Drug susceptibility testing of *Mycobacterium tuberculosis* complex by culture-based and molecular methods

California Assoc. Public Health Lab Directors

October 2016

Edward Desmond, Ph.D., D (ABMM)

Microbial Diseases Laboratory
Trend: increasingly, TB is in the foreign born.
% MDR cases in U.S. ~ 1.1%

% MDR:
- Mexico 1.8%
- Philippines 5.6%
- India 4.3%
- Vietnam 3.7%
- China 6.4%
Exploding head zone: making culture-based DST for TB reproducible and clinically relevant
How can we establish drug susceptibility test concentrations for a new drug?

Classic approach for most bacteria: do pharmacokinetic studies and figure out the serum and urine achievable levels of drug.

Test concentration could be 1/8 of the achievable level. If a bacterium in culture is inhibited by 1/8 of the achievable level, then it should respond to the drug.

Right?
TB not so simple

- A mix of intracellular and extracellular bacilli: drug concentrations and drug activity are different inside and outside the cell
- A mixture of organisms in large lesions, where drug may penetrate poorly, and much smaller lesions
- A mixture of metabolically active and dormant bacilli
- Test concentrations based on pharmacokinetics may not predict treatment success accurately
How do we decide on test concentrations for TB drug susceptibility testing?

On the shoulders of giants we can see a long way—the Canetti discrimination method.
Test concentrations: “Discrimination approach”


Test MICs of
A. Strains from untreated patients and of
B. Strains from treatment failures

Test concentration = concentration at which A. are susceptible and B. are resistant.
Establishing the usefulness of *in vitro* drug suscept. testing

Clinical trials:

Drug susceptibility result compared with clinical response
Another giant to stand on...


Nugget of wisdom: animal models are useful in predicting success or failure of treatment regimens in humans. Pharmacokinetic studies: not so much...
There are many obstacles to accurate and reproducible DST results by culture or molecular methods

- New culture-based DST methods are calibrated to match results of existing methods, but the calibration is not very precise
  - Which drug concentration in a serial 2 fold dilution scheme will give results closest to the reference method?
- Culture media contain undefined biological components such as pancreatic digest of casein or bovine albumin
  - These can vary in composition from batch to batch
- Culture-based DST is not a perfect system
Lot to lot variability in culture media sometimes affects drug susceptibility testing results.

Guthertz, L., et al. 1988 JCM 26:2338: “This study demonstrates that individual lots of components of this basal medium may vary significantly in their suitability for susceptibility testing, and failure to detect such variation may dramatically affect susceptibility profiles.”

- Medium base and (OADC) supplement contain biological materials which may vary from lot to lot.
- This may affect growth support or drug activity in media made with these components.
FIG. 1. Reduction of colony size by a new component and the effect of this component on the overall growth.
Maintaining lot to lot consistency of DST media and components

- One approach: define medium components as carefully as possible, and do pre-market testing of components to assure lot to lot consistency
  - A manufacturer’s responsibility—will they do it?
- Another approach: when a new batch of medium component is received, make up some media with new and some with old component and compare growth support and drug MICs between new and old lots
  - DST laboratory’s responsibility (an onerous one)
Consistency over time: culture-based DST vs. DNA sequencing

- Culture-based DST may yield inconsistent results due to changes in medium composition which are very difficult to avoid.
- DNA sequences are objective and not subject to subtle changes in manufactured components.
- But first the links between DNA sequences and *in vitro* and *in vivo* drug resistance must be established.
- It may take decades of work to establish the links between mutations and combinations of mutations on drug resistance.
Current issues in culture-based drug susceptibility testing

- Ethambutol—occasional differences between results by agar proportion method vs. rapid broth methods
  - Not clear which method is correct
- Pyrazinamide—false resistant results by rapid broth methods are common
  - DNA sequencing of pncA gene may be more reliable
Current issues in culture-based drug susceptibility testing, cont’d

Low level rifampin resistance: Presence of a mutation in rpoB, which causes a change in amino acid sequence, leading to an increase in MIC above that of the wild type, but <1 ug/ml in MGIT so that the culture will test as susceptible in MGIT.
Reports of treatment failure associated with "low level" resistance continue

- Williamson IJTLD 2012 16:216
- 3 New Zealand cases in which Cepheid GeneXpert detected rpoB mutation, but culture tested susceptible to rif in MGIT
- Treatment failed in these 3 cases (all were INH-resistant)
- Mutations 516 Tyr and 526 Leu were assoc. w/ rifampin MICs of 0.25 and 0.5 respectively
What to do about these drug susceptibility testing issues: my own opinions

