Role of the Laboratory in TB Diagnosis and Management

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Objectives

• At the completion of this TB webinar, participants will:
  – Be familiar with the tests to diagnose latent tuberculosis and active tuberculosis
  – Recognize the tests available to detect Mycobacterium tuberculosis in clinical specimens
  – Understand the value of molecular tests to detect TB
History of TB Diagnostics

- Robert Koch announced in 1882 that he had found a microbe, *Mycobacterium tuberculosis*, that was the cause of "White Death", a disease responsible for one-seventh of all deaths in Europe in the late part of the 1800's.
Timeline of TB Infection

Exposure

4-6 wks

Adaptive T cell response

Latent TB (LTBI)*

Yrs-decades

Lifelong Containment

Yrs-decades

Active TB

*Prevention efforts focus on detecting LTBI, most LTBI do not advance to active disease but those patients are at high risk particularly if they become immunocompromised.
<table>
<thead>
<tr>
<th>TB Infection vs. TB Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TB in the body</strong></td>
</tr>
<tr>
<td>Chest X-ray normal</td>
</tr>
<tr>
<td>Sputum not done</td>
</tr>
<tr>
<td>No symptoms</td>
</tr>
<tr>
<td>Not infectious</td>
</tr>
<tr>
<td>Not a case of TB</td>
</tr>
</tbody>
</table>
TB Algorithm

- Collect sputum specimens at 3 different times and 8 hours apart (at least one must be a first morning specimen) for AFB smear and mycobacterial culture.
- Perform MTD or NAAT test on the first smear positive sputum specimen.
Diagnosis of TB

- Clinical picture
  - History and symptoms
- Chest XRay
- Antigen Test
  - Skin test (TST)
  - Blood Test (IGRA)
- AFB (Acid Fast Bacilli) microscopy of sputum
- NAAT Testing

- Culture (up to 6 weeks)
  - Solid medium
  - Liquid (MGIT)
- Nucleic Acid Amplification Testing (NAAT)
  - Molecular probes
  - PCR
- Sensitivity Testing
- Genotyping
Requirements to get a high quality specimen to the laboratory

• Collect specimens before therapy started
• Even after a few days of therapy, AFB may be killed or numbers decreased to longer be detectable
• Specimens must be handled properly to guarantee successful cultures
• Promptly transport specimens
Specimen Type: Varies with symptoms

• Pulmonary
  – Sputum (spontaneous, induced)
  – Bronchoalveolar Lavage
• Gastric Lavage (children)
• Tissue and Body fluids (CSF, pleural, blood)
• Wounds, skin lesions (exudates)
Specimen Collection and Processing: special considerations

- Biohazard
  - Aerosol transmission
- Prevent contamination of specimen
  - Slow growth rate of TB
- Evaluate at least 3 specimens per patient
Sputum collection considerations

• Instruct patients that nasopharyngeal discharge and saliva are not sputum
• Sputum = thick, yellowish (sometimes blood-tinged) exudative material brought up from the lungs after a deep, productive cough
• First rinse mouth with mouthwash to decrease bacterial contamination
• Collect specimen into appropriate container
Sputum cont...

- About 10 ml of sputum is sufficient
- If patient cannot provide an adequate specimen then sputum induction is acceptable
  - Warm, aerosolized hypertonic salt solution
  - Be certain to label the specimen as “induced sputum”
Specimen Transport

- From the time of collection until the specimen is processed, the other bacteria present will over grow (contaminate) the slower growing *Mycobacteria* sp.
  - Speed is important
    - Courier
    - Ship cold when possible
    - Shipping cold slows bacterial growth
Specimen Processing

- If collected from a non-sterile site (sputum), then digest and decontaminate before culture
  - Kill off other microbes
  - Liquefy mucin
  - Remove organic debris
  - Homogenize tissue
- N-acetyl-L-cysteine (NALC)-NaOH method
- Concentrate specimen
Summary of Standard Diagnostic Techniques

