Landscape and Language of Molecular Diagnostics for TB Drug Resistance

Notes:

Welcome to the Association of Public Health Laboratories’ Essentials for the Mycobacteriology Laboratory Promoting Quality Practices. This presentation is “Landscape and Language of Molecular Diagnostics for TB Drug Resistance”.
1.1 Purpose

Notes:

This module will provide TB control staff, clinicians, and nurses a brief overview of basic principles of molecular biology and test platforms used for molecular testing of Mycobacterium tuberculosis as a basis for understanding how mutations are associated with drug resistance and what reporting language might be used to describe resistance results.
2. Basic Principles of Molecular Biology

Notes:

The first section of this module covers the basic principles of molecular biology. A working knowledge of these principles will help to understand how drug resistance develops in Mycobacterium tuberculosis, how molecular testing detects drug resistance, and how to interpret results of drug resistance testing.
Molecular Biology

- Study of the formation, structure, and function of macromolecules essential to life, such as nucleic acids and proteins.
- Explores the role of macromolecules in cell multiplication and transmission of genetic information.

Notes:

Molecular biology is the study of biological activity at the molecular level. It focuses on the formation, structure, and function of macromolecules essential to life, such as nucleic acids and proteins. It also explores the role of macromolecules in cell multiplication and transmission of genetic information.
To understand molecular testing, mutations, and drug resistance, we must first define a framework known as the central dogma of molecular biology. DNA (deoxyribonucleic acid) serves as the carrier of genetic information. This figure provides a simplistic overview of the flow of genetic information in a biological system. DNA replication is the production of identical DNA helices from a single double-stranded DNA molecule. Replication follows several steps that involve multiple proteins called replication enzymes. The genetic information contained within DNA is transcribed into a specific type of RNA (ribonucleic acid) called messenger RNA, or mRNA. This message, the mRNA, is translated into a particular protein. Specific parts of DNA code for specific mRNA, resulting in the translation, or formation, of specific proteins.
2.4 Characteristics of DNA

Characteristics of DNA

- Deoxyribonucleic acid
- Double-stranded
- Formed by Adenine (A), Thymine (T), Guanine (G), and Cytosine (C)
- Specifies information on messenger RNA (transcription) which is subsequently used to build protein (translation)

Notes:

This slide outlines the basic characteristics of DNA. DNA stands for deoxyribonucleic acid. DNA exists as a double stranded molecule with strands held together through complementary pairing of bases: Adenine to Thymine (A to T) and Guanine to Cytosine (G to C). DNA contains the instructions needed for an organism to develop, survive, and reproduce. To carry out these functions, DNA sequences must be converted into messages that can be used to produce proteins, which are the complex molecules that do most of the work in cells. Each DNA sequence that contains instructions to make a protein is known as a gene.
2.5 Characteristics of RNA

This slide outlines the basic characteristics of RNA. RNA stands for ribonucleic acid. RNA exists as a single stranded molecule and although there are different types of RNA, it primarily serves as a messenger (known as mRNA) that provides instructions from the DNA for protein synthesis.
This diagram illustrates how DNA codes for messenger RNA via transcription and how that messenger RNA is translated into protein. mRNA is read in sets of three bases, called codons. A codon is a combination of 3 consecutive bases within a specific gene, that specifies or encodes for a particular amino acid, as indicated in this figure by the outlined bases. Specific codons also signal the beginning or end of protein synthesis.

The three-base codons of messenger RNA are translated into a series of amino acids at a fixed point from which the "meaning" begins using the genetic code. Individual amino acids are then linked together to form a protein. The sequence of amino acids will determine the eventual shape and function of the protein. The UGG codon shown for the messenger RNA in this diagram specifies for the addition of Tryptophan (Trp). The next codon, UUU, specifies for Phenylalanine (Phe) while the GGC codon specifies for Glycine (Gly), and so forth until the whole protein is created.
2.7 Important Definitions

Let's define three important terms related to the understanding of DNA. A gene is a specific sequence of bases along a segment of DNA that provides coded instructions for synthesizing a protein or RNA molecule. Two different sequences of a gene are shown in the diagram, AAACGCGTTAGC and AAATGCCTTAGC.

A second term that is important is codon which was described in the previous slide as a set of three bases seen here within the circles.

