Drug Susceptibility Testing for *M. tuberculosis* Complex

**Drug Susceptibility Testing for *M. tuberculosis* Complex**

Duration: 47:00

Start
1. Essentials for the Mycobacteriology Laboratory: Promoting Quality Practices

1.1 Drug Susceptibility Testing for M. tuberculosis Complex

Notes:
Welcome to the Association of Public Health Laboratories Essentials for the Mycobacteriology Laboratory: Promoting Quality Practices. This presentation is Drug Susceptibility Testing for *Mycobacterium Tuberculosis* Complex.
1.2 Presentation Overview

Notes:

The goals of this module are to give some background on drug resistance, talk about conventional culture-based drug susceptibility testing, or DST, methods for *Mycobacterium tuberculosis* complex, or MTBC. We also want to talk about drug susceptibility testing turnaround times and reporting, discuss discordance in DST results, discuss ensuring quality in DST, and give a brief description of molecular methods for resistance detection.

Future core curriculum modules will focus on mycobacteriology molecular methods in greater detail.
3. Background on Resistance

Notes:

First, some background on drug resistance.
3.2 Drug Resistant TB

Let's start by defining the terms of multi-drug resistant TB, or MDR TB, and extensively drug-resistant TB, or XDR TB. MDR TB is defined as TB that is resistant to at least rifampin and isoniazid, or INH. Rifampin and isoniazid are the two most effective first-line drugs for use with treatment of TB. In 2011, in the United States, 1.3 percent of reported cases with no previously reported TB were MDR. Among cases that were previously treated for TB, the proportion of MDR was higher, at 7.8 percent. XDR TB is defined as TB that's resistant to INH and rifampin and at least one fluoroquinolone and one second-line injectable drug.
3.3 TB Treatment Regimens

Notes:

TB Treatment Regimens

Generally, there are three populations of organisms that occur in a patient with pulmonary TB. One population is metabolically active and present in large numbers. One population is less metabolically active and multiplies less rapidly. And of course, there are dormant organisms as well found in granulomas. These can be prone to sporadic bursts of replication and tend to be associated with relapses.

Treatment should address all three populations if possible. Drugs should be chosen with rapid bactericidal activity against actively multiplying organisms and drugs known as sterilizing agents, which work against more dormant populations. When a patient is diagnosed with TB, they are typically started on a drug regimen that contains four drugs: INH or isoniazid, Rifampin or RMP, ethambutol or EMB, and pyrazinamide or PZA. These four drugs are taken for two months.

At the end of two months, which is completion of the initial phase of
treatment, if the patient is HIV negative, doesn't have any cavities on the chest X-ray, and has negative AFB smears, the patient moves into the continuation phase. This involves taking INH and Rifampin for four months. If the patient has MDR TB or there is high suspicion for drug resistance, they should be treated with at least three drugs which have not been used previously. Once drug susceptibility results are available, their treatment should be individualized as appropriate.
3.4 MDR TB Treatment

MDR TB Treatment

- Approximately 2 years of therapy with a combination of first and second-line drugs
- Second-line drugs include FQ (e.g., ofloxacin, ciprofloxacin, moxifloxacin, levofloxacin), SI (i.e., capreomycin, amikacin, and kanamycin), PAS, ethionamide, cycloserine, and bedaquiline
- Regimen can become very complex depending on the extent of additional resistance beyond RMP and INH
- Second-line drugs often cause severe adverse effects and may be difficult for patients to tolerate

Notes:

MDR TB Treatment

MDR treatment lasts about two years with a combination of first and second-line drugs. Once drug susceptibility results are available, the treatment is individualized and uses drugs that the patient is susceptible to. The second-line drugs include fluoroquinolones or FQs such as ofloxacin, ciprofloxacin, moxifloxacin, and levofloxacin; secondary injectables such as capreomycin, amikacin and kanamycin; para-aminosalicylic acid or PAS; ethionamide; cycloserine; and bedaquiline. The MDR regimen can be very complex, especially if the patient is resistant to several of the first-line drugs. Second-line drugs, or secondary drugs, carry a risk for severe adverse effects including liver toxicity, hearing loss, nausea, and rashes. This may be very difficult for the patient to tolerate.
3.5 First-line Drugs and Mechanisms of Action

Notes:

This slide depicts the mechanism of action of the first-line anti-tuberculous drugs. INH and ethambutol act in different ways to inhibit the cell loss synthesis of MTBC. INH is bactericidal and bacteria static, and it inhibits the synthesis of mycolic acids. Ethambutol is bacteria static, and it disrupts the synthesis of arabinogalactan, which is a major structural component of the mycobacterial cell wall. Rifampin is a bactericidal drug that inhibits RNA synthesis. PCA is a bactericidal drug that disrupts the plasma membrane and energy metabolism of MTBC. Genetic mutations that disrupt these processes can result in drug resistance.
Intrinsic Drug Resistance

- Innate ability of bacterium to resist the activity of an antimicrobial agent
- Allows tolerance of a drug or drug class
  - Efflux systems pump toxic substances out of cell and enzymes to change the drug configuration
- Intrinsic PZA resistance seen with some members of the *M. tuberculosis* complex (MTBC) due to lack of pyrazinamidase activity
  - *M. canetti*, *M. bovis* and *M. bovis* BCG (other members of MTBC are usually susceptible to PZA)

**Notes:**

Intrinsic resistance refers to the innate ability of the bacterium to resist the activity of a particular antimicrobial agent through its inherent structural or functional characteristics. This allows tolerance of a particular drug or drug class. Intrinsic drug resistance of MTBC has been attributed to its unique cell wall properties, including the presence of mycolic acids, which constitute a very hydrophobic barrier responsible for resistance to certain antibiotics. In addition, MTBC possesses beta-lactamase enzymes, which confer intrinsic resistance to beta-lactam antibiotics, while efflux mechanisms appear to play a role in resistance to antibiotics such as tetracycline and aminoglycosides.