• Direct from specimen
  – AFB Smear – cold kinyoun and fluorescent
  – Culture in broth and on solid media
  – Direct detection by NAAT

• From growth of organism
  – Probe (accuprobe)
  – Biochemicals
  – 16S ribosomal RNA
  – Sensitivity Testing
TB Specimen Processing

Specimen

Culture
- Probe
- Biochemical
- Sensitivity
- Genotyping
- NAAT

Smear Positive
Laboratory Tests: Non-specific

• AFB smear
  – Semi-quantitative as a measure of patient infectiousness

• Culture
  – Liquid and solid media (up to 6 weeks)
  – Automated commercial systems widely used
  – Semi-quantitative
Diagnosis of TB: AFB Smear Microscopy

• Make a "smear" on a slide
• Stain for acid-fast bacteria
  – Cold Kinyoun
  – Ziehl Neelsen
  – Fluorochrome
    (Auramine-Rhodamine)
Diagnosis of TB: AFB Smear Microscopy

- **Strengths**
  - Easy, fast, cheap (ZN)

- **Weakness**
  - 50-60% of TB patients are smear negative
    - Need at least 10,000 CFU/ml sputum for positive result
  - Cannot differentiate *Mycobacteria* species
Importance of acid-fast bacilli smear microscopy as a primary diagnostic tool

- Initial diagnosis
- Monitoring treatment
- Determination of time to release from isolation
How sensitive is the smear?

- Peterson et. al. JCM 1999 vol. 37:3564-68.

<table>
<thead>
<tr>
<th>Number of specimens</th>
<th>Direct AFB smear sensitivity</th>
<th>Concentrated AFB smear sensitivity</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>353 culture positive for Mycobacteria</td>
<td>34%</td>
<td>58%</td>
<td>Direct smear cannot be relied on</td>
</tr>
<tr>
<td>208 cultures positive for <em>M. tuberculosis</em></td>
<td>42%</td>
<td>74%</td>
<td>Concentrated smear most reliable</td>
</tr>
<tr>
<td>Analysis of 3 specimens per patient</td>
<td>81%</td>
<td>91%</td>
<td>Concentrated smear is still the most reliable</td>
</tr>
</tbody>
</table>
Direct detection of TB in the specimen

- MTD test – Genprobe – transcription mediated amplification
- In house developed Nucleic Acid Amplification test (NAAT)
- GeneXpert Cepheid NAAT
### Interpretation of NAAT Result

<table>
<thead>
<tr>
<th>Smear</th>
<th>NAAT</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td><strong>Presumed Positive TB</strong>, No Additional Testing</td>
</tr>
<tr>
<td>+</td>
<td>--</td>
<td>If first sputum specimen: smear positive and NAAT-negative, repeat on one additional specimens, if negative then presume negative for TB.</td>
</tr>
<tr>
<td>--</td>
<td>+</td>
<td>Additional specimens (limit 2). <strong>Presumptive positive for TB</strong> if the subsequent specimen positive</td>
</tr>
<tr>
<td>--</td>
<td>--</td>
<td><strong>Presumptive negative for TB</strong>. Two specimens recommended.</td>
</tr>
</tbody>
</table>
TB NAAT Algorithm Respiratory Specimen

**Smear Positive**

- **NAAT**
  - **Positive**:
    - Presumed TB, pending culture results
  - **Negative**:
    - Use clinical judgment to determine whether to begin therapy while awaiting culture results and determine if additional diagnostic testing is needed.
    - Consider testing another specimen (not to exceed a total of two).
    - If a second specimen is smear positive, NAAT negative, the patient is presumed to have an infection with non-tuberculous mycobacteria, pending culture results.

