Management of discordant *Mycobacterium tuberculosis* resistance tests

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Credit, where credit’s due
Discordance

• “A state of non-harmony or non-agreement”

• Non-agreement between
  – Different genotypic tests
  – Genotypic and phenotypic tests
  – 1 or more of genotypic and/or phenotypic tests and clinical response to treatment
Genetic mutation gives rise to resistance

**GENOTYPIC RESISTANCE**

- Detected by molecular testing e.g. Xpert, LPA, sequencing

**Problems:**
- Genotype-phenotype relationship incompletely understood
- Geographic variability in distribution of mutations
- Heteroresistance and mixed infections
- Not all genetic mechanisms for resistance are known

MTB fails to grow or survives in presence of antibiotic

**PHENOTYPIC RESISTANCE**

- Detected by culture-based testing e.g. MGIT, MODS, Sensititre

**Problems:**
- Slow
- Complex for some drugs (MICs close to critical concentration)
- Controversy over critical concentrations
- Biosafety
- In vivo correlation with in vitro results is not known for some drugs

Patient fails to respond to therapy with the drug

**CLINICAL RESISTANCE**

- Usually undetected but may result in failure of smear conversion, treatment failure or relapse

**Problems:**
- Too late!
Heteroresistance

• Sensitive and resistant *M. tuberculosis* in a single clinical sample
• Resistance mutations arise from a single clone
• Spontaneous or driven by antibiotic selection pressure
• Picked up by LPA, molecular phenotyping (MIRU-VNTR) or by genome sequencing
• Assaying single samples doesn’t allow us to define full extent of heteroresistance, nor the significance of various mutations
Dynamic Population Changes in *Mycobacterium tuberculosis* During Acquisition and Fixation of Drug Resistance in Patients

Gang Sun,¹,² Tao Luo,¹,³ Chongguang Yang,¹ Xinran Dong,² Jing Li,³ Yongqiang Zhu,⁴ Huajun Zheng,⁴ Weidong Tian,² Shengyue Wang,⁴ Clifton E. Barry III,⁵ Jian Mei,³ and Qian Gao¹

![Diagram](image)

**Untreated**

19 months therapy

- 4 INH mutations
- 3 in *katG*
- 1 in *inhA* promoter

24 months therapy

- Expansion of *katG* D94N
- Suggesting superior fitness

J. Infect Dis 2012;206:1724-33
Mixed Populations

• Presence of drug-sensitive and drug-resistant populations of different clonality in the same person

• Driven by high rates of infection & re-infection in TB-endemic populations

Identification of multiple *M. tuberculosis* infections in sputum samples

March 2000 to June 2002 (HIV co-infection < 10%)

186 patients

- Male 126 and Female 60

- 61 patients infected with Beijing strains
  
  - Male 40 and Female 21

  - Single infection
    
    26 (43%) patients infected with a Beijing strain only
    
    Male 19 and Female 7

  - Mixed infection
    
    35 (57%) patients infected with a Beijing strain and a non-Beijing strain.
    
    Male 21 and Female 14


Slide courtesy of Paul van Helden
Rifampicin action & resistance

• Inhibits DNA synthesis by binding to RNA polymerase
• RNA polymerase is encoded by the \textit{rpoB} gene
• 95% of rifampicin resistance due to SNP in the 81bp rifampicin resistance determining region (RRDR)
  – alters RNA pol structure, inhibiting rifampicin binding
• RRDR is the target for Xpert MTB/RIF and GenoType MTBDR\textit{plus} line probe assay
Rifampicin Resistance Determining Region (RRDR)

* M. tuberculosis rpoB gene
Codons are numbered according to the rpoB gene of Escherichia Coli
Xpert MTB/RIF Molecular Beacons

Rifampicin Sensitive Xpert

All 5 probes & *B. globigii* control amplify (fluoresce)

Rifampicin Resistant Xpert

Probe B (green) fails to amplify

Trouble shooting Xpert MTB/RIF: False-positive rifampicin resistance

• Procedural
  – preparation of specimen
  – delay in running the assay
  – air bubbles

• Very low Mycobacterial load

• Delay to reach cycle threshold ($C_T$) rather than dropout
  – $C_T$ delay <4 sensitive
  – $C_T$ delay >5 resistant
  – $C_T$ delay 4.1 – 4.9 difficult to interpret

