Molecular Detection of Drug Resistance
Molecular Detection of Drug Resistance

INTRODUCTION
Why the Increased Interest in Molecular Diagnostics for TB?

• Goal of TB elimination and recognized need for more rapid diagnosis
• Updated CDC guidelines for use of NAAT in the diagnosis of TB (June 2009)
• Advances in technology allowing the ability to simultaneously detect *M. tuberculosis* complex (MTBC) and mutations associated with drug resistance
• FDA market authorization of GeneXpert MTB/RIF assay (2013)
• Decreasing price and increased access to platforms/reagents for laboratory developed tests (LDT)
Why the Increased Interest in Molecular Diagnostics for TB? (2)

- Shift in laboratory workforce and loss of expertise for conventional TB testing methodologies
- Molecular diagnostics are commonplace for other diseases; can capitalize on skill sets of existing molecular workforce and infrastructure
- Global roll-out of GeneXpert MTB/RIF assay
- WHO policy statements for line probe assays and GeneXpert MTB/RIF
- Incorporation of molecular diagnostics in drug resistance surveys around the world
Advantages of Molecular Detection of Drug Resistance

• Phenotypic methods are not timely due to slow growth of MTBC
  – Turnaround times can be extended, requiring weeks to months from specimen receipt

• Molecular assays can reduce turnaround time to hours to days

• Can provide information on drug resistance in cases where phenotypic results are unavailable (e.g., no growth, contamination, or mixed with NTM)
Advantages of Molecular Detection of Drug Resistance (2)

- Phenotypic methods are known to be an imperfect “gold standard”
- In some cases, molecular results may be more reliable than phenotypic results (e.g., mutations resulting in low level but clinically significant RMP resistance)
- Some drugs can be difficult to test phenotypically
  - Ethambutol
    - Microcolonies → ??
    - False susceptibility in broth reported
  - Pyrazinamide
    - Acidified liquid media
    - Sensitive to over inoculation resulting in false resistance
- Phenotypic testing methods for secondary drugs are not standardized
Considerations for Use of Molecular Assays

- Molecular assays do not yet replace phenotypic drug susceptibility testing (DST)
- Not all laboratories have infrastructure or need to perform testing in-house
- High complexity assays requiring subject matter expertise
- With the exception of GeneXpert MTB/RIF, molecular assays for the detection of drug resistance are LDT or research use only (RUO)
- Positive predictive value decreases in populations with low prevalence of resistance
- Referral for molecular detection of drug resistance is available for specimens or isolates
Mutations and Drug Resistance

• Not all mechanisms of resistance are known
  – The lack of a mutation ≠ susceptibility

• Not all mutations are associated with phenotypic drug resistance
  – Mutation could be “silent”- no change in amino acid
  – Mutation may not cause significant change in protein expression, structure, or function
Mutations and Drug Resistance (2)

• Association of a mutation with drug resistance necessitates data related to its correlation with phenotypic drug resistance, functional genetics, and clinical outcomes

• Association with clinical outcomes is difficult due to multidrug therapy used to treat TB disease
Intrinsic Drug Resistance

- Hydrophobic cell envelope (permeability barrier)
- Drug efflux systems which can pump toxic substances out of cell
- Drug modifying enzymes can alter the drug configuration
- Intrinsic PZA resistance seen with some members of the MTBC due to lack of pyrazinamidase activity
  - *M. canettii*, *M. bovis* and *M. bovis* BCG (other members of MTBC are usually susceptible)
Development of Drug Resistance

• Spontaneous mutations occur in the DNA of all cells
  – Mutations can change the structure of a protein that is a drug target
  – Protein still functions, but is no longer inactivated by the drug
  – Thus, TB can grow in the presence of the drug

• Resistance is linked to large bacterial populations
  – Mutants resistant to any drug naturally occur on average once in every $10^8$ cells
  – Pulmonary TB — cavities often contain $10^7 – 10^9$ organisms
  – By using two antibiotics, chances for both targets to be mutated and resistant to both drugs is extremely small ($10^{-8} \times 10^{-8} = 10^{-16}$)
  – This is the rationale for treatment regimens with more than one drug
Other Factors Influencing the Development of Drug Resistance

