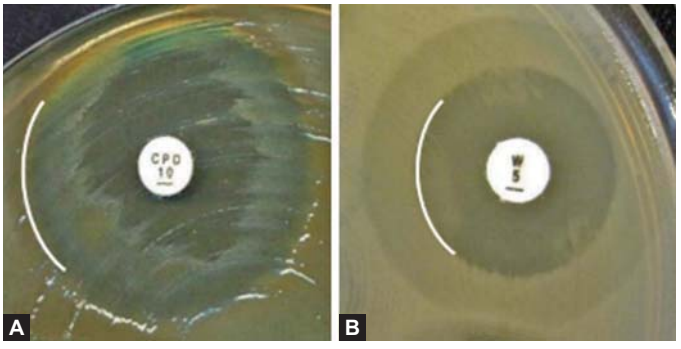
- **Fuzzy zone:** If smearing of the colonies is observed in naked eyes inside the zone edge, then only the inner colony-free zone should be measured. Make sure that plate is held against a dark background about 30 cm from the naked eye against reflected light. Plate should not be examined with transmitted light or by a magnifying glass **(Fig. 10.12)**.
- **Double zone:** When double zones are observed, the growth is checked for purity and the test is repeated, if necessary. If the culture is pure, the inner zone is measured as shown in **Figure 10.13**.
- **Swarming:** If a thin veil of swarming growth (with irregular margin) of *Proteus* occurs into an otherwise obvious zone of inhibition, it should be ignored and measurement is taken from the outer margin **(Fig. 10.14A)**.[3] However, if uniform growth of double zone is observed, then it may represent smearing of the colonies and in such case only the inner colony free zone should be measured **(Fig. 10.14B)**.



Fig. 10.12: Reading of fuzzy zone (measure the colony-free inner zone).



Fig. 10.13: Reading of double zone (if purity is confirmed, measure the colony-free inner zone).



Figs. 10.14A and B: Measuring zone of inhibition of *Proteus*: **A.** irregular inner indicates swarming, measure outer zone, **B.** Inner regular zone measure the inner colony free zone.

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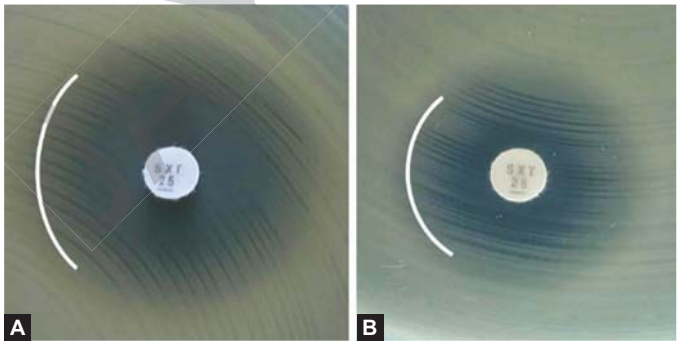
- **Penicillin/*S. aureus* (Zone-Edge test):** The interpretation is dependent on the edge of the zone, whether sharp or fuzzy (Fig. 12.1, Chapter 12).
  - The test is considered as **positive** if the zone edge is sharp or like a "cliff" indicating β-lactamase production and reported as 'R'.
  - The test is considered as **negative** if the zone edge is fuzzy or like a "beach" indicating no β-lactamase production and reported as 'S'.
- **MH blood agar:** The MHBA plates are read from the front against white background with the lid removed and illuminated with reflected light **(Fig. 10.10 and Table 10.6)**.
  - The plate is tilted back and forth to better differentiate between hemolysis and growth.
  - The hemolysis is usually free from growth, therefore the zone of growth inhibition, should be measured, but not the zone of hemolysis **(Fig. 10.15)**.
- **For cotrimoxazole:** Presence of antagonists in MHA medium may allow some growth within the inhibition zone surrounding the cotrimoxazole disk. Therefore, slight growth (≤20% of the lawn of growth) should be disregarded, and the more obvious margin is measured to determine the zone diameter **(Fig. 10.16)**.

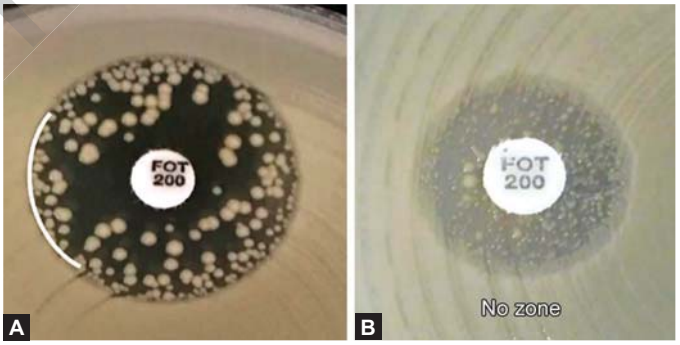


Fig. 10.15: Measurement of zone diameter in a Mueller-Hinton blood agar plate.

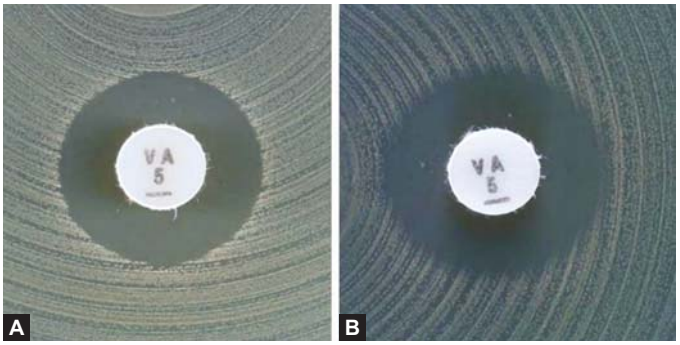


Figs. 10.16A and B: Interpretation of inhibition zones for *Stenotrophomonas maltophilia* with cotrimoxazole; **A and B.** An outer zone can be seen, therefore the outer zone edge is read and interpreted according to the breakpoints.[2]

  - This rule should also be applied when testing trimethoprim and sulfonamides.
  - According to EUCAST, if a zone edge is seen, then the growth within the inhibition zone is ignored and read the outer zone diameter.
  - For example, testing against various organisms such as Enterobacterales, *Acinetobacter, Stenotrophomonas, Burkholderia cepacia* complex, *Staphylococcus, S. pneumoniae, Haemophilus* and *Neisseria meningitidis*.
- **Fosfomycin/*E. coli*:** The isolated colonies within the inhibition zone should be ignored and the outer zone edge is measured **(Fig. 10.17)**.
- **Enterobacterales/temocillin** or **mecillinam:** The isolated colonies within the inhibition zone should be ignored and the outer zone edge is measured.
- **Inducible clindamycin resistance**: Detected by antagonism of clindamycin activity by a macrolide agent. Erythromycin and clindamycin disks are placed adjacent. D-shaped flattening of clindamycin zone or colonies inside clindamycin zone of inhibition on the side adjacent to erythromycin is suggestive of a positive test (Fig. 12.3, Chapter 12).
- **Vancomycin/enterococci**: The reading is taken only after 24 h of incubation, under the transmitted light.
  - If **sharp zone edge** is found and without any colonies in the inhibition zone, it should be reported as susceptible if the zone diameter is above the susceptible breakpoint **(Fig. 10.18)**.



Figs. 10.17A and B: Examples of inhibition zones for *Escherichia coli* with fosfomycin: **A.** Ignore all colonies and read the outer zone edge; **B.** Record as no inhibition zone.[2]



Figs. 10.18A and B: Interpretation of inhibition zones for *Enterococcus* spp. with vancomycin: **A.** Sharp zone edge. Report susceptible if the zone diameter is in the susceptible range; **B.** Fuzzy zone edge or colonies within zone. Confirm by PCR or report resistant even if the zone diameter is in the susceptible range.[2]

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- If the **zone edge is fuzzy**, or any colonies grown within the zone, it is reported as resistant (regardless of the zone size) or confirmed with PCR or a MIC-based method.

LIMITATIONS OF KB-DD METHOD[1-5]

The Kirby-Bauer disk diffusion (KB-DD) method has several limitations, as discussed below.

1. DD Breakpoints Not Available[1-5]

Though KB-DD method is the most common AST method performed worldwide, it has not been standardized for certain drug-bug combinations (see the highlight box below), for which the susceptibility can only be determined by performing a MIC based AST method.

DD breakpoints are not available in CLSI

The gram-negative organisms, for which KB-DD breakpoints are not available include:

- Nonfermenters other than *P. aeruginosa, Acinetobacter,* or *Stenotrophomonas,* i.e. other Non-Enterobacterales
- Levofloxacin or ofloxacin for *Salmonella*
- Colistin or Polymyxin B for Enterobacterales, *Pseudomonas* or *Acinetobacter*
- Ticarcillin-clavulanate and Chloramphenicol for *Stenotrophomonas*

The gram-positive organisms, for which KB-DD breakpoints are not available include:

- Vancomycin and Teicoplanin for *S. aureus*
- Lipoglycopeptide, Daptomycin and Tedizolid for *S. aureus, Enterococcus*, Viridans streptococci, βH streptococci
- Penicillins, cephalosporins (except ceftaroline), carbapenems for pneumococcus
- Penicillin, ampicillin for Viridans streptococci
- Carbapenems for Viridans streptococci, pneumococcus, βH streptococci

Misleading DD Results[1-5]

Certain antimicrobials that are clinically ineffective for specific organisms, may produce big zone of inhibition when tested by KB-DD with or without available breakpoint and falsely appear to be *in vitro* susceptible. There is a high likelihood that these drugs will not be effective *in vivo*. Therefore, such results should ideally be disregarded and not included in the patient report. Examples of such organism/antimicrobial agent combinations include:

- *Salmonella* and *Shigella* isolates when tested for first- and second-generation cephalosporins, cephamycins, and aminoglycosides.
- MRSA isolates when tested for penicillins, β-lactam/β-lactamase inhibitor combinations, antistaphylococcal cephalosporins.
- *Enterococcus* isolates when tested against aminoglycosides (except for high-level), clindamycin, cotrimoxazole and cephalosporins.
- *Intrinsic resistant (IR) agents*[1,8]*:* The bacterial species may occasionally (1–3% of cases) produce a zone of inhibition when tested against certain antimicrobial agents to which that are known to be intrinsically resistant.
  - Such *in vitro* susceptibility result may occur due to method variation, mutation, or low levels of resistance expression.
  - However, there is a very high chance that these drugs will not be effective *in vivo*. Therefore, such results should be viewed with caution and ideally be disregarded.
  - The IR agents may be wrongly tested by those laboratories which perform AST on the basis of colony morphology, without the identification result being available.
  - The likelihood of erroneously reporting IR agent as false-susceptible is high for the laboratories which follow the practice of reporting DD on the basis of *only bigger or small zones* without strictly abiding to the clinical breakpoints.

Testing Repeat Isolates[1-5]

Repeat AST testing on subsequent isolates of the same organism from the same patient is usually not recommended. However, bacterial isolates that are initially tested susceptible to an antimicrobial agent may become intermediate or resistant after the initiation of antimicrobial therapy—e.g., vancomycin-susceptible isolates of *S. aureus* may become vancomycin intermediate during the course of prolonged therapy. Therefore, laboratory should perform the AST on repeat isolates of the same species recovered from a similar body site in order to detect whether the resistance has been developed or not. However, when to perform AST on repeat isolates is decided by certain associated factors, as recommended by CLSI.

- **Time gap:** Ideally, AST should only be repeated if the subsequent isolate of the same species has been recovered from the same body site after 3–4 days.
- **Organism/antimicrobial agent:** Development of resistance during therapy although may be seen with any organisms/antimicrobial agents, but has been noted most frequently in:
  - Cephalosporins in organisms with inducible *AmpC* production, e.g. *Enterobacter, Citrobacter*, and *Serratia* spp.
  - *P. aeruginosa* with all antimicrobial agents.
  - Staphylococci with fluoroquinolones and vancomycin.
- Patient **not improving**/not responding to the ongoing therapy with drug(s) tested susceptible.
- **Severity** of the patient's condition: e.g., repeat testing is warranted for a repeat isolate from a blood culture of a premature infant.
- **Consultation:** Laboratory should discuss with the clinical team before deciding on performing AST on repeat isolate.

Modifications of KB-DD tests performed for detecting specific antimicrobial resistance and supplemental (not routine) tests have been discussed in Chapter 12.

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10.2 Dilution Methods

INTRODUCTION[1,2,8,9]

In dilution methods, susceptibility of an organism is tested against serial dilution (i.e. range of doubling concentrations) of the antimicrobial agent to determine the MIC.

- **Minimal inhibitory concentration (MIC):** It is the lowest concentration of the drug at which growth of organism is inhibited *in vivo* and no visible growth is appreciated *in vitro*. It has to be differentiated from another related terminology, called MBC.
- **Minimal bactericidal concentration (MBC):** It is the lowest concentration of the drug at which the organisms are killed or become nonviable. MBC of a drug is either same or higher than its MIC.
- The concept of MBC applies to only bactericidal drugs, but not to bacteriostatic drugs. However, MIC applies to both cidal and static drugs.

Types: Based on the nature of the culture medium used for testing, dilution methods can be classified into:

- **Broth dilution:** Here, Liquid medium such as Mueller-Hinton broth is used as growth medium. It can be further classified into two types, based on the testing format used.
  - Broth tube macrodilution (BTM): Test is performed in test tubes.
  - Broth microdilution (BMD): Test is performed in microtiter plate.
- **Agar dilution:** Here, solid medium such as Mueller-Hinton agar is used as growth medium.

Components: Both broth and agar dilution tests comprise the following components:

- Preparation of antimicrobial agent dilutions.
- Preparation of culture (growth) medium.
- Inoculum preparation followed by.
- Performing and interpreting the test result.

The method of preparation of antimicrobial agent is similar for both the dilution tests and therefore discussed together, whereas the remaining steps are discussed separately.

ANTIMICROBIALS USED FOR DILUTION TEST[1,2,8,9]

The drug powders used for dilution tests should be of only analytical grade, not the parenteral formulation available for treatment.

Drug Potency[1,8]

Potency of a drug formulation (expressed as percentage or in µg/mg or IU/mg of powder) is dependent on following factors:

- Assay purity, measured by methods such as high-performance liquid chromatography (HPLC).
- Measured water content.
- Active fraction of a drug which are supplied in salt forms, instead of free acid or base: i.e. % of drug available as free acid or base instead of salt.

Calculation of Drug Potency[8]

The information about the potency of a drug formulation is usually available in the "*certificate of analysis*" provided by the manufacturer. However, if the information on only above three individual components is provided, potency can still be calculated by the below formula.

- **Potency (µg/mg)** = [Assay purity (µg/mg) × active fraction] × [1–water content].
- **For assay purity and potency:** A value provided in percentage can be expressed in µg/mg by the formula = [10 × (that %)]. Example, 95% = 95 µg/100 µg = 950 µg/1,000 µg = 950 µg/mg.
- **For water content and active fraction:** A value provided in % = [(that %)/100] for analysis.

