Considerations for Conventional Drug Susceptibility Testing and Molecular Detection of Drug Resistance

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National TB Conference
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Drug Susceptibility Testing (DST) of MTBC

- Guide in choice of chemotherapy—provide the best chance of cure
- Detect drug resistance or confirm the emergence of drug resistance when a patient fails to show a satisfactory bacteriologic response to treatment; guide the choice of treatment with different drugs
- Offer appropriate treatment to contacts
- Use to estimate the prevalence of primary and acquired drug resistance in a community
Evolution and Genetic Basis of Drug-Resistant TB

- Mutations that confer R occur spontaneously and independently.
- Within wild-type (not previously exposed to anti-TB drugs) MTB populations, small numbers of mutants are found to be R; R to >one drug is very rare.
- The tiny populations of these inherently R mutants are easily treated during appropriate multi-drug regimens.
- Selection of these naturally occurring drug-R mutant by inadequate TB treatment will cause the population of MTB bacteria to become increasingly drug R.
Predictors of Drug Resistant TB

- Previous episode of TB treatment
- Progressive clinical and/or radiographic findings while on TB therapy
- Origin from, history of residence in, or frequent travel to a region/country with high rates of drug R
- Exposure to an individual with infectious drug-RTB
Objectives of TB Treatment

- Cure the patient—prevent mortality, limit morbidity, prevent the development of relapse
- Stop TB transmission—render the patient non-infectious as rapidly as possible
- Prevent the emergence of drug resistance
Drug Susceptibility Testing (DST) of MTBC – Classic Definitions

- Critical concentrations of anti-TB drugs were adopted by international convention. The critical concentrations represent the lowest concentrations of drugs that inhibit 95% of “wild strains” of MTBC that have never been exposed to the drugs, while at the same time not inhibiting the strains of MTBC that have been isolated from patients who are not responding to therapy and are considered resistant.
Drug Susceptibility Testing (DST) of MTBC – Classic Definitions

- **Resistance** = Growth of >1% of an inoculum of bacterial cells in the presence of a “critical” concentration of anti-TB drug; “decrease in susceptibility of sufficient degree to be reasonably certain that a strain is different from a sample of wild strains of human type that have never come into contact with the drug”
### Critical Concentration

- **Ideally** is the lowest concentration of a drug that:
  - inhibits growth of all susceptible strains AND
  - allows growth of all resistant strains

- It is difficult to find a drug concentration that actually meets this definition; we settle for the concentration that **BEST DISCRIMINATES** between susceptibility and resistance

- Critical concentrations may differ depending on test medium
Critical Concentrations and Categorical Results

- Most common methods used for DST of MTBC (e.g., MGIT, Trek, agar proportion) generally test susceptibility at critical concentration

- Commonly reported as categorical result of resistant (R) or sensitive (S)
  - Sometimes a borderline result may be provided
  - Additional concentrations may be tested (e.g., isoniazid)
Commercial Broth Systems

- Selection of critical (testing) concentrations based on comparison of results with agar proportion = “equivalent critical concentrations”
- Much more rapid results (5-7 days) than agar proportion (21-28 days)
- FDA cleared for first-line drugs
  - MGIT – IRES, Z
  - TREK – IRE, Z
- Published evaluations of second-line drugs
Current Practice for First-line Drugs

- Broth-based methods are routine and widely available
  - Results generally available within 28 days of specimen receipt in laboratory
- Molecular assays (RMP, INH) are available in a few jurisdictions – Laboratory developed tests (LDT) or Research use only (RUO) tests
  - Performed directly on clinical specimens or on culture isolates
  - Results available within 1–2 days
First-line drug testing: U.S. Laboratories Participating in MPEP*

*Model Performance Evaluation Program (free program to assess participating laboratory’s drug susceptibility testing process for *M. tuberculosis* Complex. Participation is voluntary and individual laboratories are not identified in the aggregates reports generated for each shipment.
Minimum Inhibitory Concentration

- Definition: The lowest concentration of drug that inhibits visible growth of bacteria after incubation
  - Commonly referred to as MIC

- Increasing interest in establishing MICs for clinical isolates by testing a series of antituberculosis drug dilutions

- Methods might include laboratory developed tests for using automated broth systems (e.g., MGIT), agar proportion, or Thermoscientific Sensititre® MYCOTB MIC Plate
Minimum Inhibitory Concentration (2)

- Result might be reported as categorical result of resistant (R) or sensitive (S) if breakpoints have been established or simply as the MIC with no interpretation.

- Series of concentrations for each drug and breakpoints need to be established for each drug.

- Interpretive criteria for MIC results need to be established and the clinical utility of actual MIC results, not simply categorical results, for use in tuberculosis therapy needs to be evaluated.