- Metabolism of bacilli shifted to dormancy
  - Impaired/ decreased drug uptake by *M. tuberculosis* cell
- Penetration of drugs to various body sites
  - Suboptimal drug concentration at some body sites
    - Less than therapeutic concentration
- Impaired drug absorption due to underlying host conditions such as HIV/AIDS, diabetes
- Treatment with inappropriate drugs, combinations or dosages
- Interrupted or irregular treatments
- Incomplete treatments
  - Limited duration (e.g., stop early because feels good)
  - Required number of doses not taken
Primary vs. Acquired Drug Resistance

• Primary—Drug resistant at start of treatment, patient never treated in past thereby implying transmission of drug-resistant bacilli

• Acquired—Drug susceptible at start of treatment, becomes drug resistant during treatment
Molecular Detection of Drug Resistance

DRUGS AND ASSOCIATED GENETIC TARGETS
TB Primary Drugs and Mechanisms of Action

- **Isoniazid (1952)**
  - Inhibits cell wall synthesis

- **Ethambutol (1961)**
  - Inhibits cell wall synthesis

- **Pyrazinamide (1952)**
  - Exact target unclear
  - Disrupts plasma membrane
  - Disrupts energy metabolism

- **Rifampin (1966)**
  - Inhibits RNA synthesis

**DNA Coiling, Transcription, and Translation**
- RNA Polymerase
- DNA Gyrase
- mRNA
- Ribosome
- Protein

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

**Mycobacterium tuberculosis**

**ATP Synthesis**
- ATP
- Cell Wall

National Institute of Allergy and Infectious Diseases (NIAID)
Rifampin

• Rifampin (RMP) is the most important drug in treatment of TB

• Targets the $\beta$-subunit of the RNA polymerase to inhibit the initiation of transcription

• RMP resistance (RMP-R) can be a marker for MDR TB as mono-rifampin resistance is not common

• $rpoB$ most common gene involved in RMP-R
  - $\sim$96% of RMP-R strains carry mutations in the 81 base pair rifampin resistance determining region (RRDR)
  - Secondary site for resistance at codon 176
Common Mutations Found in the RRDR

Codon changes, in-frame deletions, in-frame insertions

From S. Ramaswamy and J.M. Musser. 1998. Tubercle and Lung Disease 79:3-29
Considerations for Detecting RMP-R

• Some mutations are associated with low-level but presumably clinically significant RMP-R that may be missed by commercial broth systems
  – Examples: Leu511Pro, Asp516Tyr, His526Asn, His526Leu, Leu533Pro, and Ile572Phe (outside the RRDR)
  – May result in discordance between phenotypic and molecular methods
  – Concern that missing RMP-R could result in poor clinical outcomes

• Some mutations that are associated with low-level RMP-R may retain rifabutin susceptibility
  – Examples: Asp516Val and His525Leu
Isoniazid

- Isoniazid (INH) is commonly used to treat contacts with latent TB infection
- Prodrug that must be activated by catalase peroxidase encoded by $katG$
  - Affects mycolic acid biosynthesis
- $inhA$ and $katG$ are the most common genes associated with INH resistance (INH-R)
  - Most common mutations associated with high-level INH-R are found in $katG$
    • Ser315Thr and Ser315Asn
  - Most common mutations associated with low-level INH-R are found in the $inhA$ promoter region
    • C-15T, T-8C
Considerations for Detecting INH Resistance

- Mutations in *inhA* promoter region are associated with cross-resistance to ethionamide
- ~15% of INH-R strains do not have an *inhA* or *katG* mutation
- Analysis of additional loci may improve detection of INH-R
  - *fabG1*(mabA)
  - *ahpC*
  - *kasA*
  - *ndh*
  - *furA*
  - *mshA*
Pyrazinamide