Illustration (Drug Potency Calculation)

Suppose, the certificate of analysis of the product of a drug mentions the following informations: (1) Assay purity (by HPLC): 97.0%; measured water content: 10.5% (w/w), active fraction 100%
Then before analysis of the potency, the above parameters must be converted to the desired format.

- *Assay purity* (by HPLC): 97% = 97 µg/100 µg = 970 µg/1,000 µg = 970 µg/mg
- *Measured water content:* 10.5% (w/w) = 10.5/100 = 0.105
- *Active fraction:* 100% = 100/100 = 1.0 (which means, it is supplied as free acid/base and not as salt)

Then, the potency of the drug can be calculated as= (Assay purity) × (active fraction) × (1 – water content)
Potency = 970 × 1.0 × (1 – 0.105) µg/mg
= 877 µg/mg or 87.7%

Conversion of Drug Potency or Activity[1,8]

Potency of a drug is usually expressed in terms of 'µg/mg', which is also termed as 'activity'.

- But for some antimicrobial agents such as penicillin G, polymyxin B, colistin, streptomycin, etc., the activity or potency of the drug is expressed as 'units/mg'.
- In such case, before preparation of stock solution using the formula involving potency, the activity values in 'units/mg' must be converted into 'µg/mg' using appropriate conversion factor.

Illustration for conversion of potency or activity

For colistin, the activity is usually 30,000 units/mg; which can be considered same as 0.03333 µg/unit, i.e. 1 mg = 30,000 units; i.e. 1 µg = 30 units; i.e. 1 unit = 1/30 µg = 0.03333 µg

- Therefore, the activity in 'µg/mg' = activity in 'units/mg' × 0.03333 µg/unit
- Similarly, activity in 'µg/mg' = activity in 'units/mg' ÷ 30 units/µg

For polymyxin B, the activity is 10,000 units/mg = 0.1 µg/unit. i.e. 1 mg = 10,000 units; i.e. 1 µg = 10 units; i.e. 1 unit = 1/10 µg = 0.1 µg

- Therefore, the activity in 'µg/mg' = activity in 'units/mg' × 0.1 µg/unit
- Similarly, activity in 'µg/mg' = activity in 'units/mg' ÷ 10 units/µg

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Reconstitution of a Drug from Powder[1,8]

For performing the dilution test, the drug powder should be reconstituted with a diluent in order to obtain a desired concentration of the drug solution.

Solvent vs diluent: It is important to note the difference between a solvent and a diluent for reconstitution of a drug.

- *Solvent:* The antimicrobial powder is initially solubilized using a minimum amount of a designated solvent, e.g. dimethyl sulfoxide (DMSO), water, phosphate buffered saline (PBS), methanol, glacial acetic acid.
- *Diluent:* The subsequent final preparation of the stock solution and further dilution to make working solution are performed using a recommended diluent, e.g. DMSO, water, PBS, etc.
- *Note:* The solvent and the diluent for a drug can be same or different. The list has been provided in CLSI M100 document.

Drug weight: The weight of the dry powder to be dissolved in a fixed volume of diluent is calculated as follows:

$$\textbf{Drug weight (mg)} = \frac{[\text{Volume (mL)} \times \text{Concentration (µg/mL)}]}{\text{Potency of the drug (µg/mg)}}$$

Volume of diluent: The volume of diluent to be used to dissolve a fixed weight of the dry powder of drug is calculated as follows:

$$\textbf{Diluent volume (mL)} = \frac{[\text{Weight (mg)} \times \text{Potency (µg/mg)}]}{\text{Concentration (µg/mL)}}$$

After the above calculations are made, the best way to proceed further is to weigh the drug powder first and then the respective diluent is calculated and reconstituted.

Illustration

Prepare 100 mL of a drug solution containing 640 µg/mL of antimicrobial agent with drug powder that has a potency of 800 µg/mg

- First the amount of the drug needed is calculated
- Weight of drug needed = (100 mL × 640 µg/mL)/800 µg/mg = 80 mg of drug

But while measuring the drug, if the actual drug measured becomes 90 mg. Then, the revised calculation for the volume of the diluent needed would be

- Volume of diluent (mL) = (90 mg × 800 µg/mg)/640 µg/mL = 112.5 mL
- i.e. 90 mg of drug is needed in 112.5 mL of diluent to make a solution of 640 µg/mL

Note: It is recommended that the dry weight of the drug used for reconstitution should always be above 10 mg. Use of lesser amount of drug may be erroneous

The reconstitution of the drug is performed in two steps—preparation of stock solution, followed by preparation of working solution.

Preparation of Stock Solution[1,8]

The stock solution of an antimicrobial agent refers to the first reconstituted solution prepared from a dry powder, which is usually prepared at a higher concentration (preferably at least 10 times) than the actual concentration to be tested.

- As some antimicrobial agents have limited solubility, knowledge on specific solvent and diluent to be used for each antimicrobial agent is important (provided in CLSI M100).[1]
- First, the calculated weight of the drug powder is dissolved in minimum amount of recommended solvent.
- Then the remaining volume is achieved by the addition of appropriate diluent.
- The antimicrobial stock solution need to be prepared in an aseptic condition, but does not need membrane filtration. However, the diluent may be sterilized priorly by autoclaving or filtration, if needed.

Storage of Stock Solution[8]

The aliquots of stock solutions are prepared in small split volumes and stored in deep freezer (–20°C or preferably at –60°C) to prevent thawing and re-freezing which deteriorate the drug.

- The volume of each aliquot can be customized by the laboratory based on their weekly throughput.
- The stock solution aliquots preferably should be thawed only when needed to prepare the working dilutions.
- Each aliquot once thawed should be used completely and should not be put back in the refrigerator.

Range of MICs to be Tested in Dilution Methods[1,2,8]

The range of concentrations of an antimicrobial agent to be tested should be two-dilutions on either side of the **lowest susceptible (S_L) and highest resistant (R_H)** breakpoint MICs. The S_L and R_H breakpoint MICs are determined based on the clinical breakpoints of an antimicrobial agent for all the organism groups where it is supposed to be tested, as mentioned in the reference documents such as CLSI M100, CLSI M45, EUCAST, FDA, etc.

Illustration

Vancomycin has CLSI breakpoints available for five organism groups—*Staphylococcus* (S ≤ 2, R ≥ 32), *Enterococcus* (S ≤ 4, R ≥ 32), pneumococcus (S ≤ 1), βH streptococci (S ≤ 1), and Viridans streptococci (S ≤ 1)

- Here, the S_L and R_H breakpoint MICs for vancomycin would be 1 and 32 µg/mL
- Therefore, the MIC range for vancomycin to be tested would be two-dilutions on either side: i.e. from ≤0.25 to ≥128 µg/mL
- The drug dilution wells used will be in the range of 0.25–64 µg/mL as no growth in 0.25 µg/mL well means MIC of ≤0.25 µg/mL and growth in 64 µg/mL well means MIC of ≥128 µg/mL

Once the range of MICs to be tested is finalized, the working solutions are prepared accordingly.

Preparation of Working Solution[8]

The working solution refers to the actual concentration of the drug solution that will be used for performing the dilution test. It is prepared from the stock solution by making appropriate dilutions.

Dilution factor: While performing the dilution test, when an antimicrobial agent is mixed along with growth media with inoculum (e.g. in microtiter well for BMD or without

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inoculum (e.g. agar plates for agar dilution), the drug gets further diluted. This dilution factor needs to be pre-calculated and kept into account while calculating the range of the working solution to be prepared.

Illustration

Dilution factor (for BMD)

In BMD, the most common format involves addition of 25 µL of drug and 25 µL of inoculum to 50 µL of growth media in a microtiter well to make a total of 100 µL. Therefore, the dilution factor for the drug introduced in well is 100 µL/25 µL, i.e. **four**

Dilution factor (for agar dilution)

Here in each of the agar plate, 2 mL of drug solution is added to 18 mL of molten media at 45–50°C and then poured into the plate. Therefore, the dilution factor introduced for drug solution = 20 mL/2 mL = **10**

Range of working solution: The range of concentration of drug in the working solution to be prepared is obtained after multiplying the range of MICs to be tested with the dilution factor.

Range of working solution = range of MICs to be tested × dilution factor of the drug.

Illustration

As per the above two illustrations, the MIC range for vancomycin to be tested is from 0.25 to 64 µg/mL and the dilution factor for BMD is 4 and agar dilution is 10

- Therefore, the range of working solution to be prepared for BMD will be from 0.25 × 4 to 64 × 4 = 1 to 256 µg/mL
- Similarly, the range of working solution to be prepared for agar dilution will be from 0.25 × 10 to 64 × 10 = 2.5 to 640 µg/mL

AGAR DILUTION METHOD[8]

In this method, a range of agar plates are prepared with each plate added with different concentration of the drug in doubling dilutions.

Medium for Agar Dilution[8]

Mueller-Hinton agar (MHA) is the recommended medium routinely used in agar dilution for most of the nonfastidious organisms.

- For certain fastidious and nutritionally exacting organisms, MHA enriched with 5% sheep blood is recommended. For *Neisseria gonorrhoeae*, GC agar base with 1% defined growth supplement is used.
- Here, cation-adjustment for MHA, (i.e. by addition of calcium or magnesium cations) is not needed. This step is necessary for broth dilution test.

Preparation of Agar Dilution Plates[8]

As discussed before, the drug plates for agar dilution test are prepared by adding 2 mL of working drug solutions of corresponding dilutions to 18 mL of molten MHA at 45–50°C for the range of MIC dilutions to be tested.

There are specific supplements which need to be added to MHA for certain antimicrobial agents.

- 2% NaCl when testing *Staphylococcus* and oxacillin.
- 25 µg/mL glucose-6-phosphate when testing fosfomycin.

Inoculation of Agar Dilution Plates[8]

The initial inoculum of the test organism is prepared either by direct colony suspension or broth culture method (as discussed before) and turbidity is matched to 0.5 McF; i.e. 1–2 × 10^8 CFU/mL or approximately 10^5 CFU/µL.

- **Final inoculum:** The concentration of organism in the spot is recommended to be 10^4 CFU/spot. Therefore, an appropriate dilution of the initial inoculum suspension should be made depending on the volume of inoculum to be spotted. So, the initial inoculum has to be diluted to ten times to obtain the final inoculum (i.e. approximately 10^4 CFU/µL).
- **Checker-board squares:** Multiple checker-board squares are prepared over the back surface of agar dilution plates **(Fig. 10.19)**.
- **Spot inoculation:** A volume of 2 µL of final inoculum is spread over the agar surface of each drug containing plates to make a 5–8 mm diameter spot.
- **Growth control plate:** One or two drug-free MHA plate is also inoculated—one at the beginning and one at the end of the drug containing plates.
- **Purity check plate:** In addition, a nonselective medium like blood agar is inoculated in parallel to check for purity of the inoculum.
- **Timing:** The spot must be prepared within 15 minutes of preparation of 0.5 McF inoculum and should be allowed to air-dry at room temperature for <30 minutes before incubating the plates at 35 C± 2°C.
- **Advantage:** Because of the checker-board format of testing, multiple isolates can be simultaneously tested in agar dilution test.

End-Point Determination[8]

After an incubation of 16–18 h, the plates are examined in the following manner to determine the MIC.

Growth Control Plates

The growth control plate is examined first to look for the viability of the organism in the inoculum.



Fig. 10.19: Agar dilution method showing spot inoculations in checker-board square format.

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- If growth control plates have no growth or insufficient growth, the plates may be reincubated for some extended time.
- If still have no growth, the test must be repeated for that isolate using a fresh growth.

Purity Check Plate

The reading of the purity plate is performed to look for presence of single type of colonies. If mixed colony morphotypes are observed in the purity plate, the test for that isolate need to be repeated with fresh inoculum.

Drug-containing Plates (Calculation of MIC)

For each isolate tested, the drug containing plates are looked for presence of visible colonies (as appreciated with unaided eye) at the spots, sequentially starting from the plate with lowest concentration of drug.

- The lowest concentration of the drug plate where no visible growth is observed is considered as the MIC of the drug for that isolate **(Fig. 10.20)**.
- In case of agar dilution methods, the MBC of the drug is observed to be same as its MIC.

Agar dilution methods is not recommended for antimicrobial agents such as daptomycin and organisms such as *Haemophilus* species.

Agar dilution plates can generally be used within five days, when stored in sealed plastic bags at 2–8°C. However, for certain agents such as tigecycline, freshly prepared agar dilution plates are recommended.

Modification of Agar Dilution Test[1,8]

Colistin Agar Test[1,8]

Instead of testing a full range of MICs, only a small range of MICs around susceptible breakpoint can be tested by agar dilution to determine the isolate to be susceptible or resistant. The classical example is colistin agar test. Here, MHA with concentration of 1, 2 and 4 µg/mL of colistin are tested (susceptible breakpoint is 2 µg/mL).

Screen Agar Test[1,8]

This is another modification of agar dilution test, where instead of testing a range of antimicrobial dilutions using multiple plates to obtain the exact MIC, only a single plate with a fixed drug concentration is tested to determine the isolates are susceptible or resistant.

- The drug concentration to be tested in screen agar is usually kept between the susceptible and resistant clinical breakpoints.
- Common examples where screen agar test is performed are given below. The detail has been discussed in Chapter 12.
  - Oxacillin screen agar (MHA with 6 µg/mL of oxacillin): Used for *Staphylococcus aureus*.
  - Vancomycin screen agar (brain heart infusion agar with 6 µg/mL of vancomycin): Used for *Staphylococcus* species

BROTH MICRODILUTION TEST (BMD)[1,8,9]

Broth microdilution test (BMD) is the most commonly used dilution method globally. Here, the test is performed by adding the organism inoculum onto the microtiter plates containing a serial dilution of antimicrobial agent solutions.

Medium for Testing BMD[1,8,9]

In general for bacterial isolates, cation-adjusted Mueller-Hinton broth (CAMHB) is the medium recommended for commonly isolated, rapidly growing, aerobic or facultative anaerobic organisms. There are certain organisms, for which special media are recommended for BMD, such as:

- For fastidious organisms, CAMHB added with 2.5–5% lysed horse blood (LHB) is used.
- For *Haemophilus* species, *Haemophilus* test medium (HTM) broth is used.
- For fungal AST, Roswell Park Memorial Institute (RPMI) 1640 broth is the preferred medium.

Preparation of Medium[1,8,9]

CAMHB is prepared in double strength (2X), as the growth medium gets diluted by two-folds in the microtiter well while performing the BMD.

- **Dilution factor:** While performing BMD, the most common format used is addition of 25 µL of drug and 25 µL of inoculum to 50 µL of growth media in the microtiter wells to make a total of 100 µL. So, a dilution factor for growth media introduced in well is 100 µL/50 µL, i.e. two.

