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TITLE: Antimicrobial susceptibility testing

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Slide 1: Introduction

Hello, my name is Mark Lee. I am a Clinical Microbiology Fellow at the University of California Los Angeles. Welcome to this Pearl of Laboratory Medicine on "Antimicrobial susceptibility testing."

Slide 2: Microbial pathogens and infectious diseases

Bacterial pathogens, like *Staphylococcus aureus*, can cause skin and wound infections, sepsis, and pneumonia, among others.

While our body's own immune system can typically ward off and maintain control of invading bacteria under normal conditions, there are instances where the balance is tipped in favor of the pathogen, resulting in an infection.

This is where anti-infective drugs like penicillin or vancomycin come into the picture. These are antimicrobial agents against bacteria that can inhibit growth and help the body clear infections

Slide 3: Modes of action

There are several classes of antimicrobial agents against bacteria.

Some agents, like the cell wall-targeting beta-lactams, are bactericidal; meaning, these agents kill the organism outright. Others, like the protein synthesis-targeting aminoglycosides, are bacteriostatic; meaning, these agents inhibit growth and proliferation, but does not necessarily kill the organism.

While some cases of bactericidal agents are more effective than bacteriostatic ones, in most instances, either type of agent is sufficient to give the immune system the boost that it needs.

Slide 4: Rationale for antimicrobial susceptibility testing

The identity of the invading bacteria is important, since some bacteria are predictably susceptible to certain antimicrobials.

For example, *Streptococcus pyogenes*, also known as Group A Strep, is susceptible to penicillin and has no known resistance mechanism against this drug. Thus, patients with a Group A Strep infection can be treated with penicillin without the need for antimicrobial susceptibility testing.

Then there are bacteria that are predictably resistant to certain antimicrobial agents. This is due to their intrinsic resistance to those particular drugs. An example is *Pseudomonas aeruginosa* and ampicillin. *Pseudomonas aeruginosa* is intrinsically resistant to ampicillin due to the presence of chromosomal beta-lactamases. Therefore, other antimicrobial agents are used to treat patients with this infection.

The challenge is when susceptibility is not predictable due to acquired resistance.

A good example is *Staphylococcus aureus*, which used to be predictably susceptible to penicillin, and its derivative drugs like methicillin. Patients with *Staphyloccocus aureus* infection used to be treated with these drugs. However, emergence of acquired resistance, like penicillin hydrolyzing enzymes and penicillin-binding proteins has led to the rise of methicillin resistant *Staphylococcus aureus*, commonly known as MRSA. Patient with MRSA infections are usually treated with non-penicillin drugs like vancomycin or daptomycin.

However, many *Staphylococcus aureus* isolates do not possess one of those acquire resistance mechanism, and are still susceptible to penicillin. These are known as methicillin susceptible *Staphylococcus aureus*, or MSSA. Patients with MSSA infections can be treated with penicillins, like oxacillin or nafcillin.

Beta lactam drugs are preferred over vancomycin due to better patient outcome in critically ill patients. Thus, classifying a *Staphylolococcus aureus* isolate as MRSA or MSSA is important in proper management of patients.

In order to assess whether an isolate has an acquired resistance to a particular antimicrobial agent, an *in vitro* antimicrobial susceptibility test is performed.

Slide 5: Routine laboratory testing methods

This typically involves exposing the isolate to a panel of antimicrobial agents, usually antimicrobials in the hospital formulary, and determining at which drug concentration growth is inhibited for each of the drugs tested.

The results of the susceptibility test will provide guidance to clinicians on which drugs to use, which to avoid, and which to alter the normal dosing regimen for successful patient outcome. This is a routine service provided by the microbiology laboratories.

There are various methods to test antimicrobial susceptibility. These include phenotypic tests like broth microdilution, disk diffusion, and Etest, as well as, tests to detect the presence of resistance genes or enzymes. For this presentation, the two commonly used tests, broth microdilution and disk diffusion, will be discussed in detail.

Slide 6: BMD – preparing the inoculum

The antimicrobial susceptibility testing begins with preparation of the inoculum.

The first step is to select 2-3 isolated colonies with a sterile cotton swab, usually from an overnight culture, grown on non-selective media.

Second, suspend the colonies in saline or sterile water

Third, adjust the suspension to obtain the desirable turbidity, usually 0.5 McFarland standard, which can be read by a turbidity meter.