- Ethambutol (EMB): if the TB culture is susceptible to INH, then a susceptible result for EMB is reliable.
  - In critical cases, where there is INH resistance, consider doing an MIC with the Sensititre system or in MGIT:
    - If MIC is <4, consider susceptible
    - If MIC is 4, consider borderline
    - If MIC is >4, consider resistant
What to do about these drug susceptibility testing issues: my own opinions, cont’d

**Pyrazinamide (PZA):**

- When PZA monoresistance is found, report but also either:
  - Repeat, making sure to avoid over-inoculation.
  - Have pncA gene and promoter region sequenced. This is probably the most accurate way of determining PZA susceptibility/resistance.
What to do about these drug susceptibility testing issues: my own opinions, cont’d

Detecting low-level rifampin (RIF) resistance: do we need to do so routinely?

- INH-susceptible strains likely to be RIF susceptible as well
- When there is INH resistance, consider molecular testing to detect rpoB mutation
  - Molecular testing could be GeneXpert or sequencing
- Low-level RIF resistance is uncommon and it may be unnecessary to look for it routinely, except in more difficult-to-treat cases
Trend: Molecular methods enable rapid detection of drug-resistant TB

GeneXpert is being used internationally

GeneXpert and DNA sequencing are becoming a new standard of practice in the USA
Suspicion of drug resistance → request for molecular testing for drug resistance

- History of previous treatment
- Foreign born from country with increased drug resistance
- Patients not responding well to treatment
- Patients known to have been exposed to a person with MDRTB
Steingart, K., et al. 2013. Cochrane Collaboration Report (meta-analysis) for detecting M. tb, 88% sensitivity compared with culture (98% for smear + and 68% for smear neg) for detecting rifampin resistance, sensitivity of 94% and specificity of 98%. 

Cepheid Gene Xpert, cont’d
Due out soon: GenXpert Ultra:
more sensitive than previous generations:

- LOD equivalent to 10 – 100 CFU/ml
- Four newly designed probes detect mutations in rpoB gene
- *Mtb* detecting probes targeting IS6110 and IS1081 were added.
- None of the 3 RIF-S synonymous rpoB Q513Q (1) and F514F (2) mutant samples were detected as RIF-R
- Cepheid may not seek FDA clearance in USA
One caution about GeneXpert Mtb/RIF

- Occasional false report of rifampin resistance due to silent or synonymous mutation
- GeneXpert molecular beacon probes will hybridize with normal wild-type sequence
- When there is a silent or synonymous mutation the amino acid sequence of the RNA polymerase is not altered, so rifampin susceptibility is not affected
- GeneXpert will detect the mutation and report it as resistant
<table>
<thead>
<tr>
<th>Rifampin Phenotype</th>
<th>Resistant</th>
<th>Susceptible</th>
<th>Total</th>
<th>98% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xpert Rifampin-R</td>
<td>9*</td>
<td>6**</td>
<td>15</td>
<td>90% Sensitivity</td>
</tr>
<tr>
<td>Xpert Rifampin-S</td>
<td>1</td>
<td>418</td>
<td>419</td>
<td>99% Specificity</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>424</td>
<td>434</td>
<td>2% Prevalence</td>
</tr>
</tbody>
</table>

**60% PPV**

100% NPV

Overall, in the USA, 18-20% of rpoB mutations detected by GeneXpert are silent mutations not associated with rifampin resistance (unpublished results from CDC and California laboratory)
<table>
<thead>
<tr>
<th>Specimen</th>
<th>Probe</th>
<th>rpoB Mutation</th>
<th>Texas RMP</th>
<th>CDC RMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A*</td>
<td>No Amplification</td>
<td>S</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>Phe514Phe</td>
<td>S</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>Phe514Phe</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>Phe514Phe</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>Phe514Phe</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>6</td>
<td>B</td>
<td>Asp516Tyr &amp; Asp549Asn</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>7</td>
<td>C</td>
<td>Ser522Leu</td>
<td>R/S</td>
<td>S**</td>
</tr>
<tr>
<td>8</td>
<td>D</td>
<td>His526Tyr</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>9</td>
<td>D</td>
<td>His526Asp</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>10</td>
<td>E</td>
<td>Ser531Leu</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>11</td>
<td>E</td>
<td>Ser531Leu</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>E</td>
<td>Ser531Leu</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>E</td>
<td>Ser531Leu</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>14</td>
<td>E</td>
<td>not tested</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>E</td>
<td>Leu533Pro</td>
<td>R/S</td>
<td>S**</td>
</tr>
<tr>
<td>16</td>
<td>none</td>
<td>Ile572Phe</td>
<td>R</td>
<td>S**</td>
</tr>
</tbody>
</table>

Note that GXP probe B mutation was not associated with rifampin resistance.