The third term is locus, which refers to a region of interest in a gene.
2.8 Reading Frame

Each mRNA sequence begins with a start codon that signals initiation of translation and the first amino acid in a polypeptide chain. The most common start codon is AUG.

Each mRNA sequence ends with a stop codon that signals termination of the synthesis of a protein. Common stop codons include UAA, UAG or UGA.
2.9 Genetic Code

Notes:

Multiple codons can code for the same amino acid, which demonstrates the redundancy of the genetic code. For example, the nucleotides AAU and AAC both code for the amino acid Asparagine, while nucleotides AAA and AAG both code for the amino acid Lysine. Redundancy is beneficial because if a mutation occurs in a single nucleotide, the protein may not be altered because the same amino acid could be incorporated.
2.10 Knowledge Check One

Each codon codes for a specific:

- A) Gene
- B) Amino acid
- C) Protein

Notes:
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Now that we’ve covered some basic principles of molecular biology, we will discuss how mutations in the DNA and RNA sequence can develop.
3.2 Mutations

Notes:

Before we talk about mutations, it’s important to know that a typical or common version of a gene is referred to as wild-type. A mutation is a change in the DNA sequence that results in variation from previous generations that can be transmitted to subsequent generations. These mutations can range in size from a single base to large segments of DNA.

The possible causes of mutations include mutagenic agents such as UV light and chemicals or spontaneous mutations due to errors in the replication process.
3.3 Types of Mutations

Notes:

There are several types of mutations including point, frameshift, insertion and deletion. Definitions are provided and will be further explained on the following slide. Click each term to learn more about these types of mutations.

A point mutation is a change in a single base in the DNA sequence, an A, T, C or G. Point mutations are sometimes referred to as Single Nucleotide Polymorphisms, or 'SNPs' (pronounced 'snips'). A SNP is a variation in a base pair in a DNA sequence. While a SNP in the coding region of a gene may change the amino acid, a point mutation in a promoter or regulatory region would only result in a nucleotide change.

A missense (or non-synonymous) mutation occurs when the base change results in coding for a different amino acid. This change may effect the structure and function of the resulting protein thus changing the wild-type or what might be typical.

A silent (or synonymous) mutation is one in which the DNA sequence is changed but the resulting amino acid sequence remains the same. Therefore, the structure or function of the resulting protein is typically not affected. Redundancy in the genetic code explains the potential for silent mutations.

A nonsense mutation is a base change that results in a STOP codon. The synthesis of
the resulting protein is prematurely cut short and may result in the protein having an altered structure and function.

A frameshift mutation occurs with the addition or loss of a number of DNA bases that is not a multiple of three. For example, one, two or five nucleotides. The addition or loss of bases shifts the original frame of three nucleotides or codons in which the message was read. The resulting protein is usually nonfunctional.

In an insertion mutation a single base or set of bases is added into the DNA sequence. Depending on the location and number of bases inserted, the inserted DNA may alter the function of the resulting protein.

A deletion can occur when a single base or set of bases is removed from the DNA sequence. Depending on the location and number of base deletions, the deleted DNA may alter the function of the resulting protein.
Let’s examine the potential impact of different types of mutations. In the example included here, the normal message is indicated in the first line. Here the word “the” is the start of the message similar to the start codon and the word “end” is the end of the message similar to the stop codon in the mRNA. The normal message would read “The man saw the dog hit the can” and then the message ends.

When a point mutation is introduced as in the second line, the message now reads “The man saw the dot hit the can.” The point mutation changes the normal word “dog” to the word “dot.”

A deletion mutation as in line three, changes the message to “The man saw the hit the can.” In this example a whole word has been deleted. Similarly, a deletion mutation can cause the elimination of one or more amino acids in a protein sequence thereby potentially impacting the structure or function of a protein.

In the next example on the fourth line, an insertion results in the message “The man saw the fat dog hit the can” with the introduction of a whole new word. Similarly an insertion mutation can change the message for a protein sequence.

A frame shift is included on line five. A frame shift results from the insertion or deletion of
a number of nucleotides that is not a multiple of three. For example, 1, 2, or 5. This insertion or deletion shifts the original frame of three nucleotides in which the message was read. In the example included here, a deletion of one nucleotide results in the message “The man saw the” with a resulting shift in reading frame changing the message to one that is unable to be interpreted. Similarly, a frameshift mutation will cause a change in the resulting protein sequence such that it is completely different from what would normally be encountered (in other words, wild-type) and will likely not be functional.

