Marshfield Labs™

Antimicrobial Agents

Antimicrobials, Susceptibility Testing, and Antimicrobial Resistance: A Primer

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Objectives

1. Describe the modes of action of the major antimicrobial classes.

2. Discuss the types of data that are used when setting S/I/R breakpoints.

3. Explain the principles of agar dilution, broth dilution, disk diffusion, agar gradient, and nucleic acid amplification methods.

Scope

- While antimicrobial agents and susceptibility tests are universally used against microbial pathogens, *this lecture focuses on rapidly growing (16-24hr), aerobic bacteria*

- That said, the techniques discussed are generally applicable to other classes of pathogens with the appropriate modifications.

Antimicrobial Agents

Targets of Antimicrobial Agents

- Cell wall synthesis
- Cell membrane integrity
- Protein synthesis
- DNA replication
- RNA transcription
- Folate synthesis

Aminoglycosides

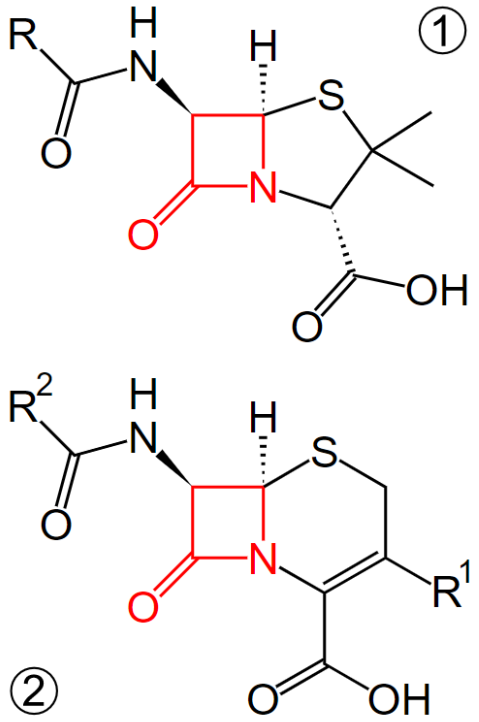
- Gentamicin, tobramycin, amikacin
  - Inhibit protein synthesis
  - Active against Gram negative, ± Gram positive aerobic bacteria
- Resistance mechanisms
  - Modifying enzymes
  - Altered target
  - Decreased uptake

ß-lactam Antibiotics

- Largest group of antibiotics
- Target: Penicillin Binding Proteins in peptidoglycan layer

- Mechanisms of resistance
  - ß-lactamase
  - Altered PBP*
  - Decreased uptake

*PBP, penicillin binding proteins. Enzymes responsible for cell wall synthesis.



1. Penicillin ring. 2. Cephalosporin ring. Core ß-lactam ring in red.

(Fvasconcellos 19:02, 23 October 2007 (UTC), Public domain, via Wikimedia Commons. Accessed 10/6/2022.)

Examples of ß-lactams

- Penicillins
  - Penicillin, ampicillin, oxacillin, piperacillin
- Cephalosporins 4 generations
  - Cefazolin (1^{st}), cefuroxime (2^{nd}), ceftazidime (3^{rd}), cefepime (4^{th})
- Carbapenems
  - Imipenem, meropenem, ertapenem
- Remember: there are many more ß-lactam drugs

ß-lactam/ß-lactamase Inhibitor Combinations

- Mechanism
  - The inhibitor binds to, and thus inactivates, the ß-lactamase enzyme leaving the ß-lactam agent unharmed

First generation*	Second generation*
Amoxicillin/clavulanic acid	Ceftolozane/tazobactam
Ampicillin/sulbactam	Ceftazidime/avibactam
Piperacillin/tazobactam	Imipenem relebactam
	Meropenem/vaborbactam
*Informal terminology	

Fluoroquinolones

- Examples: ciprofloxacin, levofloxacin

- Mechanism
  - Inhibits DNA gyrase
  - Broad GP & GN activity

- Resistance mechanisms
  - Decreased uptake
  - Altered target
  - Efflux pump

- Altered DNA gyrase due to point mutations is most common; easily developed with treatment.

Glycopeptides

- Example: Vancomycin
- Inhibits cell wall synthesis of the Gram positive peptidoglycan layer
- Not active against Gram negatives
- Does not pass through the intestine, so oral dosing is used against *C. difficle* colitis
- Resistance due to inactivating enzymes

Lincosamides, Macrolides & Oxazolidinones

- Examples
  - Macrolide: erythromycin
  - Lincosamide: clindamycin
  - Oxazolidinone: linezolid
- Good Gram positive bacterial activity
- Inhibits protein synthesis
- Resistance due to ribosomal modification and efflux pumps

Lipopeptides

- Example: Daptomycin
- Alters cell membrane integrity
- Effective against Gram positive bacteria
- Resistance, rarely seen, occurs due to cell membrane alterations

Nitrofurantoin

- Targets multiple essential sites
- Excreted in the urine, it is used only for UTI
- Has good activity against common UTI agents
- Another bacterially-activated prodrug, resistance occurs due to mutations in the activating enzymes.