- Pyrazinamide (PZA) has important sterilization activity that shortens the duration of treatment when used in combination with RMP
- A prodrug that is converted to its active form, pyrazinoic acid, by a pyrazinamidase encoded by \( pncA \)
- Inactivation of pyrazinamidase/nicotinamidase due to \( pncA \) mutations is a major cause of PZA resistance (PZA-R)
Considerations for Detecting PZA-R

- 70-90% of PZA-R isolates have a $pncA$ mutation
  - Detection of mutations within $pncA$ may be more reliable than current phenotypic testing
- $pncA$ consists of 187 codons (186 amino acids + stop codon)
- No “hotspot” for mutations; genetic variations throughout
- $M. \text{bovis}$ and $M. \text{bovis}$ BCG are naturally resistant to PZA due to a His57Asp substitution within $pncA$
Ethambutol

- Ethambutol (EMB) inhibits the *embABC* encoded arabinosyl transferases required for formation of a major cell wall component

- *embB* most common gene associated with EMB resistance (EMB-R)
  - 50-60% of EMB-R isolates have mutations at codon 306
    - Met306 → Val, Leu, Ile
  - Other mutations
    - Asp354Tyr
    - His406 → Ser, Asp
    - Met423Ile
Considerations for Detecting EMB-R

- Silent (e.g., Leu355Leu) and neutral (e.g., Glu378Ala) mutations that do not confer drug resistance have been detected.

- Mutations in embA, embB, and embC reported in clinical isolates of *M. tuberculosis* resistant to EMB.

- Variability in minimal inhibitory concentration (MICs) for mutations at codon 306 and 406.
Fluoroquinolones

• Common drugs: moxifloxacin (MOX), levofloxacin (LVX), ofloxacin (OFL), ciprofloxacin (CIP)
• Most effective second-line drugs for patients with MDR TB
• Inhibit DNA gyrase that is essential for DNA replication, transcription, and recombination
• \textit{gyrA} is the most common gene involved in FQ resistance (FQ-R)
  – \textit{gyrA} mutations in the quinolone resistance determining region (QRDR)
• \textit{gyrB} mutations also associated with resistance
Common *gyrA* and *gyrB* Mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gyrA</em></td>
<td>Gly88Ala</td>
</tr>
<tr>
<td></td>
<td>Ala90Val</td>
</tr>
<tr>
<td></td>
<td>Ser91Pro</td>
</tr>
<tr>
<td></td>
<td>Asp94 $\rightarrow$ Asn, Ala, Gly, His, Tyr</td>
</tr>
<tr>
<td><em>gyrB</em></td>
<td>Asn538Asp</td>
</tr>
<tr>
<td></td>
<td>Glu540Val</td>
</tr>
<tr>
<td></td>
<td>Arg485Cys + Thr539Asn</td>
</tr>
<tr>
<td></td>
<td>Asp500 $\rightarrow$ His, Asn</td>
</tr>
<tr>
<td></td>
<td>Asn538Lys</td>
</tr>
<tr>
<td></td>
<td>Glu540Asp</td>
</tr>
</tbody>
</table>

*Patterns of cross-resistance may vary*
Considerations for FQ-R

• Effect of *gyrA* mutation combinations
  – Thr80Ala: Susceptible
  – Ala90Gly: Resistant
  – Thr80Ala + Ala90Gly: Susceptible

• Limit of detection and variability in FQ DST results
  – Some FQ-R populations may not be detected using molecular methods

• Patterns of cross-resistance vary among mutations

• Clinical relevance not well understood

• Some laboratories may reflex to MOX MICs when certain *gyrA* mutations are observed

• Some *gyrB* mutations may be associated with low-level MOX-R (but not consistently)
Second-line Injectables

