Role of Molecular Methods in Tuberculosis Diagnosis and Treatment

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Objectives

- Describe molecular tests for the diagnosis of tuberculosis and the detection of drug resistance in *Mycobacterium tuberculosis* complex (MTBC)

- Use case-based scenarios to explain the use of molecular test results and to illustrate the benefits and limitations of these tests
“Rules of the Lab”

- No lab test is perfect
- Do not order a lab test if you are not ready to deal with the result
- Treat the patient, not the lab test

For TB—There is a lot we still need to learn about DST and molecular detection of drug resistance

Discordance
**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; currently requires nucleic acid amplification (NAA).

- **Probe**: piece of DNA that hybridizes specifically to a target nucleic acid sequence.
What is 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

Direct Detection of MTBC in Clinical Specimens; Nucleic Acid Amplification (NAA) Tests

- Objective is to detect/identify MTBC directly from clinical specimens and avoid the weeks required for culture
  - Rapid turnaround time of 24 to 48 hours after specimen receipt
- 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
NAA Tests 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; Research Use Only)
  - Hain Lifescience Genotype® MTBDRplus and MTBDRsl
  - Cepheid GeneXpert® MTB/RIF

- Laboratory developed tests or LDT (e.g., DNA sequencing, Loop-mediated isothermal amplification [LAMP], and real-time PCR assays)
NAAT for TB Diagnosis

- Becoming standard of practice
- 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

- Potential benefits – impact on therapy, public health measures, respiratory isolation, invasive procedures

- MMWR, 2009, 58:7-10
Limitations and Considerations

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

q Specificity
  ❌ Platform dependent

q Does not replace need for culture
  ❌ Culture still needed for conventional DST, genotyping

q Amplicon cross contamination in open systems

q 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
What tests are being used for molecular detection of drug resistance?

- Laboratory developed tests (LDT)
  - DNA sequencing
  - Real-time PCR assays

- Non-FDA approved tests (Research Use Only [RUO])
  - Genotype® MTBDRplus and MTBDRsl - Hain Lifescience
  - Cepheid GeneXpert® Xpert MTB/RIF
Genotype MTBDR\textit{plus}(“HAIN test”)

- NAA and hybridization-based test use immobilized DNA probes on nitrocellulose membranes (line probe assay [LPA])
- Colorimetric change indicates hybridization
- “Read” the bands to determine MTBC or not and to detect resistance-associated mutations for RMP and INH
Hain/Line Probe Assays

Advantages

• Works on processed specimens
• Rapid Assay
• Improved instrumentation for analysis and documentation of results
• Used in laboratories now

Disadvantages

• Multiple beacons 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

- 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
# Cepheid GeneXpert

## Advantages
- Rapid Assay (<4 hrs)
- Sample is unprocessed sputum
  - Hospitals
- Multi-use platform
- Automated start to finish
- Real-time PCR assay for detection of TB
- Closed system

## Disadvantages
- Proprietary instrument and cartridges
- Multiple beacons needed to cover overlapping regions
- Silent mutations may result in false predication of resistance
- Not customizable by user

### Specimens
- But not lysed cells/extracted DNA

### Isolates
Conventional (Sanger) DNA Sequencing

- PCR Amplification of target regions
- DNA Sequencing
- Sequence Analysis
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
- Reagent cost
- Throughput limitations
  - sample # vs loci #

Specimens vs Isolates
Most commonly observed \textit{rpoB} mutation:

TCG\rightarrow TTG Ser531Leu
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
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
Sample ID: 20102356

Well: B7
PSQ run: RDDR08092010
Entry ID: katG
Sequence library: katC_pyrO_Seq (2010-07-19, 11:00:50 AM)
Query sequence: CCACAGG

Result: AGC-ACA Ser315Thr katG seq1
Quality: Good

Hit 1: AGC-ACA Ser315Thr katG seq1
Score: 100
Identities: 5/5 (100%)
Gaps: 0/5 (0%)
E-value: 9.20e-004

Sequence

Score: 100
Identities: 8/8 (100%)
Gaps: 0/8 (0%)
E-value: 1.37e-005

Hit 2: T-8C inhA seq1
Score: 76.3
Identities: 7/8 (88%)
Gaps: 0/8 (0%)
E-value: 1.37e-005

Sequence

Score: 76.3
Identities: 7/8 (88%)
Gaps: 0/8 (0%)
E-value: 1.37e-005
CDC’s MDDR Service
(Molecular Detection of Drug Resistance)

- Implemented in September 2009 (CLIA compliant)
- Comprehensive 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
Criteria for MDDRT Testing
Version 2.0*

- Isolate or NAAT (+) sediments (not raw specimen)
- High-risk patients (RMP-R, MDR TB)
  - From population with high rates of drug resistance
  - Exposed to DR case
  - Failing therapy
- Cases of public health importance
  - Impact on public health measures & public health response
- Known RMP Resistance
  - Conventional or molecular test by submitter
- Mixed or non-viable cultures
- Other Reasons