Rifamycins

- Example: rifampin
- Inhibits RNA polymerase
- Resistance develops quickly due to point mutations
- Most useful in combination therapy of serious MRSA infections

Sulfonamides & Trimethoprim

- Broad spectrum
- Acts by competitive inhibition of folic acid synthesis
- Resistance occurs due to point mutations inhibiting drug binding
- Sulfa drugs and trimethoprim act at different points of synthesis. Given together, they help prevent resistance
  - Trimethoprim/sulfamethoxazole

Tetracyclines

- Examples: Tetracycline, minocycline
- Broad spectrum
- Has been relegated to 2nd line and specialty use
- Doxycycline and minocycline slightly more active than tetracycline
- Resistance, due to a variety of mechanisms, is widely seen

AST

(Antimicrobial Susceptibility Test)

When to Test

- What evidence is there of infection?
  - Lab, clinical
- Severity of the infection vs drug side effects
- Pathogenic potential
  - CNS vs *S. aureus*
- Predictability of a response
  - Predictably S or R: do not test
  - Variably S: test

Method Standardization: Keys to Success

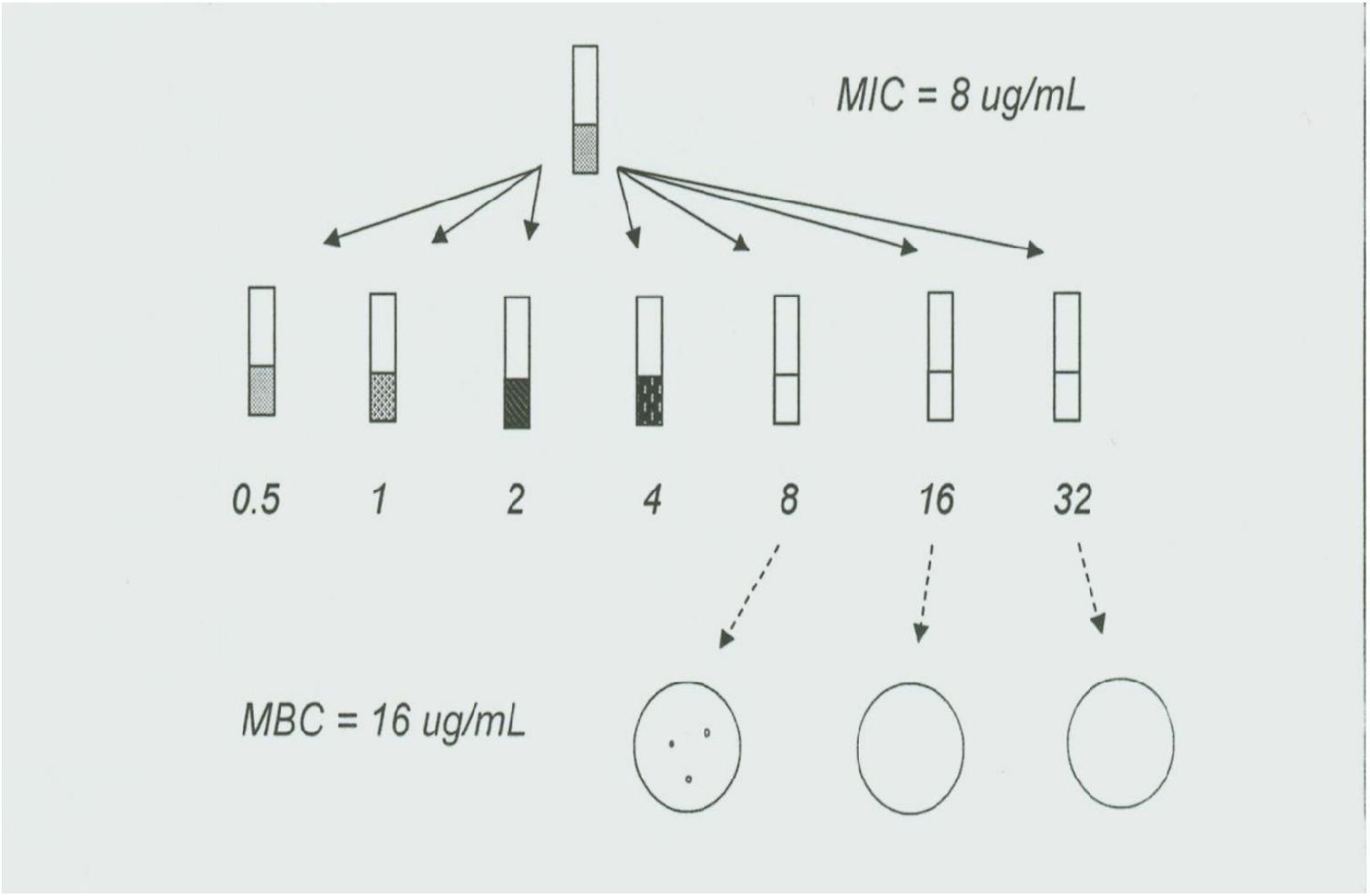
- Using standard AST methods helps ensure
  - Clinically accurate S/I/R interpretations
  - Reproducible inter- and intra-laboratory results

- Deviations can lead to both falsely susceptible (i.e. a Very Major error) and falsely resistant (Major error) results
  - Inoculum is > 0.5 McFarland → false resistance
  - Lawn on a KB plate isn't confluent → false susceptibility