• Probe E involvement or >1 probe involved (D + E)

• Extra-pulmonary specimens

Trouble shooting Xpert – False-negative ‘susceptible’ in mixed infection or heteroresistance

• Need 65-100% resistant strain DNA to be picked up

• Resistant strains partially inhibiting hybridization, would only need small concentration of sensitive strain amplicon to boost probe signal into normal range

• More common in hyperendemic regions
GenoType MTBDRplus version 2.0

95% RIF-RES encoded for by 4 mutations which cause high level resistance with MICs > 16μg/ml

Corresponding Xpert Probe

Probe B

Probe D

Probe E
Examples of GenoType MTBDRplus
Sensitive & Resistance profiles
## Trouble shooting GenoType MTBDRplus

<table>
<thead>
<tr>
<th>Error</th>
<th>False positive ‘resistant’</th>
<th>False negative ‘susceptible’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procedural</td>
<td>Hybridization</td>
<td>Hybridization</td>
</tr>
<tr>
<td></td>
<td>Bands too dark</td>
<td>Bands too light</td>
</tr>
<tr>
<td>Cross contamination</td>
<td>Cross contamination leads to overcalling</td>
<td>Some mutations at very end of amplified sequence (L533P) can be missed</td>
</tr>
<tr>
<td></td>
<td>hetero-resistance/mixed infections</td>
<td>(earlier version)</td>
</tr>
<tr>
<td>Interpretation</td>
<td>Subjective reading error or scanner error</td>
<td>Slide courtesy of Yonas Ghebrekristos</td>
</tr>
</tbody>
</table>

**Faint Amplification Control (AC)**
Culture–based DST

- Critical concentration of drug at which susceptible strains don’t grow & resistant strains do
- Agar proportion method considered reference standard
- Future MIC testing would give more accurate information e.g. Sensititre

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>9</th>
<th>10</th>
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<tbody>
<tr>
<td>A</td>
<td>OFL 32</td>
<td>MXF 8</td>
<td>RIF 16</td>
<td>AMI 16</td>
<td>STR 32</td>
<td>RFB 16</td>
<td>PAS 64</td>
<td>ETH 40</td>
<td>CYC 256</td>
<td>INH 4</td>
<td>KAN 40</td>
<td>EMB 32</td>
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<tr>
<td>B</td>
<td>OFL 16</td>
<td>MXF 4</td>
<td>RIF 8</td>
<td>AMI 8</td>
<td>STR 16</td>
<td>RFB 8</td>
<td>PAS 32</td>
<td>ETH 20</td>
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<td>KAN 20</td>
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<tr>
<td>C</td>
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<td>MXF 2</td>
<td>RIF 4</td>
<td>AMI 4</td>
<td>STR 8</td>
<td>RFB 4</td>
<td>PAS 16</td>
<td>ETH 10</td>
<td>CYC 64</td>
<td>INH 1</td>
<td>KAN 10</td>
<td>EMB 8</td>
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<tr>
<td>D</td>
<td>OFL 4</td>
<td>MXF 1</td>
<td>RIF 2</td>
<td>AMI 2</td>
<td>STR 4</td>
<td>RFB 2</td>
<td>PAS 8</td>
<td>ETH 5</td>
<td>CYC 32</td>
<td>INH 0.5</td>
<td>KAN 5</td>
<td>EMB 4</td>
</tr>
<tr>
<td>E</td>
<td>OFL 2</td>
<td>MXF 0.5</td>
<td>RIF 1</td>
<td>AMI 1</td>
<td>STR 2</td>
<td>RFB 1</td>
<td>PAS 4</td>
<td>ETH 2.5</td>
<td>CYC 16</td>
<td>INH 0.25</td>
<td>KAN 2.5</td>
<td>EMB 2</td>
</tr>
<tr>
<td>F</td>
<td>OFL 1</td>
<td>MXF 0.25</td>
<td>RIF 0.5</td>
<td>AMI 0.5</td>
<td>STR 1</td>
<td>RFB 0.5</td>
<td>PAS 2</td>
<td>ETH 1.2</td>
<td>CYC 8</td>
<td>INH 0.12</td>
<td>KAN 1.2</td>
<td>EMB 1</td>
</tr>
<tr>
<td>G</td>
<td>OFL 0.5</td>
<td>MXF 0.12</td>
<td>RIF 0.25</td>
<td>AMI 0.25</td>
<td>STR 0.5</td>
<td>RFB 0.25</td>
<td>PAS 1</td>
<td>ETH 0.6</td>
<td>CYC 4</td>
<td>INH 0.06</td>
<td>KAN 0.6</td>
<td>EMB 0.5</td>
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<tr>
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<td>OFL 0.25</td>
<td>MXF 0.06</td>
<td>RIF 0.12</td>
<td>AMI 0.12</td>
<td>STR 0.25</td>
<td>RFB 0.12</td>
<td>PAS 0.5</td>
<td>ETH 0.3</td>
<td>CYC 2</td>
<td>INH 0.03</td>
<td>POS</td>
<td>POS</td>
</tr>
</tbody>
</table>