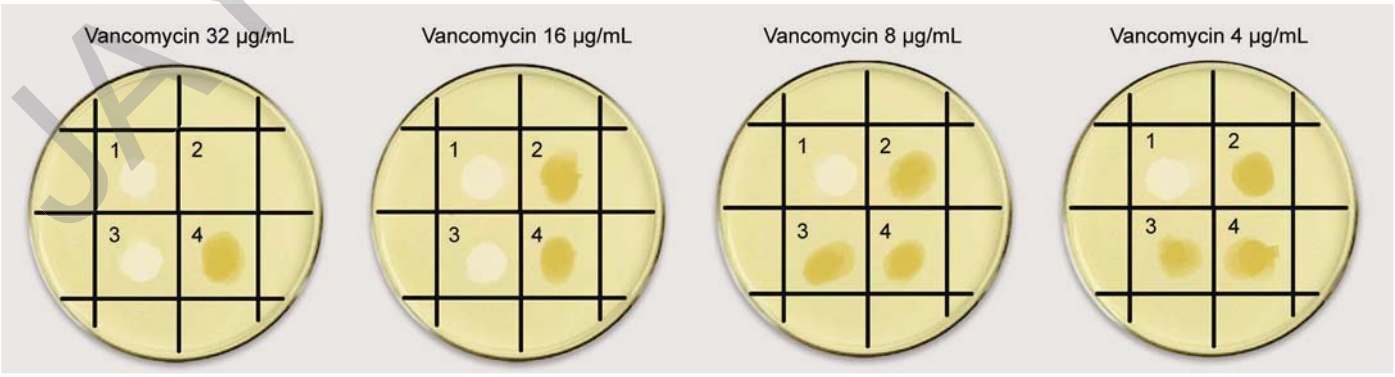

Fig. 10.20: Agar dilution for MIC detection of *Enterococcus faecium* against vancomycin. [Isolate 1: MIC-≤4 µg/mL (susceptible); Isolate 2: MIC-32 µg/mL (resistant); Isolate 3: MIC-16 µg/mL (Intermediate); Isolate 4: MIC ≥64 µg/mL (resistant); Interpretation is based on CLSI M100 2022 [S (≤4 µg/mL), I (8–16 µg/mL), R (≥32 µg/mL)].

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- If the molecular weight of CAMHB is 21 g, then 21 g is dissolved in 1 liter of water to obtain single strength medium.
- For 2X media, 42 g has to be dissolved in 1 liter.

- As CAMHB is available as **dry powder**, it is possible to prepare 2X medium, and as a result, drug and inoculum can be prepared in diluent and normal saline respectively.
- In contrast, the **RPMI-1640 medium** is available primarily in broth form only, therefore it is not possible to make a 2X medium. In such case, both drug and inoculum need to be prepared in the RPMI-1640 only. This will prevent the further dilution of growth medium while performing the BMD test.
- **Supplements:** Specific supplements may need to be added to CAMHB for testing specific antimicrobial agents, such as:
  - 2% NaCl when testing oxacillin against *Staphylococcus.*
  - 0.002% polysorbate-80 when testing dalbavancin, oritavancin, and telavancin.
  - Calcium when testing daptomycin to obtain a final concentration of 50 µg/mL.
- **Storage:** The microtiter plates added with broth and antimicrobial agent mixture are stored in freezer at ≤–20°C (preferably at ≤–60°C).

Preparation of Inoculum for Testing BMD[1,8,9]

For BMD, the method of inoculum preparation and its dilution is different than that for agar dilution method. The steps for BMD are described below:

- **Primary inoculum:** The primary inoculum prepared for bacteria is matched to turbidity of 0.5 McF, i.e. 1.5 (range, 1–2) × 10^8 CFU/mL.
- **Final concentration:** For broth dilution, the final concentration of organism required in the microtiter well is 5 × 10^5 CFU/mL.
- **Dilution factor:** So, the total dilution needed in the microtiter well from primary inoculum is 1.5 × 10^8 CFU/mL ÷ 5 × 10^5 CFU/mL, i.e. 300.
  - The dilution factor of the inoculum introduced in the microtiter well while it is mixed along with growth media and antimicrobial agent is calculated.
  - As in routine, 25 µL of drug and 25 µL of inoculum are added to 50 µL of growth media to make a total of 100 µL in the microtiter well. So, the dilution factor for inoculum introduced in well is 100 µL/25 µL, i.e. four.
  - Thus, the dilution which must be made in the primary inoculum, before it is dispensed into the microtiter well is 300/4, i.e. 75.

Illustration (Inoculum Preparation)

A primary inoculum is prepared in normal saline, at 0.5 McF; i.e. 1.5 × 10^8 CFU/mL

- Final inoculation is then prepared by diluting the primary inoculum by 75 times—by mixing 10 µL of primary inoculum to 740 µL of normal saline, i.e. the organism load would be 1.5 × 10^8 CFU/mL ÷ 75 = 2 × 10^6 CFU/mL
- Then 25 µL of inoculum is added along with 25 µL of drug and 50 µL of growth media in the microtiter well to make a total of 100 µL, i.e. four times dilution
- So final concentration in the well would be 2 × 10^6 CFU/mL ÷ 4 = 5 × 10^5 CFU/mL (as recommended)

Inoculation of the Microtiter Wells[1,8,9]

The microtiter plates (containing growth media and serial dilution of drug solution) are removed from the freezer for thawing. The thawed plates must be inoculated and incubated within 4 h from the time they are removed from the freezer. The plates if not used after thawing cannot be refreezed.

- **Drug wells:** Each drug well (containing 50 µL of growth media and 25 µL of drug solution) is added with 25 µL of inoculum.
- A **growth control well** is used for detecting viability of the organism, where only the growth medium and inoculum are added without the drug.
- A **sterility control well** (with growth medium only) is also used to rule out contamination of the growth medium.
- The inoculated microtiter plates are incubated at 35 ± 2°C for 16–20 h. Extended incubation of 20–24 h is recommended for streptococci, *Haemophilus, Neisseria* or vancomycin/*Enterococcus* (or 48 h for anaerobes).

Steps to Perform for Broth Microdilution

Following are the steps to prepare microtiter plates for broth microdilution and perform the test

- One microtiter plate can be used to test 8 isolates simultaneously, i.e. one row of 12 wells for each isolate
- The first well is marked as 'growth control well'
- Remaining 11 wells can be used for drug dilution
- Any one well in the full plate can be marked as 'sterility control well' or 'medium control well'
- **Addition of growth medium:** 50 µL of double strength CAMHB is added to each drug well, growth control well and sterility control well
- **Addition of drug dilutions:** 25 µL of working drug solution is added in their respective wells, but not in growth control and sterility control wells (25 µL & 50 µL of water/saline is added in growth control and sterility control well respectively in place of drug solution)
- Till this stage, microtiter plates are prepared and stored in deep freezer (–20°C or –60°C)
- On the day of testing, the plates are thawed and brought to room temperature
- **Addition of inoculum:** 25 µL of inoculum in normal saline (5 × 10^5 CFU/mL, i.e. 1/75 times dilution of 0.5 McF) is added to each well except 'sterility control well'

Determination of End points (MIC and MBC)[1,8,9]

After the recommended duration of incubation, the microtiter plates are examined in unaided eye, sequentially starting from the well with lowest concentration of drugs.

- The growth control and sterility control wells are read first. **Growth control** well should have acceptable growth (definite turbidity or button) and the **sterility control well** should have no appreciable turbidity.
- Then the **drug wells** are visually inspected for presence of growth (i.e. turbidity).
- **End point (MIC):** Then the MIC is determined, which is the lowest concentration of the antimicrobial agent that completely inhibits organism growth in drug wells as detected by the unaided eye.
- **MBC:** Subsequently, the minimum bactericidal concentration (MBC) can also be determined by

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subculturing on solid medium from the wells where the turbidity is not found.

- MBC refers to the lowest concentration of the drug that makes the organism nonviable so that it does not grow on subculture plates.
- For broth dilution tests, the MBC is either equal to or higher than MIC.
- MBC can be calculated for bactericidal drugs, but not for bacteriostatic drugs. Estimation of MBC may be of significance in certain situations like immunocompromised state.

Broth dilution method is not recommended for certain antimicrobial agents such as fosfomycin.

Special Considerations While Reading[1,8,9]

Special considerations need to be taken for the following type of growth, while taking the reading of BMD microtiter plates.

Trailing Growth[1,8,9]

End-point of BMD is regarded as the lowest concentration of drug where turbidity is not present, i.e. the organism is inhibited.

- But, in certain situations, there may be presence of some amount of persistent growth in the drug wells even at further higher concentrations (especially in form of button of growth).
- This is termed as "trailing growth" which usually makes the determination of end-point difficult whether to the take the reading of MIC at the trailing well or well with no growth.
- In such a case, it is recommended to take the MIC reading at the concentration of the well where trailing begins **(Fig. 10.21D)**.
- **Examples:** Trailing growth is more commonly observed for gram-positive cocci when testing chloramphenicol, clindamycin, erythromycin, linezolid, tedizolid, and tetracycline.
- When testing for **cotrimoxazole**, slight growth may also be appreciated in higher drug concentrations as a result of unavoidable antagonists in the medium. Here, the end-point is taken at the concentration of well in which there is ≥80% reduction in growth compared with the growth control well.

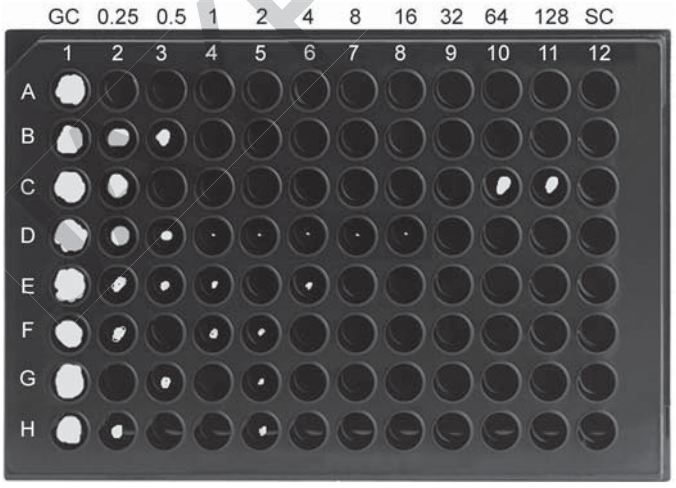

Fig. 10.21: Interpretation of broth microdilution results (schematic), see highlight box for explanation.

Skipped Wells[1,8,9]

There may be a rare instance, where growth is not appreciated at a well with lower drug concentration but turbidity is appreciated in well with higher drug concentration. Such a finding is termed as "*skipped wells*" **(Figs. 10.21E to H)**.

- Single skipped well in a BMD is considered acceptable. In this situation, the MIC reading is taken ignoring the single skipped well **(Figs. 10.21E and F)**.
- If skipped wells are observed on ≥2 wells either together or with intervals, across multiple concentrations of the drug, this is considered unacceptable, MIC is not reported for that isolate/drug and test is repeated **(Figs. 10.21G and H)**.

Paradoxical Growth (Eagle Effect)[1,8,9]

It is characterized by the ability of certain isolates to reconstitute growth in the presence of higher concentrations of antimicrobial agent, while being fully susceptible at lower concentrations.

- Instead of consistent growth across the wells, as seen in trailing growth, there is a distinct drop-off in growth and then a distinct regrowth further up the concentration gradient.
- In this situation, the MIC reading is taken ignoring the growth in higher dilutions **(Fig. 10.21C)**.
- This phenomenon has been observed in cell wall acting antimicrobial agents such as echinocandins and certain β-lactams.
- The mechanism underlying this phenomenon has been attributed to alteration in cell wall content and structure as a compensatory response to antimicrobial drug stress.

There is no clear connection between *in vitro* paradoxical growth or trailing growth of an isolate and *in vivo* resistance.

Explanation of Figure 10.21

In this illustration, the broth microdilution test is conducted for vancomycin for eight isolates (isolate A to H). The drug concentration tested range from 0.25 to 128 µg/mL. Column 1 wells are for growth control (GC) and column 12 wells are for sterility control (SC).

- Turbidity is seen in all growth control wells of column 1 and no turbidity is seen in all sterility control wells of column 12
- A: MIC ≤0.25 µg/mL (case of limiting dilution or off-scale MIC)
- B: MIC 1.0 µg/mL
- C: MIC 0.5 µg/mL (growth at C10 and C11 wells are due to paradoxical phenomena and are ignored)
- D: MIC 1.0 µg/mL (growth at D4 to D8 wells are due to trailing growth and are ignored)
- E: MIC 8.0 µg/mL (E5 is a single skipped well, so MIC reading is taken at E7)
- F: MIC 4.0 µg/mL (F3 is a single skipped well, so MIC reading is taken at F6)
- G: MIC reading will not be taken because of intermittent skipped wells at G2 and G4
- H: MIC reading will not be taken because of continuous skipped wells at H3 and H4

The broth microdilution methodology described in this book has been adapted from CLSI and NCDC guidelines

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Table 10.7: Comparative overview of broth microdilution methodologies.

	CLSI method[8]		NCDC broth microdilution protocol[25]	Method recommended in this book
Stock drug solution				
Preparation	Prepared in recommended diluent, e.g. distilled water		Same as for CLSI	Same as for CLSI
Working drug solution				
Preparation	Working solution is prepared in growth medium, i.e. CAMHB (**may lead to some dilution** of CAMHB due to addition of water)		Same as for CLSI	Working solution is also prepared in recommended diluent, e.g. distilled water
	Drug dilutions are prepared by transferring from previous working solution to next working solution serially, i.e. **increased risk of multiplication of pipetting error**		Same as for CLSI	Drug dilutions are prepared by transferring from stock solution to all the working solution tubes, i.e. **no risk of multiplication of pipetting error**
	Working solution is prepared at 2 times the desired concentration to be tested		Working solution is prepared at 4 times the desired concentration to be tested	Same as for NCDC
Addition to wells	**Using pipette** 50 µL drug solution is added to each drug well	**Using inoculator** 100 µL drug solution is added to each drug well	25 µL drug solution is added to each drug well But not to growth control well and medium/sterility control well	25 µL drug solution is added to each drug well In addition, 25 µL saline is added to growth control well, 50 µL saline is added to medium/sterility control well
Growth medium				
Strength	CAMHB is prepared in single strength		Same as for CLSI	CAMHB is prepared in double strength
Addition to wells	There is no additional steps of addition of growth medium to well. Both antimicrobial dilutions and inoculum should be prepared in CAMHB		50 µL of CAMHB is added to each drug well, 75 µL to growth control well and 100 µL to medium/sterility control well	50 µL of double strength CAMHB is added to each drug well, growth control well and medium/sterility control well
Organism Inoculum				
Initial inoculum	Prepared in normal saline and adjusted to 0.5McF turbidity (Considered 1×10^8 CFU/mL)		Prepared in normal saline and adjusted to 0.5McF turbidity (Considered 1.5×10^8 CFU/mL)	Same as for NCDC
Final inoculum	**Using pipette** Diluted 1:100 times in water/saline/broth (i.e. 1×10^6 CFU/mL)	**Using inoculator** Diluted 1:20 times in water/saline/broth (i.e. 5×10^6 CFU/mL)	Diluted 1:75 times in CAMHB, i.e. 10 µL of initial inoculum in saline + 740 µL of CAMHB (i.e. 2×10^6 CFU/mL) (**may lead to some dilution** of CAMHB due to addition of saline)	Diluted 1:75 times in saline, i.e. 10 µL of initial inoculum in saline + 740 µL of saline (i.e. 2×10^6 CFU/mL)
	If inoculum is prepared in water/saline, it will lead to **significant dilution** of growth medium			
Addition to wells	**Using pipette**	**Using inoculator**	4 times dilution in well (i.e. 5×10^5 CFU/mL)	Same as for NCDC
	2 times dilution in well (i.e. 5×10^5 CFU/mL)	10 times dilution in well (i.e. 5×10^5 CFU/mL)		
	50 µL of final inoculum is added to each drug well to make 100 µL in total	10 µL of final inoculum is added to each drug well to make 110 µL in total (so, dilution becomes **11 times** instead of 10)	25 µL of final inoculum is added to each drug well and growth control well, but not to medium/sterility control well	Same as for NCDC

with some modifications so as to make the procedure easy to perform **(Table 10.7)**.