- Amikacin (AMK), kanamycin (KAN), and capreomycin (CAP)
- Most common genes involved in resistance
  - $rrs$ (16S rRNA)
  - $eis$ (aminoglycoside acetyltransferase)
  - $tlyA$ (encodes methyltransferase that modifies 16S and 23S rRNA)
- Considerations for detecting resistance
  - Other mutations (some unknown) may cause resistance
  - Inconsistent cross-resistance patterns
  - Variable critical concentrations for CAP
# Common *rrs*, *eis* and *tlyA* Mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rrs</em></td>
<td>A1401G</td>
<td>Variable cross-resistance patterns reported (KAN, AMK, and CAP)</td>
</tr>
<tr>
<td></td>
<td>C1402T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G1484T</td>
<td></td>
</tr>
<tr>
<td><em>eis</em></td>
<td>C-14T</td>
<td>KAN</td>
</tr>
<tr>
<td></td>
<td>G-10A</td>
<td>KAN</td>
</tr>
<tr>
<td></td>
<td>G-37T</td>
<td>KAN</td>
</tr>
<tr>
<td><em>tlyA</em></td>
<td>C insertion in 218L</td>
<td>CAP</td>
</tr>
<tr>
<td></td>
<td>Asn236Lys</td>
<td>CAP</td>
</tr>
<tr>
<td></td>
<td>Leu150Pro</td>
<td>CAP</td>
</tr>
<tr>
<td></td>
<td>ΔAla codon 23</td>
<td>CAP</td>
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</table>
Most Common Loci with Mutations Conferring Resistance

<table>
<thead>
<tr>
<th>Drug</th>
<th>Common Gene/Loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampin</td>
<td><em>rpoB</em></td>
</tr>
<tr>
<td>Isonizaid</td>
<td><em>inhA, katG</em></td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td><em>pncA</em></td>
</tr>
<tr>
<td>Ethambutol</td>
<td><em>embB</em></td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td><em>gyrA, gyrB</em></td>
</tr>
<tr>
<td>Second-line Injectables</td>
<td><em>eis, rrs, tylA</em></td>
</tr>
</tbody>
</table>
Caveats for Results Interpretation

• Finding a mutation does not always result in phenotypic resistance

• A wild type sequence does not confirm a strain is susceptible

• We do not know every possible locus leading to resistance for any antibiotic
Molecular Detection of Drug Resistance

NEW ANTI-TB DRUGS
Bedaquiline (Sirturo™)

- In 2012, FDA approved bedaquiline (BDQ) for use as part of combination therapy to treat adults with pulmonary MDR TB when other alternatives are not available.
- CDC recommends that positive monthly follow-up cultures from patients on BDQ be tested for BDQ resistance.
- Mutations generated in *atpE* lead to BDQ resistance in vitro, but these have not been yet been identified clinically.
- Clofazamine resistant strains of MTBC have shown cross-resistance to BDQ as a result of up-regulation of an efflux pump.
- Currently, resistance to BDQ is determined using minimal inhibitory concentration (MIC).
  - Increases in MIC have been seen in patients who failed to convert or relapsed.
Other New Drugs

- **Delaminid**
  - Marketing authorization granted in 2014 by the European Medicines Agency for use in MDR TB treatment
  - A nitro-dihydro-imidazoxazole derivative that inhibits mycobacterial cell wall synthesis

- **PA-824**
  - Bicyclic nitroimidazole active against both replicating and latent *M. tuberculosis*
  - Resistance to PA-824 is commonly mediated by loss of a specific glucose-6-phosphate dehydrogenase (FGD1) or its deazaflavin cofactor F$_{420}$

- **MIC testing and mechanisms of resistance being evaluated**
Molecular Detection of Drug Resistance

SEQUENCE-BASED MOLECULAR METHODS
### Considerations for Sanger Sequencing

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Long sequence reads</td>
<td>• Labor intensive</td>
</tr>
<tr>
<td>• &gt;500bp</td>
<td>• Hands-on</td>
</tr>
<tr>
<td>• Easy to customize</td>
<td>• Equipment cost and maintenance</td>
</tr>
<tr>
<td>• Ability to detect mixed sequences</td>
<td>• Reagent cost</td>
</tr>
<tr>
<td>• LOD ~30%</td>
<td>• Throughput limitations</td>
</tr>
<tr>
<td>• Ability to find new mutations</td>
<td>• Sample # vs loci #</td>
</tr>
<tr>
<td>• Actual DNA sequence is determined</td>
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</table>