0.5 McFarland is roughly 1 to $2x10^8$ CFU per mL.

Slide 7: BMD – inoculating the MIC plate

The next step is the inoculation.

This can be accomplished using various inoculation apparatus. A manual disposable tray inoculator is shown on the slide.

The 0.5 MacFarland suspension is diluted further and poured into the inoculation tray. The inoculator is dipped into the inoculation tray, which collects about 10 µl of the suspension per needle, labeled A on the slide. This is then inoculated into a 96-well MIC plate containing the antimicrobial agents and broth medium. The final concentration of organism in each well is about 4 to 5 x 10^5 CFU per mL. Some difficult to grow bacteria may require additional supplementation in the broth.

The inoculated tray is then incubated, typically at 35°C in ambient air for most organisms for 18 to 24h, but some may require longer incubation and/or carbon dioxide.

Slide 8: BMD – reading the MIC plate

At the end of the incubation period, the MIC plate is placed on top of a viewing device for manual read. Growth in each of the wells is visually checked. Automated reading devices are commercially available.

The first well with growth inhibition for each antimicrobial agent tested is recorded, as indicated by the red arrow for linezolid. In this example, *Enterococcus faecium* was tested with a linezolid MIC of 2 µg/ml, and vancomycin MIC of > 32 µg/ml.

Slide 9: DD – inoculating the agar plate

Another common method of antimicrobial susceptibility testing is disk diffusion. As with the broth microdilution test, a 0.5 MacFarland suspension is made. A sterile cotton swab is submerged in the suspension, excess liquid drained, and streaked on a Muller-Hinton agar plate to make a lawn of bacteria, as shown in step 1.

After letting the plate dry for few minutes, filter disks impregnated with the antimicrobial agents of interest are placed on the plate, as shown is step 2. Forceps or dispensers can be used.

Once all the disks have been placed, the plate is incubated under the appropriate growth conditions.

As with the broth microdilution method, some difficult to grow bacteria can require additional supplementation. The inoculated plate is incubated at 35°C in ambient air for most organisms for up to about 16 to 18h hours, but some may require longer incubation and/or carbon dioxide.

Slide 10: Zone diameter

In contrast to broth microdilution, where a range of serial dilution of the antimicrobial agent is tested, in disk diffusion test, a single impregnated disk per agent is placed on the plate. The principle behind disk diffusion is, as the name would suggest, the gradual diffusion of antimicrobials from the disk out through the agar, creating a gradient, as shown in panels A and B. The closer to the disk, as illustrated with black arrows, the higher the concentration of the antimicrobial, and further out from the disk, as illustrated with white arrows, the lower the concentration.

From the gradient created by the diffusion, a zone of inhibition forms depending on the susceptibility of the organism, as shown in panel A. The zone of inhibition is measured in mm. Some resistant strains can actually grow up to the disk as shown in panel B. In these cases, the zone is typically reported as the diameter of the disk, usually 6 mm, but some laboratories may report as 0 mm.

Slide 11: DD – measuring the zone diameter

To measure the zone size, the plate is held against a dark background, and the zone margins are measured by a ruler to the nearest whole mm.

In this example, *Pseudomonas aeruginosa* was tested with a tobramycin zone diameter of 25 mm, as shown in A, and ceftazidime zone diameter of 32 mm, as shown in B.

Slide 12: Interpreting the results

The MIC or zone diameter itself is not particularly informative without some interpretation. This is where breakpoints, also referred to as interpretive criteria, come into play.

Breakpoints basically categorize an isolate as susceptible, intermediate, or resistant to a particular antimicrobial agent based on the MIC value or disk zone diameter.

In the broth microdilution example, the *Enterococcus* isolate with a vancomycin MIC of > 32 would be categorized as resistant, and thus, would be a vancomycin-resistant Enterococcus, otherwise known as VRE. The clinician receiving this result would know not to treat this patient with vancomycin, and instead, may treat with linezolid since it tested susceptible.

Various organizations establish breakpoints for organisms commonly encountered in clinical settings. In the US, breakpoints are established by the Clinical Laboratory Science Institute or CLSI and by the US FDA, while EUCAST breakpoints are followed in Europe.

Slide 13: Other routine testing methods

Other routine test methods include the use of automated systems, as shown in panel A, and E-test strips, as shown in panel B.

Automated antimicrobial susceptibility test systems are commonly used in microbiology laboratories due to their ease, shorter turnaround time, and high volume capacities. Usually, a suspension of the isolate is inoculated into a card or cartridge containing a set of antimicrobial agents in couple of concentrations, placed inside the automated platform, and incubated.