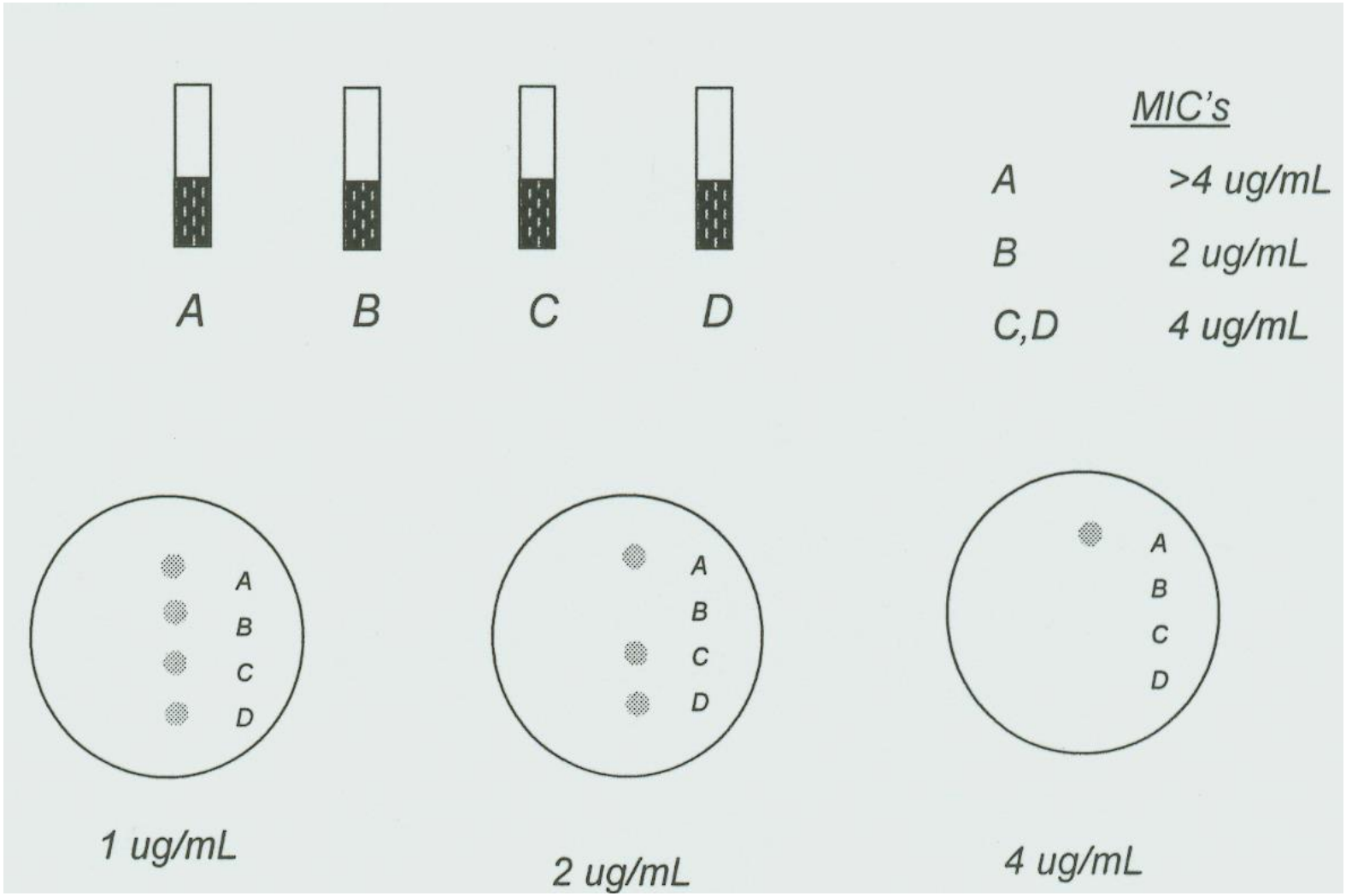
AST Variables

- Medium composition – Mueller Hinton, cation-adjusted (± 5% sheep blood)
  - pH & and buffering capacity
  - Thymidine and cation concentrations
  - Osmolarity
- Inoculum concentration – 0.5 McFarland
- Atmosphere – ambient (≈ 0.05% CO_2) vs 5% CO_2
- Agar depth
- Temperature – 35°C
- Incubation time – typically between 16 & 24h