- RVCT currently equipped to accept categorical results (‘R’ or ‘S’) and cannot accept MIC (i.e., concentration of drug).
In November 2009, TREK Diagnostic Systems launched a new Sensititre® MIC plate for the susceptibility testing of Mtb.

- The 96 well microtiter plate contains 12 first and second line drugs and has a minimum of 7 dilutions per drug.
- The plate is relatively simple to prepare and inoculate.
- The plate can be read manually using a view box or the Sensititre Vizion® System with the SWIN software platform.
- Cost ~$60 per test not counting tech time
Standard MYCOTB Microtiter Plate Setup

**SENSITITRE® CUSTOM PLATE FORMAT**

<table>
<thead>
<tr>
<th>Plate Code:</th>
<th>MYCOTB</th>
</tr>
</thead>
</table>

<table>
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**ANTIMICROBICS**

- OFL: Ofloxacin
- MXF: Moxifloxacin
- RIF: Rifampin
- AMI: Amikacin
- STR: Streptomycin
- RFB: Rifabutin
- PAS: Para-aminosalicylic acid
- ETH: Ethionamide
- CYC: Cycloserine
- INH: Isoniazid
- KAN: Kanamycin
- POS: Positive Control
- EMB: Ethambutol

- Inoculum: $5 \times 10^5$ cfu/ml
- Shelf Life: 6 mo.
- Plates per Box: 10
Growth in MYCOTB Microtiter Plate

OFL MXF RIF AMI STR RFB PAS ETH CYC INH KAN EMB
Laboratory Testing Algorithm

1. Process Specimen
   - 1 day
2. AFB Microscopy
3. Inoculate Media
   - 2 to 6 weeks
4. Culture Positive
5. Species Identification
   - 1 to 3 weeks
6. Drug Susceptibility
7. Molecular Detection of Drug Resistance
Detecting mutations associated with resistance

**DNA Sequencing**
- *Conventional sequencing*
- *Pyrosequencing*

**Liquid Hybridization**
- *Molecular Beacons (e.g., Xpert)*

**Solid-Phase Hybridization**
- *Line Probe Assays (e.g., Hain)*
Conventional DNA Sequencing

- PCR Amplification of target regions
- DNA Sequencing
- Sequence Analysis
Pyrosequencing

- Direct DNA sequencing of PCR products
- Unique chemistry
- Instrument carries out DNA sequencing reaction and analysis <2 hrs

**Sample ID:** 20102356

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<td>Entry ID</td>
<td>katG</td>
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<td>Sequence library</td>
<td>katG_pyro_Seq (2010-07-19, 11:00:50 AM)</td>
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<td>Query sequence</td>
<td>CCACAGG</td>
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**Result:** AGC-ACA Ser315Thr katG seq1
**Quality:** Good

**Hit 1:**

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**Query:** 1 CCACA 5 (?)
**Library:** 1 CCACA 5
### CDC MDDR Service: Drugs and Genes for Panel

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<tr>
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<td>Ethambutol</td>
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<tr>
<td>Isoniazid</td>
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<td>Pyrazinamide</td>
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<tr>
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<td>Fluoroquinolones</td>
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<td>Amikacin, Kanamycin, Capreomycin</td>
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<tr>
<td></td>
<td></td>
<td>Kanamycin</td>
<td></td>
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<td></td>
<td></td>
<td>Capreomycin</td>
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<td></td>
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<td><strong>MDR TB</strong></td>
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<td></td>
<td><strong>MDR TB</strong></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>tlyA (coding region)</td>
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</tbody>
</table>

<table>
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<tr>
<th>Sanger</th>
<th>inhA (-15, -8)</th>
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<th>pncA (promoter and coding regions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>katG (Ser315)</td>
<td></td>
<td></td>
<td>gyra (coding region)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>rrs (nt1401/1402,1484)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>eis (promoter region)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>tlyA (coding region)</td>
</tr>
</tbody>
</table>

### National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention
Division of Tuberculosis Elimination
GeneXpert

- Automated platform
- 4 module system
- Independent module design
  - Different test cartridges can be run at the same time (e.g., TB, influenza, MRSA)
- Bar code scanner
- Laptop with GeneXpert software
- Printer for printing results
- Uninterruptable power source (UPS)
**What is the GeneXpert MTB/RIF Assay?**

