TB Nurse Case Management
Waukesha, Wisconsin
March 31 – April 2, 2009

New Methods for Laboratory Diagnosis and Drug Resistance
Michelle Hulse, MD
April 2, 2009

New Methods for Laboratory Diagnosis and Detection of Drug Resistance
David Warshauer, PhD
Deputy Director, Communicable Diseases
Wisconsin State Laboratory of Hygiene
April 2, 2009
Laboratory Diagnostics Available
Now in the U.S.

• Fluorescence Smear Microscopy
• Automated liquid culture systems
• Nucleic acid amplification assays
• Interferon gamma release assays
• Broth Drug Susceptibility assays
• Rapid identification methods

Diagnostics in the Pipeline

• Molecular detection of drug resistance
• Microscopic Observation Drug Susceptibility (MODS)
• Phage-based tests to detect drug resistance
• Lipoarabinomannan (LAM) detection in urine
• Breathalyser screening test
Nucleic Acid Amplification

19th/20th Century Traditional Algorithm

1. **Process Specimen**
   - 24 hours

2. **AFB Smear Microscopy**

3. **Inoculate Media**
   - 2 to 6 weeks

4. **Species Identification**
   - 2-3 weeks

5. **Drug Susceptibilities**

Courtesy Tom Shinnick, PhD
21st Century Algorithm

- Process Specimen
  - AFB Smear Microscopy
    - Inoculate Media
      - Species Identification
        - Drug Susceptibilities
          - Genetic Tests

Amplification based Tests

- 24 hours
- 2 to 6 weeks
- 2-3 weeks

Nucleic Acid Amplification Tests

- FDA-cleared for use with respiratory specimens
  - Amplified *M. tb* Direct Test® (MTD): Gen-Probe, Inc.
    - Amplicor® *M. tuberculosis* (MTB): Roche Diagnostics
  - Commercial tests available outside US
    - BD ProbeTec™ MTB Direct Detection
    - COBAS® Amplicor® MTB Test
    - COBAS® TaqMan® MTB Test

- Home-brew tests
- Off-label use of FDA-cleared tests
Amplified *M. tb* Direct Test® (MTD): Gen-Probe, Inc.

- Smear positive and smear negative specimens
- Transcription mediated amplification
- Ribosomal RNA target
  - Multiple copies
- rRNA $\rightarrow$ cDNA
- Detection using a labeled MTB complex-specific DNA probe
- Assay time 2.5-3 hours

Amplicor® *M. tuberculosis* (MTB): Roche Diagnostics

- Smear positive specimens only
- PCR
- Target—584-bp region of the gene encoding for 16S rRNA
- Labeled amplified products detected
- Assay time 4-6 hours
CDC *M. tb* NAA Testing Performance Evaluation Program-TB MPEP

Amplification Procedure Used for Direct Detection of *M. tb*

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Number of Laboratories</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gen Probe MTD</td>
<td>63</td>
</tr>
<tr>
<td>Roche Amplicor</td>
<td>13</td>
</tr>
<tr>
<td>In-house</td>
<td>9</td>
</tr>
</tbody>
</table>

Number of Laboratories: N=65

Courtesy Laurina Williams, PhD

**Nucleic Acid Amplification Tests**

- Turnaround time of 8 to 48 hours
- Detect *M. tuberculosis* complex NA
- Do not distinguish live and dead bacilli
- Sensitivity
  - >95% for AFB smear-positive TB patients
  - 55-75% of AFB smear-negative, culture-positive TB patients
NAAT Performance – Respiratory Specimens

Table 1. Pooled values* (95% confidence intervals) of diagnostic odds ratio (DOR), sensitivity, and specificity of five commercial nucleic acid amplification tests (NAATs).