3.5 Knowledge Check Two

The mutation shown below is an example of a:

<table>
<thead>
<tr>
<th>Normal DNA sequence</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATG TTC GAT</td>
<td>ATG TGC GAT</td>
</tr>
</tbody>
</table>

- A) Point mutation
- B) Insertion mutation
- C) Deletion mutation

Notes:
There is no audio on this slide.
4. Mutations and Drug Resistance in *Mycobacterium tuberculosis*

Notes:

Now that we’ve discussed mutations, let’s examine how mutations in *Mycobacterium tuberculosis* can lead to drug resistance.
4.2 Mechanisms of Action of the Primary Anti-tuberculosis Drugs

Notes:

Next, we are going to describe the mechanism of action of the primary antituberculosis drugs. The two most important drugs with well-known mechanisms of resistance are rifampin (RIF) and isoniazid (INH).

Rifampin is considered the most important first-line drug in treatment regimens for tuberculosis. Rifampin acts by inhibiting RNA synthesis also known as transcription and ultimately leading to cell death.

Resistance to this drug can be used to predict multidrug-resistant (MDR) TB because rifampin resistance is frequently associated with additional resistance to isoniazid.
4.3 Potential Impact of Mutations

Mutations may or may not impact drug resistance. Some mutations will have no effect on drug resistance. While other mutations may change protein structure and inhibit drug activity or binding of the drug to the target, change the expression level to overcome effects of the drug or affinity for a drug and result in different levels of resistance depending on the mutation itself.
4.4 Development of Drug Resistance

Notes:

Mutants resistant to any one drug occur naturally at a rate of about one in every 10 to the sixth to 10 to the eighth cells. That's one in a million to one in a hundred million! The very unlikely chance of encountering cells with simultaneous mutations in two or more resistant determinants is the rationale behind the recommendation for the use of multiple drugs during TB treatment. However, erratic, incomplete, or inappropriate treatments can lead to the proliferation of drug-resistant tuberculosis.
4.5 How Drug Mutants Occur

The concept of how drug resistant mutants occur in a large bacterial population is depicted visually in the following figure. Starting in the upper left hand quadrant, if a large bacterial population is treated only with a single drug, in this case isoniazid (I), the isoniazid-resistant bacteria are able to survive and multiply. As that population increases in size, spontaneous mutations can develop that lead to rifampin (also known as rifampicin) (R) resistance in addition to the isoniazid resistance. If the drug rifampin is then added to the isoniazid treatment, a situation may arise where multidrug-resistant (rifampin and isoniazid) mutants are able to grow and proliferate.
4.6 Considerations for Detection of Rifampin Resistance

There are several considerations for detecting rifampin resistance. First, over 95% of clinical isolates resistant to rifampin have a mutation in a particular locus within the rpoB gene of M. tuberculosis referred to as the rifampin resistance determining region or RRDR.

Second, when using molecular testing to detect rifampin resistance, one must consider that some mutations are associated with low-level, but clinically significant, rifampin resistance. An isolate possessing certain rifampin-resistance associated mutations may show susceptibility when using an automated broth-based drug susceptibility test (DST) method. Growth-based testing for susceptibility in TB generally involves exposing an isolate to a single concentration of antituberculosis drug (known as the critical concentration) to see if the organism can grow in the presence of the drug and is resistant.

Mutations that lead to low-level rifampin resistance can result in discordance between molecular and growth-based DST results due to low level resistance close to the critical concentration. Isolates with these types of mutations may test as susceptible. However, literature case reports indicate these mutations may result in clinically significant rifampin resistance.

Notes:

It is critical to confirm results from Xpert MTB/RIF by DNA sequencing.
Third, silent mutations such as Phe514Phe and Arg529Arg may also be detected by molecular methods. Isolates with these mutations are susceptible to rifampin by growth-based testing and the mutations are not associated with resistance. Of note is that the presence of these mutations may lead to false prediction of resistance if the mutation is detected by a non-sequencing based test such as the Xpert MTB/RIF assay. It is critical to confirm results from Xpert MTB/RIF by DNA sequencing.

4.7 Isoniazid

Notes:

Isoniazid (also called INH) is used as a first-line agent in the treatment of both TB disease and TB infection (latent TB).