**Smear Negative**

- **NAAT**
  - **Positive**:
    - Use clinical judgment to determine whether to begin therapy while awaiting culture results and determine if additional diagnostic testing is needed.
    - **NAAT Positive**: A patient can be presumed to have tuberculosis, pending culture results, if two specimens are NAA positive.
  - **Negative**:
    - Use clinical judgment to determine whether to begin therapy while awaiting results of culture and other diagnostic tests.
    - Currently available NAA tests are not sufficiently sensitive to exclude the diagnosis of TB in AFB smear negative patients suspected of having TB.
Diagnosis of TB: Culture

- **Solid Media Culture**
  - Agar Middlebrook 7H10/7H11
  - Egg based Lowenstein-Jensen
- **Liquid – Broth Culture**
  - 7H9
  - Commercial broth and monitoring systems
    - Becton Dickinson MGIT
    - ThermoScientific, TREK Diagnostic Systems, Versa TREK Myco
- **Use a solid and a liquid media**
Laboratory Tests: Specific

- Biochemical tests
  - Require sub-culture
  - Ex. Niacin, Nitrate, Tween 80 Hydrolysis, 68 Catalase

- High performance liquid chromatography (HPLC) of cell wall mycolic acids

- Molecular probes
  - Culture confirmation
  - Direct from growth in broth or on slant

- DNA sequence analysis
  - 16S rRNA gene
Molecular Probes for Mycobacteria identification

- **MYCOBACTERIUM TUBERCULOSIS** Complex Culture Identification Test – For identification of *M. tuberculosis*, *M. bovis*, *M. bovis* (BCG), *M. africanum*, *M. canetti*, *M. microti* etc. isolated from culture.

- **MYCOBACTERIUM AVIUM** Culture Identification Test - For the identification of *Mycobacterium avium* isolated from culture.

- **MYCOBACTERIUM INTRACELLULARE** Culture Identification Test - For the identification of *Mycobacterium intracellulare* isolated from culture.

- **MYCOBACTERIUM AVIUM Complex** Culture Identification Test - For the identification of Mycobacterium avium complex (*M. avium*, *M. intracellulare*, and other members) isolated from culture.

- **MYCOBACTERIUM GORDONAE** Culture Identification Test - For the identification of *Mycobacterium gordonae* isolated from culture.

- **MYCOBACTERIUM KANSASII** Culture Identification Test - For the identification of *Mycobacterium kansasii* isolated from culture.
## Molecular Probe Performance Characteristics

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. avium</em></td>
<td>99.3%</td>
<td>100%</td>
</tr>
<tr>
<td><em>M. intracellulare</em></td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><em>M. avium complex</em></td>
<td>99.9%</td>
<td>100%</td>
</tr>
<tr>
<td><em>M. gordonae</em></td>
<td>98.8%</td>
<td>99.7%</td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td>92.8%</td>
<td>100%</td>
</tr>
<tr>
<td><em>M. tuberculosis complex</em></td>
<td>99.2%</td>
<td>99.0%</td>
</tr>
</tbody>
</table>
Biochemical tests for *M. tuberculosis* complex

- 8 species make up the complex
  - *Mycobacterium tuberculosis*
  - *Mycobacterium africanum*
  - *Mycobacterium bovis*
  - *Mycobacterium bovis (BCG)*
  - *Mycobacterium microti*
  - *Mycobacterium canettii*
  - *Mycobacterium pinnipedii*
  - *Mycobacterium mungi*

- Differentiate by biochemical testing
  - Niacin
  - Nitrate
  - Others
Antimicrobial susceptibility testing

• Required for all MTB complex patients
  – Absolute concentration
  – Resistance ratio
  – Proportion

• Recommended for some NTM species
Drug susceptibility testing of *M. tuberculosis*

- Culture based DST remains the gold std
  - Reliable for INH & Rif, inconsistent for Ethambutol resistance

- Genotypic methods
  - Sequencing
  - Line probe hybridization assays (commercial)
  - Molecular beacons (GeneXpert)
  - Loop mediated isothermal amplification
Countries that had reported at least one XDR-TB case by end 2010

The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.}

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Genotyping

• MMWR Controlling TB in the US Nov. 2005

• Refers to procedures to identify *M. tuberculosis* isolates that are identical in specific parts of the genome

