Improving MGIT PZA Susceptibility Testing – A Multi-laboratory Evaluation of Alternative Inoculum Dilutions

Glenn P. Morlock
Frances Tyrrell
James Posey

10th National Conference on Laboratory Aspects of Tuberculosis
April 18, 2017
Major Factor Affecting PZA DST

- **Inoculum size**
  - Large inoculum reduces PZA activity
    - Much more pronounced than other drugs
    - Increase from $10^6$ to $10^7$ cells/ml
      - PZA became inactive
    - $10^7–10^8$ cells/ml
      - pH increase of 1 & 1.5 units, respectively
    - $10^6$ cells / ml (commonly used inoculum)
      - pH increase of 0.3 units
  - Explanation for pH increase
    - Ammonia produced by deamination of PZA
PZA False Resistance

- **Increasingly common**
  - Concurrent with transition from BACTEC 460 to MGIT
    - Co-incidence?

- **Bactec 460**
  - Minimal (no supplement) media
  - Radiometric detection
    - $^{14}$C-labeled CO$_2$

- **Mycobacteria Growth Indicator Tube (MGIT)**
  - Enriched (supplemented) media
    - Fluorometric detection
      - Oxygen depletion from bacterial respiration
PZA Susceptibility Testing in MGIT: Problem Summarized

- Very temperamental
  - Inoculum too large
    - Media is neutralized
      - False resistance
  - Inoculum density too low
    - Test will terminate prior to a result
  - Inoculum density just right
    - Accurate result
Study Hypotheses

1. Over-inoculation causes:
   - False resistance
   - Poor reproducibility

2. Lower inoculum density will:
   - Mitigate false resistance rate
   - Improve reproducibility

3. Inoculum effect not universal
   - Some strains highly responsive
   - Others recalcitrant
Study Design

- **3 inoculum schemes**
  - Standard protocol ("BD")
  - 2 reduced inoculum protocols ("A" and "C")

- **10 laboratories**
  - 9 extramural
  - CDC LB/ART

- **20 isolates**
  - 15 unique strains
  - 5 duplicates
Standard Inoculation Protocol “BD” Method

Inoculum

Seed Tube

37°C

If Day 3, 4, or 5

Day 1 or 2

1 to 10 dilution

37°C

no dilution

Control Tube

No PZA

100 µg/ml PZA

37°C

Growth = Resistant

No Growth = Susceptible

1:5 Dilution
Experimental Protocol “A”

Inoculum

Seed Tube

37°C

Day 1 or 2

1 to 25 dilution

Control Tube
No PZA

37°C

Growth = Resistant

1 to 2.5 dilution

100 µg/ml PZA

37°C

No Growth = Susceptible
**Experimental Protocol “C”**

1. **Inoculum**
   - Seed Tube
   - 37°C

2. **Day 1 or 2**
   - 1 to 50 dilution

3. **Control Tube**
   - No PZA
   - 37°C

4. **100 µg/ml PZA**
   - Growth = Resistant
   - 37°C

5. **Growth**
   - No Growth = Susceptible
Selection Criteria for Study Strains

- **Phenotypic**
  - Minimum Inhibitory Concentration (MIC) results from previous studies

- **Genotypic**
  - *pncA* sequence
    - Sanger
    - Whole Genome
Data Collected

- **Quantity**
  - Per participant
    - 3 inoculum densities x 20 isolates = 60 tests
      - In duplicate = 120 tests
  - In Total
    - 1200 tests