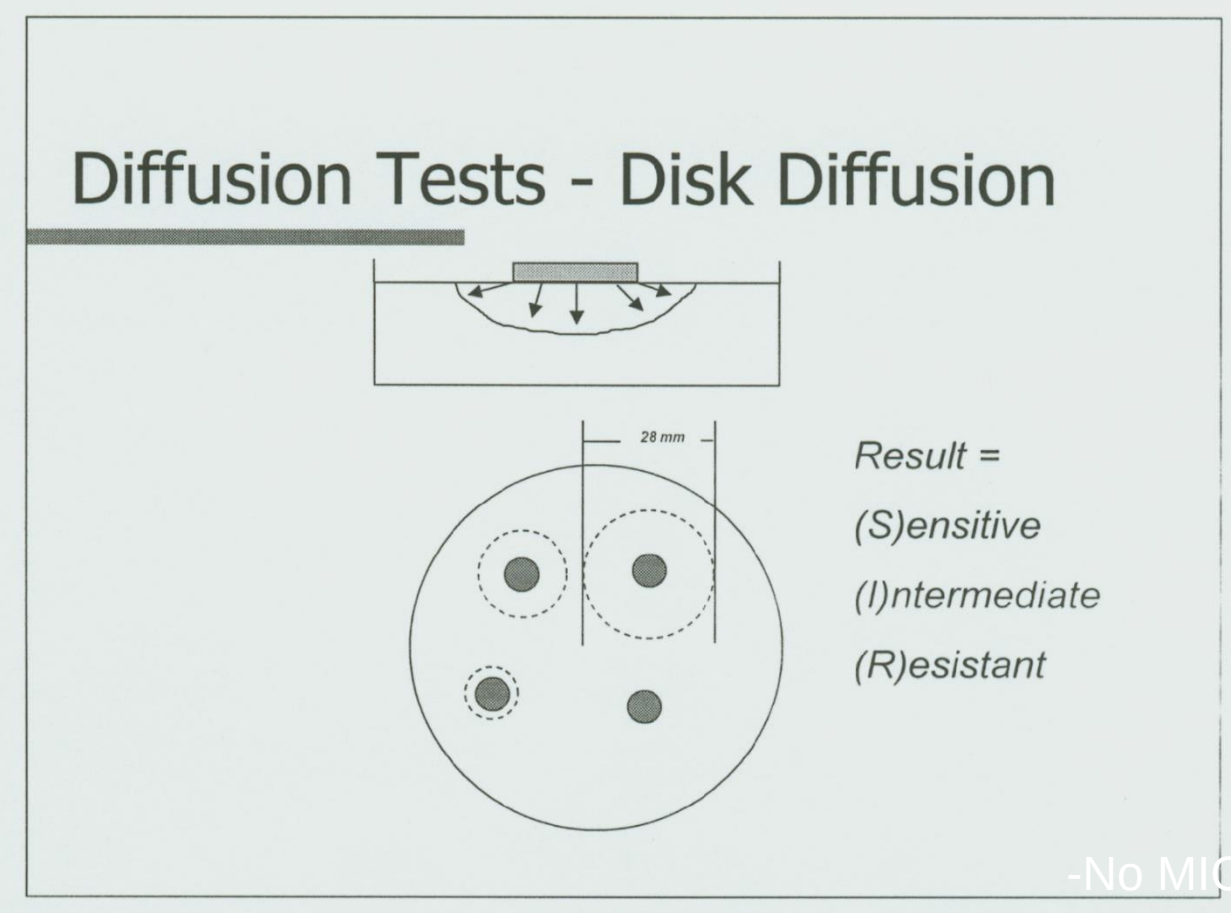
Methods of Testing: Broth Dilution



Methods of Testing: Agar Dilution

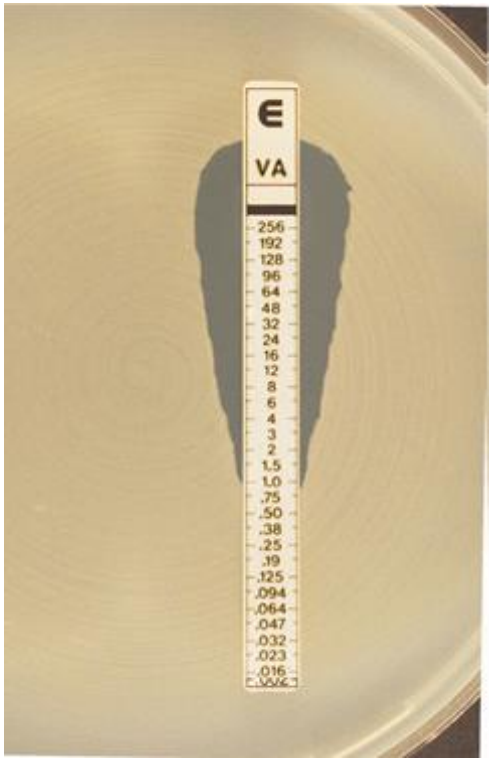


Methods of Testing: KB



Methods of Testing: ETEST

- ETEST AKA Epsiolmeter
- Underside of the strip has a gradient of the dried drug
- Placed onto the agar the drug diffuses, *maintaining the dilution gradient*
- Use
  - Set up: Disk diffusion
  - Read: MIC
- Important: Once placed, do not move the strip!



Accurate AST Results: Some Challenges

- Pre-analytic phase
  - Adequate collection and transport (GIGO principle)
- Analytic phase
  - This is biology and biochemistry = relative imprecision
  - Trades some accuracy for clinical lab workflow
- Post analytic phase
  - Patient to patient variability
    - Antibiotic distribution, metabolism and excretion
    - Drug/drug interaction
    - Immune status
    - Patient compliance (outpatients)

Inhibitory (MIC) vs. Bactericidal (MBC) Testing

- MBC tests play a role where antimicrobial bacteriocidal action is needed:
  - Endocarditis
  - Osteomyelitis
  - Immunosuppression
- MBC, measured in µg/mL, is the minimum concentration that kills the strain *in vitro*.
  - Typically several dilutions higher than MIC; never lower
- MBC testing is not routinely done

