ADVANCES IN LABORATORY
TB DIAGNOSIS

American Lung Association Building
55 West Upper Wacker Drive, #800
Chicago, Illinois  60601
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John F. Nawrocki, Ph.D.
Section Chief, Molecular Diagnostics/Mycobacteriology
Illinois Department of Public Health
2121 West Taylor Street
Chicago, IL  60612
DISCLOSURES

“I have no disclosures of any relevant financial relationships”
LEARNING OBJECTIVES

- Understand advances in the early and rapid detection of drug-resistant Mycobacterium tuberculosis in primary specimens.
- Compare rapid (molecular) versus conventional techniques. Why you have to wait for confirmation of TB infection.
- Be aware of molecular strain genotyping being used to track spread of infections.
RAPID ROUTINE SPUTUM TESTS

1. Detection of Acid Fast Bacilli (AFB) by Fluorescence Microscopy (Smear Analysis)

2. Rapid Direct Detection of the *Mycobacterium tuberculosis* Complex by Real-Time Polymerase Chain Reaction (PCR)

3. Identification of mutations associated with drug resistance (sequence analysis)
HOW TO COLLECT SPUTUM SPECIMENS

- Collect early in the morning if possible
- Collect sputum, not saliva.
- The optimal volume to collect is 5-7 mLs.
- Seal the tube to prevent leakage.
- Label the tube with the patient name as it appears on the requisition.
SMEAR ANALYSIS:

TURN AROUND TIME

- Performed on Day of Specimen Receipt

- Negative
  
  Positive (grade resulted: 1+, 2+, etc.)
  
  Equivocal (1-2 Acid Fast Bacilli detected)
AURAMINE RHODAMINE FLUORESCENCE STAIN: HIGH POWER
REAL-TIME PCR ASSAY

- Detects the *M. tuberculosis* complex (*MTBC*) directly in the sputa.
- Does not distinguish *M. tuberculosis* from other members of the complex (i.e., *M. bovis* or *M. Bovis BCG*).
- Does not distinguish live from dead organisms.
- In most laboratories, only preformed on respiratory specimens.
WHICH SPUTUM SPECIMENS ARE TESTED BY PCR?

- All New AFB+ Sputa
- *M. tuberculosis* Patients Not Responding to Therapy
- AFB Negative sputum specimens (Upon Request; only 1 of 3 specimens tested)
Cepheid GeneXpert MTB/RIF Is the Newest FDA-Cleared Assay

- Detects MTB in <2H, ("same-day rule-in) enables immediate patient intervention
- Moderately complex
- Direct sputum claim; respiratory only
- Rifampicin resistance also identified
Cepheid GeneXpert MTB/RIF Is the Newest FDA-Cleared Assay

- ADD SPUTUM TO THE GeneXpert CARTRIDGE

- LOAD THE CARTRIDGE ON THE SMARTCYCLER INTERFACED WITH A COMPUTER
GeneXpert Automation

Raw Biological Sample
Loaded into Cartridge
Up to 5mL

Target Organisms are Concentrated, Isolated, and Washed

Cells and Organisms Lysed to Release their DNA

DNA Molecules Mixed with Amplification and Detection Chemicals

DNA Molecules Captured, Purified and Concentrated

Mixture Delivered to Integrated Reaction Tube for Amplification and Detection

DNA Molecules Captured, Purified and Concentrated
Xpert® MTB/RIF Targets

- The primers and probes detect MTB-complex and RIF resistance by amplifying 81 base pair of the *rpoB* gene core region.
- There are five molecular beacons (Probes A – E) for mutations within the rifampin-resistance determining region (RRDR). Each molecular beacon is labeled with a different fluorophore.
- If sample contains MTB sample, all probes will amplify. If any one of probes does not amplify due to mutation, Xpert MTB/RIF will detect mutation in *rpoB* gene.
Simultaneous Detection of MTB and rpoB gene mutation associated with Rif Resistance
GeneXpert Clinical Performance: Detection of TB

- Only 1.2% of tested samples (13/1,126) were non-determinate on the first attempt.

Source: Cepheid Xpert MTB/RIF Package Insert
GeneXpert Clinical Performance: Detection of RIF Resistance

Xpert MTB/RIF Performance vs. DST

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xpert MTB/RIF Test</td>
<td>94.7% (18/19)</td>
<td>99.0% (404/408)</td>
</tr>
</tbody>
</table>

Source: Cepheid Xpert MTB/RIF Package Insert

DST = Gold Standard Culture Assay
Xpert MTB/RIF test to complement the management of Airborne Infection Isolation (AII) of suspected TB patients based on the following statements:

• Airborne infection isolation (AII) precautions may be discontinued when contagious TB disease is considered unlikely and either:
  • Another diagnosis is made that explains the clinical syndrome or
  • The patient has three consecutive sputum smears negative for acid-fast bacilli on microscopy.