Lee J. AAC 2014;58(1):11
Principle of culture-based DST

## Trouble shooting phenotypic DST

<table>
<thead>
<tr>
<th>False Resistance</th>
<th>False Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Too low critical concentration of drug or loss of antibiotic potency</td>
<td>Too high critical concentration drug</td>
</tr>
<tr>
<td>Inoculum too high</td>
<td>Inoculum too low</td>
</tr>
<tr>
<td>Contamination with either NTM or DR-TB</td>
<td></td>
</tr>
</tbody>
</table>
National algorithm for diagnosis of pulmonary tuberculosis
Xpert MTB/RIF

MTB Present

Rifampicin-S

2nd Specimen Microscopy

6m RIFAFOUR

Rifampicin-R

2nd Specimen Culture & LPA to confirm RIF-R

If Rif-R

Phenotypic DST to 2nd line drugs & INH

MTB Absent

If HIV infected

2nd Specimen Culture & LPA [or DST]

If Rif-R

Phenotypic DST to 2nd line drugs & INH
## Concordant Rifampicin Resistance

<table>
<thead>
<tr>
<th>XPERT</th>
<th>LPA</th>
<th>Pheno-DST</th>
<th>Scenario</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>R</td>
<td>R</td>
<td>&gt;95% cases</td>
<td>&gt;90% of cases <em>rpoB</em> mutations are high level RIF resistant (MICs&gt;16ug/ml) &amp; detected by MGIT DST at a critical concentration of 1ug/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>These are S531L, H526Y, H526D, D516V (all specifically detected by LPA)</td>
</tr>
</tbody>
</table>

Slide courtesy of N Beylis
## Discordant Rifampicin Results (1)

**XPERT-R : LPA-R : Pheno-DST-S**

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncommon</td>
<td>• Disputed <em>rpoB</em> mutations in RRDR may be detected by Xpert &amp; LPA</td>
</tr>
<tr>
<td>Affects &lt;5-10% of all <em>rpoB</em> mutations in the RRDR</td>
<td>• Effect on DST varies - low level resistance or susceptible, depending on SNP</td>
</tr>
<tr>
<td></td>
<td>• Needs confirmation by <em>rpoB</em> sequencing and MIC testing</td>
</tr>
</tbody>
</table>

Adapted from slide by N Beylis
## Discordant Rifampicin Results (2)

### XPERT-R : LPA-S : Pheno-DST-S/R

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncommon but increasingly recognized</td>
<td>• False Xpert-R or false LPA-S must be decided by <em>rpoB</em> sequencing</td>
</tr>
<tr>
<td></td>
<td>• GSH/Greenpoint study of 100 patients over 12m*</td>
</tr>
<tr>
<td></td>
<td>- XPERT-R was FALSE in 77% (no <em>rpoB</em> mutation)</td>
</tr>
<tr>
<td></td>
<td>- LPA-S was FALSE in 23%</td>
</tr>
<tr>
<td></td>
<td>• Heteroresistance or mixed population</td>
</tr>
<tr>
<td>If XPERT-R is real</td>
<td>• Phenotypic DST result depends on whether the <em>rpoB</em> mutation confers high level resistance (Pheno DST-R) or low level resistance (Pheno-DST-S/R)</td>
</tr>
</tbody>
</table>

*Ghebrekristos Y et al. Union TB conference, Cape Town, 2015*
Sequencing: L533P mutation detected (not picked up on LPA on previous versions)