- ✓ Specimens
- ✓ Isolates
Sanger Sequencing Results

| Wednesday, February 05, 2014 2:04 PM |
| Project: DST01062014_rpoB.sgq Contig 1 |

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<thead>
<tr>
<th>Mtb WT rpoB p2p.seq(1&gt;543)</th>
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<tr>
<td>20142000_rpoB_r_02_A04.ab1(83&gt;500)</td>
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<tr>
<td>20142000_rpoB_f_01_A03.ab1(84&gt;510)</td>
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<tr>
<td>20142000_rpoB_f_01_A01.ab1(145&gt;499)</td>
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</tr>
<tr>
<td>gttgacccacaaagccgactgtgccccgctg</td>
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</table>
Considerations for Pyrosequencing

Advantages

• Rapid DNA sequencing results
• Ability to detect specific mutations
• Transparent with pyrogram displayed
• Easy to customize
• High throughput

Disadvantages

• Limited to short sequence reads
  • <100bp
• Labor intensive
  • Hands-on
• Problem with homopolymer stretches (i.e., >3 of the same nucleotide)
• New mutations may need to be confirmed by Sanger sequencing
• More difficult to detect mixed populations than Sanger sequencing

☑ Specimens  ☑ Isolates
Pyrosequencing Workflow

- PCR: ~2h
- ssDNA prep: 45 min
- PSQ: ~1-2 hrs
Pyrosequencing Results

Sample ID: positive

Well: A2
PSQ run: PYRO01032014
Entry ID: rpoB_seq1
Sequence library: Mutations_rpoB_pyro_Seq1 (2010-07-06, 9:32:04 AM)
Query sequence: TCTTCGGCACCACGCGCATGAGCAGCAATTCTACGGACGAGCAGCAACCCGCTGTGCGGGTGGAAAAACAGCGACGACTGTGCGCGCGGCCC

Result: WT rpoB_seq1
Score: 100
Quality: Good
Information: Low score discrimination between best and second best hit.

Hit 1: WT rpoB_seq1
Score: 100
Identities: 60/60 (100%)
Gaps: 0/60 (0%)
E-value: 1.65e-057
Query 1: TCTTCGGCACCACGCGCATGAGCAGCAATTCTACGGACGAGCAGCAACCCGCTGTGCGGGTGGAAAAACAGCGACGACTGTGCGCGCGGCCC 60 (93)
Library 1: TCTTCGGCACCACGCGCATGAGCAGCAATTCTACGGACGAGCAGCAACCCGCTGTGCGGGTGGAAAAACAGCGACGACTGTGCGCGCGGCCC 60

Hit 2: Ser522Leu rpoB_seq1
Score: 97.6
Identities: 59/60 (98%)
Gaps: 0/60 (0%)
E-value: 1.65e-057
Query 1: TCTTCGGCACCACGCGCATGAGCAGCAATTCTACGGACGAGCAGCAACCCGCTGTGCGGGTGGAAAAACAGCGACGACTGTGCGCGCGGCCC 60 (93)
Library 1: TCTTCGGCACCACGCGCATGAGCAGCAATTCTACGGACGAGCAGCAACCCGCTGTGCGGGTGGAAAAACAGCGACGACTGTGCGCGCGGCCC 60
Next Generation Sequencing (NGS)

- Whole genome sequencing (WGS)
- Targeted amplicon-based sequencing
NGS Using Ion Torrent

Polymerase integrates a nucleotide.

Hydrogen and pyrophosphate are released.