BROTH TUBE MACRODILUTION (BTM) METHOD[1,8]

In BTM method, the test is performed in sterile test tubes instead of microtiter wells. The methodology of BTM is similar to that discussed for BMD, with some differences which are discussed below.

Preparation of Drug–Medium Mixture[1,8]

Serial two-fold doubling dilution of antimicrobial agents are prepared directly in the growth medium (CAMHB) to obtain 1 mL of final volume of broth.

- The concentration of the antimicrobial agent gets diluted by two times, as equal volume (1 mL) of inoculum is added to the growth medium.
- Therefore, the working drug solution must be prepared in double strength as compared to the range of MIC to be tested.

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Preparation of Inoculum[1,8]

The inoculum is also prepared directly in the growth medium, instead of normal saline.

- The **initial inoculum** is prepared and matched with turbidity of 0.5 McF, i.e. 1.5×10^8 CFU/mL.
- **Dilution factor:** It is diluted 150 times by adding 10 µL of initial inoculum to 140 µL of growth medium to obtain 1.5×10^8 CFU/mL ÷ 150, i.e. 10^6 CFU/mL. This is the final inoculum to be used for testing.
- **Final inoculum:** When equal volume (1 mL) of inoculum is added to the growth medium with antimicrobial agent (1 mL), the inoculum is getting diluted two times in the test tube, i.e. 5×10^5 CFU/mL. This is the recommended final inoculum turbidity to be present in the test tube.

The end-point determination (calculation of MIC and MBD of the drug) is performed in the way similar to that discussed for BMD **(Fig. 10.22)**.

Broth tube macrodilution being cumbersome is less preferred now-a-days. A modified BTM test has now being used for testing AST for colistin, called as **colistin broth disk elution test,**[1,8] where the source of antimicrobial agent is disk, rather than working solutions prepared from drug powder. This will be discussed subsequently in Chapter 12.

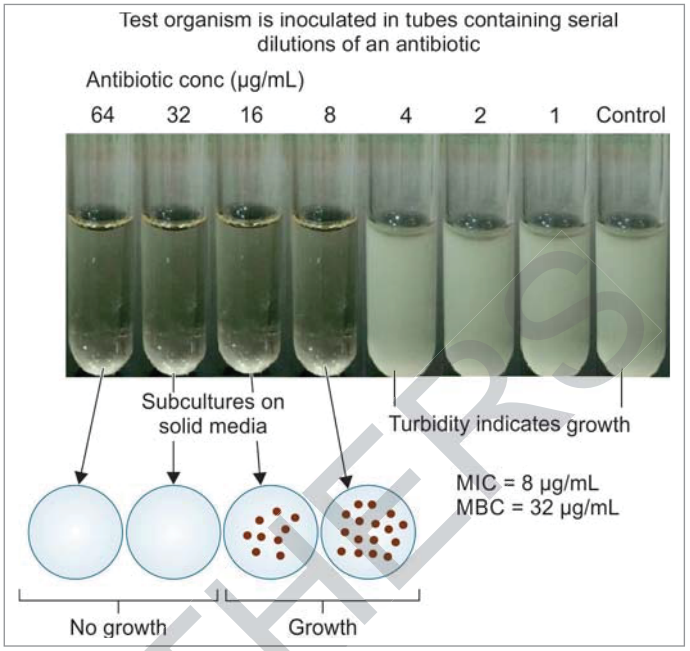

Fig. 10.22: Broth tube macrodilution (BTM) method.

Another example of broth disk elution is synergy testing of aztreonam and ceftazidime-avibactam, which is also a type of modified broth tube macrodilution method.

10.3 Epsilometer Method

This is a method of MIC determination using the principles of both dilution and diffusion of drug into the medium. It is also called as **gradient diffusion method**.

- It consists of a thin, inert and non-porous plastic/paper strip which contains immobilized predefined gradient (serial dilution) of antimicrobial agent concentrations along its length with an MIC reading scale in µg/mL.
- It is applied to the center of a lawn inoculum of an organism by a sterile forceps, with the printed area faced up.
- The immobilized antimicrobials immediately transfer into the agar to form a stable, continuous, exponential gradient of antibiotic concentrations around the strip.
- Following incubation of the test organism, an elliptical zone of inhibition is produced surrounding the strip.
- The antimicrobial concentration at which the ellipse edge intersects the strip, is taken as MIC value **(Fig. 10.23)**.

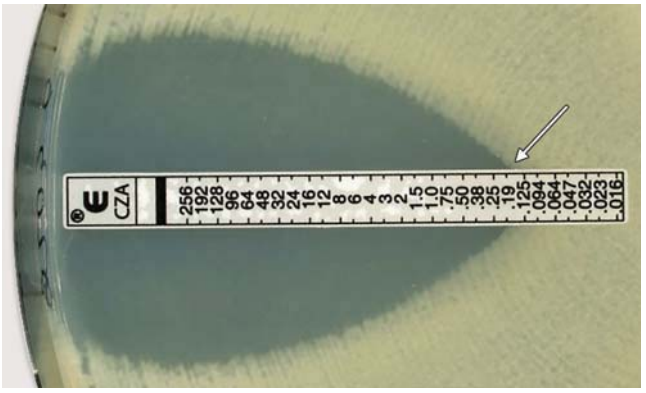

Fig. 10.23: Epsilometer or E-test.[10]

FACTORS INFLUENCING READING OF E-TEST[10]

Reading of E-test can be at times challenging, may be influenced by various factors—organisms, antimicrobial agents, resistance mechanism exhibited and technical factors.

Effect of Organisms on Reading of E-test

The reading of E-test may be influenced by the growth characteristics of the organism **(Fig. 10.24)**.

- **Hemolysis:** Only the growth edge should be read as MIC ignoring the hemolysis **(Fig. 10.24A)**.
- **Swarming:** Only the growth edge should be read as MIC ignoring the swarming **(Fig. 10.24B)**.
- When testing for ***S. pneumoniae*** **and β-lactams**, any colonies present inside the ellipse or hazy growth near the margin should be considered into account and only the inner zone edge is measured as MIC **(Figs. 10.24C and D)**.

Effect of Various Antimicrobial Agents[10]

Various antimicrobial agents may influence the reading of E-test, which include **(Fig. 10.25)**:

- **Bactericidal drugs:** Presence of any hazy growth, macro or microcolonies inside the ellipse should be considered into account and only the inner zone edge should be measured as MIC **(Figs. 10.25A and B)**.

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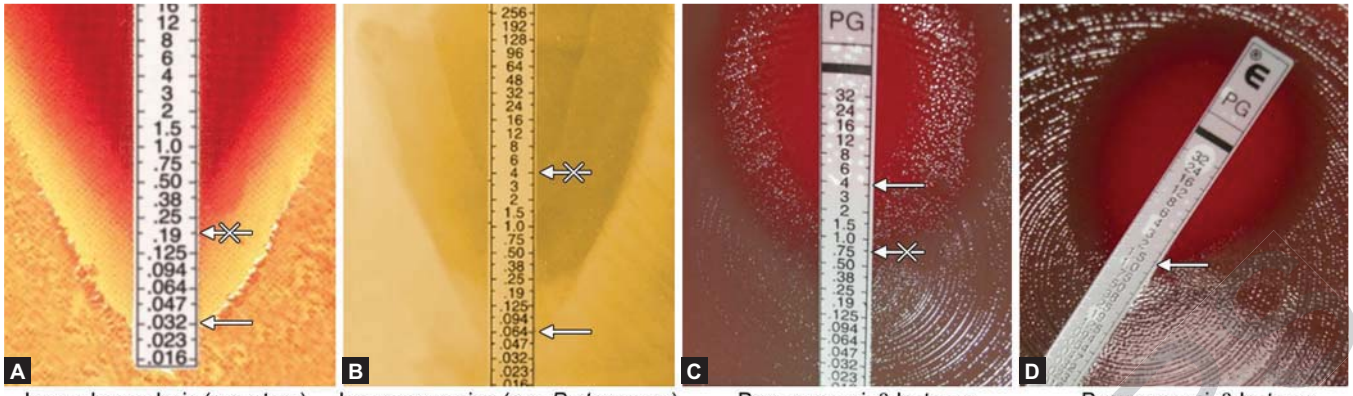
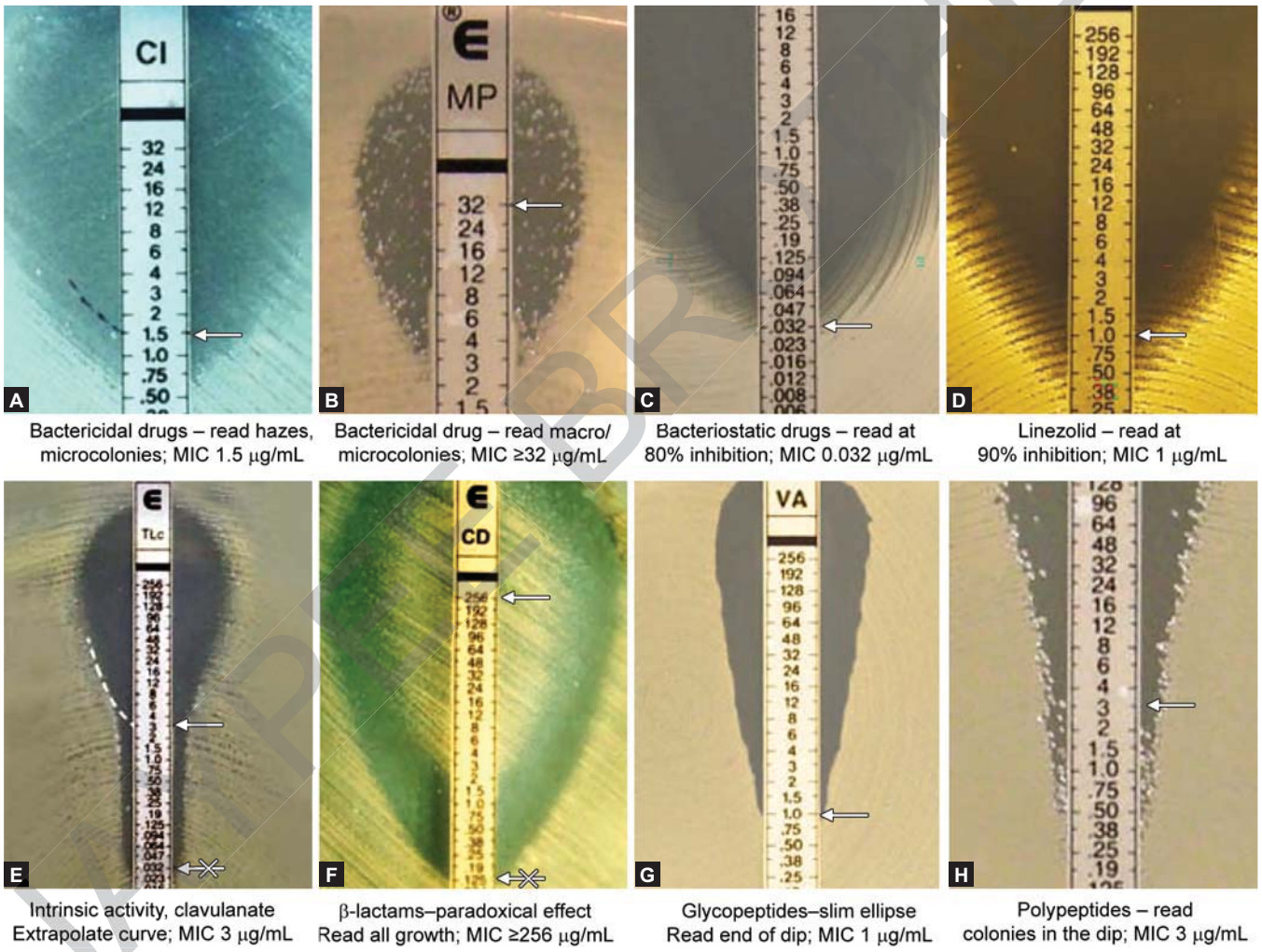


Figs. 10.24A to D: Effect of organism on reading of E-test.[10]



Figs. 10.25A to H: Effect of various antimicrobial agents on reading of E-test.[10]

- **Bacteriostatic drugs:** Minimal hazy growth, inside the ellipse should be ignored and reading is taken at 80% of growth inhibition **(Fig. 10.25C)** and for linezolid, at 90% of growth inhibition **(Fig. 10.25D).**
- **Clavulanate:** The intrinsic activity of clavulanate should be ignored. The curve of the ellipse is '*extrapolated*' and MIC is taken at the point where the curve bisects the strip **(Fig. 10.25E)**.
- **β-lactams:** The *'paradoxical effect'* seen with β-lactams should be considered and only the outer zone edge should be measured as MIC **(Fig. 10.25F)**.
- **Glycopeptides:** The '*slim ellipse*' may be produced some times. In such case, the MIC is taken at the intersecting point of the ellipse where the dip ends **(Fig. 10.25G)**, provided the slim area is devoid of colonies.

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- **Polypeptides:** '*Any colonies*' grown within the ellipse should be taken into consideration and only the inner zone edge should be measured as MIC **(Fig. 10.25H)**.

Effect of Resistance Mechanisms[10]

The reading of E-test may be influenced by presence of various resistance mechanisms **(Fig. 10.26)**. In such case, presence of any growth near the intersecting point of ellipse or discrete colonies present inside the ellipse zone should be taken into consideration and only the inner zone edge should be measured as MIC.

- Glycopeptides resistance in *S. aureus* or enterococci **(Fig. 10.26A)**.
- Oxacillin resistance in *S. aureus* **(Fig. 10.26B)**.
- Carbapenem resistance in *K. pneumoniae* **(Fig. 10.26C)**.
- β-lactamase induction by clavulanate **(Fig. 10.26D)**.

