Overview of Molecular Methods for the Identification of *Mycobacterium tuberculosis* Complex and Mutations Associated with Drug Resistance

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June 13, 2011
Objectives

- Describe the purpose of molecular diagnostics
- Discuss new molecular diagnostics, their utilization, and potential testing algorithms
- List the benefits and limitations of molecular TB diagnostics
Inoculate Media
Species Identification
Drug Susceptibility
2 to 3 weeks

21st Century Algorithm

Amplification-based Tests

Process Specimen
1 day

AFB Microscopy

Inoculate Media
2 to 6 weeks

Culture Positive

Species Identification

Drug Susceptibility

Molecular DST
Why the increased interest in molecular diagnostics for TB?

- Goal of TB elimination and recognized need for more rapid diagnosis
- Use of assays by some public health laboratories for rapid detection of mutations associated with rifampin (RMP) and isoniazid resistance (LDT and RUO)
- 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
Why the increased interest in molecular diagnostics for TB? (2)

- Decreasing price and increased access to platforms and reagents for LDT

- Shift in laboratory workforce and loss of expertise for conventional TB testing methodologies

- Globally
  - Foundation for Innovative New Diagnostics (FIND) successful demonstration projects
  - WHO Scientific and Technical Advisory Group (STAG) endorsement of Genotype® MTBDRplus line probe assay and Cepheid GeneXpert® MTB/RIF assay
Important Definitions

- **Clinical specimen** - material taken directly from the patient (e.g., sputum, CSF, pleural fluid); may be “raw” specimen or may be “processed” specimen (e.g., sediment)

- **Isolate** - organism isolated (i.e., grown) from culture of a clinical specimen (e.g., an LJ tube with MTBC growth)

- **Direct detection** - detection of RNA or DNA sequences of interest in organisms present in a clinical specimen

- **Probe** - piece of DNA that hybridizes specifically to a target nucleic acid sequence
Another Important Definition: Nucleic Acid Amplification (NAA)

- Exponential amplification of a specific sequence of nucleic acid

- NAA helps to increase the sensitivity of the assay especially when only a few organisms may be present

- Two most common types
  - Polymerase Chain Reaction (PCR)
  - Transcription Mediated Amplification (TMA)

- Amplified nucleic acid product (amplicon) detected by specific DNA probe or analyzed by DNA sequence analysis

1. Direct detection in clinical specimen
   a) Is it \textit{M. tuberculosis} complex (MTBC) or not MTBC?
   b) If MTBC, are mutations associated with drug resistance present?

2. Identification of acid-fast organism in positive culture
   a) Is it MTBC or not MTBC?
   b) If not MTBC, is it a common nontuberculous mycobacterium (NTM)?

3. Detection of resistance-associated mutations in MTBC isolate from culture
   a) Are mutations commonly associated with RMP and INH resistance present? (i.e., rapidly detecting MDR TB)
   b) Are mutations associated with second-line drug resistance present?
AccuProbe® (Gen-Probe, Inc.)

- DNA probe for identification of specific mycobacteria after growth is detected in culture
- Does not require NAA
  - “Amplification” of the target takes place in culture
  - More organisms and therefore many copies of the target 16s rRNA sequence

What does “probe positive for MTBC” mean?
Direct Detection of MTBC Using NAA Tests (1)

- Identify genetic material unique to MTBC directly in clinical samples.

- Positive result demonstrates the presence of MTBC
  - Does not distinguish live and dead bacilli

- Negative result does not necessarily mean the absence of MTBC
  - Inhibition of amplification
  - Target below the limit of detection

- Rapid turnaround time of 24 to 48 hours after specimen receipt
Direct Detection of MTBC Using NAA Tests (2)

- CANNOT replace clinical judgment or be relied on as the ONLY guide for therapy or isolation practices (less than perfect sensitivity and specificity)

- Sensitivity
  - >95% for AFB smear-positive TB patients
  - 55–75% of AFB smear-negative, culture-positive TB patients

- Performance improves with increased clinical suspicion of TB
Updated Guidelines for the Use of NAAT in the Diagnosis of TB

“NAAT should be performed on at least one respiratory specimen from each patient with signs and symptoms of pulmonary TB for whom a diagnosis of TB is being considered but has not yet been established, and for whom the test result would alter case management or TB control activities”

Although NAA testing has been available as a TB diagnostic since mid-1990s, it is now recommended as standard practice.