Instrument detects change in pH
NGS Using Illumina

- DNA is fragmented for WGS
- Adapters are added (‘barcodes’)  
- 1 molecule is attached to multiple locations on a glass slide
- Molecules are amplified to a localized cluster on the glass slide by ‘bridge amplification’
- Sequencing reaction detects incorporation of sequentially added, fluorescently labeled nucleotides
Considerations for NGS

Advantages

• Rapid Assay
• Multi-use platform (could provide genotyping and drug susceptibility information at the same time)
• High throughput
• Massive amount of data can be generated
• May be cost effective, depending on batch size

Disadvantages

• Massive amount of data to be analyzed—lots of storage space needed
• How will it work for specimens?
• Expensive to perform in small batches
  • Batching may impact turn around time

Specimens  ✔  Isolates
Molecular Detection of Drug Resistance

PROBE-BASED MOLECULAR METHODS
GeneXpert MTB/RIF Assay

- Five probes are used to differentiate between the conserved wild-type sequence and mutations in the RRDR that are associated with RMP-R.
MTB detected, RIF resistance detected
Considerations for MTB/RIF Assay

Advantages

• Self-contained, single-use disposable cartridges host PCR process
  • Cross-contamination among samples is reduced

• Limited biosafety concerns

• Results within 2 hours of specimen collection

Disadvantages

• Specific mutations are not provided
  • Silent mutations could lead to false-resistance

• Potential low positive predictive value in low prevalence populations
  • Confirmatory testing needed

• Heteroresistance may be missed

☑ Specimens ☑ Isolates
Line Probe Assays

- Genotype MTBDRplus (Hain Lifescience)
  - Detects presence of MTBC and mutations associated with RMP-R and INH-R

- Genotype MTBDRsl (Hain Lifescience)
  - Detects mutations associated with resistance to FQs, second-line injectables, and EMB

- INNO-LiPA Rif TB (Innogenetics)
  - Detects presence of MTBC and mutations associated with RMP-R (91% of RMP-R isolates are also INH-resistant)
Figure 1: Rifampicin resistance region of the rpoB gene

rpoB WT1-8: rpoB wild type probes; rpoB MUT1-3: rpoB mutation probes. The numbers specify the positions of the amino acids (codons) for all mutations listed in the table. The codons for which mutation probes were designed are highlighted.
MTBDRplus Results

Conjugate Control (CC)
Amplification Control (AC)
M. tuberculosis complex (TUB)

rpoB Locus Control
rpoB wild type probe 1 (rpoB WT1)
rpoB wild type probe 2 (rpoB WT2)
rpoB wild type probe 3 (rpoB WT3)
rpoB wild type probe 4 (rpoB WT4)
rpoB wild type probe 5 (rpoB WT5)
rpoB wild type probe 6 (rpoB WT6)
rpoB wild type probe 7 (rpoB WT7)
rpoB wild type probe 8 (rpoB WT8)
rpoB mutation probe 1 (rpoB MUT1)
rpoB mutation probe 2A (rpoB MUT2A)
rpoB mutation probe 2B (rpoB MUT2B)
rpoB mutation probe 3 (rpoB MUT3)

katG Locus Control
katG wild type probe (katG WT1)
katG mutation probe 1 (katG MUT1)
katG mutation probe 2 (katG MUT2)

inhA Locus Control
inhA wild type probe 1 (inhA WT1)
inha wild type probe 2 (inhA WT2)
inha mutation probe 1 (inhA MUT1)
inha mutation probe 2 (inhA MUT2)
inha mutation probe 3A (inhA MUT3A)
inha mutation probe 3B (inhA MUT3B)
colored marker

Resistance
- R+I I R+I R+I
R = Rifampicin, I = Isoniazid
MTBDRsI Results

Conjugate Control
Amplification Control
M. tuberculosis complex

gyrA Locus Control
gyrA wild type probe 1
gyrA wild type probe 2
gyrA wild type probe 3
gyrA mutation probe 1
gyrA mutation probe 2
gyrA mutation probe 3A
gyrA mutation probe 3B
gyrA mutation probe 3C
gyrA mutation probe 3D

rrs Locus Control
rrs wild type probe 1
rrs wild type probe 2
rrs mutation probe 1
rrs mutation probe 2

embB Locus Control
embB wild type probe 1
embB mutation probe 1A
embB mutation probe 1B

colored marker

Resistance
- FLQ+AG/CP +EMB FLQ +EMB FLQ +EMB

FLQ = Fluoroquinolones
AG/CP = Aminoglycosides/Cyclic Peptides
EMB = Ethambutol
Considerations for Line Probe Assays