<table>
<thead>
<tr>
<th>Test</th>
<th>NAA method</th>
<th>AFB-</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>AFB-</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmpliSeq</td>
<td>PCR</td>
<td>117 (56 to 246)</td>
<td>0.94 (0.94 to 0.97)</td>
<td>0.83 (0.8 to 0.86)</td>
<td>77 (51 to 115)</td>
<td>0.61 (0.57 to 0.65)</td>
<td>0.97 (0.948 to 0.974)</td>
</tr>
<tr>
<td>Colos AmpliSeq</td>
<td>PCR</td>
<td>99 (56 to 173)</td>
<td>0.96 (0.95 to 0.97)</td>
<td>0.74 (0.68 to 0.8)</td>
<td>220 (144 to 333)</td>
<td>0.64 (0.59 to 0.69)</td>
<td>0.99 (0.99 to 0.994)</td>
</tr>
<tr>
<td>BGF</td>
<td>SDA</td>
<td>181 (59 to 524)</td>
<td>0.96 (0.95 to 0.98)</td>
<td>0.95 (0.94 to 0.99)</td>
<td>14 (53 to 175)</td>
<td>0.71 (0.66 to 0.76)</td>
<td>0.97 (0.964 to 0.974)</td>
</tr>
<tr>
<td>Xpert MTB</td>
<td>XPA</td>
<td>314 (199 to 595)</td>
<td>0.97 (0.95 to 0.99)</td>
<td>0.96 (0.93 to 0.97)</td>
<td>137 (140 to 310)</td>
<td>0.76 (0.7 to 0.8)</td>
<td>0.97 (0.96 to 0.974)</td>
</tr>
<tr>
<td>LGX</td>
<td>LGX</td>
<td>45 (26 to 142)</td>
<td>0.98 (0.94 to 0.98)</td>
<td>0.95 (0.94 to 0.98)</td>
<td>71 (38 to 122)</td>
<td>0.82 (0.7 to 0.86)</td>
<td>0.99 (0.97 to 0.992)</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction; SDA, strand displacement amplification; XPA, transcription mediated amplification; LGX, ligase chain reaction; DOR, diagnostic odds ratio.

*Random effect model.

Greco, S. et al Thorax 2006;61:783-790

MTD with Smear Positive Respiratory Specimens -- WSLH

<table>
<thead>
<tr>
<th>Year</th>
<th>Culture Confirmed PTB</th>
<th>Smear Positive with MTD</th>
<th>Positive MTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>39</td>
<td>17</td>
<td>16 (94%)</td>
</tr>
<tr>
<td>2007</td>
<td>37</td>
<td>19</td>
<td>19 (100%)</td>
</tr>
</tbody>
</table>
WSLH MTD sensitivity and specificity 2000-2008

Reduction in turnaround time for laboratory diagnosis of pulmonary TB by routine use of NAAT

Processing: 5 days; NAAT 4 days; broth medium monitored 7 days

NAAT (first specimen) - AFB smear and culture (3 specimens) - 797 pt [81 TB]

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sens</th>
<th>Spec</th>
<th>PPV</th>
<th>NPV</th>
<th>Mean TAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB Smear</td>
<td>70</td>
<td>98</td>
<td>79</td>
<td>96.7</td>
<td>1</td>
</tr>
<tr>
<td>NAAT</td>
<td><strong>90</strong></td>
<td><strong>100</strong></td>
<td><strong>100</strong></td>
<td><strong>98.9</strong></td>
<td><strong>2</strong></td>
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<tr>
<td>Culture x 3</td>
<td>96</td>
<td>100</td>
<td>100</td>
<td>99.6</td>
<td>18</td>
</tr>
</tbody>
</table>

Diagnostic Accuracy of Commercial Tests for TB Meningitis

(A) Commercial tests

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

56% pooled 98%

Current CDC Recommendations for NAAT

• “NAAT should be performed on at least one respiratory specimen from each patient with signs and symptoms of pulmonary TB for whom a diagnosis of TB is being considered but has not yet been established, and for whom the test result would alter case management or TB control activities”

MMWR, 2009, 58:7-10

CDC Algorithm

• Collect respiratory specimens for smear and culture
• Collect at least one specimen, preferably the first for NAA
• Interpret NAA test results in correlation with the AFB smear results
Challenges to Implementing NAAT

- NAAT adds significant cost to the laboratory
- In current algorithm, NAAT is an add-on test
- Low volume test
- The overall costs and benefits of NAAT may vary by program
- Optimal, cost-effective testing regimens have not yet been developed
Research Needs for Future Advancements

- Studies to develop, evaluate, and select the most effective and efficient NAAT algorithms
- Develop and evaluate tests for non-respiratory specimens
- Develop tests with improved performance and ease-of-use
- Develop tests that will enhance the diagnosis of TB in children
- Develop multiplex assays that can detect *M. avium* complex, *M. kansasii* and other NTM