- Rapid molecular platform with results available within 2 hours of test start
- Results answer 2 questions simultaneously
  - Are MTBC present in the sample from the patient?
  - If MTBC are detected, are mutations associated with rifampin (RIF) resistance present?
<table>
<thead>
<tr>
<th>Advantages</th>
<th>Considerations</th>
</tr>
</thead>
<tbody>
<tr>
<td>▪ Much faster in detecting TB than culture</td>
<td>▪ Optimal testing criteria should be established</td>
</tr>
<tr>
<td>▪ Significantly faster in detecting RIF-resistant TB than conventional DST (hours vs. weeks)</td>
<td>▪ Only detects RIF-resistance so additional testing is needed for other first and second-line drugs</td>
</tr>
<tr>
<td>▪ Easy to handle</td>
<td>▪ Does not replace the need for culture and conventional DST</td>
</tr>
<tr>
<td>▪ Minor infrastructure requirements and no significant biosafety concerns</td>
<td></td>
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</table>
Possible Results for MTB/RIF Assay

1) MTB NOT DETECTED
2) MTB DETECTED/ Rif Resistance NOT DETECTED
3) MTB DETECTED/ Rif Resistance DETECTED
4) MTB DETECTED/ Rif Resistance INDETERMINATE
5) ERROR
6) INVALID
7) NO RESULT

Note: Rif Resistance DETECTED, Rif Resistance NOT DETECTED, or Rif Resistance INDETERMINATE will be displayed only in MTB DETECTED results and will be on a separate line from the MTB DETECTED result.
Line Probes utilize ‘reverse hybridization’

Samples are labeled ‘probes’ against immobilized sequences (wild type, control, specific mutations)
Important Things to Remember About Molecular Tests

- Finding a mutation does not always result in phenotypic resistance.
- A wild type sequence does not necessarily mean a strain is susceptible and should not be reported as such.
- We do not know every possible locus leading to resistance for any antibiotic.

RVCT currently accepts results for categorical conventional DST and cannot accept results from molecular assays for detection of mutations associated with resistance.
Molecular Diagnostics in the TB Laboratory Workflow

1. Direct detection in clinical specimen
   a) Is it *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 1 to 2 days after specimen receipt
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
  - Cepheid GeneXpert® MTB/RIF

- Laboratory developed tests or LDT (e.g., DNA sequencing, Loop-mediated isothermal amplification [LAMP], and real-time PCR assays including molecular beacons)
Reading a Laboratory Report
### Example - Public health lab
(elsewhere on report – sputum; collected 4/14/2011 [Thursday]; received in lab 4/14/2011)

<table>
<thead>
<tr>
<th>Test</th>
<th>Date</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>### Culture</td>
<td>5/17/2011</td>
<td>Mycobacterium tuberculosis complex</td>
</tr>
<tr>
<td>### Culture</td>
<td>5/17/2011</td>
<td>Method for ID: Gen Probe</td>
</tr>
<tr>
<td>### Culture</td>
<td>5/17/2011</td>
<td>See previous positive culture</td>
</tr>
<tr>
<td>### MTBC DNA PCR</td>
<td>4/18/2011</td>
<td>Positive for MTBC DNA</td>
</tr>
<tr>
<td>ME – Microscopic Exam</td>
<td>4/16/2011</td>
<td>Many</td>
</tr>
<tr>
<td>ME – Microscopic Exam</td>
<td>4/16/2011</td>
<td>Acid fast bacilli seen on</td>
</tr>
<tr>
<td>ME – Microscopic Exam</td>
<td>4/16/2011</td>
<td>Concentrated smear</td>
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</tbody>
</table>
**Example - Public health lab**

(elsewhere on report – sputum ; collected 5/31/2011 [Tuesday])

<table>
<thead>
<tr>
<th>Test</th>
<th>Date</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>### AFB Smear (Conc., Fluorochrome)</td>
<td>6/2/2011</td>
<td>No acid fast bacilli seen</td>
</tr>
<tr>
<td>### Amplified Mycobacterium Tuberculosis Direct Test (MTD)</td>
<td>6/2/2011</td>
<td>Positive for M. tuberculosis complex rRNA</td>
</tr>
<tr>
<td>### AFB culture</td>
<td>6/20/2011</td>
<td>AFB detected (ZN smear positive)</td>
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<tr>
<td>### Organism ID</td>
<td>6/22/2011</td>
<td>Probe positive for Mycobacterium tuberculosis complex</td>
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Example - Public health lab
(elsewhere on report – sputum; collected 6/30/2010 [Thursday])

<table>
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<tr>
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<tbody>
<tr>
<td>AFB Smear</td>
<td>7/1/2010</td>
<td>Acid fast bacilli present 10-90/F (fluorochrome stain)</td>
</tr>
<tr>
<td>AMTD Test</td>
<td>7/5/2010</td>
<td>Positive</td>
</tr>
<tr>
<td>Culture</td>
<td>7/7/2010</td>
<td>Mycobacterium tuberculosis complex Detected by DNA Probe</td>
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</table>
Example - Public health lab
(elsewhere on report – sputum; collected 5/31/2011 [Tuesday])