Intrinsic PZA resistance is seen with some members of the TB complex due to lack of pyrazinamidase activity. Pyrazinamidases are required to activate PZA to its active drug form, pyrazinoic acid. Because some members of the TB complex lack this enzyme, they're naturally resistant to PZA. *Mycobacterium canetti*, *M. bovis* and *M. bovis* BCG are considered naturally resistant to PZA,
3.7 Selection of Drug Resistant Mutants in TB

**Notes:**

Selection of Drug Resistant Mutants in TB

There can be spontaneous mutations occurring that confer resistant to TB drugs, just like resistance to any antimicrobial. Some of these mutations don’t change the protein produced but merely alter the structure enough so that the drug no longer targets it. When this happens, the organism is not killed but continues to grow even in the presence of the drug. These naturally occurring mutations can occur in large populations of organisms, typically, once they get to be ten to the sixth to ten to the eighth cells within the population. As a reference, in cavities of TB in the lungs, the organisms can reach ten to the seventh to ten to the ninth organisms, so in populations such as this, the mutations could definitely start to occur. This drives the multi-drug standard for treatment. When you use multiple drugs, you’re less likely to find a population that develops these naturally occurring resistance to all of the drugs.
This slide shows mutation rates for MTBC to three TB first-line drugs. Spontaneous mutations develop as bacilli proliferate to high numbers. You can see the different mutation rates for rifampin, INH, and PZA in this table.
3.9 How Drug Resistance Develops

Notes:

Here's an illustration of how drug resistance develops. The empty circles represent drug-susceptible organisms and the lettered circles represent organisms that are resistant to specific drugs. If an appropriate multi-drug regimen is started, as illustrated at the top, all drug-susceptible and drug-resistant bacilli are killed. Drug-resistant mutations are selected when therapy is inadequate; for instance, when a single drug, in this case isoniazid is used to treat a large population of bacilli, the treatment with a single drug kills the majority of the bacilli in the population but a small number of mutants resistant to the drug will continue to multiply.
3.10 How MultiDrug Resistance Develops

Notes:

This is an illustration of how multi-drug resistance would develop. Again, if the treatment regimen is inadequate and a single drug, rifampin, is added, further selection of multi-drug resistant organisms can occur. Let's talk about what happens during inadequate therapy. We've got a population of organisms that's INH resistant. These continue to grow unchecked, leading to an abundance of INH-resistant organisms. Remember, there can be spontaneous mutations in a large population for a different drug. Let's say there's a new mutation that encodes for rifampin resistance. Since the patient is not responding to therapy, a single drug, rifampin, is added to a failing regimen. Those organisms resistant only to INH are killed. However, since there is a subpopulation now resistant to both drugs, this group can proliferate. Now we have selected for multi-drug resistant organisms.
3.11 Other Factors Influencing Development of Drug Resistance

Notes:

This slide shows some other factors that may influence the development of drug resistance. If the patient is treated with inappropriate drugs or the wrong combination of drug, or inadequate doses, then resistance can develop. Interrupted or irregular treatments can cause drug resistance. If the bacteria become dormant, they won’t take in the drug and the drug won’t be effective. Some drugs don’t penetrate to certain body sites, such as within tissues or macrophages. Cells in these areas won’t be affected by the drug, or the drug might be at a lower or subtherapeutic concentration.
Primary Versus Acquired Drug Resistance

Primary: Strain is drug resistant at start of treatment, patient never treated in past
- implies transmission of drug resistant bacilli

Acquired: Strain is drug susceptible at start of treatment, becomes drug resistant during treatment

Notes:

Primary Versus Acquired Drug Resistance

Primary resistance is when the organism expressed resistance to a drug from the beginning of treatment when the patient has never been treated in the past. This indicates that the patient got their resistant organism from someone else. Acquired resistance is when an organism originally susceptible to the drug becomes resistant during treatment. This can occur for a variety of reasons, including inadequate therapy or through irregular compliance with taking the drugs.
3.13 DST of MTBC is Essential

Drug susceptibility testing for MTBC is essential. Just like with any antimicrobial susceptibility testing, the results can guide therapy so patient stands the best chance for cure. Even though treatment regimen is started as soon as the diagnosis is made, continuing with testing is critical, as it can either detect drug resistance in the original bug, allowing for individualization of the therapy, as well as allowing for appropriate treatment of contact cases. If a patient is not showing improvement on therapy originally thought to be effective, then repeating DST on subsequent isolate can detect acquired resistance. Having DST results also allows for the public health practitioners to determine the prevalence of drug-resistant strains in a community.
4. Conventional Methods

Notes:

Let's talk about conventional or culture-based susceptibility test methods.
4.2 Critical Concentration

Notes:

Let’s define critical concentration. DST of MTBC typically involves testing the susceptibility of an organism against the critical concentration of a drug. By definition, the critical concentration represents the lowest concentration of a drug that inhibits 95% of wild-type strains never exposed to antituberculous drugs while not inhibiting growth of strains isolated from patients not responding to therapy and considered resistant. These critical concentrations have been adopted and are used worldwide.
4.3 Critical Concentration

Notes:

More on Critical Concentration

In an ideal world, the critical concentration would be the concentration of drug at which all susceptible strains would fail to grow and all resistant strains would grow. So if you saw a colony on a drug-containing plate, you would know that the bacteria were drug resistant. If there were no colonies, you would know the bacteria were drug susceptible. However, the world is not ideal and it is difficult to find a drug concentration that actually meets this definition. So we often have to settle for the concentration that best discriminates between susceptible and resistant strains.
4.4 Determining Critical Concentrations

This slide shows how critical concentrations were determined. The experiment involves comparing presumably drug susceptible bacteria from previously untreated patients, in the dotted lines, to presumably resistant bacteria from patients who failed therapy, which are solid lines. The experiment looks at percent of susceptible and resistant bacteria that are inhibited at different drug concentrations. The best discrimination between susceptible and resistant strains is shown by the vertical line. This is the concentration that is used for the critical concentration.

In this case, it's 40 micrograms per ml for rifampin on the left, and 0.2 micrograms per ml for INH on the right. For rifampin, the susceptible strains are easily distinguished from the resistant strains over a wide range of drug concentrations. For INH, susceptible strains are also easily distinguished from resistant strains but over a much narrower range of drug concentrations. For the laboratory, this means that care must be taken to precisely make the drug containing media. Too little drug could lead to false resistant results, whereas too much drug could lead to false susceptible results.
4.5 Difficulty of Determining Critical Concentrations

Notes:

This slide shows some difficulties encountered when determining critical concentrations. For ethambutol, the picture is more complicated. For many of the drug concentrations tested, there is little or no discrimination between susceptible and resistant strains. Even at the most discriminatory concentration, the critical concentration shown as two micrograms per ml on this graph, there is some concern that the strains may be incorrectly classified as susceptible or resistant. Because the critical concentration can't discriminate as well between susceptible and resistant strains, you may get different results if the assay conditions are changed even slightly, or different results between different labs.
4.6 Equivalent Concentrations

Notes:

It was found that critical concentrations varied, depending on the media used. The critical concentrations were originally determined using LJ media. When Middlebrook 7H10 and 7H11 agar and the commercial broth systems came along, they needed to determine an equivalent concentration that could correlate to the LJ results.
### 4.7 Recommended Equivalent Test Concentrations for First-line Drugs

![Recommended Equivalent Test Concentrations for First-line Drugs](image)

#### Notes:

This slide shows the equivalent concentrations used in Middlebrook agar proportion testing and the commercial broth systems. You have different concentrations of drug depending on which system or method you use. INH concentrations are different for agar proportion than for the automated broth systems. Rifampin concentrations are the same for all test methods. PZA concentrations differ between the two commercial methods.
4.8 Recommended Concentrations for Second-line Drugs

Here we see the recommended drug concentrations used for second-line drug testing using agar proportion and the MGIT system. The MGIT system is not FDA-approved for second-line drugs. If you want to use the MGIT for second-line drugs, you would have to perform a full in-house validation to satisfy CLIA recommendations.

<table>
<thead>
<tr>
<th>Drug</th>
<th>System and Concentration (µg/mL)</th>
<th>7H10 Agar</th>
<th>7H11 Agar</th>
<th>MGIT*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td></td>
<td>4.0</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td></td>
<td>5.0</td>
<td>6.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Capreomycin</td>
<td></td>
<td>10.0</td>
<td>10.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td></td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Levofoxacin</td>
<td></td>
<td>1.0</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>Ethionamide</td>
<td></td>
<td>5.0</td>
<td>10.0</td>
<td>5.0</td>
</tr>
<tr>
<td>PAS</td>
<td></td>
<td>2.0</td>
<td>8.0</td>
<td>-</td>
</tr>
<tr>
<td>Rifabutin</td>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Streptomycin</td>
<td></td>
<td>2.0 and 10.0</td>
<td>2.0 and 10.0</td>
<td>1.0 and 4.0</td>
</tr>
</tbody>
</table>

* Most concentrations listed are based on multicenter studies. Systems are not cleared by the FDA for testing second-line drugs (except Streptomycin).
4.9 Critical Concentration Differs from Minimum Inhibitory Concentration

Notes:

This slide discusses the difference between critical concentration and minimum inhibitory concentration, or MIC. Under some circumstances, laboratories may perform tests to establish an MIC for TB drugs. The MIC is the lowest concentration of drug that prevents visible growth of MTBC in a series of broth dilutions of the drug. Usually with MIC results, you will report the numerical MIC result which would be a drug concentration in micrograms per ml and then use established breakpoints to interpret the results. This differs from using critical concentrations in which a single drug concentration is tested and a categorical result of resistant or susceptible is reported.
4.10 Recommended Panel for DST

Recommended Panel for DST

- Initial MTBC isolates from ALL patients should be tested for susceptibility against four first-line drugs
  - INH, RMP, EMB, and PZA
- Isolates resistant to RMP or any two first-line drugs, should be tested against second-line drugs
  - Minimally, second-line panel should include at least one FQ, amikacin, kanamycin, and capreomycin
- DST should be repeated after 3 months if patient remains culture positive

Notes:

Recommended Drug Testing to Perform for MTBC

An initial isolate from each new TB patient should be tested against the first-line drugs, which are INH, rifampin, ethambutol, and PZA. Isolates that are resistant to rifampin or any two of the first-line drugs should be tested against second-line drugs. Minimally, the second-line panel should include one fluoroquinolone and the injectable drugs, which are amikacin, kanamycin, and capreomycin. DST should be repeated after three months if the patient remains culture positive.
**4.11 DST Should Always be Performed on a Pure Culture**

- Indirect DST is performed after growth is identified as MTBC.

