Landscape and Language of Molecular Diagnostics for TB Drug Resistance
Purpose

This module will provide:

• A brief overview of basic principles of molecular biology

• An introduction to mutations and their association with drug resistance in *Mycobacterium tuberculosis*

• An overview of methods used in molecular testing of *M. tuberculosis*

• Examples to aid interpretation of molecular results
I. Basic Principles of Molecular Biology
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
Central Dogma of Molecular Biology

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)

[Image of DNA structure]

Characteristics of RNA

- Ribonucleic acid
- Single-stranded
- Formed by Adenine (A), Cytosine (C), Guanine (G), and Uracil (U)
- Transcribed from DNA by an enzyme called RNA polymerase
- RNA transcribed from a gene is messenger RNA (mRNA)

Amino Acids and Proteins

- Amino acids are the building blocks of proteins
- mRNA is read in sets of three bases, called codons
- Each codon encodes one amino acid in the protein

http://bio1152.nicerweb.com/Locked/media/ch17/central_dogma.html
**Gene**

A distinct sequence of bases along a segment of DNA that provide the coded instructions for synthesizing a protein or RNA molecule.

**Codon**

A combination of three consecutive bases within a gene that specifies a particular amino acid.

**Locus**

A region of interest in a gene.
Reading Frame

- Begins with a start codon that signals initiation of translation.
- Most common start codon is AUG.
- Translation continues, three bases at a time, each triplet sequence coding for a single amino acid.
- Ends with a stop codon that signals termination of the synthesis of a protein.
- Common stop codons are UAA, UAG, or UGA.

http://www.ucl.ac.uk/~sjjgsca/translation.html
### Genetic Code

<table>
<thead>
<tr>
<th>First letter</th>
<th>Second letter</th>
<th>Third letter</th>
<th>Key:</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>U</td>
<td>U</td>
<td>Ala = Alanine (A)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>C</td>
<td>Arg = Arginine (R)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>A</td>
<td>Asn = Asparagine (N)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>G</td>
<td>Asp = Aspartate (D)</td>
</tr>
<tr>
<td></td>
<td>UUU</td>
<td>U</td>
<td>Cys = Cysteine (C)</td>
</tr>
<tr>
<td></td>
<td>UUC</td>
<td>C</td>
<td>Gin = Glutamine (Q)</td>
</tr>
<tr>
<td></td>
<td>UUA</td>
<td>A</td>
<td>Glu = Glutamate (E)</td>
</tr>
<tr>
<td></td>
<td>UUG</td>
<td>G</td>
<td>Gly = Glycine (G)</td>
</tr>
<tr>
<td></td>
<td>CUU</td>
<td>U</td>
<td>His = Histidine (H)</td>
</tr>
<tr>
<td></td>
<td>CUC</td>
<td>C</td>
<td>Ile = Isoleucine (I)</td>
</tr>
<tr>
<td></td>
<td>CUA</td>
<td>A</td>
<td>Leu = Leucine (L)</td>
</tr>
<tr>
<td></td>
<td>CUG</td>
<td>G</td>
<td>Lys = Lysine (K)</td>
</tr>
<tr>
<td></td>
<td>AUU</td>
<td>C</td>
<td>Met = Methionine (M)</td>
</tr>
<tr>
<td></td>
<td>AUC</td>
<td>A</td>
<td>Phe = Phenylalanine (F)</td>
</tr>
<tr>
<td></td>
<td>AUA</td>
<td>G</td>
<td>Pro = Proline (P)</td>
</tr>
<tr>
<td></td>
<td>AUG</td>
<td>G</td>
<td>Ser = Serine (S)</td>
</tr>
<tr>
<td></td>
<td>AUU</td>
<td>U</td>
<td>Thr = Threonine (T)</td>
</tr>
<tr>
<td></td>
<td>AUC</td>
<td>C</td>
<td>Trp = Tryptophan (W)</td>
</tr>
<tr>
<td></td>
<td>AUA</td>
<td>A</td>
<td>Tyr = Tyrosine (Y)</td>
</tr>
<tr>
<td></td>
<td>AUG</td>
<td>G</td>
<td>Val = Valine (V)</td>
</tr>
</tbody>
</table>