It inhibits cell wall synthesis by interfering with production of mycolic acid, a major component of the Mycobacterium tuberculosis cell wall.

The most common genes associated with INH resistance are katG and inhA. Most mutations associated with high-level isoniazid resistance are found in katG while the most common mutations associated with low-level resistance are found in the inhA promoter region.
4.8 Considerations for Detection of Isoniazid (INH) Resistance

Considerations for Detection of Isoniazid (INH) Resistance

- About 15% of isoniazid resistant (INH-R) strains do not have a mutation detected in *inhA* or *katG*
- Mutations in the *inhA* promoter region (nucleotide change only) are associated with cross-resistance to the drug ethionamide
- Additional loci where mutations associated with INH-R have been reported include:
  - *fabG1* (*mabA*)
  - *ahpC*

Notes:

When using molecular testing for detection of isoniazid resistance, it must be taken into consideration that approximately 15% of isoniazid-resistant isolates do not have a detectable mutation in *inhA* or *katG*. Also, mutations in the *inhA* promoter region (nucleotide change only) are associated with cross-resistance to the drug ethionamide. Additional loci where mutations associated with isoniazid resistance have been detected include *fabG1* (*mabA*) and *ahpC*.
4.9 Considerations for Detection of Ethambutol (EMB) Resistance

Considerations for Detection of Ethambutol (EMB) Resistance

- 30 to 70% of EMB-resistant strains have a mutation in *embB*
- Resistance mutations have also been found in *embC* and *embA* genes
- Not all mutations associated with resistance to EMB are known
- Results from molecular testing may be discordant with results observed with growth-based testing methods
- All EMB mono-resistant isolates should be questioned as EMB mono-resistance is rare and is most commonly associated with resistance to isoniazid

Notes:

Ethambutol (EMB) used in initial combination antituberculosis therapy minimizes the risk of drug resistance development to companion first line drugs, particularly isoniazid. Thirty to seventy percent of EMB resistant strains have a mutation in *embB*, however, mutations have also been found in *embC* and *embA* genes. Not all mutations associated with resistance to EMB are known. Molecular testing results may be discordant with growth-based testing method results. EMB mono-resistant isolates should be questioned as EMB mono-resistance is rare, and EMB resistance is commonly associated to isoniazid.
4.10 Considerations for Detection of Pyrazinamide (PZA) Resistance

Notes:

Pyrazinamide (also called PZA) has important sterilizing activity that shortens the duration of TB treatment when used in combination with rifampin. It is a prodrug that is converted to its active form, pyrazinoic acid, by a pyrazinamidase encoded by pncA, which can also be pronounced “pink A”. Inactivation of pyrazinamidase due to pncA mutations is a major cause of PZA resistance. Seventy to ninety percent of PZA resistant isolates have a pncA mutation.

Detection of mutations within pncA may be more reliable than using growth-based testing due to methodologic issues involved with growth-based tests for PZA. Not all mutations in pncA are associated with resistance. M. bovis and M. bovis BCG are naturally resistant to PZA due to a His57Asp substitution within pncA.

• 70–90% of PZA resistant isolates have a pncA mutation
• Detection of mutations within pncA may be more reliable than using growth-based testing especially to detect resistance
• *M. bovis* and *M. bovis* BCG are naturally resistant to PZA due to a His57Asp substitution within pncA
4.11 Considerations for Detection of Fluoroquinolones (FQ) Resistance

Notes:

Fluoroquinolones (FQ) are the most effective second-line drugs for patients with MDR TB. Commonly used FQs include: moxifloxacin (MOX), levofloxacin (LVX), ofloxacin (OFL), and ciprofloxacin (CIP). FQs inhibit DNA gyrase, an enzyme, that is essential for DNA replication, transcription, and recombination.

\(\text{gyrA}\) is the most common gene involved in FQ resistance. \(\text{gyrA}\) mutations are found in the quinolone resistance determining region (QRDR). \(\text{gyrB}\) mutations are also associated with resistance.

Heteroresistance, which refers to the presence of both drug-resistant and drug-susceptible sub-populations can result in discordance between molecular and growth-based tests due to differences in the limit of detection of the specific assay used. For example, a subpopulation of resistant organisms may not be detected using molecular methods but will grow in the presence of the drug and is resistant. Patterns of cross-resistance may also vary among mutations.