- It is essential to ensure that MTBC cultures are pure; contaminating bacteria are known to harbor intrinsic resistance and potentially cause false-resistant MTBC DST results.

- It is recommended that broths also be subcultured to 7H10/7H11 and blood agar to assess purity and colony morphology.

- If a culture is mixed with NTM or other bacteria, laboratories can attempt to re-isolate the MTBC.

**Notes:**

DST should always be performed on a pure culture. Indirect DST is performed on culture growth after a growth is identified as MTBC. When performing a DST, it’s critical that you ensure that the isolate is pure. If it’s mixed with bacteria or nontuberculous mycobacteria, you could get false resistance showing up. It’s recommended that you subculture the inoculum used for DST to 7H10 or 7H11 solid media and also a blood agar plate to assess the purity and colony morphology of the strain used for the inoculum. If a culture is mixed with nontuberculous mycobacteria or other bacteria, laboratories can attempt to reisolate the TB.
### 4.12 Culture-based Methods for DST

<table>
<thead>
<tr>
<th>Culture-based Methods for DST</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Table]</td>
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</tbody>
</table>

#### Notes:

So here's some information about the different culture-based methods currently in use for DST. As you can see, only the MGIT and the VersaTREK systems are FDA-cleared. Indirect agar proportion is the standard method developed in the laboratory. This assay is not FDA-cleared because there aren't commercially available kits that include all of the reagents and components for this test method. The TREK Sensititre method is a broth microdilution method performed in a microtitre plate. MIC results would be reported using this method, and it is for research use only at this time.
4.13 Procedure for Agar Proportion DST

Notes:

Here's the procedure for agar proportion testing. First, a bacterial suspension of known concentration is spread onto the solid media. There would be a growth control quadrant or tube that contains no drug. Other quadrants or tubes would contain critical concentrations of the antituberculous drugs. PZA can't be tested using the agar proportion method. Solid media doesn't support the low pH conditions necessary for PZA testing. The plates or tubes are incubated for three weeks, and growth is observed each week. The isolate is considered resistant if the number of colonies on the drug-containing media is greater than or equal to one percent of the colonies on the growth control media that doesn't contain drugs.
4.14 Analysis of Results from Agar Proportion Method

Notes:

Here you see an example of an agar proportion plate. The growth control quadrant is on the top right. It contains 90 colonies. The INH quadrant is on the top left. It contains 30 colonies. This isolate would be considered resistant to INH because the number of colonies in the INH quadrant is more than one percent of the colonies in the growth control well. To be exact, it's 33 percent resistant.

Again, in the bottom left quadrant, the isolate is resistant to rifampin. The isolate is considered to be susceptible to streptomycin because there's no growth in the streptomycin-containing quadrant on the bottom right.
4.15 Commercial Broth Systems

**Commercial Broth Systems**

- Selection of critical concentrations based on comparison of results with agar proportion to determine equivalent critical concentrations
- More rapid turnaround time results (4-13 days) than agar proportion (21-28 days)
- Continuous, automated signal detection (e.g., fluorescence for MGIT, pressure changes for VersaTREK) by instrument reducing hands-on time
- Published evaluations of second-line drugs (no FDA-cleared methods)

**Notes:**

As we mentioned before, there are commercial systems using broth media that can be used for DST. The critical concentrations of the broth systems may be different than those for agar-based methods. Equivalent concentrations of the drugs had to be determined for the broth methods. The major benefit to the broth systems is that it’s a more rapid turnaround time, anywhere from one-fifth to about one-half of the time it takes to get results for the agar proportion method. Since the systems are continuously monitored, you don't have to keep reading the media. The system will alert you when it detects a certain level of growth in the assay. There are no FDA-cleared commercial broth systems for second-line drug testing.
4.16 Commercial Broth Systems

This chart shows the difference between the commercial broth systems with regards to how the signal is detected, the turnaround times, how the result is reported, and the drugs available in each test system. As you can see, the turnaround times for the different assays are fairly comparable. For detecting culture growth, the MGIT uses a fluorescent detection method and the VersaTREK uses head space pressure changes in the bottle. The Sensititre system is a 96-well plate so you're visualizing examining for growth. Both the MGIT and VersaTREK systems are reported as a categorical, susceptible or resistant result. The Sensititre system can report a MIC. Most of the systems can test the usual first-line drugs. PZA is not included in the Sensititre assay, but this assay does include many other second-line drugs. As mentioned before, you can test second-line drugs on the MGIT but it would be considered a lab-developed test and it would require full validation.
**4.17 Broth-based DST MGIT 320/960**