MMWR, 2009, 58:7-10
NAA Assays for Direct Detection of MTBC

- FDA-approved for use with respiratory specimens
  - Amplified MTD® (*Mycobacterium tuberculosis* Direct) Test: Gen-Probe, Inc.

- Non-FDA approved tests (RUO or not available in U.S.)
  - Hain Lifescience Genotype® MTBDRplus and MTBDRsl
  - Innogenetics INNO-LiPA Rif.TB
  - COBAS® TaqMan® MTB Test
  - Cepheid GeneXpert® MTB/RIF
  - Akonni TruArray® MDR-TB
  - AutoGenomics Infinity® MDR-TB

- Laboratory developed tests or LDT (e.g., DNA sequencing, Loop-mediated isothermal amplification [LAMP], and real-time PCR assays including molecular beacons)
Testing Algorithms:
NAAT for direct detection of MTBC in respiratory specimens

- Test all specimens or all patients
- Test all acid-fast smear-positive specimens
  - Most common algorithm in PHL—all new smear positive specimens and smear negatives on request
- Base testing on communication with healthcare provider, for example:
  - Don’t test smear positives when classic TB symptoms and history are present
  - Do test smear negatives when clinical suspicion of TB is high
Challenges to Implementing NAAT

- Adds reagent and personnel costs to the laboratory
- Add-on test and culture still required for additional analysis
- Low volume test in many laboratories and batching may “defeat” purpose of offering rapid test
- Unidirectional workflow to prevent cross contamination has implications for infrastructure and equipment
- Overall costs and benefits may vary by program
- Need to determine optimal testing algorithms
Who should be tested?

- CDC recommends NAAT on first sputum of all patients suspected of TB for whom the test result would alter case management or TB control activities
  - NAAT should not be ordered routinely when clinical suspicion of TB is low.
  - PPV higher with high clinical suspicion

- Definition of a “suspect” case can vary among clinicians

- TB programs, clinicians, and laboratorians must collaborate to develop criteria/definition for patients to be tested

Courtesy of David Warshauer, PhD
NAAT Limitations and Considerations

- **Sensitivity**
  - Reduced for smear negative specimens and some specimen types?
  - Do you want to “rule in” or “rule out”?
  - Platform dependent

- **Specificity**
  - Platform dependent

- **Does not replace need for culture**
  - Culture still needed for conventional DST, genotyping

- **Amplicon cross contamination in open systems**

- **Cost and sustainability**
  - Expense can limit utilization
Molecular Detection of Drug Resistance (Molecular DST)

- Examining DNA of specific genes for mutations known to be associated with phenotypic resistance
  - Mutations in what genes are associated with resistance?
  - Where are the mutations within the gene?
  - Some areas are “hot spots”—resistance determining regions

- DNA sequence examined may be important for protein expression, code for the protein itself, or code for rRNA
Different mutations within a gene can cause different levels of resistance \textit{in vitro}.

Some mutations not associated with resistance as determined in the laboratory:
- Do these mutations have any clinical significance?
- Problems with conventional DST

Output from the assay depends on the platform:
- Not everything is a molecular beacon!
- Need to understand platform to understand limitations
What tests are being used for molecular detection of drug resistance?

- Laboratory developed tests (LDT)
  - DNA sequencing
  - Loop-mediated isothermal amplification (LAMP)
  - Real-time PCR assays (including molecular beacons)

- Non-FDA approved tests (Research Use Only [RUO] or not available in U.S.)
  - Genotype® MTBDRplus and MTBDRsl - Hain Lifescience
  - Innogenetics INNO-LiPA Rif.TB
  - Cepheid GeneXpert® Xpert MTB/RIF
  - Akonni TruArray® MDR-TB
  - AutoGenomics Infinity® MDR-TB
Conventional DNA Sequencing