Low and high level resistance may be observed with MOX.
4.12 Considerations for Detection of Second-Line Injectable Resistance

Common second-line injectable drugs include amikacin (AMK), kanamycin (KAN), and capreomycin (CAP). These drugs inhibit protein synthesis. The most common genes involved in second-line injectable resistance are rrs, eis, and tlyA which can also be pronounced "till A".

It must be taken into consideration that not all mechanisms of resistance are known, there are variable cross-resistance patterns between the second-line injectable drugs, as well as variable critical concentrations used for growth-based testing of capreomycin.
4.13 Limitations of Molecular Testing

Notes:

As with all laboratory tests, molecular testing has some limitations.

Not all mechanisms of resistance are known and therefore the lack of detection of a mutation does not necessarily imply that the organism is susceptible to a given drug. The sensitivity of the loci examined for each drug should be considered. Also, not all mutations correlate with growth-based resistance.

Another limit of molecular testing is that it may not detect emerging resistance in a bacterial population with a mix of wild type and mutants, commonly referred to as heteroresistance. This is due to the limit of detection associated with molecular tests.

In addition, the clinical significance of many mutations is unknown, new mutations can be detected, and different mutations may result in varying levels of resistance.

Our knowledge concerning molecular testing continues to expand. Evolving molecular methods such as Next Generation Sequencing (NGS) (including whole genome sequencing) may improve sensitivity and increase the amount of data available but may also provide new challenges as well.
An isolate that does not have an *inhA* or *katG* mutation is susceptible to isoniazid.

- [ ] True
- [x] False
5. Detecting Mutations Associated with Drug Resistance in M. tuberculosis

Notes:

Now that we’ve discussed how mutations in *M. tuberculosis* can lead to drug resistance, let’s look at some of the more common molecular tests used to detect mutations associated with drug resistance.
5.2 Growth-Based Testing for Drug Susceptibility

Notes:

There are two testing approaches for drug resistance: growth-based testing and molecular testing.

Growth-based testing is used to determine if an isolate grows in the presence of antituberculosis drugs. If >1% of the bacterial population or (>10% for PZA) grows in the presence of the critical concentration of the drug (as compared to growth in the absence of drug), it is considered resistant.

The images shown are a set of BBL Mycobacteria Growth Indicator Tubes, often referred to as MGIT tubes and the MGIT growth-monitoring instrument. In order to perform DST, a MGIT tube containing the antituberculosis drug is inoculated and then inserted into the MGIT instrument. Bacterial growth above a specific threshold indicates the isolate tested is resistant to the drug.

Advantages of growth-based testing include the following: currently considered the gold standard for drug susceptibility testing (DST), it is sensitive and specific, growth can be observed, and the results are quantifiable. Some considerations for this approach include slow growth - requiring weeks to months from specimen collection to obtain a DST result, differences in results obtained from the same clinical specimen between laboratories and different methods, difficulty in testing some drugs, and the need for
expertise to conduct the testing.

The increasing use of molecular tests have brought new issues to light regarding growth-based DST including the concept that different mutations can result in variable growth-based results.

5.3 Molecular Testing for Drug Resistance

Molecular testing for drug resistance detects mutations. Mutations are identified by comparing an organism’s sequence to a wild-type or reference sequence.

There are several advantages of molecular testing: Rapid turnaround time for results; the ability to provide some information on drug resistance in cases where the results of growth-based testing are unavailable, for example when there is no growth or contamination; and testing can be performed using either Mycobacterium tuberculosis isolates or clinical specimens.

There are few considerations for molecular testing such as not all mechanisms of drug resistance are known, not all mutations detected are associated with growth-based drug resistance, absence of a mutation does not necessarily mean susceptibility, and the limit of detection can vary among tests.
At this time, molecular testing does not replace growth-based testing such as DST. Although as mentioned, there may be instances where molecular results are the only ones available.

5.4 Molecular Tests to Detect Mutations

Notes:
In this module, we will focus on molecular tests commonly used in the United States to detect mutations in the DNA sequence of *M. tuberculosis*. Specifically, a DNA probe-based technique (Cepheid Xpert® MTB/RIF) and sequencing techniques (Sanger sequencing and Pyrosequencing) will be discussed.
5.5 Non-sequencing Based Techniques

The first category of molecular tests for detecting mutations in M. tuberculosis that we will discuss are considered non-sequencing based techniques.