Tuberculin Skin Test

- Developed in 1907 by Dr. Charles Mantoux
- Intradermal injection of TB “purified protein derivative” (PPD)
  - Positive reaction produces a measurable induration
- Used effectively in U.S. for most of 20th century
Requirements for a replacement for TST

- High sensitivity and specificity
- Reliable in immunosuppressed
- Single, objective lab test
- Simple implementation
- Cost effective

Interferon Gamma Release Assays (IGRAs)
Interferon Gamma Release Assays

• Blood tests for detecting M. tuberculosis infection
  – Sensitized white blood cells will release IFN-gamma in response to contact with TB antigens

• Do not differentiate latent infection from active disease

No Cross-reactivity to BCG and Most NTMs

<table>
<thead>
<tr>
<th>Complex</th>
<th>Tuberculosis</th>
<th>Antigens</th>
<th>Strains</th>
<th>Environmental</th>
<th>Antigens</th>
<th>ESAT 6</th>
<th>CFP 10</th>
<th>ESAT 6</th>
<th>CFP 10</th>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>M. tuberculosis</td>
<td>+</td>
<td>+</td>
<td>M. abscessus</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>M. africanum</td>
<td>+</td>
<td>+</td>
<td>M. avium</td>
<td>-</td>
<td>-</td>
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<tr>
<td>M. bovis</td>
<td>+</td>
<td>+</td>
<td>M. intracellulare</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BCG substrain</td>
<td></td>
<td></td>
<td>M. kansasii</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>gothenburg</td>
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<td>M. marinum</td>
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<td>tokyo</td>
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<td>M. szulgai</td>
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<td>danish</td>
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<td>M. terrae</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>glaco</td>
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<td>-</td>
<td>M. vaccae</td>
<td>-</td>
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<td></td>
<td></td>
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<tr>
<td>montreal</td>
<td>-</td>
<td>-</td>
<td>M. xenopi</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>pasteur</td>
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<td>-</td>
<td>M. xenopi</td>
<td>-</td>
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</tr>
</tbody>
</table>
**T-SPOT.TB Test Kit**

- Flexible, 96-well format
  - Twelve, 8-well strips
  - 4 wells used per patient; 24 patients per kit
  - Positive and Negative control for each patient test
- Utilizes standard blood collection tubes
- No special lab equipment required

**Performing the T-SPOT.TB Test**

- Peripheral blood mononuclear cells (PBMCs) are separated from whole blood and washed
  - Removes any source of background interference

- Washed PBMCs are counted to ensure a *standardized number of cells* are added to the assay

- Blood must be processed within 8 hours of collection
The Science behind T-SPOT™
Simplified, validated ELISpot method

1. Collect white cells using BD CPT tube or Ficoll extraction.
2. Add white cells and TB antigens to wells. Effector T cells release interferon gamma.
3. Incubate, wash and add conjugated second antibody to interferon gamma.
4. Interferon gamma captured by antibodies.
5. Add substrate and count T SPOTs.

Interpretation of Results

- **Negative**
  - Nil Control
  - ESAT-6 Panel A
  - CFP 10 Panel B

- **Positive**
  - Positive Control
Interpretation of Results

• Results are interpreted by subtracting the spot count in the NIL control well from the spot count in each of the antigens, according to the following algorithm:

  • The test result is **Positive** if (ESAT-6 minus NIL) and/or (CFP-10 minus NIL) ≥ 8 spots

  • The test result is **Borderline** (equivocal) where the highest of (ESAT-6 minus NIL) or (CFP-10 minus NIL) spot count is 5, 6 or 7 and retesting by collecting another sample is recommended

  • The test result is **Negative** if (ESAT-6 minus NIL) and/or (CFP-10 minus NIL) ≤ 4 spots. This includes values less than zero.

QuantiFERON® -TB Gold In-Tube

Stage 1 – Blood Stimulation and Harvesting

• After blood collection, mix QuantiFERON® blood tubes thoroughly by shaking vigorously for 5 seconds.
Stage 1 – Blood Stimulation and Harvesting

• As soon as possible, and within 16 hours of collection, incubate tubes upright at 37°C for 16-24 hours.

