How Traditional Techniques Complement Molecular Approaches:

The Future of TB Testing

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Key points about new technology tests for agents of infectious disease

1. Microbes are living, evolving creatures; genetic elements change

2. Validation of new technology usually still requires culture confirmation

3. Antibiotic resistance factors are unstable and may be too numerous for reliable molecular detection

4. If tests are not 100% (and none are) and false negative results are serious, culture is still essential
Sunnyvale woman back from India has hard-to-treat case of TB

A 30-year-old Sunnyvale woman, recently back from a stay in India, is in an isolation unit at Stanford Hospital with a tough-to-treat strain of tuberculosis, and health officials are scrambling to find any people with whom she may have come into close contact.

The CDC has identified 44 people from 16 states who were within two rows of the woman on American Airlines Flight 293 from New Delhi to Chicago's O'Hare airport on Dec. 13. On Thursday, the CDC sent health officials in the 16 states a list of the names and asked for help in having them tested for TB.
**Mycobacterium tuberculosis**

- Worldwide: >9 million new cases/yr
- Worldwide: ~5,000 deaths occur daily (1.8 million/yr)
- US: 14,517 cases of TB reported in 2004
- ~19 million TB cultures performed worldwide – need 60 million by 2015
  - 12.2 million in developing countries
  - 6.4 million in developed countries
- Drug resistance is increasing (MDR & XDR)
Prevalence of TB & HIV co-infected pts.
## DOT Therapy for TB

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug S TB</th>
<th>MDR-TB (4+ drugs)</th>
<th>XDR-TB (5+ drugs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (INH)</td>
<td>✓</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>Rifampicin (RIF)</td>
<td>✓</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>Pyrazinamide (PZA)</td>
<td>✓</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Ethambutol (EMB)</td>
<td>✓</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Streptomycin (STM)</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>Aminoglycoside</td>
<td>✓</td>
<td></td>
<td>✓ (R)</td>
</tr>
<tr>
<td>Fluoroquinolone</td>
<td>✓</td>
<td></td>
<td>✓ (R)</td>
</tr>
<tr>
<td>Ethionamide (ETH)</td>
<td>+/-</td>
<td></td>
<td>+/-</td>
</tr>
<tr>
<td>$p$-aminosalicylic acid (PAS)</td>
<td>+/-</td>
<td></td>
<td>+/-</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>+/-</td>
<td></td>
<td>+/-</td>
</tr>
<tr>
<td>Linezolid</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>
Adverse Drug Reactions

- Hepatitis
- Eye damage
- Rash
- Peripheral neuropathy
- Nausea, vomiting
- Arthritis, gout
- Ototoxicity, imbalance, hearing loss
- Renal toxicity
- Thrombocytopenia
- Drug interactions
Challenges to diagnostic testing for TB

Good news
• M. tuberculosis is a true pathogen; detection or recovery of one organism indicates disease

Bad news
• Many samples have very low numbers of AFB
• On smears, most mycobacteria look alike
• Patients become infected but may not develop disease; antibody not useful; IGRA tests not specific
• TB can infect almost any tissue, which require different processing procedures
• TB grows unusually slowly in culture; 7-42 days
Conventional broth enhanced method

Decontaminate & concentrate sputum (refrigerated floor centrifuge)

Incubate up to 4 weeks in instrument or manual read up to 8 weeks

Inoculate liquid & solid media; make smear
Stain the slides

Examine the slides – 3-5 minutes or more each

Most mycobacteria look alike in smears; identification of TB not possible by smear

Ziehl-Neelsen stain

Auramine stain
Susceptibilities Are Necessary

Organisms are emulsified in broth and added to antibiotic-containing media for an additional growth period.