This slide shows the inoculation procedure for the MGIT systems. There are multiple options for preparing the inoculum for the assay. You can use a positive MGIT tube or you can use a cell-suspension prepared from growth on solid media. You can also use a seed tube, which is specially grown for the inoculation purpose. The organism suspension is diluted 1 to 100 prior to inoculating the growth control tube. The organism suspension is used undiluted to inoculate the drug tubes. The standard kit for MGIT includes components for testing streptomycin, INH, rifampin, and ethambutol. Other kits are available to allow you to test higher concentrates of INH and streptomycin. The PZA kit is also sold separately. The broth has a pH adjusted to 5.9 because PZA requires a lower pH for testing.
4.18 Alternative Inoculum Preparation for MGIT

This slide describes how to prepare inoculum from solid media growth for the MGIT system. This protocol is included in the package insert. Cell suspensions are made and diluted to a turbidity equivalent of a 0.5 McFarland standard. A 1 to 5 dilution is made for inoculating the drug-containing tubes; growth control is 1:100 dilution of the 0.5 McFarland standard. Lin, et al. (2009) described a similar method for preparing inoculum from MGIT broth, based on turbidity. Improved reproducibility was reported (JCM 47:3630).

Notes:

This slide describes how to prepare inoculum from solid media growth for the MGIT system. This protocol is included in the package insert. Cell suspensions are made and diluted to a turbidity equivalent of a 0.5 McFarland standard. A 1 to 5 dilution is made for inoculating the drug-containing tubes. A 1 to 100 dilution is made for inoculating the growth control tube. Lin, et al., in 2009, described a similar method for preparing inoculum from MGIT broth based on turbidity. This method improved reproducibility, and it's a good alternative method. Because it's different from the package insert, a full validation would be necessary in your laboratory.
4.19 VersaTREK

- Same system used for mycobacteria growth and detection from sputum, sterile body fluids, and blood can be used for DST
- Instrument configurations holding 240 or 528 bottles
- Sophisticated LIMS interface
- Bottles can be inoculated by pipette or needle
- Available PZA tested at 300 ug/mL

Notes:

The VersaTREK system is also a broth-based system. The same system is used to grow and detect mycobacteria from clinical specimens and to do DST. The system can hold 240 or 528 bottles and it has a sophisticated capability to interface with your laboratory information system. In this system, the bottles can be inoculated either by a pipette or a needle. The equivalent concentration of PZA is 300 micrograms per ml.
4.20 Confirming Resistance from Broth Systems

Notes:

Confirming Resistance from Broth Systems

So when you get a resistant result, what do you do to confirm it? You should examine growth from purity plates to check for contaminating organisms. Also, examine growth from the drug-containing vial to determine the consistency. You would expect to see clumps or granular growth from MTBC. Perfuse turbidity might indicate contamination. You want to prepare a smear from the tube. You'd want to see acid-fast organisms. With TB, you might see cording. Check for non-acid fast organisms in the smear. Subculture the tube to 7H10 or 7H11 plate and examine the colony morphology. You would want to see pure growth of MTBC. You would want to repeat testing from pure growth if there are indications that the inoculum was mixed or contaminated.
4.21 Sensititre MYCOTB MIC Plate

Notes:

Another method for TB drug susceptibility testing is the Sensititre plate. This is a 96-well microtiter plate containing a panel of 12 first- and second-line antituberculous drugs. The plate contains a minimum of seven dilutions per drug. A bacterial suspension of known concentration (1X10^5 cfu/ml) is prepared and inoculated into wells of the plate. Growth can be examined manually using a view box, or also using the Sensititre Vizion® System. Resistant results can be detected in as little as 7-10 days.
4.22 Growth in MYCOTB MIC Plate

Notes:

In this slide, we see an example of the TREK Sensititre MYCOTB mycodilution plate. Each column has a serial dilution of different drug. There are 12 antituberculous drugs total. Using rifabutin and PAS as examples, we'll circle the first well or the lowest concentration of drug showing inhibition of growth. How would you read and report the MIC for rifabutin? It's 8 micrograms per ml. And PAS? There's no growth in any of the wells, so the MIC is less than 1.5 micrograms per ml. Plates should be checked at 10 days for growth and rechecked at 21 days. The MIC is recorded as the lowest antibiotic concentration that inhibits visible growth. Growth can be seen as turbidity or deposit of cells in the bottom of the well.
4.23 Limitations of MIC Testing for MTBC

Notes:

While we're very comfortable reporting MICs for other bacterial drug susceptibility testing, this type of testing is still in development for MTBC. We don't have standardized methods, and none of the MIC assays are FDA-cleared. We don't have established breakpoints with interpretive criteria. There's not robust published data on how MIC results correlate with patient outcomes. At this time, we don't know how MIC results correlate with the critical concentration results that have been reported for years. More studies are needed so that we can compile standards for MIC testing.
5. Turnaround Time and Reporting

Notes:

Let's talk about turnaround times and reporting.
5.2 Recommended Turnaround Time for First-line DST

- Although indirect agar proportion is considered the standard method for DST, it is not a rapid method.
- Initial isolates of MTBC should be tested against a panel of first-line drugs using a rapid commercial broth system.
- CDC recommends that DST results should ideally be available to the submitter within 28 days of specimen receipt.

Meeting the recommended turnaround time necessitates the use of a rapid commercial broth system.