• Along with epi investigation, genotype used to confirm transmission
CDC program for genotyping *M. tuberculosis* isolates

- DNA Fingerprinting – Restriction Fragment Length Polymorphism (RFLP)
<table>
<thead>
<tr>
<th>Test</th>
<th>Maximum turnaround time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy for acid-fast bacilli</td>
<td>≤24 hours from specimen collection or, if test is performed offsite, ≤24 hours from</td>
</tr>
<tr>
<td></td>
<td>receipt in laboratory; if latter, time from specimen collection to laboratory receipt</td>
</tr>
<tr>
<td></td>
<td>should be ≤24 hours</td>
</tr>
<tr>
<td>Nucleic acid amplification assay</td>
<td>≤48 hours from date of specimen collection</td>
</tr>
<tr>
<td>Mycobacterial growth detection by culture</td>
<td>≤14 days from date of specimen collection</td>
</tr>
<tr>
<td>Identification of cultured mycobacteria</td>
<td>≤21 days from date of specimen collection</td>
</tr>
<tr>
<td>Drug susceptibility testing</td>
<td>≤30 days from date of specimen collection</td>
</tr>
<tr>
<td>Drug susceptibility testing of second-line</td>
<td>≤4 weeks from date of request</td>
</tr>
<tr>
<td>drugs</td>
<td></td>
</tr>
</tbody>
</table>
What are the expected TAT?

<table>
<thead>
<tr>
<th>Test</th>
<th>Standard</th>
<th>SHL</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB smear</td>
<td>&lt;24 h</td>
<td>7 h</td>
</tr>
<tr>
<td>NAAT</td>
<td>&lt;48 h</td>
<td>&lt;24 h</td>
</tr>
<tr>
<td>Growth in culture</td>
<td>&lt;14 d</td>
<td>NA</td>
</tr>
<tr>
<td>ID of culture</td>
<td>&lt;21 d</td>
<td>14 d</td>
</tr>
<tr>
<td>Sensitivity Testing</td>
<td>&lt;30 d</td>
<td>21 d</td>
</tr>
</tbody>
</table>
Tuberculin Skin Test (TST)

- In routine use since 1910
- TST is the most used test for *M. tuberculosis* infection in U.S.
- Delayed type hypersensitivity reaction to PPD, a polyvalent mycobacterial antigen mixture
TST Pro’s Con’s

Advantages
• Inexpensive
• Good performance
• No special equipment
• Long history of experience

Limitations
• Reader variability
• “Boost” response
• Low specificity
  – Cross reaction with BCG and NTM
• Low sensitivity
• Need for 2 visits
Interferon Gamma Release Assays:

- **Principle:**
  - Persons exposed to *M. tuberculosis* develop T-cells (lymphocytes that recognize and respond to TB-specific antigens.
IGRA (continued)

• When stimulated with TB-specific antigens, these primed T-cells release the cytokine, interferon-gamma (IFN-γ)
IGRA (continued)

- The released IFN-$\gamma$ can then be detected and serves as an indirect indicator of exposure to TB.
First a blood sample is collected from the patient. The white blood cells are separated and then put into the wells of a 96-well microtiter plate. The plates are pre-coated with high affinity antibodies (Y) to Interferon-gamma (IFN-\(\gamma\)), a cytokine released by effector T-cells when fighting TB infection.

Antigens from the TB organism are then added to the wells with the white blood cells to provoke IFN-\(\gamma\) secretion from any effector T cells primed against TB. The antigens are selected to be unique for \(M.\) \(tuberculosis\) so that only responses of T cells specific for TB are measured.

Antigen specific responding T cells (\(\rightarrow\)) release the cytokine (\(\star\)), which is captured in the immediate vicinity of the T cells, by the antibodies lining the bottom of the well.