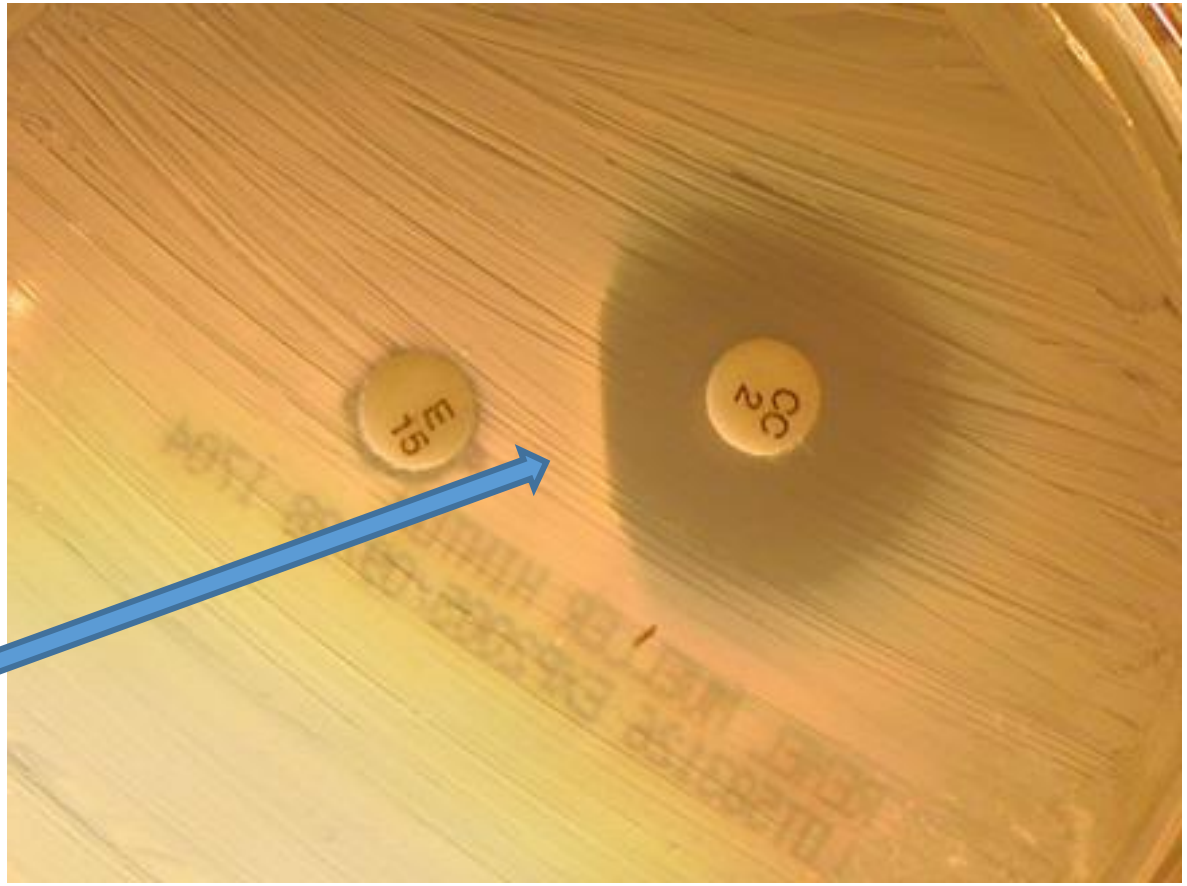
MLS in Staph, Strep

- The Problem
  - In some strains *erm* may be inducible* *in vitro* by macrolides but not clindamycin
  - An inducible *erm*$^{+}$ strain appears macrolide-R / clindamycin-S, A Very Major error (reporting S on an R strain)
- The Solution
  - Test clindamycin in the presence of erythromycin
    - MIC
    - Disk diffusion, the D-zone test

* Meaning the gene is not always expressed, but can be turned on by exogenous molecules

D test

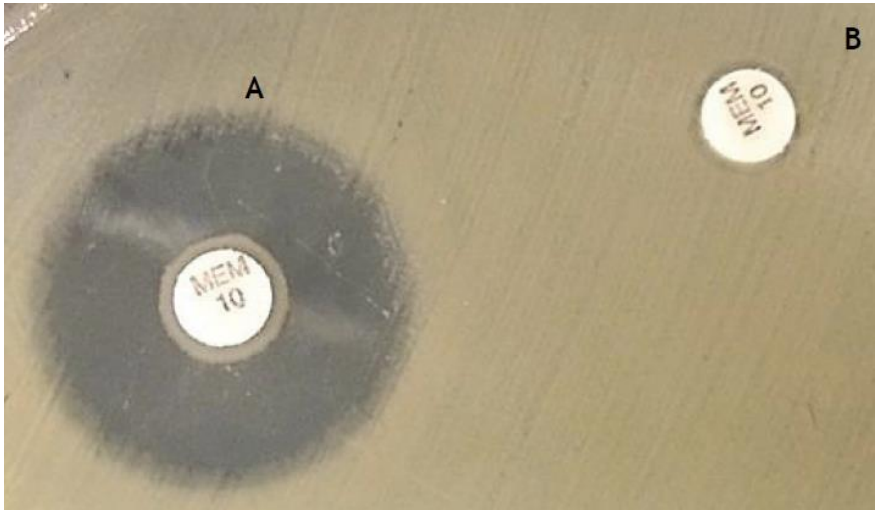
Sub-inhibitory level of E induces *erm*, allowing growth and a flattening of the zone around CC to form a “D” shaped zone



Modified Carbapenemase Inhibition Method (mCIM) Test

- mCIM used to identify carbapenemases in carbapenem-R Enterobacterales and *P. aeruginosa*
  - Inoculate suspect strain in 2mL TSB with a 10µg MEM disk; incubate 4h
  - Lawn a 0.5McF suspension of *E. coli* 25922 onto a MH plate
  - Lift the MEM paper disk out of the TSB and place it on the *E. coli* lawn
  - Incubate overnight
  - Measure zones and interpret

mCIM



A. Negative (≥ 19mm)
B. Positive (6-15mm)

Note: A zone of 16-18mm is indeterminate and requires further testing

CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 32nd ed. pp. 138-139. 2022. Clinical and Laboratory Standards Institute. Wayne PA

FDA-Cleared Rapid AST

- Genotypic and phenotypic platforms

- Pros
  - Shorter turn-around time
  - Direct from the positive bottle
  - Usually includes microbial ID as well
  - Self-contained, sample to answer technologies
  - Can lead to shorter length of stay, better Abx stewardship

- Cons
  - Relatively expensive direct test cost
  - Limited lists of species identified
  - Positive blood cultures only
  - NATs only: Pos/Neg results, not MIC values; limited gene targets

FDA-Cleared NATs

(Microbial targets not discussed.)