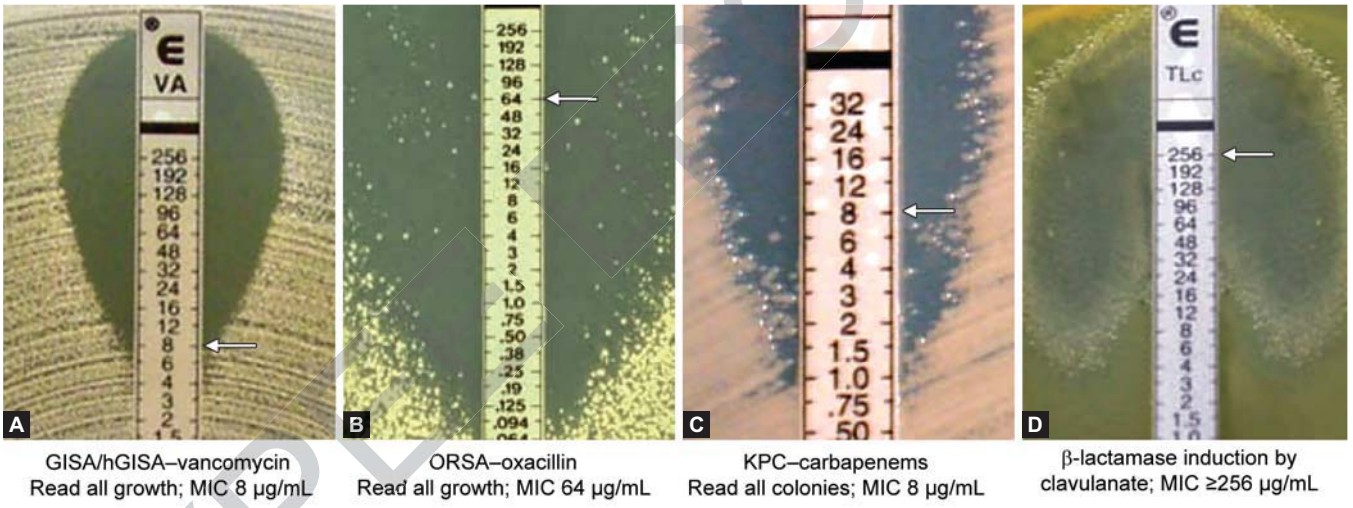
Effect of Technical Issues[10]

Presence of any technical issues may be a challenge while taking the reading of E-test. In such case, the general rules which need to be followed are given below **(Fig. 10.27)**.

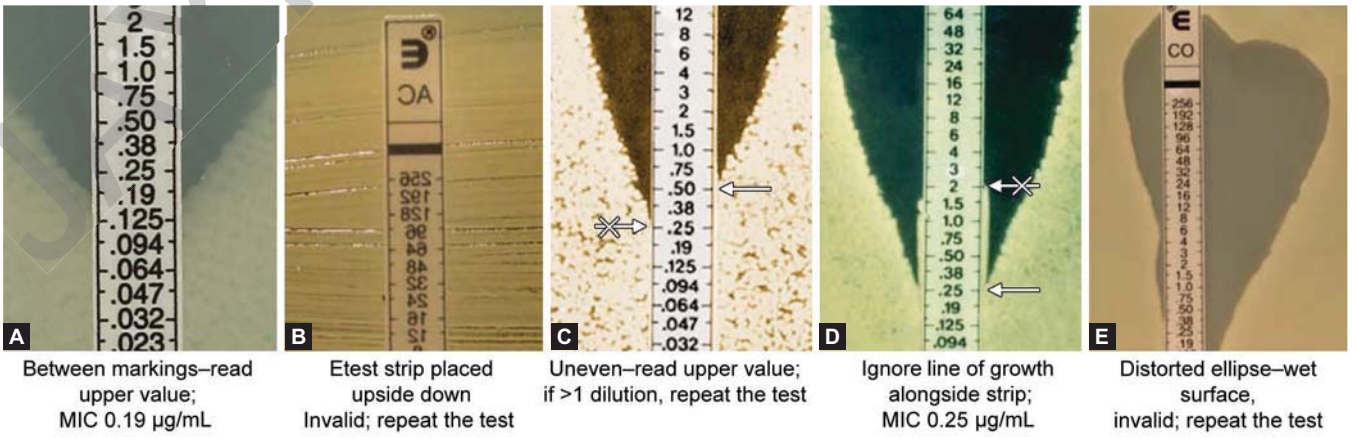
- **Intersecting between dilutions:** If the point of intersection of the ellipse lies in between two adjacent dilutions, then the higher dilution is taken as MIC **(Fig. 10.27A)**.
- **Placed upside down:** If the strip is placed upside down, then the result should be considered invalid and the test is repeated **(Fig. 10.27B)**.
- **Uneven intersecting:** If both sides of the ellipse intersect at different point of dilution, then the higher point of dilution is taken as MIC **(Fig. 10.27C)**.
- Presence of **any line of growth** along the side of the strip should be ignored and the intersecting point of ellipse is taken as MIC **(Fig. 10.27D)**.
- **Distorted ellipse:** If the ellipse is distorted which usually occurs if the surface of the MHA plate is wet, then the result should be considered invalid and the test is repeated **(Fig. 10.27E)**.

READING OF E-TEST RESULTS FOR FUNGI[10-11]

CLSI also provides reference documents for antifungal susceptibility testing. It has disk diffusion document for yeast (M44)[12] and non-dermatophyte filamentous fungi (M51).[13] It also has separate broth dilution document for yeasts (M27)[14] and filamentous fungi (M38).[15] Reading of E-test results for yeasts (e.g., *Candida*)[10] and molds[11] have been depicted in **Figures 10.28 and 10.29**.



Figs. 10.26A to D: Effect of various resistance mechanisms on reading of E-test.[10]



Figs. 10.27A to E: Effect of technical issues on reading of E-test.[10]

(GISA, glycopeptide intermediate *Staphylococcus aureus*; hGISA, heterogenous glycopeptide intermediate *S. aureus*; ORSA, oxacillin resistant *S. aureus*)

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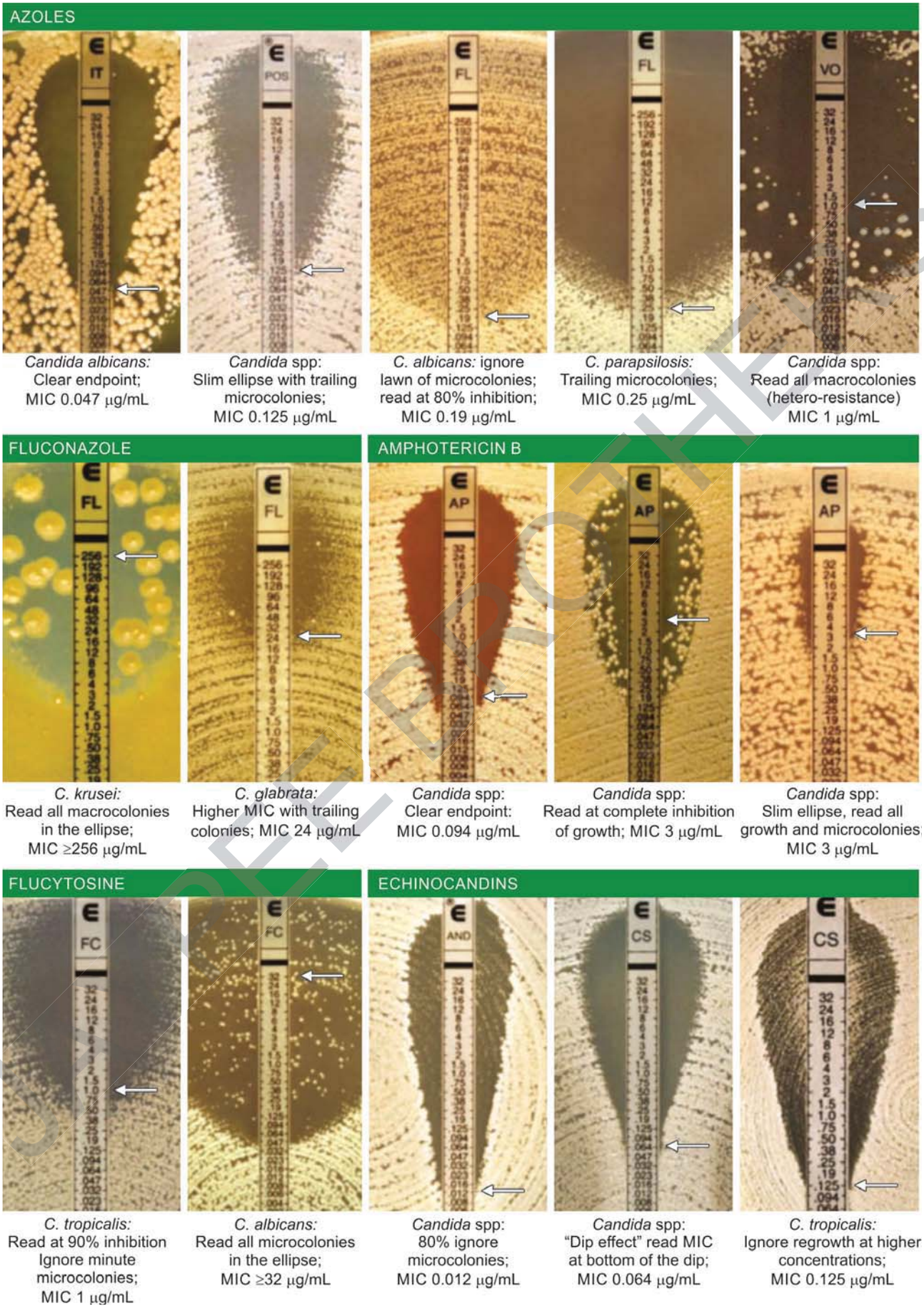

Fig. 10.28: Reading of E-test results for yeast (e.g. *Candida*).[10]

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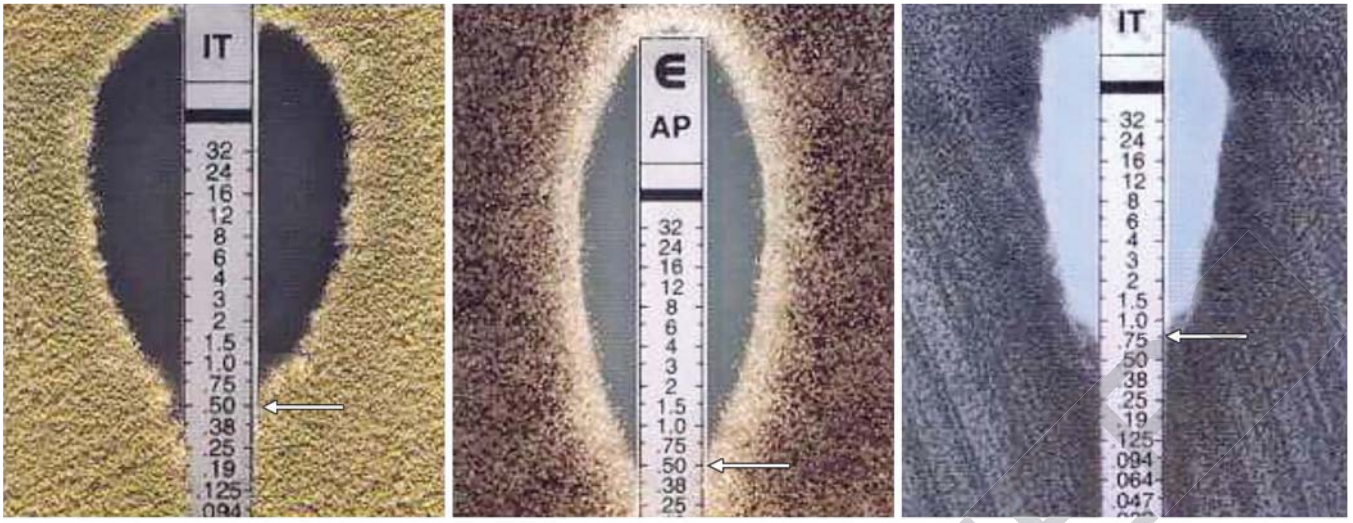

A. flavus, read at 72 hours, MIC 0.5 µg/mL *A. niger*, read at 48 hours, MIC 0.5 µg/mL *A. fumigatus*, read at 24 hours, MIC 0.75 µg/mL

Fig. 10.29: Reading of E-test results for molds (e.g. *Aspergillus*).[11]

10.4 Automated AST Methods

There are three automated AST instrument available globally, which can be classified on the basis of principles.

- Based on broth microdilution: Phoenix and Microscan WalkAway systems.
- Based on modified broth microdilution coupled with growth curve algorithm: VITEK2 system.

VITEK2 AUTOMATED AST SYSTEM[16-19]

VITEK2 instrument is the most common automated AST system used in India. It can also be used for microbial identification, which has been discussed in Chapter 9.

Procedure (VITEK2 AST)[16-19]

The procedure of inoculum preparation for performing AST in VITEK2 is as follows:

- **Suspension tubes:** A total of two suspension tubes are needed for AST testing—ID suspension tube and AST suspension tube. The same two tubes are also needed when intending to perform ID and AST together.
- **ID suspension tube:** Inoculum is prepared by direct colony suspension method in a polystyrene test tube, using 3 mL of 0.45% sterile saline provided by the manufacturer. The inoculum turbidity is matched to 0.5–0.63 McF using DensiCHECK.
- The **AST suspension** tube is prepared by transferring 145 µL (for gram-negatives) or 280 µL (for gram-positives and yeasts) of 0.5 McF inoculum (1.8–2.2 McF for yeasts) from the ID suspension tube to 3 mL of 0.45% saline. This is done in two ways–either manually or automatically by the instrument.
  - *For VITEK2 compact,* both ID and AST suspensions are prepared **manually** and loaded.
  - *Whereas for VITEK2 60/XL,* the uninoculated AST suspension tube containing 3 mL of 0.45% sterile saline is loaded into the instrument along the inoculated ID suspension tube and then the inoculation into the AST suspension tube from the ID suspension tube happens **automatically** inside the instrument.

From the AST suspension tube, the drug wells present in the VITEK card are inoculated automatically by the instrument.

Principle (Growth Curve Algorithm)[18-19]

VITEK2 system works on the principle of modified broth microdilution (mBMD) test, coupled with growth curve algorithm.

- The **VITEK2 AST card** is a single, self-contained disposable card, comprises of 64 wells—at least one growth control well and the remaining drug wells.
  - The **drug wells** are pre-coated with a range of increased concentration of various antimicrobial agents along with proprietary growth medium in lyophilized/freeze dried form **(Fig. 10.30)**.

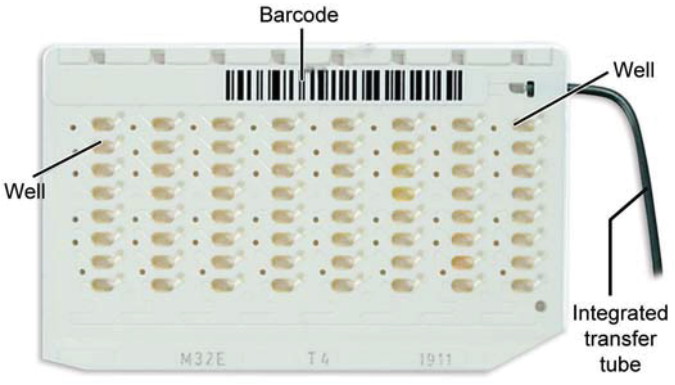

Fig. 10.30: VITEK2 AST card.