- **Type**
  - Categorical
    - Resistant
    - Susceptible
## Study Strains

### Characteristics of 20 M. tuberculosis strains.

<table>
<thead>
<tr>
<th>Isolate No. (duplicate)</th>
<th>Susceptibility to PZA (100 µg/ml) (multiple tests)</th>
<th>pncA mutation</th>
<th>Other Drug Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Consistently</td>
<td>Predominately</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>1</td>
<td>Susceptible</td>
<td>—</td>
<td>C 195 → T</td>
</tr>
<tr>
<td>4</td>
<td>Resistant</td>
<td>—</td>
<td>A(-11) → G</td>
</tr>
<tr>
<td>7</td>
<td>Susceptible</td>
<td>—</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>Susceptible</td>
<td>—</td>
<td>None</td>
</tr>
<tr>
<td>11 (23)</td>
<td>Susceptible</td>
<td>—</td>
<td>None</td>
</tr>
<tr>
<td>12 (24)</td>
<td>—</td>
<td>Susceptible</td>
<td>None</td>
</tr>
<tr>
<td>13 (25)</td>
<td>—</td>
<td>Susceptible</td>
<td>None</td>
</tr>
<tr>
<td>14</td>
<td>Resistant</td>
<td>—</td>
<td>T 37 → C</td>
</tr>
<tr>
<td>16 (26)</td>
<td>—</td>
<td>Susceptible</td>
<td>None</td>
</tr>
<tr>
<td>17</td>
<td>—</td>
<td>Resistant</td>
<td>A 139 → G</td>
</tr>
<tr>
<td>18</td>
<td>Susceptible</td>
<td>—</td>
<td>C 509 → T</td>
</tr>
<tr>
<td>19</td>
<td>Inconclusive</td>
<td>Inconclusive</td>
<td>C 244 → T</td>
</tr>
<tr>
<td>20</td>
<td>Susceptible</td>
<td>—</td>
<td>None</td>
</tr>
<tr>
<td>21 (27)</td>
<td>Inconclusive</td>
<td>Inconclusive</td>
<td>G 82 → A</td>
</tr>
<tr>
<td>22</td>
<td>Susceptible</td>
<td>—</td>
<td>A 110 → T</td>
</tr>
</tbody>
</table>
“Predominately”
PZA Susceptible with Wild Type PncA

“The Likely Suspects”

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Susceptible or Resistant by Method</th>
<th>“BD”</th>
<th>“A”</th>
<th>“C”</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>2</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>3</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>14</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>14</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>10</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>14</td>
<td>6</td>
<td>17</td>
</tr>
</tbody>
</table>

**Note**
- Appreciably effected by inoculum dilution
"Predominately" PZA Resistant with *pncA* Mutation "The Trouble Maker"

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Susceptible or Resistant by Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BD</td>
</tr>
<tr>
<td></td>
<td>S</td>
</tr>
<tr>
<td><strong>17</strong></td>
<td>2</td>
</tr>
</tbody>
</table>

- MIC near critical concentration
- Thr 47 Ala *pncA* mutation
  - known to give discrepant PZA results
  - Also INH and RIF resistant
- Consideration
  - Strains borderline resistant at critical concentration
  - Likely to be impacted by inoculum density
  - Isolate 17 supports this contention
Algorithmically Assigned Predicted Results

- 82% True Susceptible
- 18% True Resistant

Note
- Inconclusive Isolates Excluded
Predicted Results vs Study Results I

Predicted

- True Susceptible: 82%
- True Resistant: 18%

“BD” Method

- False Resistant: 21.1%
- False Susceptible: 17.1%
- No result: 0.7%
- No result: 0.7%
Predicted Results vs Study Results II

Predicted

- True Susceptible: 82%
- True Resistant: 18%

“A” Method

- True Susceptible: 76.4%
- True Resistant: 14.2%
- False Resistant: 3.7%
- False Susceptible: 3.3%
- No result: 5.7%
Predicted Results vs Study Results III

Predicted

- True Susceptible: 82%
- True Resistant: 18%

“C” Method

- False Resistant: 3.1%
- False Susceptible: 5.6%
- No result: 2.8%
- 79.2%
Study Results Comparison I

“BD” Method

- True Susceptible: 21.1%
- True Resistant: 17.1%
- False Resistant: 0.7%
- False Susceptible: 0.7%
- No result: 61.1%

“A” Method

- True Susceptible: 76.4%
- True Resistant: 14.2%
- False Resistant: 5.7%
- False Susceptible: 3.7%
- No result: 3.3%
Study Results Comparison II

“BD” Method
- True Susceptible: 61.1%
- True Resistant: 17.1%
- False Resistant: 21.1%
- False Susceptible: 0.7%
- No result: 0.7%

“C” Method
- True Susceptible: 79.2%
- True Resistant: 14.5%
- False Resistant: 2.8%
- False Susceptible: 14.5%
- No result: 3.1%
Study Results Comparison III

“A” Method

- True Susceptible: 76.4%
- True Resistant: 14.2%
- False Resistant: 3.3%
- False Susceptible: 5.7%
- No result: 3.1%

“C” Method

- True Susceptible: 79.2%
- True Resistant: 14.5%
- False Resistant: 2.8%
- False Susceptible: 5.6%
- No result: 3.1%
Data Analysis
Precision and Accuracy