<table>
<thead>
<tr>
<th>Test</th>
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<th>Result</th>
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</thead>
<tbody>
<tr>
<td>AFB smear</td>
<td>6/2/2011</td>
<td>Acid fast bacilli seen: numerous</td>
</tr>
<tr>
<td>### Amplified Mycobacterium Tuberculosis Direct Test</td>
<td>6/2/2011</td>
<td>Positive</td>
</tr>
</tbody>
</table>
| ### HAIN Test GenoType MTBDRplus          | 6/8/2011 | No rpoB point mutation detected
No katG point mutation detected
inhA point mutation detected |
| ### AFB culture                           |          | Pending                                                               |
| ### Organism ID by PRA                    | 6/14/2011| Mycobacterium tuberculosis complex                                    |
MTB-RNA, Direct

Source: SPUTUM
MTB-RNA, Direct DETECTED Not detected

Qualitative detection of Mycobacterium tuberculosis complex rRNA is determined using the Amplified Mycobacterium tuberculosis Direct (MTD) test, manufactured and distributed by Gen-Probe, Inc. This test is recommended with initial pulmonary AFB smear and culture requests on untreated patients suspected of having tuberculosis. The MTD test is specific for, but does not differentiate among, members of the M tuberculosis complex. A negative result does not exclude the presence of infection with M.tuberculosis, and should be correlated with culture results.

This assay is FDA approved for use on sputum sample. The performance characteristics of this assay on non-respiratory specimens have been determined by Diagnostic Laboratory Services.

Collection Site or Method: SPUTUM

Acid Fast Smear And Culture

Acid Fast Smear:
4+ "ACID FAST BACILLI"

On respiratory samples, MTB Direct Amplification testing by PCR should be considered if clinical symptoms consistent with infection by Mycobacterium tuberculosis is present.

AFB Culture Status: FINAL

AFB Culture: AFB present in culture AFTER 18 DAYS

This isolate is confirmed by reference lab as XDR MTB.

M. tuberculosis complex DNA Probe
Result: POSITIVE
**Tests** | **Results** | **Reference Values**
---|---|---
**MTB, Nucleic Acid Amplification**
Source: MTB, Nucleic Acid Amplification | SPUTUM | Not detected

Qualitative detection of *Mycobacterium tuberculosis* complex rRNA is determined using the Amplified *Mycobacterium tuberculosis* Direct (MTD) test, manufactured and distributed by Gen-Probe, Inc. This test is recommended with initial pulmonary AFB smear and culture requests on untreated patients suspected of having tuberculosis. The MTD test is specific for, but does not differentiate among, members of the *M. tuberculosis* complex. A negative result does not exclude the presence of infection with *M. tuberculosis*, and should be correlated with culture results.

This assay is FDA approved for use on sputum sample. The performance characteristics of this assay on non-respiratory specimens have been determined by Diagnostic Laboratory Services.

Collection Site or Method: **SPUTUM**

**Acid Fast Smear and Culture**

Acid Fast Smear:
2+ "ACID FAST BACILLI"

On respiratory samples, MTB Direct Amplification testing by PCR should be considered if clinical symptoms consistent with infection by *Mycobacterium tuberculosis* is present.

AFB Culture Status: **FINAL**

AFB Culture:
- **POSITIVE** by conventional method.
- **AFTER 25 DAYS**
- Not MTB complex, no further ID
- Call lab for further identification and susceptibility if clinically indicated.

**M. tuberculosis complex DNA Probe**

Result (Isolate 1): **NEGATIVE**
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<th>Results</th>
<th>Reference Values</th>
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Qualitative detection of Mycobacterium tuberculosis complex rRNA is determined using the Amplified Mycobacterium tuberculosis Direct (MTD) test, manufactured and distributed by Gen-Probe, Inc. This test is recommended with initial pulmonary AFB smear and culture requests on untreated patients suspected of having tuberculosis. The MTD test is specific for, but does not differentiate among, members of the M. tuberculosis complex. A negative result does not exclude the presence of infection with M. tuberculosis, and should be correlated with culture results.

This assay is FDA approved for use only on respiratory specimen. The performance characteristics of this assay on non-respiratory specimen has not been determined and is unknown.

Collection Site or Method: SPUTUM

**Acid Fast Smear and Culture**

Acid Fast Smear:
1+ "ACID FAST BACILLI"

On respiratory samples, MTB Direct Amplification testing by PCR should be considered if clinical symptoms consistent with infection by Mycobacterium tuberculosis is present.

AFB Culture Status: FINAL

AFB Culture:
CULTURE NEGATIVE FOR ACID FAST BACILLI

NOTE: A positive smear and negative culture may indicate a lack of viability due to treatment or for other unknown reasons. If clinically indicated, recollection and culture is suggested.

*** FINAL REPORT ***

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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.