*June 2012
MDDR Service Description

- Pyrosequencing
  - βRMP \((rpoB)\) and INH \((katG, inhA)\)
- Sanger Sequencing*
- Conventional DST performed in parallel

MDDR Service:
Sanger Sequencing
Drugs and Genes for Panel

- Rifampin
- Isoniazid
- Ethambutol
- Pyrazinamide
- Fluoroquinolones
- Amikacin, Kanamycin, Capreomycin
- Kanamycin
- Capreomycin
- RmpB (81bp region)
- InhA (-15)
- KatG (Ser315)
- EmbB (Met306, Gly406)
- PncA (promoter and coding regions)
- GyrA (coding region)
- Rrs (nt1401/1402, 1484)
- Eis (promoter region)
- TlyA (coding region)

MDRTB

XDR TB
MDDR V2.0 Algorithm

- **Isolate or NAAT(+) Sediment Received for MDDR**
  - Molecular Analysis (PSQ; PSQ then Sanger; Sanger)*
  - 2-3 day turn-around time

- **Conventional DST**

- **Molecular Results (Interim Report[s])**
  - ~35 day turn-around time

- **Molecular + Conventional DST Results (Final Report)**

*based on information supplied on request form
How to report results?

Weighing Genotypic versus Phenotypic Results

1. The term ‘Gold Standard’ can be misleading.
2. New, previously uncharacterized or poorly characterized mutations:
   - Reported as clinical significance unknown
   - Anecdotal information may be reported
3. Functional genetic analysis is necessary to definitely determine effect of mutation on resistance
4. Need to develop standardized reporting language
Results for Molecular Detection of Drug Resistance; Conventional Drug Susceptibility Test in progress.

<table>
<thead>
<tr>
<th>Locus (region) examined</th>
<th>Result</th>
<th>Interpretation (based on in-house evaluation of 254 clinical isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB (RRDR)</td>
<td>No mutation</td>
<td>Probably Rifampin susceptible. (96% of RIF-R isolates in our in-house evaluation of 254 clinical isolates have a mutation at this locus.)</td>
</tr>
<tr>
<td>inhA (promoter)</td>
<td>No mutation</td>
<td>Isoniazid resistant. (100% of isolates in our in-house evaluation of 254 clinical isolates with this mutation are INH-R.)</td>
</tr>
<tr>
<td>katG (ser315 codon)</td>
<td>Mutation: AGC&gt;ACC; Ser315Thr</td>
<td></td>
</tr>
</tbody>
</table>

* A negative result (e.g., no mutation) does not rule out contributory mutations present elsewhere in the genome.
Other Example Interpretive Comments

- No mutations in *inhA* or *katG*
  
  Cannot rule out INH resistance. (90% of INH-R isolates in our in-house evaluation of 254 clinical isolates have a mutation at one or both of these loci.)

- Ser531Leu mutation in *rpoB*
  
  Rifampin resistant. (100% of isolates in our in-house evaluation of 254 clinical isolates with this mutation are RMP-R)

- Gly285Cys mutation in *katG*
  
  Cannot rule out INH resistance. The mutation found has not been detected previously and the significance of this mutation regarding predicting resistance to INH is unknown.
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 (review)

- Not all mechanisms of resistance are known and the lack of a mutation ≠ susceptibility
- Limited genes and sites are targeted
- Emerging resistance (mixed populations) may not be detected; limit of detection
- Not all mutations are associated with phenotypic resistance
  - Silent (synonymous) mutations—no change in protein (e.g., Phe514Phe and Arg528Arg in \textit{rpoB})
  - Neutral polymorphisms (e.g., \textit{gyrA} codon 95 may be Ser or Thr)
Limitations and Considerations (review 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 SAT critical concentration (e.g., Leu511Pro in rpoB)
- Clinical utility—Do results impact patient care? Will clinicians “trust” these results or “wait for the conventional DST result?”
- Expertise of staff and clinicians
  - Output from the assay depends on the platform; Need to understand platform to understand limitations
- Educational partnerships (laboratory, program, and clinicians) need to be developed
Understanding Discordance

- Between different phenotypic DST results
  - e.g., MGIT 960 and agar proportion

- Between phenotypic and genotypic (molecular) results

- Between different genotypic results
  - e.g., GeneXpert and Sanger sequencing
What causes discordant DST results?

- “Human error/lab error”
  - Transcription, labeling errors
  - Cross contamination/specimen mix-up

- Different “inoculum”/bacterial population
  - e.g., isolates from different specimens; sampling from same specimen; original isolate vs. subculture
  - Size of inoculum/clumps
  - Different growth kinetics

- Different method or media
  - “equivalent” critical concentrations
  - “calling” result too soon

- The “bug”- MIC is close to the critical concentration
  - Evaluations performed with “highly resistant” bugs
What causes discordance between molecular and phenotypic DST results?