- Low accuracy
  - Low precision

- Low accuracy
  - High precision

- High accuracy
  - Low precision

- High accuracy
  - High precision
Accuracy Comparison

- "BD" Method: 78.3% Accurate, 21.7% Inaccurate
- "A" Method: 90.5% Accurate, 9.5% Inaccurate
- "C" Method: 94.1% Accurate, 5.9% Inaccurate
Precision Comparison

- Precise & Accurate
  - BD method: 72.2
  - A method: 87.6
  - C method: 93.5

- Precise & Inaccurate
  - BD method: 1.3
  - A method: 6.9
  - C method: 2.9

- Not Precise
  - BD method: 12.6
  - A method: 5.5
  - C method: 3.6

- < 2 Results
  - BD method: 1.3
  - A method: 5.2
  - C method: 8.5

Legend:
- BD method
- A method
- C method
Conclusions I

- Inoculum size critical
  - Too high
    - Can produce false-resistant result
  - Just Right
    - Accurate result
  - Too little
    - Accurate Result
      - Delayed
    - No result
      - Test terminated

- Ideal Inoculum a difficult goal
  - Dilution not universal solution
    - Can be detrimental
      - Very delayed or no result
MGIT PZA Test = PZA mono-resistant
What do you do?

PZA mono-resistant

M. bovis?

Yes

M. Bovis is naturally resistant to PZA

No

PZA Mono-resistance is rare

Be Suspicious

Consider False Resistance
MGIT PZA Test = PZA mono-resistant Considerations

- Consider False Resistance
- Possibility of Over Inoculation
- Action Pathways

No Validation required

Within Protocol?
MGIT PZA Test = PZA mono-resistant Considerations

Within Protocol?

Yes

- Repeat Test
  - Use “younger” seed tube
    - Day 1 vs. day 2
      - Day 3 vs. day 4 or 5
    - Record hours past positive
  - Avoid clumps
    - Allow time to settle
    - Aspirate from top

MGIT PZA Test = PZA mono-resistant Considerations

- Consider False Resistance
- Possibility of Over Inoculation
- Action Pathways
  - No Validation required
  - Within Protocol?
  - Outside Protocol?
  - Validation required
MGIT PZA Test = PZA mono-resistant
Considerations

Within Protocol?

**Yes**

- Repeat Test
  - Use “younger” seed tube
    - Day 1 vs. day 2
      - Day 3 vs. day 4 or 5
    - Record hours past positive
  - Avoid clumps
    - Allow time to settle
    - Aspirate from top

Outside Protocol?

**Yes**

- Repeat Test
  - Use reduced inoculum
  - Use smaller volume
  - Use standardized inoculum
- Use Modified Test
  - Reduced inoculum

---

Acknowledgements

- **Co-investigators**
  - Frances Tyrrell
  - James Posey

- **Enablement**
  - Angela Starks
  - Tracy Dalton
  - David Temporado

- **Participating Laboratories**

- **Whole Genome Sequencing**
  - Paige Chopra
  - Lauren Cowen

- **Intangible Support**
  - Entire Laboratory Branch
The Devil is in the Seed Tubes and When to Use Them

- **BD Protocol**
  - “Day 0” = day seed tube positive
  - Use on “day 1 or 2”
  - Theoretically
    - Positive @ 23:00
      - May be used from hours 1 to 49 past positive
    - Positive @ 01:00
      - May be used from hours 23 to 71 past positive
    - Using 18 hour / 1 generation
      - 0 to 4 generations difference
BD Protocol May Be Too Imprecise?

- **Too large a time window**
  - Considering temperamentality of test system
    - Differing growth rate between isolates

- **Is there a better measurement?**
  - Smaller time window
    - May be constrained by work processes
  - Growth Units value
    - Not linear but may still be better
  - Calculation using both factors
    - May compensate for differences in growth rates
Algorithm for Assigning Predicted Categorical Result: Susceptible, Resistant or Inconclusive?

Susceptible N = 10

If pncA Mutation?

Yes: Non-Synonymous or regulatory?

Yes: Resistant N = 1

No: Susceptible N = 3

Yes: PZA MIC Predominately ≤ 100 µg/ml?

No: PZA MIC Consistently > 100 µg/ml?

Yes: Resistant N = 2

No: Inconclusive N = 3

Yes: PZA MIC Predominately > 100 µg/ml?

Yes: Resistant N = 1

No: Susceptible N = 3