• AFB Smear positive, Xpert MTB/RIF negative is consistent with presence of non-tuberculosis mycobacteria (NTM).
  • Ruling out contagious TB, specimens can be tested by microscopy, NAA, or a combination of the two.
WHO Endorsement of Xpert MTB/RIF

The Test that is Changing the World

It’s a development that the world has been waiting for, for literally decades. It’s something that has the potential of truly revolutionizing the way we deal with TB today.

Dr. Mario Raviglione
Director, Stop TB, WHO
Laboratory Considerations for Use of Cepheid Xpert® MTB/RIF Assay

This document is intended to guide laboratories on integrating the Cepheid Xpert® MTB/RIF assay into existing TB testing practices.
DRUG RESISTANT GENOTYPING ASSAYS WERE DEVELOPED FOR THE FIRST LINE DRUGS

- RIFAMPIN
- INH
- PZA
- ETHAMBUTOL
CAPILLARY GEL ELECTROPHORESIS TO IDENTIFY THE SEQUENCE OF
DRUG-RESISTANT MUTATIONS IN THE RIFAMPIN RPO GENE

PARTIAL DEPICTION OF THE 240 BASE PAIR FRAGMENT WITH “HOT SPOTS” FOR MUTATIONS

- WILD: CTG AGC CAA TTC ATG GAC TCG CAC AAG TCG CTG
- MUTANT: CCG ACC CTA TTC ATT GTC CAG GAC CAG TTG CCG
TESTING CAVEATS

- FALSE NEGATIVES

Detects only 95% of Rifampin resistant MTB
5% of strains have mutations not detected by the assay.

- CONFIRMATION USING THE GOLD STANDARD CULTURE-BASED ASSAY REQUIRED
## GENE TARGETS FOR THE OTHER DRUGS

<table>
<thead>
<tr>
<th>DRUG</th>
<th>GENE</th>
<th>% SPECIFICITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td>kat G</td>
<td>75-90%; Codon 315</td>
</tr>
<tr>
<td></td>
<td>inhA</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>mabA</td>
<td>8-20%</td>
</tr>
<tr>
<td>PZA</td>
<td>pncA</td>
<td>72-95%</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>embB</td>
<td>47-69%; Codon 306</td>
</tr>
</tbody>
</table>
### Results of Our Year-Long Study

<table>
<thead>
<tr>
<th></th>
<th>Rifampin</th>
<th>Isoniazid</th>
<th>Pyrazinamide</th>
<th>Ethambutol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correctly Called Resistant/Total</td>
<td>39/39</td>
<td>59/74</td>
<td>35/38</td>
<td>17/19</td>
</tr>
<tr>
<td>Correctly Called Sensitive/Total</td>
<td>57/57</td>
<td>42/42</td>
<td>60/63</td>
<td>55/60</td>
</tr>
<tr>
<td>Total Called Correctly</td>
<td>96/96</td>
<td>101/116</td>
<td>95/101</td>
<td>72/79</td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(87.1%)</td>
<td>(94.1%)</td>
<td>(91.1%)</td>
</tr>
<tr>
<td>False Positive Rate</td>
<td>0.0%</td>
<td>0.0%</td>
<td>3.0%</td>
<td>6.3%</td>
</tr>
<tr>
<td>False Negative Rate</td>
<td>0.0%</td>
<td>12.9%</td>
<td>3.0%</td>
<td>2.5%</td>
</tr>
</tbody>
</table>
# Sensitivities

<table>
<thead>
<tr>
<th>FIRST LINE DRUG</th>
<th>Published Sensitivity</th>
<th>IDPH Sensitivity</th>
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</thead>
<tbody>
<tr>
<td>RIFAMPIN</td>
<td>95%</td>
<td>100%</td>
</tr>
<tr>
<td>INH</td>
<td>75-90%</td>
<td>87.1%</td>
</tr>
<tr>
<td>PZA</td>
<td>72-95%</td>
<td>94.1%</td>
</tr>
<tr>
<td>ETHAMBUTOL</td>
<td>47-69%</td>
<td>91.1%</td>
</tr>
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</table>
Rapid Sputum Assays are Preliminary

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>False Negative for Detection</td>
<td>Sensitivity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Smear Negative Sputum</td>
<td></td>
</tr>
<tr>
<td>False Positive for Detection</td>
<td>Laboratory Error</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Contamination</td>
<td></td>
</tr>
<tr>
<td>False Negative for Drug Resistance</td>
<td>Only a few sites (base pairs) causing mutation are tested</td>
<td>Many sites that are known to cause mutation are not tested</td>
</tr>
<tr>
<td>False Positive for Drug Resistance</td>
<td>Mutations do not always translate into drug resistant phenotypes</td>
<td>Silent mutations Polymorphisms</td>
</tr>
</tbody>
</table>
RESEARCH CASE STUDY #1