- BioFire BCID2
  - VRE
  - MRSA
  - GN ESBL
  - GN carbapenemases
  - GN colistin

- ePlex BCID GP, GN & Fungal
  - VRE
  - MRSA
  - GN ESBL
  - GN carbapenemases

- Verigene BC-GP, BC-GN
  - VRE
  - MRSA
  - GN ESBL
  - GN carbapenemases

FDA-Cleared Phenotypic Tests

- Accelerate Pheno BC
  - GP & GN bacteria ID & AST
  - Yeast ID
  - ≈ 2h ID, ≈7h AST

- Affinity Biosensors
  - GN AST only
  - ≈ 4.5h

Interpretive Criteria (IC) i.e. Breakpoints

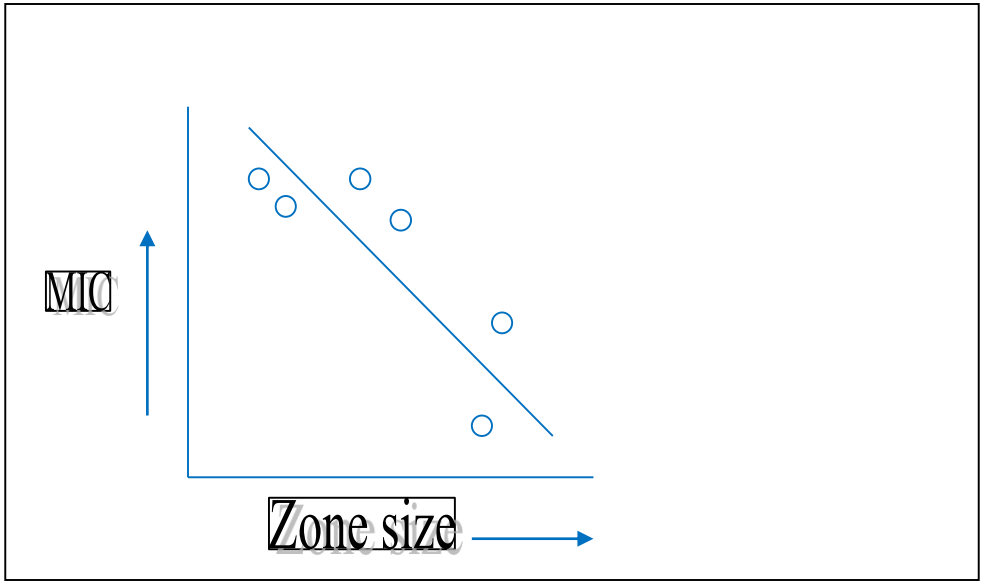
- So now you have a number, what's next?
  - Use published ICs!

- Organizations such as the CLSI & EUCAST periodically review newly published AST study data to create and update ICs.

- What are ICs you ask?
  - Simply put, an IC converts a number, the MIC or KB zone size, into experimentally validated susceptible, intermediate or resistant (S/I/R) interpretations for clinical use.

MIC – KB Correlation

- MIC & zone size
  - An inversely proportional relationship
  - Must have high degree of correlation for KB to be valid



Interpretive Categories

- Susceptible: Infection predicted to respond
- Intermediate
  - Buffer zone against analytic imprecision
  - Some activity, not enough to clear the infection alone
  - Unclear division between S & R populations
- Resistant: Predicted to not respond
- Susceptible, Dose Dependent (SDD)
  - Susceptible *only if* the alternate, higher dosing allowed by the FDA is used
  - Not available for most antimicrobials

IC Development

- Factors considered include:
  - Innate bug-drug interactions
  - Pharmacokinetics ie *PK* (how the body manages a drug)
  - Pharmacodynamics ie *PD* (how the bacteria responds to the drug)
  - Clinical outcomes data
  - Computer modeling e.g. Monte Carlo simulation
  - Likely site(s) of infection

Table 2A. Zone Diameter and MIC Breakpoints for Enterobacterales

Testing Conditions	Routine QC Recommendations (see Tables 4A-1 and 5A-1 for acceptable QC ranges)
Medium: Disk diffusion: MHA Broth dilution: CAMHB; iron-depleted CAMHB for cefiderocol (see Appendix I)[1] Agar dilution: MHA **Inoculum:** Broth culture method or colony suspension, equivalent to a 0.5 McFarland standard; positive blood culture broth for select antimicrobial agents with disk diffusion (see general comment [5]). **Incubation:** 35°C ± 2°C; ambient air Disk diffusion: 16-18 hours Dilution methods: 16-20 hours	*Escherichia coli* ATCC®[a] 25922 *Pseudomonas aeruginosa* ATCC® 27853 (for carbapenems) *Staphylococcus aureus* ATCC® 25923 (for disk diffusion) or *S. aureus* ATCC® 29213 (for dilution methods) when testing azithromycin against *Salmonella enterica* ser. Typhi *or Shigella* spp. Refer to Tables 4A-2 and 5A-2 to select strains for routine QC of β-lactam combination agents. When a commercial test system is used for susceptibility testing, refer to the manufacturer's instructions for QC test recommendations and QC ranges.

Refer to Tables 3A, 3B, and 3C for additional testing, reporting, and QC for Enterobacterales.

General Comments

(1) For disk diffusion, test a maximum of 12 disks on a 150-mm plate and no more than 6 disks on a 100-mm plate; disks should be placed no less than 24 mm apart, center to center (see M02,[2] Subchapter 3.6). Each zone diameter should be clearly measurable; overlapping zones prevent accurate measurement. Measure the diameter of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disk (see the *M02 Disk Diffusion Reading Guide*[3]). Hold the Petri plate a few inches above a black background illuminated with reflected light. The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye. Ignore faint growth of tiny colonies that can be detected only with a magnifying lens at the edge of the zone of inhibited growth. Strains of *Proteus* spp. may swarm into areas of inhibited growth around certain antimicrobial agents. With *Proteus* spp., ignore the thin veil of swarming growth in an otherwise obvious zone of growth inhibition. With trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore, disregard slight growth (20% or less of the lawn of growth) and measure the more obvious margin to determine the zone diameter.