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- The **growth control well** (or positive-control well or drug-free well) contains only the growth medium, without any antimicrobial agent.

- In a reference **BMD test**, a range of all the serial doubling dilutions of an antimicrobial agent are tested to obtain the true/actual MIC, with the end-point being taken as presence of growth/no growth or % reduction in growth as compared to growth control after overnight incubation.
- However, in the **modified BMD algorithm** implicated in automated AST such as VITEK2, only a few selected intermittent drug concentrations are tested and based on the growth pattern obtained, the **projected MIC** for all the doubling dilution is estimated. Thus, in this algorithm, a projected MIC is determined, rather than the true MIC.
- **For example,** in case of amikacin/*P. aeruginosa*, the BMD would test from a range of 1 µg/mL to 64 µg/mL with a total of seven dilution wells. But the mBMD in VITEK card tests for only intermittent drug concentrations at 2, 4, 16 and 48 µg/mL. The growth pattern for these 4 drug wells is then used to predict the projected MIC value of the isolate ranging over all the seven dilutions (i.e. from 1 to 64 µg/mL).

Growth Monitoring[18-19]

VITEK2 continuously monitors the microbial growth in all the wells at every 15 minute, interval and detects the growth through turbidity measurements in terms of raw transmittance units (RTU).

- **Growth in the control well** shows that the test isolate is viable and growing at an appropriate rate to begin analysis of the various drug wells.
- **%ΔRTU of growth control well:** The change in turbidity of growth control well with time as compared its turbidity at the beginning of the test is recorded as percent change in RTU (%ΔRTU).
- **Growth in drug well:** The relative growth of the organism in each drug well is compared to the growth in the growth control well.
- **%ΔRTU of a drug well:** The change in turbidity of a drug well with time as compared to the turbidity of the growth control well is recorded as percent change in RTU.
- **Growth curve:** The growth curve of a particular drug well can be plotted with %ΔRTU (Y-axis) against hours of incubation (X-axis) **(Fig. 10.31)**.

The MIC is determined by comparing the growth pattern of the test isolate to the growth pattern of a large number of reference isolates with known MICs, present in VITEK database.

Reference MIC[18-19]

It is important to understand the concept of reference isolates and reference MIC (rMIC).

- **Reference isolates:** The manufacturer has used a large number of reference organisms (>1,800 gram-negative and >1,200 gram-negative bacteria) having various resistance mechanisms.
- **Reference MIC:** The reference isolate were tested against a wide range of drug dilutions by BMD method to determine the actual MIC, also termed as 'rMIC'.

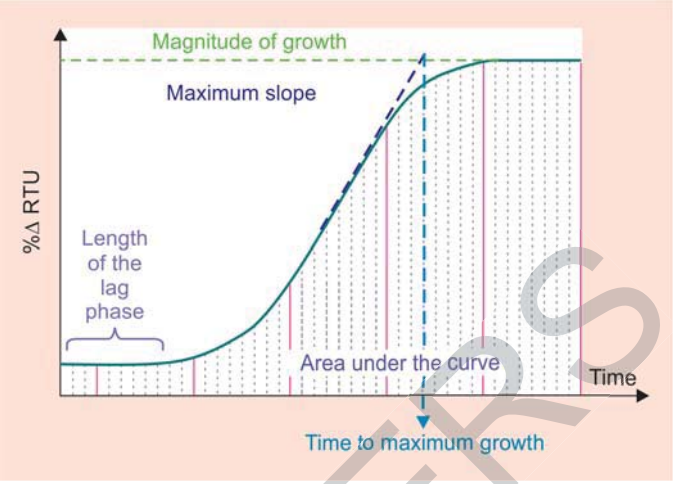

Fig. 10.31: The growth curve of a particular VITEK2 well in terms of %ΔRTU (Y-axis) plotted against hours of incubation (X-axis).

Deciding on Intermittent Drug Concentrations to be Included in AST Panel[16-19]

All the reference isolates are tested by VITEK methodology over a large range of drug concentrations (test MIC or tMIC) to obtain the **%ΔRTU** for each drug concentration tested. The %ΔRTU of all the reference isolates having range of rMICs tested at each tMIC is obtained and plotted **(Fig. 10.32)**, and compared if the %ΔRTU at any particular tMIC has distinct values for each rMIC, so that it is able to differentiate between various rMIC and subsequently predict the MIC.

Illustration[18-19]

In the illustration **(Fig. 10.32)**, the reference isolates with rMIC in the range of 4–128 µg/mL are tested at tMIC of 1–128 µg/mL and %ΔRTU is calculated (values in brackets).

- For tMIC of 1, 2, 4, 16, and 64 µg/mL, there is no clearcut distinction between %ΔRTU for various rMIC.
- But, the tMIC of 8 µg/mL can discriminate between rMIC of 4, 8 and 16 by %ΔRTU (i.e. 0.264, 0.513 and 0.782).
- Similarly, tMIC of 32 µg/mL and 128 µg/mL can also discriminate between rMIC of 16–64 and 32–128 respectively.
- So, if testing is carried out for 8, 32 and 128 µg/mL in the panel, it will be able to predict the MIC range of 4 to 128 µg/mL based on %ΔRTU.

Establishing the Database[18-19]

Subsequently the reference isolates are tested by VITEK2 method against a limited intermittent drug concentrations (which is intended to be made available in VITEK card), i.e. (test MIC or tMIC).

- **Reference growth curve:** For each reference isolate having a particular rMIC, the %ΔRTU in the growth control well and various drug dilution wells (tMIC) are obtained and plotted as growth curves **(Fig. 10.33)**.
- All reference isolates having same rMIC are plotted in the same graph.
- Separate graphs are plotted for a full range of rMIC,

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Concentration included in the prototype card (μg/mL)	1	2	4	8	16	32	64	128
MICs reference (known strains)	%ΔRTU for each MICs reference tested with each concentration							
4	0.860	0.812	0.542	0.264	0.201	0.190	0.261	0.250
8	0.923	0.896	0.772	0.513	0.299	0.225	0.271	0.243
16	0.924	0.911	0.862	0.782	0.571	0.261	0.296	0.266
32	0.930	0.913	0.885	0.841	0.718	0.513	0.370	0.277
64	0.920	0.906	0.894	0.881	0.827	0.736	0.621	0.466
128	0.958	0.953	0.943	0.928	0.911	0.846	0.797	0.675

Fig. 10.32: Selection of testing drug concentrations with respect to the calling range of MIC to obtain the projected MIC.

- Each graph depicts the growth as observed by VITEK2 system for different reference isolates of a particular organism with a particular rMIC.

Illustration[18-19]

Let us imagine a scenario where, the manufacturer intends to develop a VITEK card for an antimicrobial agent having doubling dilution ranging from ≤0.25 to ≥8.0 μg/mL (i.e. calling range), but are intending to test only three concentrations of 0.5, 2.0 and 8.0 μg/mL.

- In this scenario, the manufacturer would test a large number of reference isolates by BMD (from 0.25 to 8.0 μg/mL) to get the rMIC and also by the VITEK against the three concentrations (0.5, 2.0 and 8.0 μg/mL) of the antimicrobial agent intended to include in the panel.
- **Figure 10.33A:** For all reference isolates with rMIC of ≤0.25 μg/mL, a growth curve (%ΔRTU vs time) is plotted for growth control well (blue line), 0.5 μg/mL well (green line), 2.0 μg/mL well (yellow line) and 8.0 μg/mL well (red line). It can be noted that, as rMIC of isolate is ≤0.25 μg/mL, the growth curves of all the drug wells are flat lines.
- **Figure 10.33B:** For a different reference isolate with rMIC of 0.5 μg/mL, similar plotting of growth curve is done. It can be noted that, even if the rMIC of isolate is 0.5 μg/mL, there is some appreciable growth at the 0.5 μg/mL well (green line), which is approximately 60% of the growth in control well.
- **Figure 10.33C-F:** Similarly, for four other reference isolates with individual rMIC of 1.0, 2.0, 4.0 and 8.0 μg/mL, the growth curves are plotted. In **Figure 10.33D**, the rMIC of isolate is 2.0 μg/mL and the growth at 0.5 μg/mL well is almost 95% of that in growth control well and that at 2.0 and 8.0 μg/mL wells are nearly 50% and 8% respectively.

Relative Organism Growth[18-19]

The growth in the various drug well as compared to that in the control well expressed in % is termed as **relative organism growth**. For example, as explained above, in **Figure 10.33D** the relative organism growth at 0.5, 2.0 and 8.0 μg/mL wells are 95%, 50% and 8% respectively.

Table 10.8 depicts the relative organism growth for the three drug concentrations tested by VITEK2 against the rMIC obtained using reference test (i.e. BMD), as shown in graphs A to F of **Figure 10.33**.

By plotting the relative organism growth values over a large set of different reference isolates of same organism for the 3 drug wells (0.5, 2 and 8 μg/mL) as shown in **Table 10.8**, a **three dimensional scatter plot** can be obtained **(Fig. 10.34)**. In this scatter plot, the following informations can be deduced.

- **Each dimension** (X,Y,Z axis) represents the relative % growth in any one concentration of the antimicrobial agent tested (i.e. 0.5, 2 and 8 μg/mL).
- **Each diamond** represents a particular reference isolate whose position in the scatter plot corresponds the relative organism growth values for all three drug wells.
- **Color clusters:** The reference isolates are arranged in different color clusters, each of them represents a set of reference isolates of an organism with rMICs ranging from ≤0.25 μg/mL to ≥8.0 μg/mL.
- **For example,** the diamond for the data corresponding to **Figure 10.33B and Table 10.8** would be at the point (60%, 3%, 1%). The 60% would be plotted on the 0.5 μg/mL axis, the 3% on the 2.0 μg/mL axis, and the 1% on the 8.0 μg/mL axis. Since this organism has an MIC of 0.5 μg/mL, the color of the diamond would be grey (the diamond is shown inside a grey square).
- This scattered plot is finally used to predict the projected MIC of a test isolate subjected to VITEK AST.

Projected MIC of Test Isolate[18-19]

When the VITEK AST is performed on a test isolate using a limited set of drug concentrations, the relative organism growth value is obtained for each drug concentration. These growth values are then plotted in the reference three dimensional scatter plot **(Fig. 10.34)** to find out the location of the diamond representing the test isolate.

- **For example,** the relative organism growth values of a test isolate at 0.5, 2.0 and 8.0 μg/mL are found to be 55%, 52% and 25% respectably, and these values are plotted over the in the reference three dimensional scatter plot in **Figure 10.34**.
  - Then the location of the diamond representing the test isolate is determined in the scattered plot (represented by blue box, **Fig. 10.34**).

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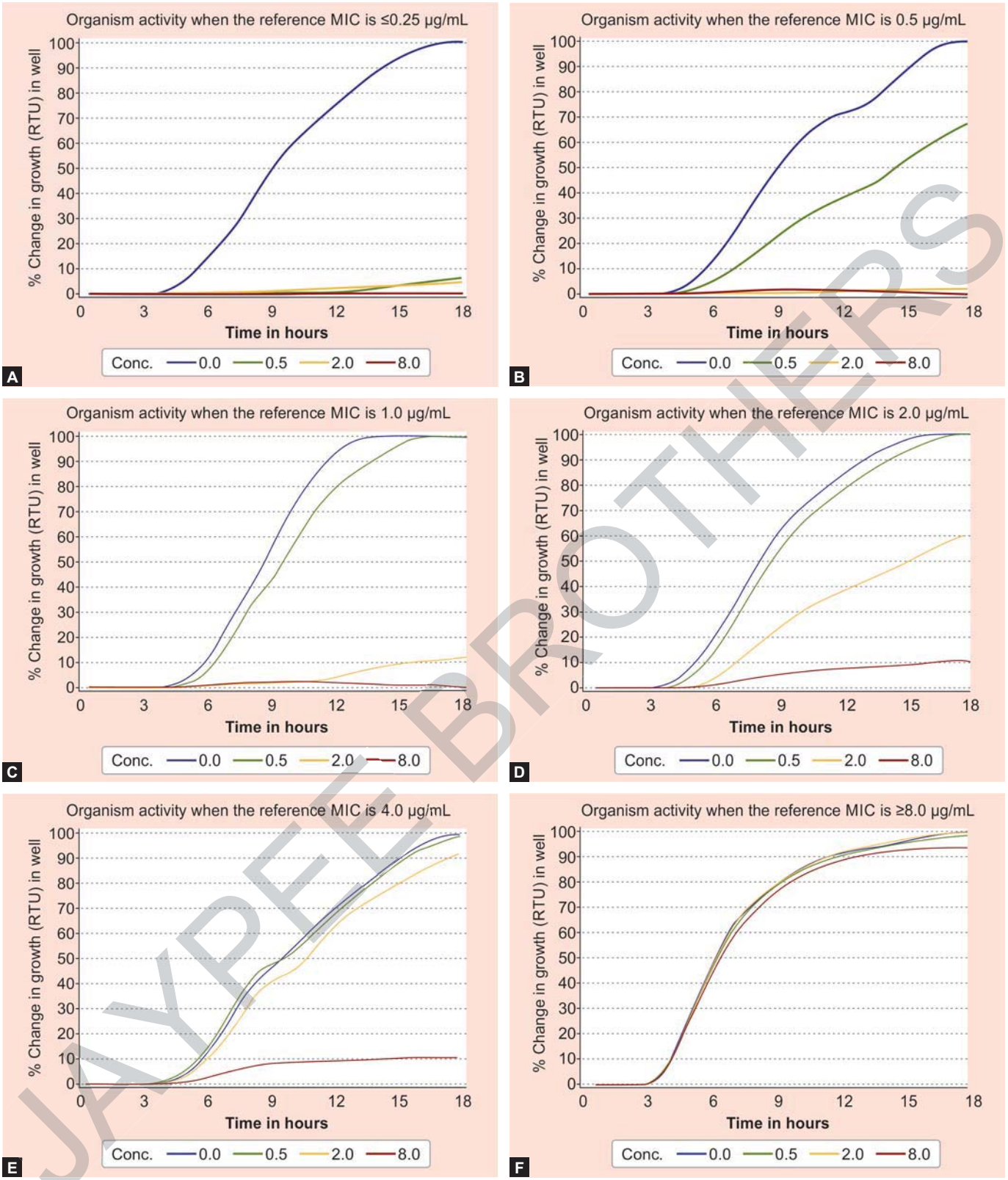

Figs. 10.33A to F: Illustrate the relationship between data generated by a VITEK® 2 system to results obtained by a reference method.

- As this location corresponds to the rMIC of 2.0 µg/mL, the projected MIC of the test isolate for this antimicrobial agent will be reported as 2.0 µg/mL.

- Thus, even if when tested for three drug wells, the VITEK2 algorithm can predict the reporting of 6 different MICs (≤0.25 to ≥8.0). Therefore, a limited set of drug wells are still sufficient to predict the MIC values for a wider range of doubling dilution.
- In simplified terms **(Fig. 10.35)**, when a clinical isolate is tested at these three drug concentration (tMIC), the growth pattern/curve for that isolate (illustrated as green graph) are compared to the reference growth pattern/curve for

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various rMIC stored in the database (illustrated as blue graph). The rMIC value for which the test curves matches with the reference curves is chosen as the projected/predicted MIC for that isolate.