Microscopic Observation Drug Susceptibility Assay (MODS)

1. Inoculate broth wells containing bacterial inhibitors, growth supplements, and antibiotic dilutions with 800 µL decontaminated sputum.
2. Examine wells through an inverted microscope starting on day 7.
Microscopic Observation Drug Susceptibility Assay (MODS)

Results usually by Day 15

Day 15

Day 7
Ideal TB Test

1. Rapid; results ready while patient is still available to start therapy
2. Sensitive: positive in patients with low numbers of TB in their sputum
3. Specific: differentiates M. tb from other species
4. Able to detect resistance to all the important drugs
5. Works on samples that are easy to obtain for patients who cannot produce sputum
6. Works with other types of specimen (CSF, Tissue)
7. Doesn’t need infrastructure or expertise to run
8. Doesn’t result in sample:sample contamination
9. Cheap
Blinded evaluation of commercial urinary lipoarabinomannan for active tuberculosis: a pilot study.

Urine lipoarabinomannan assay for tuberculosis screening before antiretroviral therapy diagnostic yield and association with immune reconstitution disease
Lawn et al. 2009. AIDS. 23:1875-.

<table>
<thead>
<tr>
<th>Results vs Sputum Culture</th>
<th>SENS</th>
<th>SPEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daley</td>
<td>20%</td>
<td>83%</td>
</tr>
<tr>
<td>Lawn</td>
<td>38%</td>
<td>100%</td>
</tr>
</tbody>
</table>
Examples of Molecular Tests for Detection of TB (not all FDA-cleared)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicor (Roche)</td>
<td>16S rRNA gene; smear +</td>
</tr>
<tr>
<td>MTD (GenProbe)</td>
<td>16S rRNA gene</td>
</tr>
<tr>
<td>Probe-Tec (BD)</td>
<td>16S rRNA &amp; IS6110; smear +</td>
</tr>
<tr>
<td>Xpert Mtb/RIF (Cepheid)</td>
<td>rpoB gene</td>
</tr>
<tr>
<td>LAMP (Eiken; ?Meridian)</td>
<td>gyrA gene</td>
</tr>
<tr>
<td>GTMD (HAIN)</td>
<td>23S rRNA gene; smear +</td>
</tr>
<tr>
<td>Gold nanoparticle probe (Taiwan)</td>
<td>IS6110 &amp; Rv3618</td>
</tr>
</tbody>
</table>

Most still require specimen decontamination & concentration
Whole Procedure of TB-LAMP

Sample preparation
- Decontamination of sputum sample with NALC-NaOH
- Boiling of the sample to lyse bacteria
- Purification of DNA using Extraction device

Amplification
- Detach the DNA-trapped Microweb tip into the reaction mixture containing Calcein reagent
- LAMP reaction at 65°C for 40min

Detection
- Detection of visible fluorescent signal
Evaluation of reverse transcription loop-mediated isothermal amplification in conjunction with ELISA-hybridization assay for molecular detection of *Mycobacterium tuberculosis*
Lee et al. 2009.  *J. Microbiol. Methods* 76:174-

**Operational Feasibility of Using Loop-Mediated Isothermal Amplification for Diagnosis of Pulmonary Tuberculosis in Microscopy Centers of Developing Countries**

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<th>SENS</th>
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<tbody>
<tr>
<td>Lee</td>
<td>94%</td>
<td>83%</td>
</tr>
<tr>
<td>Boehme Smear +</td>
<td>98%</td>
<td>99%</td>
</tr>
<tr>
<td>Smear neg</td>
<td>49%</td>
<td></td>
</tr>
</tbody>
</table>
HAIN GTMD (~5 hr TAT)
Direct Detection of Mycobacterial Species in Pulmonary Specimens by Two Rapid Amplification Tests, the Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test and the GenoType Mycobacteria Direct Test


**Table 1. Performance of the MTD and GTMD tests in 61 pulmonary specimens, using 16S rRNA sequencing as the reference method**

<table>
<thead>
<tr>
<th>Test result</th>
<th>Reference test results</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of positive specimens (no. of MTC-positive specimens)</td>
<td>No. of negative specimens (no. of MTC-positive specimens)</td>
<td></td>
</tr>
<tr>
<td>MTD</td>
<td>Positive 27 (27)</td>
<td>2 (2)</td>
<td>93.1</td>
</tr>
<tr>
<td></td>
<td>Negative 2 (2)</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>GTMD</td>
<td>Positive 28 (28)</td>
<td>3 (3)</td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>Negative 2 (1)</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