Xpert® MTB/RIF (Cepheid) is a commercial real-time PCR test. It is an automated system for identifying M. tuberculosis and detecting mutations associated with resistance to the first-line drug rifampin in the rifampin resistance determining region (RRDR) of rpoB. Advantages of this test include fast results (approximately two hours) from when the test is started in the instrument, a multi-purpose platform, and its closed system in which DNA extraction, amplification, and detection occur within a single-use disposable cartridge.

There are a few considerations with this test. First, silent mutations could lead to false prediction of resistance. Second, if there is a population of mycobacteria with mixed wild-type and resistance characteristics, Xpert may not detect the resistant population as it looks for wild-type. Finally, Xpert MTB/RIF has only been approved by FDA for testing of sputum specimens.

Line probe assays are not FDA approved and are not commonly used in the United States. Laboratories may also use what is called a “laboratory developed test (LDT)” that might be probe-based as in a real-time PCR test.
5.6 DNA Sequencing Techniques

Notes:

The second category of molecular tests for detecting mutations in M. tuberculosis that we will discuss are sequencing techniques. Sequencing is the process of determining the specific order of bases within a DNA molecule.

Two commonly used DNA sequencing techniques include Sanger and Pyrosequencing. Next Generation Sequencing (NGS) is also utilized in some laboratories. Generally there is a 2-3 day turnaround time, however, NGS may take longer. With sequencing, the resulting sequence is always compared to a reference sequence. Specific mutations may be identified that provide information on drug resistance. These results may then be used with growth-based DST results.
5.7 Knowledge Check Four

Molecular testing for drug resistance is:

- A. Relatively fast
- B. Able to provide results in cases where growth-based results are unavailable
- C. Both A and B

Notes:
There is no audio on this slide.
6. Interpreting Results from Drug Resistance Testing of Mycobacterium tuberculosis

Notes:
Now that we’ve looked at some of the common molecular tests used to detect mutations associated with drug resistance in M. tuberculosis, let’s spend some time on how to interpret results from such tests.
6.2 Interpretation of Results

Notes:

The results from molecular tests will look different from the results obtained from growth-based DST.

You may not see a definitive “susceptible” or “resistant” outcome for each target examined using molecular techniques.

When reporting sequencing results, the testing laboratory may present the proportion of total samples received that were resistant to the particular drug, given the same mutation.

The interpretation of molecular test results is dependent on the testing platform used and the drug being tested.
6.3 Interpreting Xpert® MTB/RIF Results

This table shows examples of four different results provided by Xpert® MTB/RIF. You may recall from earlier in the course that Xpert® is an automated system for identifying M. tuberculosis complex and detecting mutations associated with resistance to the first-line drug rifampin.

The first two columns shown are taken directly from the Xpert® MTB/RIF package insert while the minimum reporting language as recommended by CDC is shown in the third column. Laboratories are encouraged to enhance and customize this basic reporting language.

Please take a minute to review the table.
6.4 Understanding Sequence-based Results

Before we examine how DNA sequencing results are interpreted, let’s review the nomenclature used when presenting sequence-based results.

Mutations may be reported by describing the DNA change and, when applicable, the amino acid change that occurs at the protein level. The example shown here is for a mutation that is commonly seen in the rpoB gene of RIF resistant isolates. The wild-type codon sequence is TCG, while the mutant sequence is TTG, which is a point mutation (SNP). Some laboratories may include the codon number (which is 531) for this locus when reporting the test result, in addition to the wildtype amino acid (which is Serine) to the left of the codon number and the mutant amino acid (which is Leucine) to the right of the codon number, thus the report ‘Ser531Leu’.

Alternatively, other laboratories may represent the mutation using only the codon number and mutant amino acid: ‘531Leu’. Other laboratory reports may abbreviate the amino acid names to single letters, thus ‘S531L’.

A laboratory may choose to represent the mutation result using the codon number, the mutant amino acid, and add the mutant codon in parentheses, thus 531Leu(TTG).

If the mutation occurs before the start of the open reading frame, a negative number is
6.5 Case 1: DNA Sequencing Result Interpretation

Let us now examine DNA sequencing results from three different patients. This table shows results for an isolate obtained from the first patient. The first column of the table shows the genes and loci examined, the second column indicates whether or not a mutation was detected and describes the specific mutation, and the third column provides an interpretation.