- 64 codons for 20 amino acids (redundancy)
Knowledge Check One

Each codon codes for a specific:

A. Gene
B. Amino acid
C. Protein
II. Introduction to Mutations
Mutations

• The typical or common version of a gene is often referred to as wild-type

• A mutation is a change in the DNA sequence resulting in variation from previous generations that may be transmitted to subsequent generations

• Range in size from a single base to large segments of DNA

• Possible causes of mutations
  – Mutagens (UV-light, chemicals)
  – Spontaneous (replication errors)
Types of Mutations

- **Point mutations** (Single Nucleotide Polymorphisms or SNPs)
  - **Missense** (non-synonymous)
  - **Silent** (synonymous)
  - **Nonsense** (stop codon)
- **Frameshift**
- **Insertion**
- **Deletion**
Mutations Illustrated

**Normal message:**  AS THE MAN SAW THE DOG HIT THE CAN END ITI

**Point Mutation:**  AS THE MAN SAW THE DOT HIT THE CAN END ITI

**Deletion:**  AS THE MAN SAW THE*HIT THE CAN END ITI

**Insertion:**  AS THE MAN SAW THE FAT DOG HIT THE CAN END ITI

**Frameshift:**  AS THE MAN SAW THE *OGH ITT HEC ANE NDI TIS

Adapted From:  http://www.brooklyn.cuny.edu/bc/ahp/BioInfo/MUT/Mut.Types.html
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
III. Mutations and Drug Resistance in *Mycobacterium tuberculosis*
Mechanisms of Action of the Primary Anti-tuberculosis Drugs

- **Isoniazid (1952)**: Inhibits cell wall synthesis.
- **Ethambutol (1961)**: Inhibits cell wall synthesis.
- **Pyrazinamide (1952)**: Exact target unclear, disrupts plasma membrane, disrupts energy metabolism.

**Mycobacterium tuberculosis**

- **DNA Coiling, Transcription, and Translation**
  - RNA polymerase
  - DNA gyrase
  - mRNA
  - Ribosome
  - Protein

- **Cell Wall Synthesis**
  - Acyl Lipids
  - Mycolic Acid
  - Arabinogalactan
  - Peptidoglycan
  - Plasma Membrane

- **ATP Synthesis**

*National Institute of Allergy and Infectious Diseases (NIAID)*
Potential Impact of Mutations

- No effect on drug resistance
- Change in protein structure inhibiting drug activity (e.g., no activation of prodrug)
- Change in protein structure such that drug cannot bind to target
- Change in promoter region leading to changes in expression level to overcome the effects of drug
- Change in rRNA or modifying enzyme (change in affinity for drug)
- Different levels of resistance depending on the specific mutation
Development of Drug Resistance

- Resistance is linked to large bacterial populations
  - Mutants resistant to any drug naturally occur on average once in every $10^6$ to $10^8$ cells
  - The probability of the presence of multiple mutations resulting in resistance to different drugs is extremely small; hence the reason for a multidrug therapy
Drug-resistant mutants in large bacterial population

Monotherapy: isoniazid-resistant bacteria proliferate

Isoniazid

Isoniazid-resistant bacteria multiply to $>10^8$ and spontaneous mutations develop to rifampicin

Isoniazid-monoresistant mutants killed with addition of rifampicin, but rifampicin-resistant mutants proliferate → MDR TB
Considerations for Detection of Rifampin (RIF) Resistance

- Over 95% of isolates resistant to rifampin have a mutation in the rifampin resistance determining region (RRDR) of the \textit{rpoB} gene.

- Some mutations are associated with low-level but clinically significant rifampin resistance (RIF-R):
  - Isolate may be found susceptible using a commercial growth-based drug susceptibility testing (DST) method.
  - May result in discordance between molecular and growth-based methods.