(2) When fecal isolates of *Salmonella* and *Shigella* spp. are tested, only ampicillin, a fluoroquinolone, and trimethoprim-sulfamethoxazole should be reported routinely. **Data regarding whether amoxicillin should be used to treat shigellosis are conflicting. When reporting ampicillin results, state that treatment of shigellosis with amoxicillin might not be comparable to ampicillin, with poorer efficacy.** In addition, for extraintestinal isolates of *Salmonella* spp., a 3rd-generation cephalosporin should be tested and reported, and chloramphenicol may be tested and reported if requested. Susceptibility testing is indicated for typhoidal *Salmonella* (*S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi A-C) isolated from extraintestinal and intestinal sources. Routine susceptibility testing is not indicated for nontyphoidal *Salmonella* spp. isolated from intestinal sources. In contrast, susceptibility testing is indicated for all *Shigella* isolates.

Table 2A. Enterobacterales (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints, nearest whole mm				Interpretive Categories and MIC Breakpoints, µg/mL				Comments
			S	SDD	I	R	S	SDD	I	R	
QUINOLONES AND FLUOROQUINOLONES for *Salmonella* spp. (Please refer to Glossary I.) (Continued)											
B	Ciprofloxacin	5 µg	≥31	-	21-30^	≤20	≤0.06	-	0.12-0.5 ^	≥1	(64) Isolates of *Salmonella* spp. that test not susceptible to ciprofloxacin, levofloxacin, ofloxacin, or pefloxacin may be associated with clinical failure or delayed response in fluoroquinolone-treated patients with salmonellosis.
B	Levofloxacin	-	-	-	-	-	≤0.12	-	0.25-1^	≥2	
O	Ofloxacin	-	-	-	-	-	≤0.12	-	0.25-1^	≥2	
Inv.	Pefloxacin (surrogate test for ciprofloxacin)	5 µg	≥24	-	-	≤23	-	-	-	-	(65) Report results as ciprofloxacin susceptible or resistant based on the pefloxacin test result. Pefloxacin will not detect resistance in *Salmonella* spp. due to *aac(6')-Ib-cr*. Pefloxacin disks are not available in the United States. See comment (63).
FOLATE PATHWAY ANTAGONISTS											
B	Trimethoprim-sulfamethoxazole	1.25/ 23.75 µg	≥16	-	11-15	≤10	≤2/38	-	-	≥4/76	See general comment (2).
U	Sulfonamides	250 or 300 µg	≥17	-	13-16	≤12	≤256	-	-	≥512	(66) Sulfisoxazole can be used to represent any of the currently available sulfonamide preparations.
U	Trimethoprim	5 µg	≥16	-	11-15	≤10	≤8	-	-	≥16	
PHENICOLS											
C	Chloramphenicol	30 µg	≥18	-	13-17	≤12	≤8	-	16	≥32	(67) Not routinely reported on isolates from the urinary tract.
FOSFOMYCINS											
U	Fosfomycin	200 µg	≥16	-	13-15	≤12	≤64	-	128	≥256	(68) Disk diffusion and MIC breakpoints apply only to *E. coli* urinary tract isolates and should not be extrapolated to other species of Enterobacterales. (69) The 200-µg fosfomycin disk contains 50 µg of glucose-6-phosphate. (70) The only approved MIC method for testing is agar dilution using agar media supplemented with 25 µg/mL of glucose-6-phosphate. Broth dilution MIC testing should not be performed.

What About Reporting the Numbers?

Should the MIC or the KB zone size be routinely reported? In my opinion…

- S, I or R alone is enough for most infections
- Dangerous misinterpretation is easy (“I pick the lowest MIC drug”)
- KB zones cannot be interpreted beyond S, I, or R

Thus, routinely report only the SIR result

- Caveat: ID docs and PharmD specialists have the PK/PD training to interpret MICs in tough cases, so release MICs on request

Limitations

- *In vitro* AST “Susceptible” vs clinical response ≠ 100% correlation
  - Biological systems
    - AST
    - The microbe
    - The human patient
- Patient immune status
  - Immune system works with antimicrobial therapy
- Infection site
  - Well perfused vs necrotic
- Metabolism and excretion rates
- Patient compliance
- Physicians staying the course