THANKS TO KEN JOST FROM TEXAS STATE LAB
<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. Confirmatory testing should follow</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>
Rapid detection of drug resistant TB at CDC or CA laboratory

Uses DNA sequencing of genes associated with resistance to 1st and 2nd line drugs

Turnaround time of 1-4 days
MDDR Service: Testing Algorithm

- Entire molecular panel
- Routine agar proportion DST panel (INH, RIF, EMB, STR, FQ, AMK, KAN, CAP, ETH, PAS) and MGIT PZA
MDDR Service: Specimens

Isolates of *M. tuberculosis* Complex

- Solid media
- Positive broth cultures (e.g., MGIT)

Acid-fast smear-positive sputum sediments, with prior approval from CDC
<table>
<thead>
<tr>
<th>Drug</th>
<th>Gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIF</td>
<td>rpoB, inhA, katG, rrs, eis, rrs, tlyA</td>
</tr>
<tr>
<td>INH</td>
<td>inhA, katG, rrs, eis</td>
</tr>
<tr>
<td>KAN</td>
<td>rrs</td>
</tr>
<tr>
<td>AMK</td>
<td>rrs</td>
</tr>
<tr>
<td>CAP</td>
<td>rrs, tlyA</td>
</tr>
<tr>
<td>FQ</td>
<td>gyrA</td>
</tr>
<tr>
<td>PZA</td>
<td>pncA</td>
</tr>
<tr>
<td>EMB</td>
<td>embB</td>
</tr>
</tbody>
</table>
Pyrosequencing for detection of drug resistance

- Short run times (5 hours)
- Shorter sequences are determined than more traditional sequencing
- Can work directly on sputum sediments
- Available now as a routine service at the CA State laboratory
- Can detect resistance to INH, rif, FQ, injectables (e.g. amik, kana, capreo)
Hit 1: gyrA resistant codon 94ggc     Score: 100     Identities: 31/31 (100%)
Query 1   CCACCCGCACGGCGACGCGTCGATCTACGGC 31
Library 1  CCACCCGCACGGCGACGCGTCGATCTACGGC 31

Hit 2: gyrA susceptible     Score: 94.3     Identities: 30/31 (97%)
Query 1   CCACCCGCACGGCGACGCGTCGATCTACGGC 31
Library 1  CCACCCGCACGGCGACGCGTCGATCTACGAC 31
<table>
<thead>
<tr>
<th>Presence of M. bovis</th>
<th>pncA codons 54-61</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td>katG (codon 312-316), promoter of inhA and ahpC, and fabG1</td>
</tr>
<tr>
<td>RIF</td>
<td>rpoB (codons 507 to 533, and 176)</td>
</tr>
<tr>
<td>Quinolones</td>
<td>gyrA (codons 88 to 95)</td>
</tr>
<tr>
<td>Injectable drugs</td>
<td>rrs (positions 1397 to 1406)</td>
</tr>
</tbody>
</table>
Taking it to a new level

Predicting quantitative drug susceptibility and response to different drugs with a class of drugs within a day by DNA sequencing
Rifabutin MICs associated with various rpoB mutations  

Note rifampin MICs in red in column headings.

<table>
<thead>
<tr>
<th>RBU MIC (μg/mL)</th>
<th>None</th>
<th>531 tcg → ttg Rif &gt;8</th>
<th>516 gac → gtc Rif 0.5-8</th>
<th>526 cac → ctc Rif =2</th>
<th>526 cac → ggc Rif = 1</th>
<th>526 cac → agc Rif = 1</th>
<th>526 cac → tgc Rif 2-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥8</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>9</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td></td>
<td>6</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.125</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>≤0.0625</td>
<td>37</td>
<td></td>
<td>1 (RIF S)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Samples</td>
<td>42</td>
<td>34</td>
<td>17</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>
# MOX MIC & Mutations in **gyrA**

<table>
<thead>
<tr>
<th>MOX MIC (ug/ml)</th>
<th>Mutations ( # of strains)</th>
<th>Total # strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90gtg 94gcc 95acc 94ggc 91ccg 94cac 94tac</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1 1 1</td>
<td>3</td>
</tr>
<tr>
<td>0.75</td>
<td>6 3 1</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>1 1 1 1 1 1</td>
<td>5</td>
</tr>
<tr>
<td>1.5</td>
<td>1 2 5 1 2</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>1 3 2 1</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total # strains</td>
<td>8 6 6 13 2 4 1</td>
<td>40</td>
</tr>
<tr>
<td>Company</td>
<td>GeneXpert® MTB/RIF</td>
<td>HAIN Genotype® MTBDRplus</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td><strong>Genetic loci</strong></td>
<td>rpoB (for RMP)</td>
<td>rpoB (RMP), katG (INH), and inhA (INH)</td>
</tr>
<tr>
<td><strong>Format</strong></td>
<td>Semi-automated real-time PCR</td>
<td>Line probe assay</td>
</tr>
<tr>
<td><strong>FDA approved</strong></td>
<td>Market authorization</td>
<td>No</td>
</tr>
<tr>
<td><strong>Expected turn-around time from specimen receipt in laboratory</strong></td>
<td>1-2 working days</td>
<td>1-2 working days (depends on how often performed in lab)</td>
</tr>
</tbody>
</table>
Trend: DNA sequencing is revealing complexities of drug susceptibility and resistance which were not previously appreciated.
Expected discrepancies (1)