Automated systems can provide results within hours by measuring subtle changes in growth by turbidity, fluorescence, or colorimetric reactions.

Depending on the platform, either MIC values or breakpoint categories are reported by the automated system.

Etest is similar in concept as disk diffusion, where the impregnated drug on the Etest strip diffuses into the agar, creating a zone of inhibition. Unlike disk diffusion, Etest measures MIC and not zone diameter. In panel B, this organism has an MIC of 0.5 µg/ml for this particular agent.

Slide 14: CLSI breakpoint categories

There are 3 categories of breakpoints as defined by the CLSI.

A susceptible isolate is inhibited by the usually achievable drug concentrations when the normal dosage is used. Treatment success is likely.

An intermediate or susceptible-dose dependent isolate has an MIC approaching the upper limits attainable in the blood, and implies clinical efficacy in body sites where the drugs are physiologically concentrated or when a higher dosage can be used. It is also a buffer zone between the susceptible and resistant categories

A resistant isolate is not inhibited by the usually achievable drug concentrations with normal dosage schedule and/or fall in the MIC range where specific microbial resistance mechanisms are likely. Treatment failure is therefore likely.

Many factors are taken into consideration when establishing breakpoints

It is a very involved, complicated, and highly technical process. For the purposes of this presentation, we will briefly go over the major points.

Slide 15: Establishing MIC breakpoints

MIC breakpoints for broth microdilution are established by considering 3 different cutoff values: the wild-type cutoff, PK/PD cutoff, and clinical cutoff

To determine the wild-type cutoff, a wide range of drug concentration is tested on each isolate. The MIC results are then tabulated as a histogram as shown on the slide for *Klebsiella pneumoniae* and hypothetical drugX. The distribution is visually analyzed or run through a mathematical algorithm. This approach differentiates isolates with wild-type MIC from isolates with elevated MIC, potentially due to acquired resistance.

To establish the PK/PD cutoff, a variety of studies are conducted to investigate the pharmacokinetics, defined as how the drug is metabolized by the body, and the pharmacodynamics, defined as the relationship between the drug concentration and bacterial killing.

The goal of PK/PD studies is to determine if drug exposure time, peak drug levels, or a combination of time and concentration is the best parameter in establishing the dosing regimen.

To establish the clinical cutoff, prospective clinical trials are conducted, especially for regulatory approval purposes. Based on clinical outcomes and antimicrobial susceptibility results of isolates from patients, MIC ranges where most patients respond is assigned the susceptible category, MIC ranges where some patients respond is assigned the intermediate category, and MIC ranges where most patients fail therapy is assigned as the resistant category.

Breakpoints can be revised and updated with shifts in wild-type cutoff, new data on PK/PD, and changes in clinical outcome. In fact, organization establishing breakpoints, like CLSI and EUCAST, constantly review data and hold discussions to ascertain whether changes to an existing breakpoint are warranted, and undertake studies in re-assessing breakpoints.

Slide 15: Establishing zone diameter breakpoints

Establishing zone diameter breakpoints for disk diffusion usually requires knowledge of the MIC breakpoints established for broth microdilution. An isolate is tested with both the broth microdilution and disk diffusion methods, and the resulting MIC and zone diameter are plotted in a scattergram, as shown on the slide for the hypothetical drug X.

The zone diameter breakpoints are set to ensure maximal agreement with the MIC breakpoints and minimize discrepancies or errors. In essence, you do not want to categorize an isolate as susceptible by disk diffusion when it is resistant by broth microdilution, and vice versa.

This is shown with the 2 red arrows, where establishing the zone diameter breakpoints at ≤ 11 for resistance, 12 to 13 for intermediate, and ≥ 14 for susceptible for the hypothetical drug X, aligns the isolate with the MIC breakpoints with the least number of discrepancies.

Slide 15: Summary

Antimicrobial susceptibility testing plays a pivotal role in patient care by categorizing isolates as susceptible, intermediate, or resistant, which can guide clinicians on appropriate therapy

MIC breakpoints are established by taking microbiological, PK/PD, and clinical outcome data into consideration

Breakpoints can be revised and updated as clinical outcome and susceptibility patterns change over time

Establishing zone diameter breakpoints for disk diffusion usually requires knowledge of the MIC breakpoints

Slide 14: References

Slide 15: Disclosures

Slide 16: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on "**Antimicrobial susceptibility testing**."