Failure to detect TB in 3 infected patients. Clinical implications.....
Integrated NAAT for TB/Rif: An update

**Workflow**

- fully automated with 1-step external sample prep.
- time-to-result < 2 h (walk away test)
- throughput: up to 1-48 tests / run
- no bio-safety cabinet
- closed system (no contamination risk)

A technology platform for
- TB & Rif Resistance
- TB Quinolone resistance
- Potential for HIV viral load
Cepheid GeneXpert Mtb/RIF (RUO)*

2-Step PCR in one cartridge; high tech diagnostics in a moderate complexity format

*“Research use only” – not FDA-cleared in U.S. but CE-marked
rpoB gene sequence: 5 targets

5' - GCACCAGCCAGCTGACGCAATTCATGGACACAAACCCCGCTGTGCGGGTTGACCCACAAACGCGCAGACTGTCCGGGTGCGCTG - 3'
3' - CGTGGTCCGACTCGGAAGGTACCTGGTCTTTGTGCGGACASSAACTGGGTGTTCCGGCTGACAGCGCGAC - 5'

No mutations; TB / Rif S

1 mutation; TB / Rif R

Each probe is labeled with a different fluorophore, permitting simultaneous detection of the presence of wild type.
Software interprets the Ct’s
Rapid detection of *Mycobacterium tuberculosis* and rifampin resistance by use of on-demand, near-patient technology.

Evaluation of the Analytical Performance of the Xpert(R) MTB/RIF Assay.

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<tr>
<th>Results vs Sputum Culture</th>
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<th>SPEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smear +</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Smear neg</td>
<td>72%</td>
<td></td>
</tr>
<tr>
<td>FIND beta trial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smear +</td>
<td>99.5%</td>
<td>98%</td>
</tr>
<tr>
<td>Smear neg (x3)</td>
<td>90.2%</td>
<td></td>
</tr>
</tbody>
</table>

100%/100% Rif R
97.5%/98% Rif R
Evaluation of GeneXpert MTB/RIF on pulmonary ...samples in a high throughput lab
Naidoo, S. (Lancet Labs) ECCMID Poster 2010

MTB detection

<table>
<thead>
<tr>
<th></th>
<th>Culture +</th>
<th>Culture -</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xpert +</td>
<td>800</td>
<td>20</td>
</tr>
<tr>
<td>Xpert -</td>
<td>2</td>
<td>318</td>
</tr>
</tbody>
</table>

Sensitivity = 99.8%
Specificity = 94.1%
PPV = 97.6%
NPV = 99.4%

Rifampin resistance

<table>
<thead>
<tr>
<th></th>
<th>Rif Res</th>
<th>Rif Susc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xpert R</td>
<td>157</td>
<td>8</td>
</tr>
<tr>
<td>Xpert S</td>
<td>1</td>
<td>635</td>
</tr>
</tbody>
</table>

Sensitivity = 99.4%
Specificity = 98.8%
PPV = 95.2%
NPV = 99.8%

“U.S. = For Research Use Only. Not for use in diagnostic procedures.”
Barriers to development of new assays for TB & MDR-TB

- Areas with worst problem are least able to pay for a sophisticated assay; wealthy market needed to offset development and manufacturing costs
- Clinical trials in U.S. (FDA currently requires PMA for HIV and TB tests) would be too expensive given need for 50 positives for each target
- Samples for some patients (children, AIDS patients) and sites of infection (lymph nodes, internal organs, bone) are difficult to get

Incentives:

- Huge potential worldwide market
- Altruistic motives; help end a global scourge
Future TB “Point of Care” Diagnostics in Development
Future TB Tests

1. Must be fast, reliable, simple, inexpensive
2. Must deal with sputum, feces, tissues
3. Require validation with conventional tests
4. Require backup cultures for patient care and drug resistance testing
5. Will not be perfect