After a short incubation time the wells are washed, removing the antigens and cells from the wells. A conjugated second antibody (\(\rightarrow\)) is then added which binds to the IFN-\(\gamma\) secreted by the T cells (and captured by the primary antibody).

A substrate is then added which produces spots (\(\bullet\)) where the IFN-\(\gamma\) was secreted by T cells. The number of spots is counted.

http://www.oxfordimmunotec.com/na/healthcare/howitworks.html
1. Collect the blood sample. At the lab, PBMCs are separated from whole blood, washed, counted and inoculated into 4 separate microtiter wells.

2. PBMCs [●] and specific TB antigens [□] are added to wells pre-coated with antibodies to IFN-γ [γ] and incubated 16 to 20 hours (37°C, CO₂).

3. IFN-γ [γ] is released from activated T cells and captured. Wash wells, add secondary conjugated antibody [✓]. Incubate for one hour.

4. Wells are washed. A substrate is added which produces spots [●] where interferon gamma was secreted by T cells. Spots are counted.

http://www.oxfordimmunotec.com/How_It_works_North_America
QuantiFERON TB- Gold In-Tube

• Blood test that measures and compares amount of interferon-gamma (IFN-\(\gamma\)) released by blood cells in response to antigens
• FDA approved in May 2005 – Cellestis, Carnegie, Australia
QFT Procedure – Clinic and Lab

• Procedures in Clinic
  – Blood Collection
  – Shaking of Tubes
  – Blood Incubation
  – Plasma Separation

• Procedures in Lab
  – ELISA
  – Data Analysis
**Stage One – Blood Incubation and Harvesting**

- After blood collection, mix QuantiFERON®-TB Gold tubes thoroughly, by shaking vigorously for 5 seconds.
- As soon as possible, and within 16 hours of collection, incubate tubes upright at 37°C for 18-24 hours.
- Centrifuge tubes at 2000-3000 g (RCF) for 15 minutes.
- Harvest at least 200 µL plasma from each tube. Store in racked microtubes or uncoated microplates.

**Stage Two – Human IFN-γ ELISA**

- Add 50 µL of conjugate solution to each well. Add 50 µL of plasma or standard.
- Shake covered plate for 1 min. Incubate for 120 minutes at Room Temperature.
- Wash plate ≥ 6 times. Add 100 µL of substrate. Incubate 30 min. at Room Temperature.
- Add 50 µL of stop solution. Read absorbance within 5 min at 450nm (620-650nm ref).
- Calculate Results using QuantiFERON®-TB Gold In-Tube Analysis Software.
Data Analysis and Results

• Results are reported as
  – Positive
  – Negative
  – Indeterminate

• Indeterminate
  – Low mitogen
    • CMI response
  – High Nil
    • live vaccines
    • secondary infection
# Technology Comparison

<table>
<thead>
<tr>
<th></th>
<th>T-SPOT</th>
<th>QFTB In Tube</th>
<th>TST</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antigens</strong></td>
<td>ESAT-6 &amp; CFP10</td>
<td>ESAT-6 , CFP10, TB7.7</td>
<td>PPD</td>
</tr>
<tr>
<td><strong>Boosting effect with repeat tests</strong></td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>TAT</strong></td>
<td>16-20 h</td>
<td>16-24 h</td>
<td>48-72 h</td>
</tr>
<tr>
<td><strong>Readout units</strong></td>
<td>IFN-Gamma spot forming cells</td>
<td>International units of IFN-Gamma</td>
<td>Millimeters of induration</td>
</tr>
<tr>
<td><strong>Technology</strong></td>
<td>ELISpot</td>
<td>ELISA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Readout system</strong></td>
<td>Count of spots</td>
<td>Measurement of optical density values using an automated reader</td>
<td>Palpable induration</td>
</tr>
<tr>
<td><strong>Subjective Reading</strong></td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
CDC advises that IGRA’s can be used in all circumstances in which the TST is used, including…