Between culture-based methods: agar proportion (AP) vs. rapid broth (MGIT or VersaTrek)

Example A:

- **Ethambutol**: November 2010 MPEP– duplicate isolates with Met306Val mutation
  - 90% of labs using AP detected drug
  - 23% of labs using MGIT detected drug

Note: for most cultures, ethambutol results by AP and MGIT agree. The frequency of discrepancies is unknown. But there is a tendency for AP to detect more resistance than MGIT.
Expected discrepancies (2)
Between molecular methods (probes vs. sequencing)

Silent or synonymous mutations in the rifampin-resistance determining region (RRDR) of rpoB gene:

Cepheid GeneXpert: mutation detected, likely rifampin resistant

DNA sequencing: silent or synonymous mutation detected, likely drug susceptible
Expected discrepancies (3)

Between molecular and culture-based drug susceptibility testing methods, cont’d

“Disputed” or “low level resistance” mutations in rpoB, e.g. Asp516Tyr or Leu511Pro

- GeneXpert: mutation present, resistance predicted
- Sequencing based method: exact mutation identified, clinical significance beginning to be understood
- Culture-based DST: may test as susceptible or resistant (AP more likely to detect resistance than MGIT)
Expected discrepancies

Between molecular and culture-based drug susceptibility testing methods, cont’d

- Targeted DNA sequencing at CDC and California labs sequences only gene segments more commonly associated with drug resistance
- Less commonly, mutations may occur in other genes or gene loci, leading to resistance
  - rpoB sequencing detects 98% of Rif resistance
  - Sequencing of katG and inhA promoter detects about 90% of INH resistance
- Follow-up culture-based DST is recommended
- In the future, whole genome sequencing is expected to improve the sensitivity of molecular detection of drug resistance
Which technology gives most accurate results? It depends:

<table>
<thead>
<tr>
<th>Technology</th>
<th>Heteroresistance (mixture of S and R)</th>
<th>Silent mutation</th>
<th>Low level RIF resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture-based DST</td>
<td>☑️</td>
<td>☑️</td>
<td>☑️</td>
</tr>
<tr>
<td>Probe-based molecular (e.g. GXP)</td>
<td></td>
<td>☑️</td>
<td>☑️</td>
</tr>
<tr>
<td>DNA sequencing</td>
<td></td>
<td>☑️</td>
<td>☑️</td>
</tr>
</tbody>
</table>
Decontaminated, concentrated

sputum sediment

GeneXpert MTB/Rif
- TB pos
- Rif suscept.

MTB/Rif
- TB pos
- rpoB mutation

Acid-fast smear
- TB
- negative

Culture (DST if positive)

Retained sediment

Send retained sediment

Sediment for DNA sequencing

Detect silent and low level resistance mutations

Detect heteroresistance
Bedaquiline

- Trade name 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
- Possible drug-drug interactions are a concern:
  - BDQ is metabolized by cytochrome p450 isoenzyme, which is induced by rifampin (van Heeswijk 2014 J. Antimicrob. Chemother.)
Other new drugs

- **Delaminid**
  - In 2014, delamanid was granted a marketing authorization by the European Commission for use MDR-TB treatment.
  - Delaminid is a nitro-dihydro-imidazooxazole 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}.
  - A derivative of PA-824 (TBA-354) appears to have superior activity and stability.

- **MIC testing being evaluated and mechanisms of resistance still under discovery**
Take home points

- DNA sequencing provides rapid results not altered by subtle changes in reagents.
- Correlation between sequence changes and drug susceptibility may be complex and multifactorial for some drugs. Elucidation may require extensive study.
- Some mutations are clearly associated with resistance.
- Culture-based DST may be affected by subtle changes in culture medium ingredients.
  - Is rigorous QA by the end user practical?
  - Would more rigorous pre-market testing by manufacturer help?
- For ethambutol, clinical correlation of DST may need to be re-established.
- Testing algorithms may be designed to overcome weaknesses in current test methods.
Thank you!