For the rpoB testing result, the nomenclature TCG>TTG denotes that the nucleotide Cytosine in the codon TCG mutates to Thymine, which results in a change in the amino acid, from the wild-type Serine to Leucine.

No mutation was detected when the promoter region of the inhA gene was examined.

For katG, the nucleotide Guanine in the codon AGC is changed to Cytosine, resulting in a change in the amino acid, from the wild-type Serine to Threonine.

The mutation in the rpoB gene (Ser531Leu) is known to confer resistance to rifampin while the katG gene (Ser315Thr) mutation is known to confer resistance to isoniazid.
Therefore, the interpretation states that this isolate is resistant to both rifampin and isoniazid.

**6.6 Case 2: DNA Sequencing Result Interpretation**

<table>
<thead>
<tr>
<th>Gene examined</th>
<th>Result</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB (RRDR)</td>
<td>No mutation</td>
<td>Probably Rifampin susceptible. (97% of RIF-R isolates have a mutation at this locus.)</td>
</tr>
<tr>
<td>inhA (promoter)</td>
<td>No mutation</td>
<td>Cannot rule out Isoniazid resistance. (86% of INH-R isolates have a mutation at one or both of these loci.)</td>
</tr>
<tr>
<td>katG (Ser315 codon)</td>
<td>No mutation</td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**

This table shows results for an isolate obtained from the second patient. No mutations were found in any of the three genes associated with resistance to Rifampin and Isoniazid.

However, notice the interpretation for the rpoB result. There is a distinct difference in the language used when compared to that for inhA and katG (isoniazid resistance-associated) genes. The difference in interpretation is because the percentage of certainty at which the lack of a mutation in the genes is associated with susceptibility is higher for rifampin than it is for isoniazid, 97% vs. 86%, respectively. This is because although katG and inhA are the most common, a number of different loci are implicated in INH resistance.
### 6.7 Case 3: DNA Sequencing Result Interpretation

This table shows pyrosequencing results for an isolate obtained from a third patient. The isolate has no mutations within the RRDR of the rpoB gene. The report indicates that the isolate is "probably" susceptible to Rifampin because the vast majority of rifampin-resistant isolates (>95%) have a mutation at this locus.

The result for inhA, C-15T, is a common mutation that involves a nucleotide change in the promoter region with no amino acid change. The isolate is considered resistant to Isoniazid.

<table>
<thead>
<tr>
<th>Gene examined</th>
<th>Result</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB (RRDR)</td>
<td>No mutation</td>
<td>Probably Rifampin susceptible. (97% of RIF-R isolates have a mutation at this locus.)</td>
</tr>
<tr>
<td>inhA (promoter)</td>
<td>C-15T</td>
<td>100% of isolates with this mutation are isoniazid resistant.</td>
</tr>
<tr>
<td>katG (Ser315 codon)</td>
<td>No mutation</td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**

This table shows pyrosequencing results for an isolate obtained from a third patient. The isolate has no mutations within the RRDR of the rpoB gene. The report indicates that the isolate is “probably” susceptible to Rifampin because the vast majority of rifampin-resistant isolates (>95%) have a mutation in the RRDR.

The result for inhA, C-15T, is a common mutation that involves a nucleotide change in the promoter region with no amino acid change. The isolate is considered resistant to Isoniazid.
6.8 Interpreting DNA Sequencing Results for Other Genes/Loci

Notes:

DNA sequencing is also used to detect mutations in various other genes.

In this example, a mutation was found in gyrA. A GCG>GGC mutation was found in the Quinolone resistance determining region (QRDR), known to confer resistance to Ofloxacin.

For pncA and rrs, the statement that resistance cannot be ruled out is provided as the absence of a mutation does not absolutely rule out resistance.
6.9 What to Consider when Reading Interpretative Comments

Notes:

The interpretations of molecular testing outcomes will differ among laboratories and will depend on testing methods used and drugs being tested.

If a mutation is found, the reporting language may vary from “resistant” to “associated with resistance” and from “possibly resistant” to “probably resistant”. Some laboratories may not provide an interpretation.

In the same way, if no mutation is found, reporting language may vary from “susceptible”, or “cannot rule out resistance” to “suggests susceptibility” or “likely susceptible”.