1. Xpert: RIF-R
2. LPA: RIF-S
3. Phenotypic DST: RIF-S

FALSE susceptible LPA phenotypic result?
Sequencing: No mutation detected

1. Xpert: RIF-R
2. LPA: RIF-S
3. Phenotypic DST: RIF-S

Sequencing: No mutation detected
## Discordant Rifampicin Results (3)
**XPERT-S : LPA-R : Pheno-DST-S/R**

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Explanation</th>
</tr>
</thead>
</table>
| Uncommon as if Xpert-S, algorithm only allows 2\textsuperscript{nd} specimen for microscopy, not LPA | • False Xpert-S / false LPA-R decided by *rpoB* sequencing  
|                                                                           | OR                                                                                             |
|                                                                           | • Heteroresistance / mixed population                                                           |
## Discordant Rifampicin Results (4)

**XPERT-S : LPA-S : Pheno-DST-R**

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Explanation</th>
</tr>
</thead>
</table>
| Uncommon as if Xpert-S, algorithm only allows 2\textsuperscript{nd} specimen for microscopy, not LPA | • *rpoB* mutations outside the RRDR  
• Efflux pumps are not be detected by Xpert or LPA  
• Limit of detection for genotypic tests is above the level of the Rifampicin-R mutant in the sample |
Discordant Isoniazid Results
LPA-S : Phenotypic-DST-R

- Isoniazid resistance
  - 60-70% due to *katG* mutation (high level)
  - 10-20% due to *inhA* mutation (low level)
- Resistance engendered by non-*katG/inhA* mutation mechanisms will not be picked up

Adapted from slide by N Beylis
What does heteroresistance or mixed populations look like on the LPA?

Rifampicin and Isoniazid heteroresistance
Due to the current diagnostic approach, which relies on Xpert as the starting point, heteroresistance usually becomes apparent either once the LPA is performed, if multiple samples get through the system, or the patient presents with ‘failure to thrive’
Clinical outcomes of patients with discordant diagnostic tests, heteroresistance or mixed infections
Mixed *Mycobacterium tuberculosis* Complex Infections and False-Negative Results for Rifampin Resistance by GeneXpert MTB/RIF assays: Associated with Poor Clinical Outcomes

Nicola M. Zetola,a,c,d Sanghyuk S. Shin,h Kefentse A. Tumedi,a Keletso Moeti,b Ronald Ncube,a Mark M. Byass,a Stephen O. S. G. Mhofu, a,b,c,d Jeffrey D. Klausner,h Chawangwa Modongoa,c

- Retrospective cohort study in Botswana
- Data from the National TB Treatment Program
- Predictors of poor clinical outcome
  - Xpert RIF
    - OR 6.6 (95% CI 2.1-20.5) p<0.001
  - Mixed *Mycobacterium tuberculosis* infections
    - OR 6.5 (95% CI 1.2-48.2) p=0.03

Should Xpert RIF-S tuberculosis be interrogated further in high prevalent TB endemic setting?
Managing mixed infections

Treat for both DS-TB and DR-TB
How should we treat patients with disputed *rpoB* mutations?

- Sequenced sputum from 1\textsuperscript{st} failure or relapse
- 10.6% samples from Kinshasa and 13.1% from Bangladesh had disputed mutations
  - 511Pro, 516Tyr, 526Asn, 526Leu, 533Pro, 572Phe
Rifampin Drug Resistance Tests for Tuberculosis: Challenging the Gold Standard

Armand Van Deun,a,b Kya J. M. Aung,c Valentin Bola,d Rossin Lebeke,d Mohamed Anwar Hossain,c Willem Bram de Rijk,a Leen Rigouts,a Aysel Gumusboga,a Gabriela Torrea,a Bouke C. de Jonga

- No difference in treatment failure (63%) with 1st line TB therapy between those with disputed vs undisputed rpoB mutations
- Other smaller studies by Williamson (NZ) and van Ingen (Netherlands) similar findings
Conclusions

• Discordance between genotypic and phenotypic tests are increasingly recognized and often rely on genome sequencing to elucidate the mechanism

• High rates of *Mycobacterium tuberculosis* transmission in high endemicity populations increase the prevalence of mixed infections
Conclusions (2)

• Patients with mixed populations of *Mycobacterium tuberculosis* should be treated for both DS-TB and DR-TB

• Patients with disputed *rpoB* mutations should be treated for MDR-TB ± high dose rifampicin as clinical outcome is worse with standard treatment