Notes:

Recommended Turnaround Times for TB First-line Drugs

Although indirect agar proportion is considered the standard for MTBC-DST, it's not a rapid method. The most recent recommendations are to perform TB-DST for all initial isolates using a rapid commercial broth system. The CDC recommends that DST results be available within 28 days of receiving the initial clinical specimen. To meet these recommendations, you would need to use one of the rapid commercial broth systems.
5.3 Concerns with Current DST Practices

Notes:

Some Concerns with Current DST Practices

Does your laboratory perform drug susceptibility testing? If you don't do this testing, how do you get these test results done? To whom do you refer the testing? Another larger clinical lab? A reference lab? A state public health lab? Do they do your first- and second-line drug susceptibility testing? When would you send an isolate for second-line or third-line drugs? Most laboratories refer for DST with multiple referrals needed for a full panel of first and second-line antituberculous drugs. How often do you see resistant isolates? Are you comfortable reporting resistant results? Laboratories may lack confidence or be reluctant to report resistant results prior to confirmation. This could delay reporting of important information. Additionally, discordant results can occur within a lab, between labs, or using different methods. How are these discordant results resolved? The DST assays are complex assays. You need good training and enough experience to get consistently good results.
5.4 Considerations for Reporting DST Results

Considerations for Reporting DST Results

- Any resistance should be considered a critical value and the submitter and public health authorities should be notified immediately.
- Issue preliminary reports as results become available.

Notes:

Some Considerations when Reporting DST Results.

Any resistance should be considered a critical value, and the submitter and public health authorities should be notified immediately. Issue preliminary reports as soon as results are available. Don't wait for all drugs before issuing a report. Even if PZA is still pending, you can issue a report for the other drugs.
5.5 Considerations for Reporting DST Results (2)

Considerations for Reporting DST Results (2)

- If resistance detected, issue preliminary report describing results while concurrently confirming resistance and requesting DST with second-line drugs
  - Also indicate the test is being repeated
  - If resistance is confirmed, issue final report
- For patients with resistant isolates, consultation with reference laboratory and specialists in the management of drug resistant TB should be considered
- If the resistance does not confirm
  - Call provider to inform of discordant results
  - Issue a second preliminary report
  - Submit to CDC or other reference laboratory for testing by the same or different method (e.g., agar proportion)

Notes:

Although you want to confirm any resistant result, you should issue a preliminary report. This information needs to be relayed to the provider. Indicate the test is being repeated. Once you get confirmation, you can issue a final report. If drug resistance is detected, it's as good idea to consult with experts who have experience in management of patients with drug resistance. If the drug resistance doesn't confirm, call the provider and explain the situation. Issue another preliminary report. Submit the isolate to CDC or a reference lab for confirmation testing. It would be preferable to use a method that's different such as agar proportion.
### 5.6 Conventional DST Report

**Conventional DST Report**

- Reports should, at minimum, include the name of the drug tested and a clinically relevant interpretation such as susceptible or resistant.
- If reports indicate the concentrations tested for each drug, it should also include the testing medium and/or method used.
- If agar proportion is used, the percent resistance may also be reported.
- If more than one concentration is tested for a drug, interpretive comments may be included on reports (e.g., high and low concentrations of INH).

---

**Notes:**

Conventional DST Reporting.

Reports should, at minimum, include the name of the drug tested and a clinically relevant interpretation such as susceptible or resistant. If reports indicate the concentrations tested for each drug, it should also include the testing medium or method used. If agar proportion is used, the percent resistance may also be reported. If more than one concentration is tested for a drug, interpretive comments may be included on the reports, such as this is the high concentration or low concentration of INH.
5.7 Considerations for DST Referral Process

Considerations for DST Referral Process

- Submitting and referral laboratories should be familiar with shipping guidelines for infectious substances.
- If possible, laboratories should refer liquid cultures for DST rather than waiting for growth on solid media.
- Submitting laboratories should routinely monitor turnaround time of the referral laboratory.

Notes:

Considerations for Referring DSTs.

Submitting and referring laboratories should be familiar with shipping guidelines for infectious substances. If possible, laboratories should refer liquid cultures for DST rather than waiting for growth on solid media. Submitting laboratories should routinely monitor turnaround times of the referral laboratory.
6. Discordant Results

**Notes:**

Now we will discuss discordant drug susceptibility test results.
6.2 Discordant DST Results

Notes:
Discordant DST results can occur within the same lab, with the same method, between different labs, and between different methods. So what do discordant results indicate? Which result is correct? All of them, only one, none of them?
6.3 Reasons for Discordant DST Results

Notes:

There are several possible reasons for discordant DST results. There could be differences in the bacterial populations. Primary cultures are preferred over subcultures to avoid selection for a specific population of bacteria. Resistant populations may have different growth rates than susceptible populations. Subcultures used too early may select for susceptible populations. There may be variation in DST due to the age of the culture or stage of the growth. Over inoculation or under inoculation of the assay can cause differences in resistance patterns. Different DST methods may produce different results due to media components and other variables.
6.4 Reasons for Discordant DST Results (2)

Reasons for Discordant DST Results (2)

- Operator or laboratory error
  - Deviation from standard protocol
  - Transcription, labeling errors
- Cross-contamination
- Variability of isolate in that the MIC is close to the critical concentration tested
- Difficult drugs and lack of standardized methods for second-line DST

Notes:

Discordant DST results may be caused by operator or laboratory error, either deviation from the standard protocol or other errors. Cross contamination with another strain of TB could be a cause for discordant results. If the isolates MIC is close to the critical concentration tested, perhaps considered borderline resistant or low level resistant, it may test resistant one time and susceptible the next. The established critical concentrations for some drugs and the assays, especially for second-line drugs, are less robust, which would lead to more variability.
6.5 Standardization and Reliability Issues