- Potential for the presence of silent mutations (Phe514Phe, Arg529Arg) and false-resistance detection by some molecular methods.

\textbf{It is critical to confirm results from Xpert MTB/RIF by DNA Sequencing.}
Considerations for Detection of Isoniazid (INH) Resistance

- About 15% of isoniazid resistant (INH-R) strains do not have a mutation detected in \textit{inhA} or \textit{katG}

- Mutations in the \textit{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:
  - \textit{fabG1 (mabA)}
  - \textit{ahpC}
Considerations for Detection of Ethambutol (EMB) Resistance

- 30 to 70% of EMB-resistant strains have a mutation in \textit{embB}.
- Resistance mutations have also been found in \textit{embC} and \textit{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.
Considerations for Detection of Pyrazinamide (PZA) Resistance

- 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
Considerations for Detection of Fluoroquinolones (FQ) Resistance

- Commonly used FQs include: moxifloxacin (MOX), levofloxacin (LVX), ofloxacin (OFL), and ciprofloxacin (CIP)
- Resistance most commonly associated with mutations in the quinolone resistance determining region (QRDR) of gyrA
  - Mutations in gyrB also thought to be associated with resistance
- Heteroresistance can result in discordance between molecular and growth-based tests due to differences in the limit of detection
- Patterns of cross-resistance vary among mutations
- Low and high level resistance may be observed with MOX
Considerations for Detection of Second-Line Injectable Resistance

- Second-line injectables include: amikacin (AMK), kanamycin (KAN), and capreomycin (CAP)
- Inhibit protein synthesis
- Common genes involved in resistance: *rrs*, *eis*, and *tlyA*
  - *rrs*—AMK, KAN, and CAP
  - *eis*—KAN
  - *tlyA*—CAP
- Not all mechanisms of resistance are known
- Variable cross-resistance patterns
- Different laboratories may use different critical concentrations for growth-based testing of CAP
Limitations of Molecular Testing

• Not all mechanisms of resistance are known
• Absence of a mutation in the gene target examined does not necessarily equal susceptibility
• Not all mutations correlate with growth-based resistance
• All tests have a limit of detection
• Unknown clinical significance for some mutations
Knowledge Check Three

An isolate that does not have an \textit{inhA} or \textit{katG} mutation is susceptible to isoniazid.

A. True
B. False
IV. Detecting Mutations Associated with Drug Resistance in *M. tuberculosis*
Growth-based testing is used to determine if an isolate grows in the presence of anti-tuberculosis drugs.

- If >1% grows in the presence of critical concentration of the drug, it is considered resistant.
- >10% for PZA

**Advantages**
- Highly sensitive and specific
- Can observe growth
- Quantitative

**Considerations**
- Slow growth – weeks to months
- Discordant results between laboratories and methods
- Some drugs difficult to test
- High complexity testing
Molecular Testing for Drug Resistance

Detects mutations by comparing an organism’s sequence to a wild-type or reference sequence

Advantages

• Detects mutations in hours to days
• Provides some information on drug resistance in cases where growth-based results are unavailable (e.g. no growth, contamination)
• Isolates and clinical specimens can be tested

Considerations

• Not all mechanisms of resistance are known
• Not all mutations detected are associated with growth-based drug resistance
• Absence of a mutation does not necessarily mean susceptible
• Limit of detection
# Molecular Tests to Detect Mutations

<table>
<thead>
<tr>
<th>Test Purpose</th>
<th>Specimen Types</th>
<th>Importance</th>
<th>Platforms Available</th>
</tr>
</thead>
</table>
| Detection of Mutations Associated with Resistance | - Clinical specimens  
- MTB-positive cultures | - Rapid results for prompt patient therapy decisions | - Cepheid Xpert® MTB/RIF  
- Sanger sequencing  
- Pyrosequencing  
- Next Generation Sequencing (including whole genome sequencing)  
- Line Probe Assays |
## Non-sequencing Based Techniques