Table 10.8: Relative organism growth for the three drug concentrations tested by VITEK2 against the rMIC obtained using reference test (i.e. BMD), as shown in graphs of Figures 10.33A to F.

Organism (Fig. 10.33)	Reference MIC (µg/mL)	Antimicrobial concentration tested in the VITEK wells		
		0.5 µg/mL	2.0 µg/mL	8.0 µg/mL
Figure 10.33A	≤0.25	3%	2%	1%
Figure 10.33B	0.5	60%	3%	1%
Figure 10.33C	1.0	90%	8%	2%
Figure 10.33D	2.0	95%	50%	8%
Figure 10.33E	4.0	99%	92%	9%
Figure 10.33F	≥8.0	100%	100%	99%

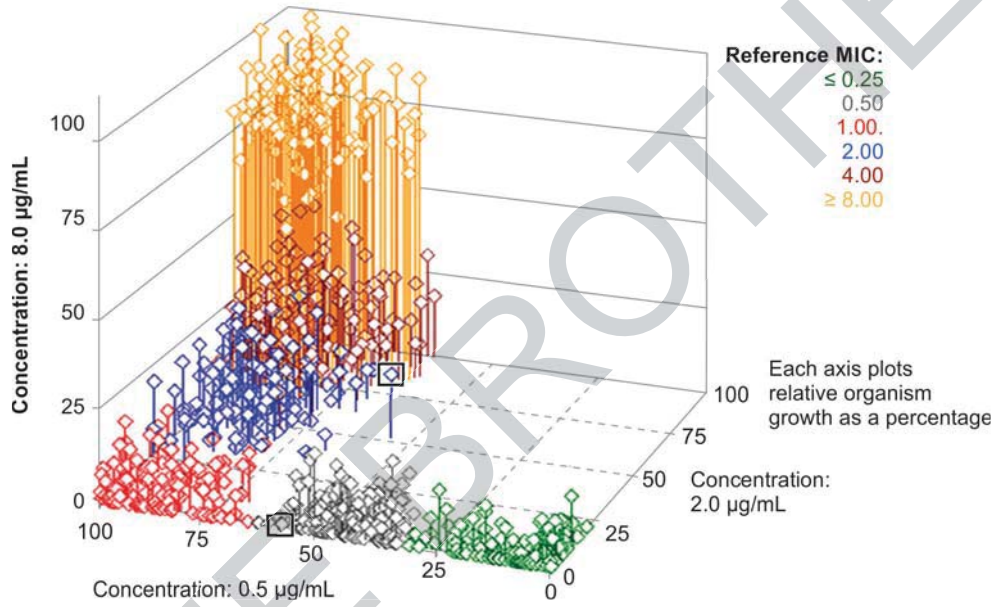

Fig. 10.34: Relative organism growth over a large set of different reference isolates of same organism; each diamond represents a single isolate.

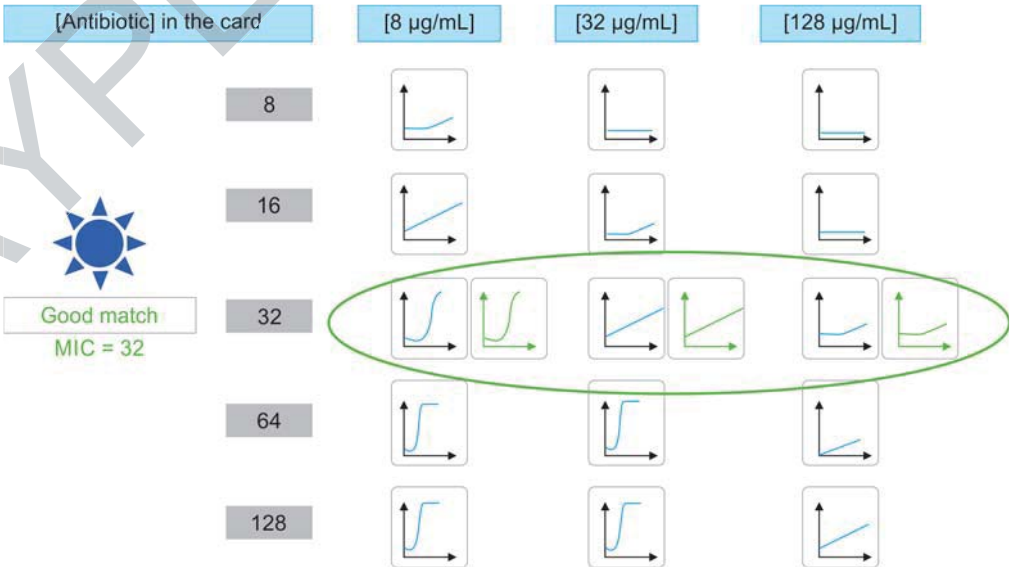

Fig. 10.35: Simplified view to use the relative organism growth to predict the MIC.

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- Based on the above concept, the manufacturer claims that it is not necessary to include drug wells for every MIC values that can be reported.
- However, manufacturers of automated AST systems are required to generate clinical trial performance data to establish agreement between their testing algorithms compared with the reference methods (e.g. FDA approval).

Interpretation of Projected MIC[16-19]

The projected MICs obtained for each antimicrobial agents are interpreted by the VITEK2 software based on the clinical breakpoints stored in the data base.

- VITEK2 software has been equipped with the clinical breakpoints as recommended by CLSI, EUCAST or other equivalent bodies.
- However, VITEK2 provides the interpretation for those organism/antimicrobial agent combinations for which FDA approval has been obtained.
- For the organism/antimicrobial agent combinations for which FDA approval has not been obtained, VITEK2 software although does not interpret the result, but has a provision to provide the MIC values. The laboratory can approach the manufacturer to obtain such MIC values and interpret manually using appropriate clinical breakpoints.

Trouble Shooting[8,16,17,20]

The following precautions need to be taken during the procedure of VITEK2 AST in order to avoid erroneous results.

- **Calibration of DensiCHECK:** The working condition of DensiCHECK should be verified using the calibration standards on a daily basis **(Table 10.3)**. The cleaning of DensiCHECK must be done once in month, by the disinfectants such as 0.5% hypochlorite, or 3–25% hydrogen peroxide or quaternary ammonium compound, but not with alcohol.
- **QC of sterile saline:** The 0.45% sterility saline is inoculated in BHI broth (daily) to rule out microbial contamination.
- **Purity and age of the colony:** Colonies should be 16–18 h old and should be isolated pure, without any contamination.
- **Storage of VITEK2 cards:** The manufacturer's instructions need to be strictly followed for storing VITEK2 cards (including while transportation).
- **Preventive maintenance**: Should be done as per manufacturer's recommendations.

Advanced Expert System[21,22]

VITEK2 software is equipped with an automated tool termed as 'Advanced Expert System (AES)', which is intended to verify and validate the AST results to make it more reliable and relevant. In addition, it also modify the interpretation of the AST results in case of any discrepancy.

- It also detects and suggest various resistance patterns/mechanisms based on the AST results obtained, using complex algorithms. It doesn't test for these resistance mechanisms per se.
- The AES knowledge base is very robust covering 141 organisms, and 4,000 phenotypes comprising of 55,000 MIC distributions.

The AES results are allotted with various colored confidence indicators, each has a particular interpretation, for which a specific action need to be taken **(Fig. 10.36 and Table 10.9)**.

- Consistent (green color).
- Consistent with correction (yellow color).
- Inconsistent (red color).
- Analysis not performed (purple color).
- No breakpoint defined (black color).

For each phenotype, the AES prepares a histogram illustrating the expected MIC distributions for each antimicrobial tested as shown in **Figure 10.37**. This histogram is available for the laboratory.

Steps of AES

AES involves five major steps:

1. **Biological validation:** Discussed below.
2. **MIC interpretation:** The obtained MIC is interpreted by using a pre-stored database of clinical breakpoints from standard sources like CLSI or EUCAST.
3. **Therapeutic corrections:** AES autocorrects the interpretation based on obtained phenotypes and set rules to better predict the vivo response.
4. **Forcing rules:** AES changes the interpretation based on the results of equivalent and surrogate agents, etc., as recommended by CLSI/EUCAST.

Table 10.9: Advanced Expert System: various results, their interpretation and action need to be taken.

Result	Interpretation	Action
Green color	Indicates isolates with MIC value of 'all' antimicrobials completely fit to an expected pattern for a defined phenotype	Such results are reliable and can be reported.
Yellow color	Indicates isolates with MIC value of 'one' antimicrobial does not fit the expected pattern for a known phenotype	Such results can be reported only after review by the clinical microbiologist.
Red color	Indicates isolates with MIC pattern of >1 antimicrobial could not be matched to that of a known phenotype. This indicates a possibility of technical errors or a new mechanism of resistance.	Such results cannot be reported and must be re-confirmed by re-testing by either same or a different method before reporting.
Purple color	Indicates organisms that do not have predefined phenotypes in the AES database, but AST results are available. The manufacturer recommends not to report such results.	It is preferable to perform alternate AST method. However, if not feasible, then the MIC values available can be interpreted using clinical breakpoints provided by CLSI/EUCAST
Black color	Indicates that no clinical breakpoints have been defined for this organism/antimicrobials combination	AST of the drug cannot be reported for that organism.

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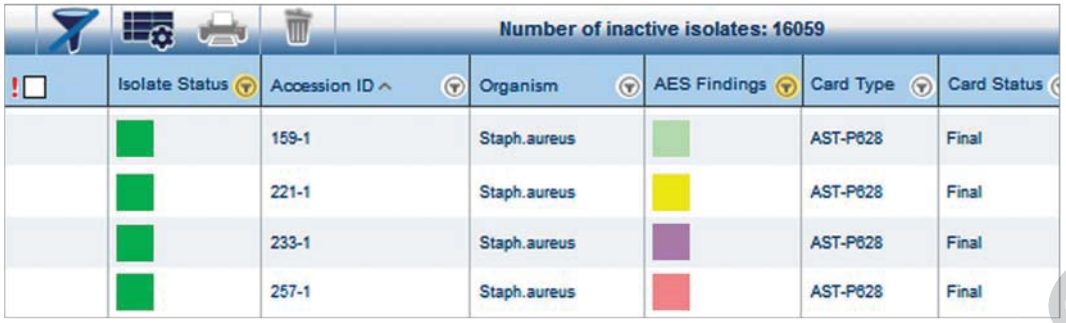

Fig. 10.36: Various types of results of Advanced Expert System.

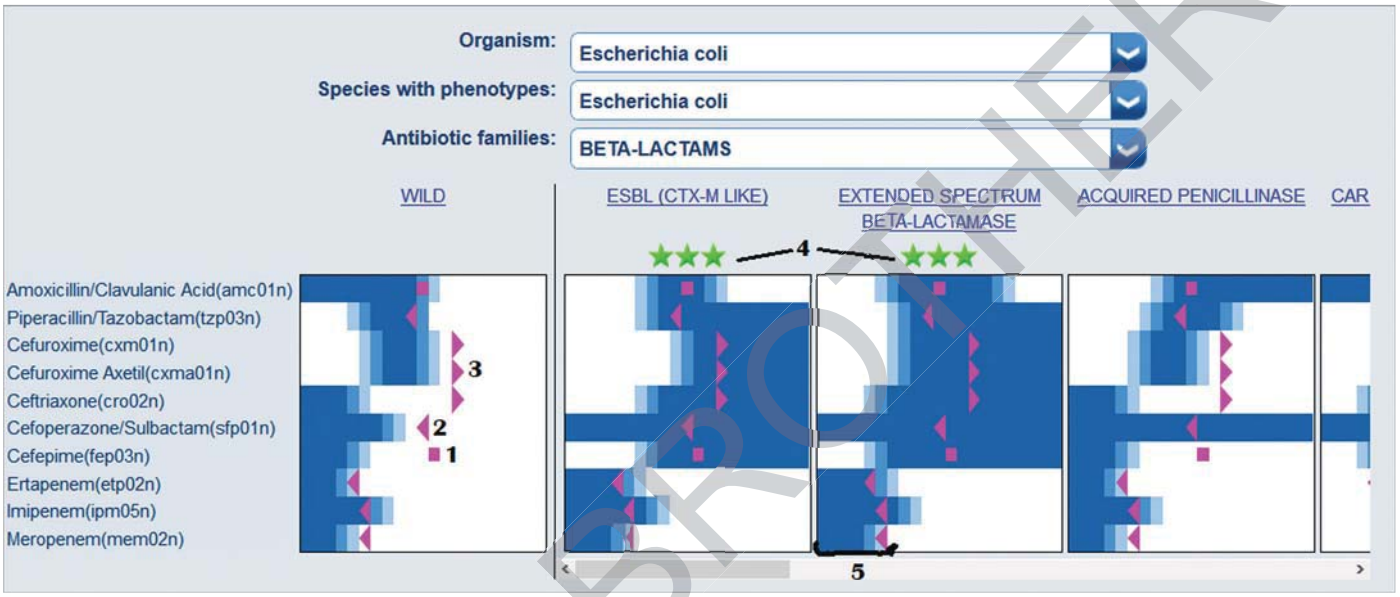

Fig. 10.37: AES showing histogram for each phenotype, illustrating the expected MIC distributions for each antimicrobial tested.
1: ■ Discrete MIC with a value that falls within the package insert calling range (e.g. 2, 4, 8).
2: ◂ Tested MIC is at the lower end of the package insert calling range (e.g. ≤1), i.e. 'less or equal to'.
3: ▸ Tested MIC is at the upper end of the package insert calling range (e.g. ≥16), i.e. 'greater or equal to'.
4: Confidence of the phenotypic match: In this example, ★★★ denotes best phenotype—the best match; Other confidence of the phenotypic match are: ★★ denotes recognized phenotype—a better match; ★ denotes possible phenotype—a good match.
5: MIC distribution dataset of an antimicrobial agent for an organism.

5. **Antimicrobial deduction:** Interpretation for the drugs which are not tested is provided by set rules or phenotype obtained.

Biological Validation

It is the most important step, which involves a matching process to identify the phenotypes in the database which most closely resembles the tested MICs for each drug class/organism combination.

- MIC distribution range for a drug/organism has been prepared using MIC dataset from large number of organisms tested. This MIC range is divided into—very typical phenotype, typical phenotype and not very typical phenotype **(Fig. 10.38)**.
- Similarly, such MIC distributions are prepared for each antimicrobial agents within the same antimicrobial class for that organism.

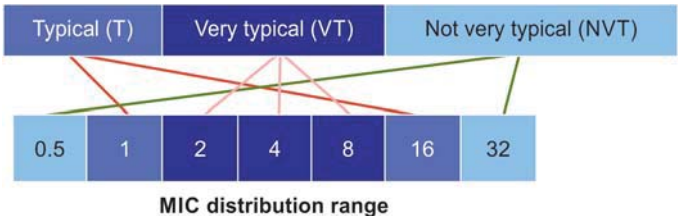

Fig. 10.38: MIC distribution dataset of an antimicrobial agent for an organism.