Currently, standards for interpretative comments and tools to aid in interpretation are not available.
For this reason, it is imperative that medical providers put the laboratory results in context with the patient’s medical history and clinical presentation. In instances where a mutation is found but there are few to no previous reports of that particular mutation, the report will say the mutation is present and is novel, but its role in conferring resistance is unknown. In such cases, growth-based DST results may help to guide therapy.
6.11 Discordant Results

Notes:
Growth-based drug susceptibility test results and molecular results can differ. The next two slides highlight common examples of possible discordance between results, examine why discordance may occur, and offer suggestions on how to interpret results.
6.12 Discordant Results: Rifampin

This table shows two scenarios of discordant results obtained when comparing results from growth-based DST and molecular methods: Xpert and sequencing.

In the first scenario, the growth based DST result indicates Rifampin susceptibility, indicated by the “S”, however, Xpert detects Rifampin resistance. Results from sequencing clarify the mutation detected is silent and therefore, has no effect on drug resistance.

In scenario 2, the growth based result indicates Rifampin susceptibility, however, Xpert indicates resistance. However, sequencing in this example identified a mutation associated with low-level RIF resistance not detected by growth based DSTs. The clinical significance of isolates with this mutation is not completely understood, but some studies have shown poor patient outcomes associated with this mutation.

These two scenarios are just examples of possible cases where discordant results may be obtained when testing for rifampin resistance.
6.13 Summary of Result Interpretation

Summary of Result Interpretation

- All laboratory tests are subject to false positives and false negatives—no laboratory test is perfect
- Not all mechanisms of resistance are known
  - Lack of mutation does not necessarily mean susceptibility
- Not all mutations cause resistance
  - Some are new or “novel”—role in resistance is unknown or less characterized
  - Some are silent or synonymous mutations
- When in doubt, call the laboratory and discuss the results
  - Most laboratory staff are very willing to provide additional information

Notes:

There are several things to keep in mind when interpreting molecular test results. First, all laboratory tests are subject to false positives and false negatives. No laboratory test is perfect. Second, not all mechanisms of resistance are known, and therefore, finding no mutation using a given molecular test does not equate to drug susceptibility. Another point to keep in mind is that not all mutations cause resistance. As we saw in our examples, some mutations detected by molecular testing are novel and their role in resistance is unknown or less characterized. Some mutations are silent or synonymous, so that the DNA sequence is changed but the resulting amino acid sequence remains the same. Therefore, structure or function of the resulting protein is not affected. Call the laboratory to discuss results when unsure of interpretation. Most laboratory staff are very willing to provide additional information.
6.14 Knowledge Check Five

Which of the following actions should a medical professional take if the interpretation for a molecular test result provided by the laboratory appears to be ambiguous or contradictory?

- A) Consult with the laboratory for clarification
- B) Consider the laboratory results in light of the patient’s clinical presentation, country of origin and travel history
- C) Consider that there are still gaps in the knowledge about all the mutations that may lead to antibiotic resistance
- D) Consider all mutations result in phenotypic resistance
- E) A, B, and C are correct

Notes:
There is no audio on this slide.
6.15 Conclusion

Notes:

In conclusion, we hope that you have learned about the basic principles of molecular biology and mechanisms of drug resistance, the association of mutations with resistance to drugs for treatment of TB, and test platforms used to detect drug resistance and reporting language.

Lastly, we hope we have left you with some key points to reflect on as you encounter drug susceptibility testing results including, that mutations may or may not result in drug resistance. Some mutations will have no effect on drug resistance. Not all mechanisms of resistance are known and interpretation of drug resistance results is not always easy, consult with the laboratory that performed the test for additional information.
6.16 Course Complete

Thank You for Participating!

Landscape and Language of Molecular Diagnostics for TB Drug Resistance

Please review the additional modules from the Association of Public Health Laboratories "Essentials for the Mycobacteriology Laboratory: Promoting Quality Practices" especially:

- Molecular Detection and Identification of Mycobacteria
- Molecular Detection of Drug Resistance


Notes:

This concludes “Landscape and Language of Molecular Diagnostics for TB Drug Resistance” which is part of a series from the APHL’s “Essentials for the Mycobacteriology Laboratory Promoting Quality Practices.” Please see the CDC and APHL websites for more information on these topics.