**Advantages**

- Works on processed specimens
- Rapid Assay
- Improved instrumentation for analysis and documentation of results

**Disadvantages**

- Open system may lead to amplicon contamination
- Silent mutations may result in false prediction of resistance
- Not customizable by user

☑ Specimens ☑ Isolates
## Comparing Molecular-based Assays

<table>
<thead>
<tr>
<th>Method</th>
<th>GeneXpert® MTB/RIF</th>
<th>HAIN Genotype® MTBDRplus</th>
<th>Sanger Sequencing</th>
<th>Pyrosequencing</th>
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<tbody>
<tr>
<td>Company</td>
<td>Cepheid</td>
<td>HAIN Lifescience</td>
<td>Laboratory developed test</td>
<td>Laboratory developed test</td>
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<tr>
<td>Genetic loci</td>
<td>rpoB</td>
<td>rpoB, katG, and inhA</td>
<td>First and second-line drugs</td>
<td>Varies but can include rpoB, inhA, katG, aphC, gyrA, and rrs</td>
</tr>
<tr>
<td>Format</td>
<td>Semi-automated real-time PCR</td>
<td>Line probe assay</td>
<td>DNA sequencing</td>
<td>DNA sequencing</td>
</tr>
<tr>
<td>FDA approved</td>
<td>Market authorization</td>
<td>No</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Expected turn-around time from specimen receipt in laboratory</td>
<td>1-2 working days (depends on how often performed in lab)</td>
<td>1-2 working days (depends on how often performed in lab)</td>
<td>1-2 working days (depends on how often performed in lab)</td>
<td>1-2 working days (depends on how often performed in lab)</td>
</tr>
</tbody>
</table>
Comparing Molecular-based Assays

• DNA probe methods such as molecular beacons (GeneXpert) or line probe assays indicate the presence or absence of a mutation
  – Line probe assays may also identify specific common mutations associated with drug resistance

• Sequencing provides more detailed information—detection of specific mutations
  – Enables correlation between specific mutations and phenotypic DST and MICs
## CDC Guidelines for Use of Xpert MTB/RIF

<table>
<thead>
<tr>
<th>GeneXpert instrument generated result</th>
<th>Interpretation of Xpert MTB/RIF result</th>
<th>Recommended “minimum reporting language”</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTB detected, RIF resistance detected</td>
<td>MTB detected within sample, mutation in <em>rpoB</em> detected</td>
<td>MTBC detected. A mutation in <em>rpoB</em> has been detected, indicating possible RIF resistance. <strong>Confirmatory testing should follow</strong></td>
</tr>
<tr>
<td>MTB detected, RIF resistance not detected</td>
<td>MTB detected, but no mutation in <em>rpoB</em> detected</td>
<td>MTBC detected. No <em>rpoB</em> mutation suggests probably RIF susceptible</td>
</tr>
<tr>
<td>MTB detected, RIF resistance indeterminate</td>
<td>MTB detected, unable to determine if there is an <em>rpoB</em> mutation</td>
<td>MTBC detected, presence of <em>rpoB</em> 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>
Confirmation of Resistance by Xpert MTB/RIF Assay

- Confirmatory testing using DNA sequencing, including genetic loci associated with MDR TB ($rpoB$, $inhA$, and $katG$) and prompt phenotypic DST
  - Silent mutations in $rpoB$ may be detected by MTB/RIF assay, producing a false resistant result
  - Also, mutations that are associated with low-level but clinically relevant RIF-R may be detected; these mutations have been associated with false susceptible results by phenotypic DST
Case # 1—Is it RMP-R?