Notes:

Standardization and Reliability Issues. Some drugs can be difficult to test. For ethambutol, microcolonies in the agar proportion method make the plates difficult to interpret. False susceptibility may be a concern with commercial broth methods. For PZA, the low pH necessary for the assay may impact growth of the organism. The assay is very sensitive to over inoculation which can cause false resistant results, especially in the commercial systems.
Notes:

Cycloserine can be difficult to test. Although it's an important second-line drug option, we don't have good susceptibility test methods available in the U.S. Data regarding optimal testing of second-line drugs are limited and standardized protocols require additional evaluation.
6.7 Variability in DST Results for EMB

Notes:

This slide demonstrates variability in DST results for ethambutol. These are ethambutol results reported by participants in CDC's Model Performance Evaluation program, or MPEP program, for six isolates of ethambutol resistant MTBC. Mutations associated with ethambutol resistance are shown in the second column, and CDC agar proportion results are shown in the third column. Participant results are stratified by DST method. If you look at Strain 2010A as an example, 41 percent of laboratories testing by agar proportion reported resistance for this isolate while only 2 percent reporting MGIT results showed resistance. None of the labs performing VersaTREK reported resistance to this isolate. Within this study, there were two mutations in particular: the methionine 306 isoleucine mutation and the glycine 406 aspartic acid mutation that confer resistance that is rarely detected in the commercial broth systems.
6.8 Variability in DST Results for PZA

Data indicate potential false PZA resistance in some automated liquid systems

<table>
<thead>
<tr>
<th>Isolate*</th>
<th>Bactec 460</th>
<th>MGIT</th>
<th>VersaTREK</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0/17 (0)</td>
<td>1/64 (2)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>B</td>
<td>0/17 (0)</td>
<td>7/62 (11)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>C</td>
<td>0/17 (0)</td>
<td>20/62 (32)</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>D</td>
<td>0/17 (0)</td>
<td>21/63 (33)</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>E</td>
<td>0/17 (0)</td>
<td>0/64 (0)</td>
<td>(0)</td>
</tr>
</tbody>
</table>

* A and E are same strain; C and D are same strain.
Bactec 460 system no longer commercially available

Notes:

This slide demonstrates variability in DST results for PZA. These are PZA results reported by participants in CDC’s Model Performance Evaluation Program for five isolates. All of these isolates tested as PZA susceptible at CDC. The Bactec 460 performed well with no resistant results; however, this system is no longer available. The MGIT and VersaTREK systems both showed varying levels of false resistant results. Sometimes the percentage of resistant reported was quite high. These data indicate potential false PZA resistance in some of the automated liquid systems.
6.9 When PZA Testing is Likely to be Repeated

Notes:

This slide shows situations in which PZA testing should be repeated. If the assay grows too quickly, the tests will need to be repeated. If there are growth units in the drug tube showing low resistance or high susceptibility, the test should be repeated. If the isolate is PZA mono resistant but not known to be \textit{M. bovis} or \textit{M. bovis} BCG, further testing should be performed. Any discordant result should be investigated, including discordancies between laboratories and discordancies between molecular and phenotypic testing.
6.10 Considerations for Detecting RMP Resistance

Notes:

This slide discusses some issues with detecting rifampin resistance. The level of resistance to rifampin can vary, depending on the mutations present in the rpoB gene. Reports of low-level but presumably clinically significant rifampin resistance are being missed by commercial broth systems. This can result in discordance between conventional and molecular test methods. There is concern that conventional methods are missing rifampin resistance that could impact clinical outcomes for patients.
6.11 Variability in DST Results for RMP

Variability in DST Results for Rifampin.

This slide shows rifampin results recorded by participants in CDC's Model Performance Evaluation Program for two isolates. These isolates contain a histidine 526 Lucein mutation and were rifampin resistant in CDC's agar proportion testing. Participant results are stratified by DST method. If you look at strain H as an example, 70 percent of laboratories testing by agar proportion reported resistance for this isolate, while only 18 percent reporting MGIT results showed resistance. None of the labs performing VersaTREK reported resistance for this isolate. These data suggest that this mutation conferring low-level rifampin resistance can be missed by commercial broth systems.

Notes:

Variability in DST Results for Rifampin.
7. Ensuring Quality

Notes:

Now we'll talk about ensuring quality for TB drug susceptibility testing.
7.2 Quality Control

Notes:
As far as quality control organisms, laboratories should include an isolate that's susceptible to all drugs being tested, such as H37Rv. Although it isn't necessary, laboratories may choose to test a resistant strain as part of their QC. Use of a single strain that is resistant to two or more drugs is not recommended due to safety risk.

Reference: CLSI M24A2
7.3 Quality Control (2)

Notes:

More on Quality Control

The drug susceptible strain should be run with each new lot of drug or media each time a new batch of in-house media is prepared, at least once a week or with each new run. If a drug-resistant QC strain is used, it may be tested less frequently.

- Drug susceptible QC strain should be run
  - For each new lot of drug or media component before use on patient isolates (Especially for Oleic Albumin Dextrose Catalase (OADC) and 7H10 powder)
  - Each time new batch of media is prepared
  - At least once a week or with each run

- If a resistant strain is used for QC, it may be tested less frequently than drug susceptible strain

Reference: CLSI M2A2
7.4 Quality Control (3)

Notes:

This slide discusses what to do with QC fails. Failure occurs when the drug-susceptible QC strain doesn’t grow in the growth control tube (i.e., no drug) or exhibits growth in the drug(s) being tested.