<table>
<thead>
<tr>
<th>Technique</th>
<th>Result(s)</th>
<th>Advantages</th>
<th>Considerations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cepheid Xpert® MTB/RIF</td>
<td>Detection of MTB</td>
<td>Rapid test (~2 hrs run time)</td>
<td>Silent mutations within the <em>rpoB</em> region may result in false prediction of resistance</td>
</tr>
<tr>
<td></td>
<td>Detection of mutations associated with rifampin resistance</td>
<td>Multi-use platform</td>
<td>May miss resistance in mixed populations</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Closed system</td>
<td>FDA approved for sputum specimens only</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>If RIF resistance detected, should be followed up with sequence-based testing for confirmation</td>
</tr>
<tr>
<td>Line probe assay (e.g., HAIN)</td>
<td>Identification of MTB</td>
<td>Rapid test</td>
<td>Silent mutations may result in false prediction of resistance</td>
</tr>
<tr>
<td></td>
<td>Detection of mutations associated with drug resistance</td>
<td>Some also detect drug resistance other than rifampin</td>
<td>Contamination risk due to open system</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Not FDA approved</td>
</tr>
</tbody>
</table>
DNA Sequencing Techniques

- Sanger, pyrosequencing, and next generation sequencing (NGS)
- Generally a 2–3 day turnaround time; NGS may take longer
- Resulting sequence is compared to a reference sequence
- Allows for specific mutations to be identified and analyzed for resistance prediction
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. A and B
V. Interpreting Results from Drug Resistance Testing of \textit{Mycobacterium tuberculosis}
Interpretation of Results

Results of molecular tests will look different from growth-based DST results.

- For molecular tests, you may not see a definitive “susceptible” or “resistant” for each target examined.
- For sequencing results, the laboratory may report the proportion of total samples received that were resistant to the particular drug, given the same mutation.
- Interpretation of molecular results is dependent on the testing platform used and the drug being tested.
Interpreting Xpert® MTB/RIF Results

<table>
<thead>
<tr>
<th>Instrument result</th>
<th>Interpretation</th>
<th>Minimum recommended reporting language*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTB DETECTED; RIF Resistance DETECTED</td>
<td>The MTB target is detected within the sample: • A mutation in the rpoB gene has been detected.</td>
<td>MTBC detected. A mutation in rpoB has been detected, indicating possible RIF resistance. Confirmatory testing should follow.</td>
</tr>
<tr>
<td>MTB DETECTED; RIF Resistance NOT DETECTED</td>
<td>The MTB target is detected within the sample: • A mutation in the rpoB gene has not been detected.</td>
<td>MTBC detected. No rpoB mutation; suggests probably RIF susceptible.</td>
</tr>
<tr>
<td>MTB DETECTED; RIF Resistance INDETERMINATE</td>
<td>The MTB target is detected within the sample: • A mutation in the rpoB gene could not be determined due to insufficient signal detection</td>
<td>MTBC detected, rpoB gene mutations cannot be accurately determined.</td>
</tr>
<tr>
<td>MTB not detected</td>
<td>MTB target is not detected within the sample.</td>
<td>MTBC not detected.</td>
</tr>
</tbody>
</table>

To be consistent with the Xpert MTB/RIF assay package insert, MTB and MTBC= Mycobacterium tuberculosis complex; and RIF and RMP = rifampin.

Understanding Sequence-based Results

### Interpreting DNA Sequencing Results: 1

<table>
<thead>
<tr>
<th>Gene examined</th>
<th>Result</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rpoB</em> (RRDR)</td>
<td><strong>Mutation:</strong> TCG&gt;TTG; Ser531Leu</td>
<td>Rifampin resistant (97% of RIF-R isolates have a mutation in the RRDR)</td>
</tr>
<tr>
<td><em>inhA</em> (promoter)</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td><em>katG</em> (Ser315 codon)</td>
<td><strong>Mutation:</strong> AGC&gt;ACC; Ser315Thr</td>
<td>Isoniazid resistant</td>
</tr>
</tbody>
</table>
Interpreting DNA Sequencing Results: 2