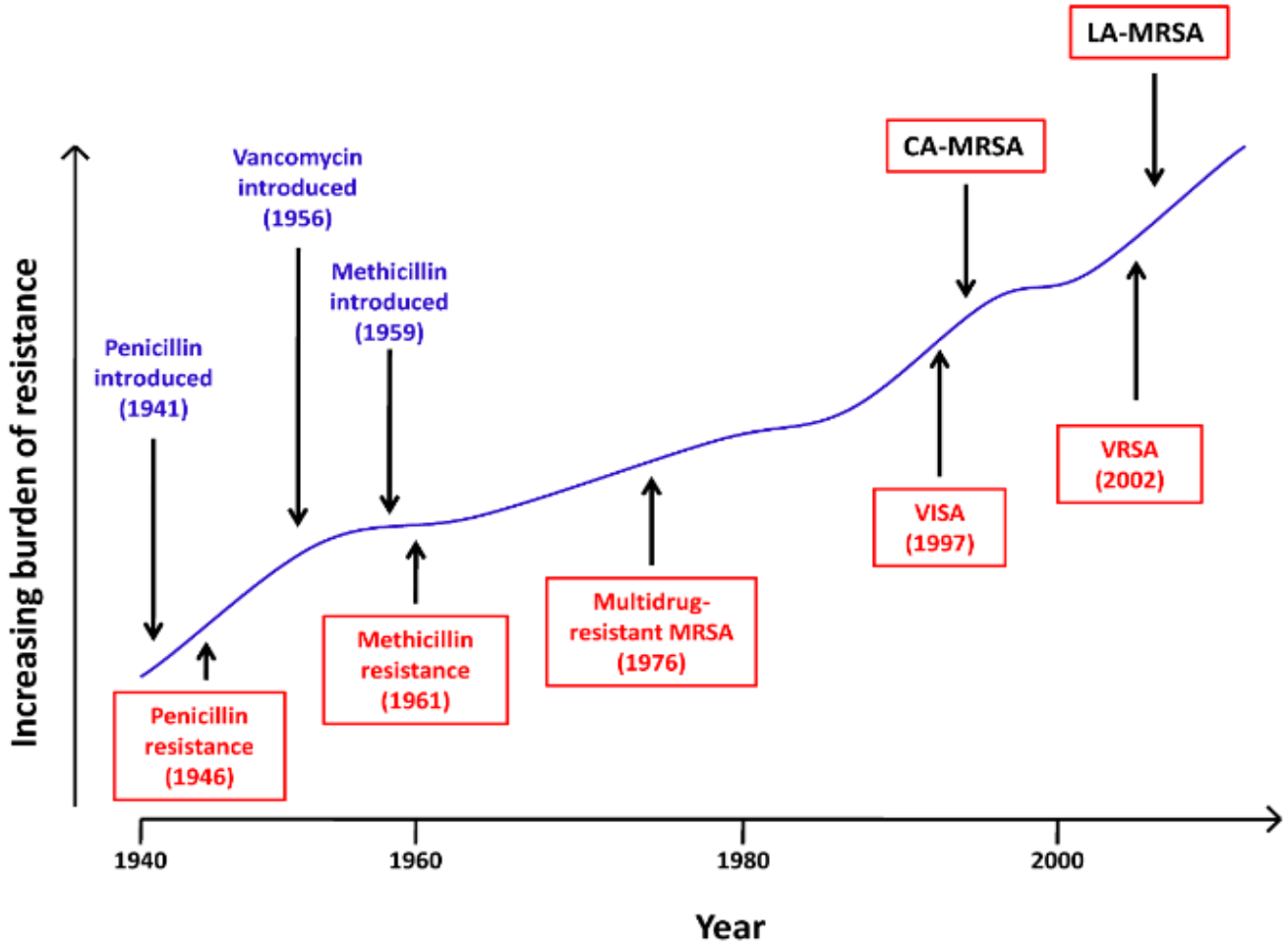
MIC.11385	Current Antimicrobial Susceptibility Test Interpretation Breakpoints	Phase I
	Effective January 1, 2024, the laboratory uses current breakpoints for interpretation of antimicrobial minimum inhibitory concentration (MIC) and disk diffusion test results. New breakpoints are implemented within three years of the date of publication by the FDA for laboratories subject to US regulations, or within three years of publication by CLSI, EUCAST or other standards development organization (SDO) for laboratories not subject to US regulations.	

Antimicrobial Resistance

Select Examples

The Post-Antibiotic Era…

S. aureus: A Case Study



Open source. Schmidt, Tracy, Kock, Marleen, Ehlers, Marthie. "Antimicrobial Resistance in Staphylococci at the Human–Animal Interface" In Antimicrobial Resistance: An Open Challenge, edited by Maria Ossiprandi. London: IntechOpen, 2015.

A Sample of Resistance Problems

- *Streptococcus pneumoniae*
  - Penicillin
- *Enterococcus*
  - Vancomycin, penicillin & ampicillin
- *Streptococcus*
  - inducible macrolide/ lincosamide / streptogramin (MLS)
- *Staphylococcus*
  - Penicillin, methicillin, MLS
- *Enterobacterales*
  - 3rd & 4th gen. cephalosporins, carbapenems
- Multiple Drug Resistant Organisms (MDRO)

Mechanisms of Resistance

- Intrinsic
  - Inherited
  - Predictable
  - AST less necessary
- Acquired
  - Unpredictable
  - AST needed

Mechanisms of Acquired Resistance

- Enzymatic drug inactivation
- Drug management
  - Drug efflux → trans-membrane pumps
  - Decreased uptake → trans-membrane porin changes
- Target alteration
  - Penicillin binding proteins or other proteins in the nascent peptidoglycan layer
  - RNA methylation
  - Ribosomal protection
  - Gyrase/topoisomerase mutations

Extended Spectrum Beta-lactamase (ESBL) Enzymes

- Evolved from narrow spectrum TEM-1 & SHV-1 cephalosporinases, now there are hundreds of variants

- ESBLs variably inactivate 1st 2nd & 3rd gen cephalosporins
  - Carried on transferable plasmids
  - Common to *E. coli, Klebsiella, Proteus*, and other Enterobacterales species
  - A major concern worldwide
  - We used to use special tests to detect ESBL+ strains; since 2010 CLSI has moved to clinically relevant ICs

ESBLs

- If not using current CLSI breakpoints (and with an AST instrument you may not be), perform a confirmatory test on “susceptible with elevated MIC”) strains
- Done by KB or broth dilution, test relies on the inhibition of the ESBL enzyme by clavulanic acid
- Test is performed using ceftazidime and cefotaxime (3rd gen cephs) with and without clavulanic acid added
- If addition of clavulanic acid increases zone size by $\geq$5 mm, or reduces MIC by $\geq$ 3 dilutions

Carbapenemase – Producing GNRs

- Enterobacterales *P. aeruginosa, Acinetobacter*, other NFGNBs
- In Enterobacterales, termed CP-CRE (carbapenemase-producing carbapenem-resistant Enterobacterales)
- Carbapenemase enzymes encoded by transferable genes:
  - KPC, NDM, VIM, OXA-48
- **Note**: GNR carbapenem resistance not always due to a carbapenemase

Carbapenem – Resistant GNRs

- High level *in vivo* resistance to carbapenems, variably to cephalosporins and penicillins
- Detected by NATs, clinically relevant ICs, or the modified CIM test
- Rules out arguably the most important drug class, the ß-lactams
- A major concern to hospital infection prevention, public health

Questions?