- Smear (+) pulmonary TB
- Hospital laboratory results
  - Xpert MTB/RIF (X2) — RMP Resistance Detected
  - DST (MGIT) — INH-R and RMP-S
- Agar proportion DST pending at public health laboratory
- Referred for DNA sequencing
  - \( rpoB \) (Phe514Phe) indicating silent mutation
Case # 1—Is it RMP-R? (2)

- Silent mutations in RDDR of *rpoB* generally not associated with RMP-R
- Xpert detected the silent mutation and the isolate is not RMP-R
- Sequencing was required to determine the cause of the discordance
Case # 2—Is it RMP-R?

- Public health laboratory DST results
  - MGIT—R to INH; S to RMP (1 µg/ml)
  - Agar proportion—100% R to INH; 80% R to RMP (1 µg/ml)
- Referred for pyrosequencing
  - \textit{rpoB}—Asp516Tyr;  RMP resistant
  - \textit{inhA}—C(-15)T;  INH resistant
- Agar proportion at reference laboratory
  - RMP (1 µg/ml) — 40% R by agar proportion
  - INH-R by agar proportion
- Observed discordance between broth and agar phenotypic DST
Case # 2—Is it RMP-R? (2)

• The *rpoB* mutation detected is associated with highly discordant DST results
  – “Low–level” or “borderline” resistance
• Probably clinically relevant resistance
• Resistance often missed by standard, growth-based systems, especially automated broth systems
  – Critical concentration may be too high to cover all clinically relevant resistance
  – The methods may need modification (e.g., prolonged incubation, larger inoculum size) to detect resistance
• Prevalence of these strains unknown
• Examples of mutations with highly discordant results
  Asp516Tyr, Leu511Pro, Leu533Pro, His526Leu, His526Ser, Ile572Phe
Molecular Detection of Drug Resistance

COMMUNICATING MOLECULAR RESULTS
Reporting Molecular Results

- Results must be provided in a format that is clear and understandable to the healthcare provider.
- Education and training are needed to understand test results and limitations:
  - Understand reporting language or interpretations provided in package insert (if available).
- Considerations for inclusion in the report:
  - Test performed
  - Method used
  - A comprehensive result (e.g., mutation including nucleotide sequence and amino acid substitution, when applicable) or just mutation detected/not detected.
- Helpful if report provides interpretive comments.
Regional Training and Medical Consultation Centers

- The CDC's Division of Tuberculosis Elimination funds five TB Regional Training and Medical Consultation Centers (RTMCCs)
- The RTMCCs are regionally assigned to cover all 50 states and the U.S. territories.
- Provide medical consultation to TB programs and medical providers

- Curry International Tuberculosis Center
- Mayo Clinic Center for Tuberculosis
- Heartland National Tuberculosis Center
- Southeastern National Tuberculosis Center
- Global Tuberculosis Institute at Rutgers, The State University of New Jersey

http://www.cdc.gov/tb/education/rtmc/default.htm
Need for a Drug Resistance Database

- Need for continued progress and tools for rapid detection of drug resistant tuberculosis
- Improve understanding of molecular basis of drug resistance and clinical relevance of mutations
- Effort requires comprehensive and curated data platform
  - Sequencing data with correlated phenotypic drug susceptibility and clinical data
- Database could inform diagnostics development and clinical decision making using molecular results
Resources

- APHL TB page
- APHL TB Core Services
- APHL Fact Sheet for Cepheid Xpert MTB/RIF Assay
- TB Education and Training Resources
- Evidence-based TB Diagnosis
- CDC TB Website
- CDC Molecular Detection of Drug Resistance (MDDR) Service
- Regional Training and Medical Consultation Centers
- CDC Model Performance Evaluation Program
- Report of Expert Consultations on Rapid Molecular Testing to Detect Drug-Resistant Tuberculosis in the United States
Journal Articles of Interest-1

Journal Articles of Interest-2

- Lin, S.Y.G., and Desmond, E.P. (2014). “Molecular Diagnosis of Tuberculosis and Drug Resistance” Microbial Diseases Laboratory, California Department of Public Health