- The test MICs are then plotted in this MIC distributions, which have three scenarios: (1) tested MIC in the on-scale calling range, (2) tested MIC in the upper off-scale calling range, and (3) tested MIC in the lower off-scale calling range **(Fig. 10.39)**.
- The test MICs are then classified into-very typical, typical and not very typical types.

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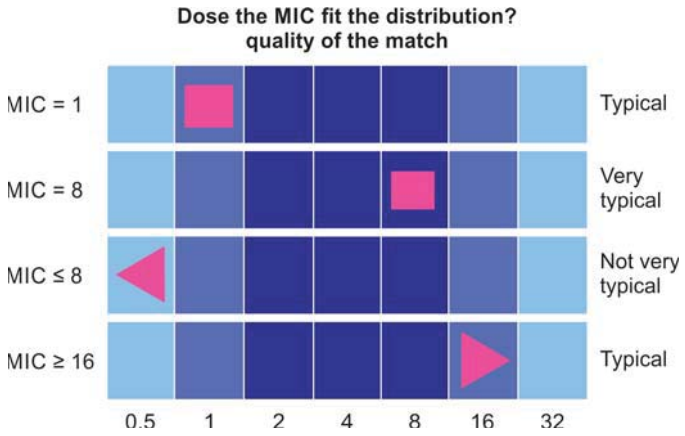

Fig. 10.39: Tested MIC in the on-scale and upper and lower off scale MIC for the calling range of the test plotted in the reference MIC distribution dataset.

- A ranking system is used to show the confidence of the phenotypic match.
- If MIC obtained for the test isolate for a particular drug is not consistent with the MIC distribution range defined for any reference phenotype for that drug-organism combination, the results are displayed with either consistent with correction or inconsistent (as explained before).

AES/Breakpoint Mismatch

There are instances when the MIC interpretation is modified by the AES software so that it does not correlate with that using clinical breakpoint provided by CLSI (or EUCAST). The change of interpretation made by AES is usually based on phenotypic MIC distribution database. Detail of this has been explained in Chapter 11.

PHOENIX AUTOMATED AST SYSTEM[23]

Phoenix system (BD Diagnostics) is the second most common automated ID and AST instrument available globally. Its use for identification has been discussed in Chapter 9.

AST panels:[23] The AST panels of Phoenix available are—NMIC and PMIC panels for gram-negative and gram-positive bacteria respectively. In addition, ID/AST combination panels are also available. The AST panel comprises of 136 wells; whereas the combo panels comprise of 51 ID wells and 85 AST wells. The AST wells are coated with several antimicrobial agents with a range of two-fold doubling dilution concentrations, in dried lyophilized form.

Principle:[23] The Phoenix AST method works on the principle of broth microdilution (BMD).

- It utilizes an optimized colorimetric redox indicator for the detection of organism growth in the presence of an antimicrobial agent in the well.
- Continuous measurements (every 20 minutes) of changes to the indicator as well as bacterial turbidity are used in the determination of bacterial growth.
- Organism identification is used in the interpretation of the MIC values according to the clinical breakpoints.

MICROSCAN AUTOMATED AST SYSTEM[24]

Microscan WalkAway system is another ID and AST instrument available globally. Its use for identification has been discussed in Chapter 9.

- **AST panels**[24]**:** There are eleven AST panels of Microscan available:
  - Four gram-negative MIC panels.
  - Four gram-negative combo panels.
  - Two gram-positive combo panels.
  - One gram-positive MIC panels.
- **Principle**[24]**:** The Microscan AST system involves broth microdilution and continuously monitors the growth of the organisms in the drug wells by turbidometric method.
  - It detects the true MIC (not the predicted MIC), determined based on the minimum concentration of drug which inhibits the growth in the drug well.
  - The AST panels of Microscan are designed in a way to accommodate more number of antimicrobial agents in the limited number of wells available. In order to do so, the dilutions tested for some of the antimicrobial agents are kept limited.
  - For example, the gram-negative panel 'Neg MIC 62' tests for about 36 drugs, with limited dilutions—21 drugs are tested at 1–2 dilutions and rest at 3–5 dilutions.
  - Testing for a limited range of dilutions may have a risk of compromising the interpretation if it does not cover the clinical breakpoints.

Table 10.10 depicts the testing dilutions and reporting (i.e. calling) range of few selected antimicrobial agents from the AST panels of VITEK. It also demonstrates whether the reporting MIC range is able to cover the CLSI and EUCAST breakpoint range or not.

Table 10.10: Testing dilutions and reporting (i.e. calling) range of few selected antimicrobial agents from various automated AST cards.

AST System	Panel	Antimicrobial agent	Testing dilutions	Reporting MIC range	Breakpoint for Enterobacterales	
					CLSI	EUCAST
VITEK	N406	Gentamicin	4, 8, 32	≤1 to ≥16	S ≤ 4, R ≥ 16	S ≤ 2, R ≥ 4
VITEK	N406	Amikacin	2, 4, 16, 48	≤1 to ≥64	S ≤ 16, R ≥ 64	S ≤ 8, R ≥ 16

Green color, breakpoint range is covered by the reporting range.

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REFERENCES

1. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing, 35th edition. CLSI supplement M100. Clinical and Laboratory Standards Institute, USA; 2025.
2. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Version 15.0. Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_15.0_Breakpoint_Tables.pdf.
3. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Disk Susceptibility Tests, 13th edition. CLSI standard M02. Wayne, PA: Clinical and Laboratory Standards Institute, USA; 2018.
4. EUCAST Disk Diffusion Test Methodology. Manual. V10.0, Jan 1, 2022. Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2022_manuals/Manual_v_10.0_EUCAST_Disk_Test_2022.pdf. Accessed on 10th April 2022.
5. EUCAST disk diffusion method for antimicrobial susceptibility testing. Reading guide. V9.0, Jan 1, 2022. Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2022_manuals/Reading_guide_v_9.0_EUCAST_Disk_Test_2022.pdf. Accessed on 10th April 2022.
6. EUCAST. Preparation of agar plates and broth for EUCAST AST. V7.0, Jan 1, 2022. Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/Media_preparation_v_7.0_EUCAST_AST_2022.pdf. Accessed on 10th April 2022.
7. Clinical and Laboratory Standards Institute (CLSI). Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria, 3rd edition. CLSI guideline M45. Wayne, PA: Clinical and Laboratory Standards Institute, USA; 2015.
8. Clinical and Laboratory Standards Institute (CLSI). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 11th edition. CLSI standard M07. Wayne, PA: Clinical and Laboratory Standards Institute, USA; 2018.
9. EUCAST Broth microdilution reading guide V4.0, Jan 1, 2022. Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2022_manuals/Reading_guide_BMD_v_4.0_2022.pdf. Accessed on 10th April 2022.
10. E-test Reading guide. bioMerieux, Inc. Available at: https://biomerieux.showpad.com/share/FEvMVbTlT68okP6hRYBUb. Accessed on 10th April 2022.
11. E-test Antifungal Susceptibility Testing of Moulds. bioMerieux, Inc. Available at: https://traveler.lsh.is/focal/gaedahandbaekur/gnhsykla.nsf/5e27f2e5a88c898e00256500003c98c2/d1b446b77ad2fbf500257c67003b9240/$FILE/Etest_-_Antifungal_Susceptibility_Testing_of_Moulds_2010_11.pdf. Accessed on 10th April 2022.
12. Clinical and Laboratory Standards Institute (CLSI). Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts, 3rd editon. CLSI guideline M44. Wayne, PA: Clinical and Laboratory Standards Institute, USA; 2018.
13. Clinical and Laboratory Standards Institute (CLSI). Method for Antifungal Disk Diffusion Susceptibility Testing of Non-dermatophyte Filamentous Fungi. Approved guideline, 1st editon. CLSI document M51. Wayne, PA: Clinical and Laboratory Standards Institute, USA; 2010.
14. Clinical and Laboratory Standards Institute (CLSI). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, 4th editon. CLSI standard M27. Wayne, PA: Clinical and Laboratory Standards Institute, USA; 2017.
15. Clinical and Laboratory Standards Institute (CLSI). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi. Approved guideline, 2nd edition. CLSI document M38-A2. Wayne, PA: Clinical and Laboratory Standards Institute, USA; 2008.
16. VITEK2 Automated instrument for ID/AST testing. Available at: https://www.biomerieuxindia.in/product/vitekr-2. Accessed on: 6th May 2022.
17. VITEK2 Compact Automated ID/AST Instrument. Available at: https://www.biomerieuxindia.in/product/vitekr-2-compact. Accessed on: 6th May 2022.
18. Michalik S. How does VITEK® 2 Generate MIC Values? bioMerieux, iNews. Oct 2017. Available at: https://www.biomerieux-microbio.com/how-does-vitek-2-generate-mic-values/. Accessed on 10th April 2022.
19. Farnham S. What is a "Real" or "True" MIC – and does VITEK® 2 provide them? bioMerieux, iNews. Feb, 2017. Available at: https://www.biomerieux-microbio.com/what-is-a-real-or-true-mic-and-does-vitek-2-provide-them/. Accessed on 10th April 2022.
20. EUCAST Quality Control. V12.0, Jan 1, 2022. Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/QC/v_12.0_EUCAST_QC_tables_routine_and_extended_QC.pdf. Accessed on 10th April 2022.
21. VITEK2 Advanced Expert System. bioMerieux, Inc. Available at: https://www.biomerieux-diagnostics.com/vitekr-2-advanced-expert-system. Accessed on 10th April 2022
22. Michalik S. Tips & Tricks for the Advanced Expert System™ (AES). bioMerieux, iNews. Sept 2017. Available at: https://www.biomerieux-microbio.com/tips-tricks-for-the-advanced-expert-system-aes. Accessed on: 6th May 2022.
23. The BD Phoenix automated identification and susceptibility testing system. Available at: https://www.bd.com/en-ca/offerings/capabilities/microbiology-solutions/identification-and-susceptibility-testing/bd-phoenix-automated-identification-and-susceptibility-testing-system. Accessed on: 6th May 2022.
24. The MicroScan WalkAway ID/AST systems and panels. Available at: https://www.beckmancoulter.com/en/products/microbiology. Accessed on: 6th May 2022.
25. Standard Operating Procedures for Antimicrobial Resistance in Priority Bacterial Pathogens under National AMR Surveillance Network (NARS-net). National Programme on Antimicrobial Resistance Containment. National Centre for Disease Control, India. 2021. Available at: https://ncdc.gov.in/WriteReadData/l892s/2592857567162548178 8.pdf. Accessed on 10th April 2022.

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This is probably the first book in India on Antimicrobial Stewardship (AMS). The first edition was a mega hit among microbiologists, infectious disease physicians, pharmacologists, clinicians, surgeons, physicians, intensivists, general practitioners and pharmacists, and nurses. The second edition includes substantial updates reflecting recent advances in AMS guidelines, clinical microbiology reporting, and the management of infectious diseases. Every chapter has been extensively revised, but the key updates incorporated are enlisted below.

- **Section-1 (Core Components of AMS):** Provides in-depth discussion on various core concepts of AMS including the integrated stewardship model, steps of implementation of AMS program, monitoring of AMS program, current scenario of AMR, and role of AMR surveillance. This new edition covers the updates in list of drugs in AWaRe classification, pathogen-directed (PD) AMS audit (in-depth discussion), definitions on antimicrobial consumption (AMC) and antimicrobial use (AMU), global and Indian data on AMU and AMR, state action plan on containment of AMR (SAPCAR), etc.
- **Section-2 (Diagnostic Stewardship)**: This section represents the HEART of this book, covers numerous facets of diagnostic stewardship such as types of turnaround time (TAT) in AMS, appropriate specimen collection, role of direct microscopy, processing and reporting of cultures, accurate identification, basics of AST and interpretation, detection of specific AMR, direct susceptibility, selective reporting, breakpoint-to-MIC quotient (BMQ), etc. This section has been extensively revised in this edition to incorporate significant updates in every chapter.
  - Chapter 11 has been thoroughly updated as per the latest guidelines from CLSI and EUCAST, incorporating several updates such as tier system of CLSI, update in the list of SDD and ECV, update in the clinical breakpoints including *Salmonella/Shigella* breakpoint table, update in the reporting of fosfomycin, aminoglycosides, CZA-ATM synergy, update on AST reporting of *B. cepacia, B. pseudomallei, Stenotrophomonas, A. baumannii*, update on treatment recommendations for invasive enterococcal infections, major update on validation of AST results, and inclusion of a major topic on "Decision to be taken on VITEK-AES modifications"
  - Chapter 8: The new modified Duke's criteria have been incorporated in Chapter 8.
  - Chapter 12 contains major updates on fosfomycin and CZA-ATM synergy testing methods, AmpC detection methods, etc.
  - Chapter 15 has undergone substantial changes—updates on BMQ and MIC guiding tables and selective reporting of *Salmonella*.

- **Section 3 (Hospital Antibiogram):** Gives comprehensive coverage on various aspects of antibiogram such as data analysis software, routine antibiogram, various types of specialized antibiograms such as enhanced, syndromic, subtraction, multifacility antibiograms, etc. A separate chapter on analysis of MDR/XDR/PDR is provided. The section underwent a major revision, enriched with several key updates such as inclusion of **validation of antibiogram** based on general acceptable S% rules (Chapter 19) and also includes an exercise on validation.
- **Section 4 (Antimicrobial Agents):** Provides a thorough discussion on various antimicrobial drugs—their PK-PD aspects, mode of action, spectrum of activity, mechanism of resistance, adverse drug effects, etc. In this new edition, several newer antimicrobials have been incorporated in every chapter of this section such as newer β-lactams and BLBLI, newer aminoglycosides, newer tetracyclines, newer quinolones, and several novel antimicrobials. A major revision has been carried on the treatment of tuberculosis chapter.
- **Section 5 (Clinical Stewardship):** This section provides an in-depth guidance on various types of antimicrobial therapy such as empiric, targeted, and prophylactic. The exhaustive tables and flowcharts in this section shall serve as a backbone in framing antibiotic policy at an individual hospital level. This section too underwent a major revision, especially on chapters on "Targeted therapy for GNB and GPC".
- **Section 6 (Miscellaneous):** Covers AMS in various special situations [e.g., OPD, outpatient parenteral antibiotic therapy (OPAT), etc.] and One Health Approach in AMS. This section underwent minor revision on various key topics.

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Dr Apurba Sastry and his team have authored several popular books which are extremely appreciated among faculty and students:

1. Essentials of Microbiology for Postgraduates (Volume 2 Systematic Bacteriology)
2. Essentials of Medical Microbiology, 5th edition, for MBBS
3. Essentials of Hospital Infection Control, 1st edition
4. Essentials of Antimicrobial Stewardship, 2nd edition
5. Essentials of Medical Parasitology, 2nd edition
6. Review of Microbiology and Immunology, 9th edition, for PG entrance examination
7. Essentials of Practical Microbiology, 2nd edition, for MBBS
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