- PCR Amplification of target regions
- DNA Sequencing
- Sequence Analysis – Compare sequence to sequence library to identify mutations
Most commonly observed \textit{rpoB} mutation:

TCG$\rightarrow$TTG  Ser531Leu
Conventional DNA Sequencing

Advantages

• Long sequence reads
  • >500bp
• Easy to customize
• Ability to detect mixed sequences
  • LOD ~30%
• Ability to find new mutations
• Actual DNA sequence is determined

Disadvantages

• Labor intensive
  • hands-on
• Equipment cost and maintenance
  • ABI3130xl ~$175k
• Reagent cost
• Throughput limitations
  • sample # vs loci #

✓ Specimens
✓ Isolates
Pyrosequencing

Direct DNA sequencing of PCR products
  • Unique chemistry - detection of released pyrophosphate
  • Visible light is generated that is proportional to the number of incorporated nucleotides

• Biotin labeled PCR product (1 strand)
• Biotinylated DNA strand captured on beads
• Beads hybridized with sequencing primer
• Instrument carries out DNA sequencing reaction and analysis <2 hrs
Sample ID: 20102356

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Hit 1: AGC-ACA Ser315Thr katG seq1

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Hit 2: T-8C inhA seq1

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Hit 3: C-15T mutation inhA seq1

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Hit 1: WT inhA seq1

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Hit 2: T-8C inhA seq1

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Hit 3: C-15T mutation inhA seq1

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<td>E-value</td>
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Pyrosequencing

Advantages
• Rapid sequencing reactions
• Easy to customize
• Ability to detect mixed sequences
• High throughput
• Actual DNA sequence is determined

Disadvantages
• Short Sequence Reads
  • <100bp
• Labor intensive
  • hands-on
• Separate PCR rx’s to sequence both strands
• Poor performance in homopolymer stretches (i.e., >3 same nucleotide)

☑ Specimens
☑ Isolates
“Molecular beacons”
Real time PCR results with molecular beacons

Probe target is amplified in a “real time PCR” – liquid hybridization

Detection of wildtype sequence, not a specific mutation
Detection of Mutations with a Molecular Beacon

**Mutant Sequence**

- WT Beacon + Fluorophore
- Quencher

**Wildtype Sequence**

- Amplicon + Fluorophore
- Loop
- Light
- WT Beacon + Quencher

**Molecular Beacon OFF**

Hybrid — Molecular Beacon ON
# Real-time PCR with Molecular Beacons

<table>
<thead>
<tr>
<th><strong>Advantages</strong></th>
<th><strong>Disadvantages</strong></th>
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<tr>
<td>• Customizable</td>
<td>• Multiple beacons needed to cover overlapping regions</td>
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<tr>
<td>• Many RT-PCR platforms available</td>
<td>• Silent mutations may result in false predication of resistance</td>
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<tr>
<td>• High Throughput</td>
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<tr>
<td>• Amplification and detection of the amplified product take place inside a sealed system</td>
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<tr>
<td>• Detection of the amplified product takes place <em>during</em> the amplification</td>
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- **Specimens**
- **Isolates**
Line Probe Assays (LPA)

- NAA and hybridization-based tests use immobilized DNA probes on nitrocellulose membranes
  - Colorimetric change indicating hybridization
  - “Read” the bands to determine MTBC or not and to detect resistance-associated mutations for RMP and INH

- HAIN Lifesciences
  - GenoType® MTB-DRplus
  - GenoType® MTBDRsl
- PCR based assay
- Detect MTBC
- Detect RMP R (rpoB gene)
- Detect INH R (inhA and katG gene)
GenoType® MTBDRs/ Assay

Heidi Albert, FIND, Uganda - US/Southern Africa Joint Research Forum on Tuberculosis March 1-2, 2010
Line Probe Assays

Advantages

• Rapid Assay
• Improved instrumentation for analysis and documentation of results
• Used in laboratories now

Disadvantages

• Multiple probes needed to cover overlapping regions
• Silent mutations may result in false predication of resistance
• Not customizable by user