• After incubation can hold up to 3 days at 2-27°C

Stage 1 – Blood Stimulation and Harvesting

• Centrifuge tubes at 2000 – 3000 g (RCF) for 15 minutes.
Stage 2 – Human IFN-γ ELISA

- Add conjugate to each well, then add plasma or standards.
- Shake plate and incubate for 120 minutes at room temperature.

* Can be automated

Courtesy Cellestis

Stage 2 – Human IFN-γ ELISA

- Wash plate 6 times. Add substrate.
- Incubate for 30 minutes at room temperature.

* Can be automated

Courtesy Cellestis
Stage 2 – Human IFN-\( \gamma \) ELISA

- Add stop solution.
- Read absorbance within 5 min at 450nm (620-650nm ref).

* Can be automated

Courtesy Cellestis

Stage 2 – Human IFN-\( \gamma \) ELISA

- Calculate results using QuantiFERON® Analysis Software.

Courtesy Cellestis
Laboratory Issues

- Processing of blood within required time frame
- Test verification more difficult
- Sufficient test volume to make economically feasible
- Transfer of cost from TB Program to the laboratory

WMLN: QFT Testing (10 Labs)
Update on IGRAs:
review of 38 studies on performance of IGRAs

Machida K, MD, PhD; Bice Zwetsloot, MSc; and Rob Navaiojo, MD, MSc

<table>
<thead>
<tr>
<th>Pooled Sensitivity &amp; Specificity of IGRAs and TST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
</tr>
<tr>
<td>QuantiFERON-Gold</td>
</tr>
<tr>
<td>QuantiFERON-Gold In-Tube</td>
</tr>
<tr>
<td>T-SPOT.TB</td>
</tr>
<tr>
<td>Tuberculin Skin Test</td>
</tr>
</tbody>
</table>

CI confidence interval

Advantages of IGRAs

• Requires a single patient visit
• Not subject to reader bias
• “Specific” TB antigens
• Not affected by prior BCG vaccination
• Controlled laboratory based test
• Objective result
• No possibility of adverse reactions in hypersensitive individuals
• Do not boost responses measured by subsequent tests
Disadvantages of IGRAs

- Blood must be processed within 8-16 hours after collection
- Errors in collection or transport of samples
- Lab variability and errors in performance or interpretation of the test
- Limited data on use in certain populations
  - Immunocompromised
  - Treatment with immunosuppressive drugs
  - Patients with hematological disorders, diabetes, malignancies
- Limited data on the use of IGRAs to determine risk for developing TB disease

CDC Guidelines for the use of IGRAs

- MMWR Dec. 16, 2005 54(RR15); 49-55.
  - Updated guidelines coming soon
- “CDC recommends that QFT-G may be used in all circumstances in which the TST is currently used, including
  - Contact investigations
  - Evaluation of recent immigrants
  - Sequential-testing surveillance programs
CDC Guidelines (Cont.)

• Specific precautions
  – Limited data in young children and immunocompromised patients
  – QFT-G sensitivity for LTBI might be less than that of TST
    ▪ Lack of a confirmatory test makes this difficult to assess
  – Ability of QFT-G to predict risk for LTBI progressing to TB disease not determined

CDC Guidelines (Cont.)

• Specific precautions (cont.)
  – Cannot differentiate infection associated with TB disease from LTBI
  – Negative QFT-G results should not be used alone to exclude M. tb infection in persons with symptoms/signs suggestive of TB disease
New Molecular Methods for Rapid Detection of Drug Resistance

Molecular Basis of DR in MTBC

<table>
<thead>
<tr>
<th>Drug</th>
<th>Gene Locus</th>
<th>Gene function</th>
<th>Percent of Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>katG</td>
<td>Catalase-Peroxidase</td>
<td>40 - 100 %</td>
</tr>
<tr>
<td></td>
<td>inhA</td>
<td>Enoyl-ACP-Reduktase</td>
<td>appr. 25 %</td>
</tr>
<tr>
<td></td>
<td>ahpC-Promoter</td>
<td>Alkyl-Hydroxid-Peroxidase</td>
<td>appr. 10 %</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>rpoB</td>
<td>β-Subunit of RNA-Polymerase</td>
<td>&gt; 90 %</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>pncA</td>
<td>Pyrazinamidase</td>
<td>appr. 95 %</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>rpsL,rss</td>
<td>ribosomal Protein S12, 16S rRNA</td>
<td>appr. 60 %, appr. 20 %</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>embB</td>
<td>Arabinosyl-Transferase</td>
<td>appr. 60 %</td>
</tr>
<tr>
<td>Chinolone</td>
<td>gyrA</td>
<td>DNA-Gyrase A</td>
<td>appr. 80-90%</td>
</tr>
</tbody>
</table>
Hain GenoType® Series
Mycobacteria