- 25 Year old male on multi-drug therapy since 6/1/09 (Rif; INH; PZA; E)
- Sputa collected 6/1/09, 6/13/09, 6/14/09, and 6/15/09
- All were AFB + (many); MTB detected in all by PCR
- Trouble encountered with drug phenotyping assay
RESEARCH CASE STUDY #1

- Patient not responding well to therapy
- Genotyping used in July to help understand why patient is not getting well.
  - Rifampin (wild type)
  - INH (wild type)
  - PZA (mutation)
  - Ethambutol (mutation; codon 306)
RESEARCH CASE STUDY #2

- Foreign student enters US in June with previous history of TB, but has no evidence of disease.
- In July student develops clinical symptoms of TB. Specimens were positive for AFB and for MTB by PCR.
- 4 drug therapy was initiated
- Return travel to homeland is based, in part, on whether the student is infected with drug-resistant MTB.
The dead line for return travel made phenotypic analysis impractical.

Genotyping was conducted on three sputa with the following results:

Rifampin (wildtype; drug susceptible)
INH (wildtype; drug susceptible)
PZA (wildtype; drug susceptible)
Ethambutol (wildtype; drug susceptible)
RESEARCH CASE STUDY #3

- Active extra pulmonary case involving lymph nodes (AFB positive/no culture/no prospects for another specimen)
- Sputum specimens negative for AFB/PCR
- Patient from India (4-5% drug resistance incidence) and travels frequently
- Genotyping from lymph node biopsy is a feasible alternative
OUTBREAK INVESTIGATION

- Resident of homeless shelter is infected with M. Tuberculosis
- Sputum specimens are AFB and PCR positive
- Sequence analysis performed for one of the infected individuals - MTB sensitive to all first line drugs
CULTURE

WHY IS IT IMPORTANT?

- Did AFB and/or PCR positive results detect live organisms (up to 42 days)?

- Identify species-confirm MTBC

- *M. tuberculosis* cultures are required for drug sensitivity/resistance assays
LIQUID CULTURES

- PROCESSED PRIMARY SPECIMENS ARE SEEDED INTO LIQUID CULTURE FOR THE ISOLATION OF MYCOBACTERIUM
- CULTURED FOR UP TO 42 DAYS
SOLID CULTURE ISOLATES PROVIDES MORPHOLOGIES
IDENTIFICATION OF MYCOBACTERIUM SPECIES IN CULTURE

- HPLC (mycolic acid analysis)

- FDA-cleared probe assays for MTBC, MAC, *M. kansaii*, and *M. gordonae*

- Commercial assays for MTBC and 17 clinically relevant non-tuberculous Mycobacterium

- Sequence analysis of the HSP65 gene; over 100 Mycobacterium species can be detected.

- Maldi TOF
REFLEX ASSAY REQUIRED: HSP65 DNA SEQUENCE ANALYSIS

- MTBC and all the usual clinically relevant species
- *M. mucogenicum*
- *M. arupense*
- *M. szulgai*
- *M. leprae*

*Will also identify non-*Mycobacterium* acid fast organisms such as *Nocardia spp.*
MYCOBACTERIUM TUBERCULOSIS COMPLEX (MTBC) DRUG RESISTANCE IN CULTURE

- Only cultures confirmed as MTBC are tested

- Liquid Culture (with and without the antibiotic) is used most often.

- Agar assays are the gold standard, but can’t be used for PZA
Average Turn Around Time is 21 days

- Wait for culture to grow
- Need to identify species
- 10-12 days to perform initial and repeat (for resistant) drug assay
TB STRAIN GENOTYPING: EPIDEMIOLOGICAL INVESTIGATIONS

- New rule mandates that laboratories send TB isolates to the IDPH for genotyping.

- IDPH sends isolates to the Michigan Dept. of Community Health-genotype assigned.

  Spoligotyping (Direct Repeats)
  Variable Number of Tandem MIRU Repeats
  Restriction Fragment Length Polymorphism
Spoligotyping

Example 1

Example 2
## MIRU Typing

### Example 1

<table>
<thead>
<tr>
<th>MIRU locus name</th>
<th>02</th>
<th>04</th>
<th>10</th>
<th>16</th>
<th>20</th>
<th>23</th>
<th>24</th>
<th>26</th>
<th>27</th>
<th>31</th>
<th>39</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of repeats</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

MIRU designation: 232234253322

### Example 2

<table>
<thead>
<tr>
<th>MIRU locus name</th>
<th>02</th>
<th>04</th>
<th>10</th>
<th>16</th>
<th>20</th>
<th>23</th>
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<th>26</th>
<th>27</th>
<th>31</th>
<th>39</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of repeats</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>11</td>
</tr>
</tbody>
</table>

MIRU designation: 14322404354b
Restriction Fragment Length Polymorphism (IS6110)
TB Genotype Management System (TB-GIMS)

- TRACKS OUTBREAKS
- IDENTIFIES CROSS CONTAMINATION