- Contact investigations
- Evaluation of recent immigrants who have had BCG vaccination
- TB screening of health care workers and other individuals in high risk settings
- IGRA is in place of (not an addition to) TST
General Benefits of IGRA over TST

• Requires only one patient visit
• Assesses responsiveness to *M. tuberculosis* antigens
• Does not boost previous responses
• Interpretation less subjective than for TST
Limitations of IGRAs

• Cross-reactivity is possible with some atypical Mycobacteria infections:
  – *M. kansasii*, *M. szulgai*, and *M. marinum*

• Testing logistics:
  – Specimen transport time
  – Requirement for specialized testing equipment

• Additional data needed in certain patient populations
  – Children, Immunocompromised, Pregnancy
BCG Vaccinated Patients

- IGRA benefit the BCG vaccinated patient
- Many false positive TST due to vaccination status
- Treatment is costly, carries risk of significant side effects
- Treatment is not always needed since most do not have LTBI
Performance of IGRAs and the TST:

An up-to-date TB Test Meta-Analysis

R Diel, R Loddenkemper and A Nienhaus
Evidence based comparison of commercial interferon-gamma release assays for detecting active tuberculosis – a meta-analysis.
Chest, 2009, Published on Dec 18, 2009 in electronic format;

Chest April 2010 137:952-968; doi:10.1378/chest.09-2350
Contact Investigations

• For persons with recent TB exposure, negative IGRA results should be confirmed with a repeat test 8-10 weeks after exposure (end of window period) per CDC. This is the same as for a negative TST.

  – “3 months interval from the diagnosis of the index case will be enough for the final decision of the infection of contacts.”

• N=25, 8 positive QFTB-G
For high risk contacts…

• When “window prophylaxis” has been started for high-risk contacts exposed to an infectious TB patient, a negative IGRA result at the end of the window period should be interpreted in light of all other clinical and epi data
  – A full course of LTBI TX should be considered even with a negative result when the rate of TB transmission to other contacts is high or when a false-negative is suspected because of immune status.
Use of IGRA
Baseline and Serial Testing

• Baseline testing with IGRA
  – Establish baseline with single negative IGRA
  – HCWs with positive IGRA result should be referred for diagnostic evaluation

• Serial testing for infection control
  – A conversion is a change from negative to positive
Cost Barrier?

— Cost-effective alternative to TST
  • Reduction in false positive test results
  • No second visit needed to complete testing
  • Two-step testing not needed
  • Reduction in rates of CXR (due to higher specificity for *M. tuberculosis*)
Are IGRAs cost effective?

• DePerio et al: Arch Intern Med. 2009
  – Use of IGRA “leads to superior clinical outcomes and lower costs than the TST and should be considered in screening non-BCG-vaccinated and BCG vaccinated new HCWs for LTBI.”

  – “Selected use of QFT-G appears to be cost effective if used in targeted fashion.”
IGRA Summary

- IGRAs are more specific than TST and are not confounded by previous BCG vaccination
  - Less unnecessary preventive treatment
- IGRA are more sensitive than TST
TB antibody tests

- Tests that detect IgG antibody to TB
- Highly variable results for sensitivity and specificity
- Do not have a roll in the diagnosis of TB
- Not FDA approved
- Recently confused with IGRA in the news.
Take Home Message

• Culture of TB remains the gold standard
• AFB smears are the most cost effective
• NAAT are sensitive and rapid but cannot differentiate between dead and viable TB
• IGRA do not differentiate between active and latent TB
• There are new tests on the horizon
Let’s not forget...
Bibliography

• Requirements to get a high quality specimen to the laboratory

• Standard diagnostic techniques for the detection and identification of Mycobacteria

• Importance of acid-fast bacilli smear microscopy as a primary diagnostic tool

• Drug susceptibility testing of M. tuberculosis

• Rapid methods for the identification and drug susceptibility testing of M. tuberculosis as they compare to the traditional methods

• CDC program for genotyping M. tuberculosis isolates

• Interferon Gamma Assays (IGRA) – Pro’s and Con’s