GenoType® Mycobacterium CM/AS
GenoType® MTBC
GenoType® MTBDRplus
GenoType® Mycobacteria Direct

GenoType® MTBDRplus

1. DNA preparation
2. PCR
3. Hybridization
4. Evaluation
GenoType® MTBDRplus

- Permits identification of the M. tuberculosis complex
- Detects resistance to rifampicin and/or isoniazid from culture growth or pulmonary smear-positive patient material
  - \textit{rpoB} gene for rifampin: Sens 98% Spec 99%
  - \textit{katG} gene for high level isoniazid resistance: Sens 89%; Spec 99%
  - \textit{inhA} gene for low level isoniazid resistance

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**Reaction zones of the GenoType® MTBDRplus**

- Conjugate Control (CC)
- Amplification Control (AC)
- M. tuberculosis complex (TUB)
- \textit{rpoB} Locus Control
- \textit{rpoB} wild type probe 1 (\textit{rpoB} WT1)
- \textit{rpoB} wild type probe 2 (\textit{rpoB} WT2)
- \textit{rpoB} wild type probe 3 (\textit{rpoB} WT3)
- \textit{rpoB} wild type probe 4 (\textit{rpoB} WT4)
- \textit{rpoB} wild type probe 5 (\textit{rpoB} WT5)
- \textit{rpoB} wild type probe 6 (\textit{rpoB} WT6)
- \textit{rpoB} wild type probe 7 (\textit{rpoB} WT7)
- \textit{rpoB} wild type probe 8 (\textit{rpoB} WT8)
- \textit{rpoB} mutation probe 1 (\textit{rpoB} MUT1)
- \textit{rpoB} mutation probe 2A (\textit{rpoB} MUT2A)
- \textit{rpoB} mutation probe 2B (\textit{rpoB} MUT2B)
- \textit{rpoB} mutation probe 3 (\textit{rpoB} MUT3)

- \textit{katG} Locus Control
- \textit{katG} wild type probe (\textit{katG} WT)
- \textit{katG} mutation probe 1 (\textit{katG} MUT1)
- \textit{katG} mutation probe 2 (\textit{katG} MUT2)

- \textit{inhA} Locus Control
- \textit{inhA} wild type probe 1 (\textit{inhA} WT1)
- \textit{inhA} wild type probe 2 (\textit{inhA} WT2)
- \textit{inhA} mutation probe 1 (\textit{inhA} MUT1)
- \textit{inhA} mutation probe 2 (\textit{inhA} MUT2)
- \textit{inhA} mutation probe 3A (\textit{inhA} MUT3A)
- \textit{inhA} mutation probe 3B (\textit{inhA} MUT3B)

- Colored marker
Possible Results

Isoniazid: Sensitive/Resistance (katG gene)

Isoniazid-sensitive M. tuberculosis strain

→ the wildtype probes show positive signal

both mutation probes are negative
Detection of Mutations with a Molecular Beacon
(Loop portion containing wildtype SQ)

- **Mutant Sequence**
- **Wildtype Sequence**
- **Amplicon**
- **Fluorophore**
- **Loop**
- **Quencher**

**Molecular Hybrid (Molecular Beacon - On)**

**Hybrid (Molecular Beacon - Off)**

California Public Health Laboratory

**Performance of Molecular Beacon Assay**
*(Initial study)*

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% Resistance</td>
<td>82.67%</td>
<td>100%</td>
<td>100%</td>
<td>98.11%</td>
</tr>
<tr>
<td><strong>RIF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% Resistance</td>
<td>97.53%</td>
<td>100%</td>
<td>100%</td>
<td>99.95%</td>
</tr>
</tbody>
</table>

In two years of using the MB assay, the overall agreement between MB and phenotypic drug susceptibility results were 95.6% for INH and 96.7% for RIF.
Thank You