- Patient results for the drug or drugs that failed QC should not be reported for that testing period.
- Testing for drugs and patient isolates affected by the QC failure should be repeated.
- Most common causes of DST QC failure include contaminated QC cultures, over or under inoculated cultures, no drug added to tubes, and instrument errors.
- Growth in drug-containing (resistant) tubes should be checked for purity using Ziehl Neelsen or Kinyoun stain of smear.

A blood agar plate could also be used to check for non-AFB contamination.
7.5 Drug Resistance Rates as a Performance Indicator

Notes:

Checking drug-resistant rates is a good performance indicator. You need to note the normal drug-resistant rates for your population as a baseline. If you start to see an increase or a decrease in the level of resistance, you'd want to investigate the reason. A false increase in resistance might be due to cross-contamination, cultures mixed with NTM or non acid-fast organisms, inappropriate drug concentrations or media preparation, or errors in interpretation. A false decrease in drug resistance might be due to inappropriate drug concentrations or media preparation, or errors in interpretation. If you have a very low rate of resistance in your area, it could be tempting to assume that a resistant result is due to contamination. You don't want to make this assumption because you could end up delaying a critical result.
Notes:

Proficiency Testing and Evaluation Programs

Maintaining technical proficiency is critical to ensure rapid and reliable DST results. As with any testing, you need to have a high enough volume to maintain your competency. If you are testing fewer than 50 isolates a year, you may want to consider referring DST to another lab. Current external quality assurance programs in the United States are lacking in ability to sufficiently assess proficiency for detection of MDR TB or second-line drug resistances. The Cap surveys do cover DST, but they don't include resistant organisms. The CDC MPEP is an excellent program which includes drug-resistant strains. Ongoing CDC studies are gathering information to further characterize mutations that confer resistance and evaluate the benefits and limitations of commercial DST systems.
8. Molecular Methods

Notes:

Let's briefly talk about molecular methods for drug susceptibility testing.
8.2 Use of Molecular Assays to Detect Resistance

Molecular methods will be covered in a separate module, but we wanted to give a brief overview of this testing. Conventional DST requires growth of the organism and turnaround time is measured in weeks. Molecular assays are usually analyzing nucleic acids of the organism. Turnaround times for molecular assays to detect mutations associated with resistance are reduced to a few hours or days. In this way, molecular assays provide a more timely guidance for clinical management, especially where there is risk for drug resistance or resistance is suspected. Molecular methods may require an isolate but clinical specimens can be analyzed by more sensitive methods. Conventional DST is still required because the genetic basis for drug resistance is not fully characterized. There may be a limitation to the sensitivity of molecular assays as well. Under some circumstances, molecular results may be more accurate. An example would be detection of mutations that confer low level, clinically significant rifampin resistance.
# 8.3 Examples of Tests for Molecular Detection of Mutations Associated with Drug Resistance

This slide shows examples of tests for molecular detection of drug resistance. The Cepheid GeneXpert MTB/RIF assay received FDA approval in 2013. This test can be run on broth sputum or digestive sputum sediment. It detects MTBC and mutations within the rpoB gene. The HAIN test is a lime-probe assay and will give information on mutations associated with rifampin and INH resistance. This test is not FDA-approved, so a full in-house validation must be done prior to using it for patient diagnostic purposes. Sanger sequencing and pyrosequencing can look for mutations in multiple genes or loci. These are considered laboratory-developed tests and require a high level of expertise.

<table>
<thead>
<tr>
<th>Company</th>
<th>Genetic loci</th>
<th>Format</th>
<th>FDA approved</th>
<th>Expected turnaround time from receipt in laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cepheid</td>
<td>rpoB (for RMP)</td>
<td>Semi-automated</td>
<td>Market</td>
<td>1-2 working days (depends on how often performed in lab)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>real-time PCR</td>
<td>authorization</td>
<td></td>
</tr>
<tr>
<td>HAIN Lifescience</td>
<td>rpoB (RMP), katG (INH), and inhA (INH)</td>
<td>Line probe assay</td>
<td>No</td>
<td>1-2 working days (depends on how often performed in lab)</td>
</tr>
<tr>
<td>Sanger Sequencing</td>
<td>Varies but can include rpoB, inhA, katG, aphC, embB (EMB), pncA (PZA), gyrA (FQ), and rrs (injectables)</td>
<td>DNA sequencing</td>
<td>N/A (laboratory developed test)</td>
<td>1-2 working days (depends on how often performed in lab)</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>Varies but can include rpoB, inhA, katG, aphC, gyrA, and rrs</td>
<td>DNA sequencing</td>
<td>N/A (laboratory developed test)</td>
<td>1-2 working days (depends on how often performed in lab)</td>
</tr>
</tbody>
</table>
8.4 Thank you for participating!

Thank you for participating!

Drug Susceptibility Testing for
*M. tuberculosis* Complex

Course complete

Notes:

This concludes the Drug Susceptibility Testing for *M. Tuberculosis* Complex presentation, which is part of a series from the Association of Public Health Laboratories Essentials of the Mycobacteriology Laboratory: Promoting Quality Practices. Please see the CDC and APHL websites for more information on the topics presented here.