<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>
### Interpreting DNA Sequencing Results: 3

<table>
<thead>
<tr>
<th>Gene examined</th>
<th>Result</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rpoB</em> (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><em>inhA</em> (promoter)</td>
<td>C-15T</td>
<td>100% of isolates with this mutation are isoniazid resistant.</td>
</tr>
<tr>
<td><em>katG</em> (Ser315 codon)</td>
<td>No mutation</td>
<td></td>
</tr>
</tbody>
</table>
Interpreting DNA Sequencing Results for Other Genes/Loci

<table>
<thead>
<tr>
<th>Gene examined</th>
<th>Result</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pncA (promoter, coding region)</td>
<td>No mutation</td>
<td>Cannot rule out PZA resistance. (86% of PZA-resistant isolates have a mutation at this locus)</td>
</tr>
<tr>
<td>gyrA (QRDR)</td>
<td>Mutation: GCG&gt;GGC: (Asp94Gly)</td>
<td>Ofloxacin resistant. (100% of isolates with this mutation are OFL-resistant)</td>
</tr>
<tr>
<td>rrs (1400 region)</td>
<td>No mutation</td>
<td>Cannot rule out resistance to injectable drugs (KAN, AMK, CAP). (55% - 91% of isolates resistant to KAN, AMK or CAP have a mutation at this locus)</td>
</tr>
</tbody>
</table>
What to Consider when Reading Interpretative Comments

- Interpretative comments will vary from laboratory to laboratory and will depend on test method used and drugs being tested.
- Reporting language may differ when a mutation is detected.
- Standards for interpretative comments and tools to aid in interpretation are currently not available.
What to Consider when Reading Interpretative Comments continued

- Laboratory results must be used in conjunction with a patient’s medical history and clinical presentation
- “Unknown” or “novel” mutations
  - Role in conferring resistance unknown
  - Growth-based DST may help
Discordant Results
## Discordant Results: Rifampin

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Result from growth-based DST</th>
<th>Results from Molecular Testing</th>
<th>Reason/ Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scenario 1</strong>—isolate has a Phe514Phe mutation</td>
<td>S</td>
<td>Xpert® MTB/RIF result</td>
<td>In this case, Xpert would be considered falsely-resistant. Silent mutations in <em>rpoB</em> do not alter the resulting amino acid and have been shown not to result in resistance.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNA Sequencing</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phe514Phe mutation detected; this is a silent mutation</td>
<td>In this case, Xpert would be considered falsely-resistant. Silent mutations in <em>rpoB</em> do not alter the resulting amino acid and have been shown not to result in resistance.</td>
</tr>
<tr>
<td><strong>Scenario 2</strong>—isolate has a Leu533Pro mutation in <em>rpoB</em></td>
<td>S</td>
<td>RIF resistance detected</td>
<td>In this case, growth-based DST results might be considered falsely-susceptible. Isolates with this mutation have MICs that are slightly higher than WT, but not high enough to signal resistant in MGIT. This mutation has been associated with poor patient outcomes (Van Deun, Aung et al. 2013).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results from molecular testing of *rpoB*
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
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 growth-based resistance
E. A. B, and C are correct
Conclusion

• Provided in this module were:
  – Basic principles of molecular biology and mechanisms of drug resistance
  – Association of mutations with resistance to drugs for treatment of TB
  – Test platforms used to detect drug resistance and reporting language

• Key points to reflect:
  – 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.
  – Interpretation of drug resistance results is not always easy, consult with the laboratory that performed the test for additional information.
Landscape and Language of Molecular Diagnostics for TB Drug Resistance

• Please review the additional modules from the Association of Public Health Laboratory’s “Essentials for the Mycobacteriology Laboratory: Promoting Quality Practices” especially:
  – Molecular Detection and Identification of Mycobacteria
  – Molecular Detection of Drug Resistance

www.cdc.gov/tb  www.aphl.org/tb