☑ Specimens ☑ Isolates
Cepheid Xpert MTB/RIF Assay
Cepheid Xpert MTB/RIF Assay

- Automated commercial system for identification of *M. tuberculosis* complex and mutations in *rpoB*
- Uses real-time PCR with molecular beacons
  - 5 probes for wild-type RRDR in *rpoB* and 1 probe for amplification control (*B. globigii*)
- Decontamination, digestion, DNA extraction, amplification, and detection in same cartridge; Limited biosafety requirements
- Results in ~2 hours
- Minimal hands on manipulation - technically simple
- Platform is random access
Rifampin Susceptible Sample

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<tr>
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<tr>
<td>Assay</td>
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### Notes

| Start Time | 2008/4/16 15:50:33 |
| End Time   | 2008/4/16 17:15:31 |
| Status     | Done |
| Error Status | OK |
| User       | support |
| SW Version | 2.1 |
| Instrument Module S/N | 7022666/600013 |

### Result View

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<tr>
<td>Test Result</td>
<td>MTB POSITIVE MEDIUM; Rif Resistance NOT DETECTED</td>
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### Analyte Name

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Xpert MTB/RIF

**Advantages**
- Rapid Assay (<4 hrs)
- Sample is *unprocessed* sputum
  - Hospitals; near-patient testing
- Multi-use platform
- Automated start to finish

**Disadvantages**
- Proprietary instrument and cartridges
- Does not detect all mutations in 81bp region
- Silent mutations may result in false predication of resistance
- Not customizable by user

- Specimens
- Isolates (But not lysed cells/extracted DNA)
Benefits of Molecular Detection of Drug Resistance

- Rapid results within days as compared to weeks for conventional testing
- Expedite further conventional testing (e.g., second-line drug susceptibility testing)
- High throughput
- Some assays are “closed systems”—reduces potential for cross contamination
- Development of technologies requiring limited biosafety infrastructure; does not require BSL-3 once DNA is extracted
- Information provided by some platforms may be used to enhance accuracy of conventional DST
Limitations and Considerations (1)

- Not all mechanisms of resistance are known
  - The lack of a mutation ≠ susceptibility
- Limited genes and sites are targeted
- Emerging resistance in mixed populations may not be detected (limit of detection)
- Not all mutations are associated with phenotypic drug resistance
  - Silent (synonymous) mutations—no alteration in protein sequence (e.g., Phe514Phe and Arg528Arg in rpoB)
  - Neutral polymorphisms (e.g., gyrA codon 95 may be Ser [AGC] or Thr [ACC])
  - Output is platform dependent
Limitations and Considerations (2)

- Still filling in gaps in knowledge about drug resistance (phenotypic and genotypic testing)

- “Gold-standard” DST may not be perfect
  - Mutations resulting in elevated MICs but S at critical concentration (e.g., Leu511Pro in \textit{rpoB})

- Clinical utility - Do results impact patient care?
  - Will clinicians “trust” these results or “wait for the conventional DST result?”

- Educational partnerships (laboratory, program, and clinicians) need to be developed
Molecular Detection of Drug Resistance (MDDR) Service

- Implemented in September 2009 (CLIA compliant)
- Clinical service to domestic TB control programs and clinicians
  - Rapid confirmation of RMP-resistant and MDR TB
  - Laboratory testing data available about SLD resistance in cases of RMP-resistant or MDR TB
- New technologies may fill the role in the future but demand exists now
- DNA sequencing, ABI 3130xl
MDDR Service: Drugs and Genes for Panel

- Rifampin
- Isoniazid
- Fluoroquinolones
- Amikacin, Kanamycin, Capreomycin
- Kanamycin
- Capreomycin
- Ethambutol
- Pyrazinamide
- rifB (81bp region)
- inhA (-15)
- katG (Ser315)
- gyrA (coding region)
- rrs (nt1401/1402,1484)
- eis (promoter region)
- tlyA (coding region)
- embB (Met306, Gly406)
- pncA (promoter and coding regions)

MDR TB

Added Oct 2010

XDR TB
MDDR Algorithm

Isolate Received for MDDR

Molecular Analysis

2 day turn-around time

Molecular Results (Interim Report)

Conventional DST

38 day turn-around time

Molecular + Conventional DST Results (Final Report)
The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Angela Starks, PhD

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