- “Human error/lab error”
- Not all mechanisms of resistance are known
  - Lack of a mutation ≠ susceptibility
- Limited genes and sites are targeted
- Emerging resistance (mixed populations)
  - May not be detected; limit of detection
- Not all mutations are associated with phenotypic resistance
  - Silent (synonymous) mutations—no change in protein
  - Neutral polymorphisms (e.g., gyrA codon 95 may be Ser or Thr)
- Output is “platform dependent”
- “Gold-standard” DST may not be perfect
  - Mutations resulting in elevated MICs but Sat critical concentration (e.g., Leu511Pro in rpoB)
What causes discordance between different molecular platform results?

- "Human error/lab error"
- Not necessarily looking at the same segment of DNA
  - looking for a particular single nucleotide polymorphism (SNP) in one codon versus looking at 30 codons
- Limited genes and sites within genes are targeted
  - katG only versus katG+inhA
- Emerging resistance (mixed populations)
  - may not be detected; limit of detection
Cases
Case # 1—Is it RMP-R? (RMP Discordance between molecular and conventional results and between 2 molecular results)

q Smear (+) pulmonary TB; prisoner

q At hospital
  Š Xpert (X2) — RMP Resistance Detected
  Š DST (MGIT) — INH-R and RMP-S

q AP DST pending at State lab

q At CDC, rpoB DNA sequence — Phe514Phe; RMP Susceptible
Case # 2—Is it RMP-R?
(RMP Discordance between molecular and conventional results)

- Pulmonary TB; Burma (Nepal camp)
- State Lab DST (MGIT) — INH-R and RMP-S
- CDC *rpoB* — Asp516Tyr; RMP resistant
- CDC AP — RMP-S
rpoB mutations associated with highly discordant DST results


q “Low–level” or “borderline” resistance
q Probably clinically relevant resistance
q Resistance often missed by standard, growth-based systems, especially automated broth systems
  β Critical concentration may be too high to cover all clinically relevant resistance, or
  β Maybe the methods need modification (e.g., prolonged incubation, larger inoculum size) to detect resistance
q Frequency of these strains unknown
q Mutations: Asp516Tyr, Leu511Pro, Leu533Pro, His526Leu, His526Ser, Ile572Phe
Asp516Tyr (CDC MDDR)
(case #2)

<table>
<thead>
<tr>
<th></th>
<th>RMP-R by DST # with mutation/Total</th>
<th>RMP-S by DST # with mutation/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original (retrospective) validation</td>
<td>2/152</td>
<td>0/102</td>
</tr>
<tr>
<td>Prospective validation</td>
<td>0/17</td>
<td>1/63</td>
</tr>
<tr>
<td>Total</td>
<td>4/253</td>
<td>4/308</td>
</tr>
</tbody>
</table>
Clinical failures associated with \textit{rpoB} mutations in phenotypically occult MDR TB

- Significant association between the presence of \textit{rpoB} mutations that are not detected in DST and treatment failure

  - 4 had \textit{rpoB} mutations (GeneXpert)
    - RRDR sequenced—Leu511Pro/Met515Ile, His526Asn/Ala532Val, Asp516Tyr, His526Leu
    - 3 of 4 were treatment failures; other was unknown
Case # 3—Is it RMP-R?

- Isolate submitted for MDDR
  - HIV+, prison, Mexico, intermittent therapy, “funky”
  - RMP on Bactec 460
- CDC rpoB—wildtype; probably RMP-S
- CDC AP—contaminated
- Resubmit isolate (A) and a newer isolate (B)
  - AP (A)—RMP-R (5%)
  - AP (B)—RMP-R (12%)
- rpoB on colonies—His536Tyr (100% of isolates with this mutation are RMP-R)
- Under limit of detection of MDDR
Case #4—Persistently smear (+) patient with drug susceptible MTBC

q MDDR
  ∅ rpoB, inhA, katG all wildtype (no mutations)
  ∅ pncA—mutation with unknown significance

q AP DST
  R to INH, S to RMP

q MGIT PZA—S

q 10-15% of INH-R isolates do not have a mutation in inhA or katG

q A mutation in pncA does not necessarily = resistance
Conclusions

- Paradigm shift in laboratory diagnosis of TB and detection of drug resistance in MTBC
  - Molecular tests for diagnosis do not replace culture
  - Molecular tests do not replace conventional DST
  - Need to develop cost-effective algorithms for incorporating new technology; timely referral

- Results from genotypic and phenotypic tests for drug resistance need to be used in conjunction with one another (may depend on drug and genetic locus)

- Molecular (genotypic) tests may
  - Elucidate “truth” in certain cases
  - Add to confusion in certain cases
  - Help us “fine-tune” conventional DST

- Communication is essential
The findings and conclusions in this report are those of the author and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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