Clinical Mycobacteriology:
TB Diagnostic Methods

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Role of the Laboratory

The Bacteriologic Examination

- Specimen collection and transport
- Specimen processing
- AFB smear microscopy
- Nucleic acid amplification testing (NAAT)
- AFB culture
- Identification of M. tuberculosis complex and nontuberculous mycobacteria from culture
- M. tuberculosis drug susceptibility testing
Case Presentation

- 21 y/o east Asian man
- Cough, sputum, fever, night sweats, and weight loss for one year.
- Never before treated for TB.
- Now a student in Washington.
- Vital signs and physical exam normal

CXR

- Bilateral upper lobe cavitary infiltrates with volume loss
- Scattered nodular infiltrates in lower lung fields
- Possible right pleural effusion

What would you do first?

- Place a tuberculin skin test
- Prescribe moxifloxacin for pneumonia treatment
- Collect sputum samples for AFB smear and culture
- Obtain chest CT scan
Clinical diagnosis is confirmed by demonstration of the organism: *M. tuberculosis*

- Smear
- Amplification of nucleic acid
- Culture

Specimen Quality is Important

- The results of tests, as they affect patient diagnosis and treatment, are directly related to the quality of the specimen collected and delivered to the laboratory.

Specimen Quality is Important

- Sputum is a challenging specimen to collect!
- A high quality specimen requires:
  - Well trained health care professionals to supervise collection
  - Well coached and informed patient
  - Alternatives should be available if the patient cannot produce sputum
- Collect specimens *before* initiating therapy.
Specimen Collection, Transport, Handling, and Processing

This module discusses pre-analytic factors contributing to specimen quality that impact patient diagnosis and treatment. The content compares collection, storage and transport of different specimen types (e.g., respiratory and non-respiratory) and explains the principles of specimen processing.

Specimen Collection: Pulmonary Disease

- **Sputum: Initial Diagnosis**
  - Collect three sputa, each ideally of 3-10 mL volume, on different days or 8-24 hours apart, at least one of which is an early morning specimen. Saliva is unacceptable.
  - Alternatives if patient cannot produce sputum
    - Sputum induction using aerosolized saline
    - Bronchoalveolar lavage, bronchial washing, lung biopsy, or other invasive procedures

<table>
<thead>
<tr>
<th>Study</th>
<th>Specimen Type</th>
<th>Total</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Overnight</td>
<td>160</td>
<td>85.0</td>
</tr>
<tr>
<td></td>
<td>Spot</td>
<td>160</td>
<td>51.8</td>
</tr>
<tr>
<td>2</td>
<td>Overnight</td>
<td>181</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>Spot</td>
<td>179</td>
<td>13.9</td>
</tr>
</tbody>
</table>

1 PANIDE et al., Indian J Tuberc 21:1974, 192

Slide courtesy of Dr. Ed Desmond, Muralial Diseases Laboratory, California State Dept. of Health Services
Specimen Collection

- Extrapulmonary disease

- **Tissue**: Send for AFB culture and smear as well as pathology and molecular studies.

- **Body fluids**: Send maximum volume attainable for AFB culture and smear. Swab drastically reduces AFB yield.

- **CSF**: AFB culture more sensitive than PCR (in house data)

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Why does it take so long to get smear results?

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Outside of Biosafety cabinet:
Prepare reagents and media

Inside Biosafety Cabinet:
Process samples, inoculate media and smears
Acid-Fast Smears

- Smear preparation: Entire sputum sample is concentrated
  - More sensitive than direct smear
  - Specimen processing requires more time, equipment & technical expertise than making a direct smear
- Fluorochrome Stain: Smear is read at low magnification
  - More sensitive than fuchsin-dye (ZN or Kinyoun) stain
  - NOT Ag-Ab complex
  - Auramine, Auramine-Rhodamine

Sensitivity and Reliability of Microscopy

Cruickshank 1952, Truant 1962

Smear negative specimen contains < 10^3 AFB per mL

<table>
<thead>
<tr>
<th>Quant. AFB on Z-N smear</th>
<th>Quant. AFB on Auramine smear</th>
<th>Acid-fast bacilli per mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2+</td>
<td>4+</td>
<td>10^7</td>
</tr>
<tr>
<td>1+</td>
<td>3+</td>
<td>10^6</td>
</tr>
<tr>
<td>Rare</td>
<td>2+</td>
<td>10^5</td>
</tr>
<tr>
<td>1+</td>
<td>1+</td>
<td>10^4</td>
</tr>
<tr>
<td>Rare</td>
<td></td>
<td>10^3</td>
</tr>
</tbody>
</table>

Reporting acid-fast smear results

Smithwick, Laboratory Manual for Acid-Fast Microscopy, CDC, 1976

<table>
<thead>
<tr>
<th>Number of AFB seen:</th>
<th>Report:</th>
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<tbody>
<tr>
<td>0</td>
<td>Negative, or No AFB seen</td>
</tr>
<tr>
<td>1-2 / 300 fields³</td>
<td>+/-</td>
</tr>
<tr>
<td>1-9 / 100 fields</td>
<td>1+</td>
</tr>
<tr>
<td>1-9 / 10 fields</td>
<td>2+</td>
</tr>
<tr>
<td>1-9 / field</td>
<td>3+</td>
</tr>
<tr>
<td>&gt;9 / field</td>
<td>4+</td>
</tr>
</tbody>
</table>

³ 800-1000X field for Kinyoun or Z-N stains, 200-250X field for fluorochrome stains
Acid-Fast Stained Smears: Interpreting Results

- Acid-fast bacilli, or AFB, or “Positive”
  - Usually *M. tuberculosis* or NTM (nontuberculous Mycobacterium)
  - *Nocardia*, other modified acid-fast organism
  - Live or dead organisms
  - Quantity should correlate with growth in culture
- No AFB seen, or “Negative”
  - Smear Negative, Culture Positive
    - $<10^3$ AFB / mL sputum (fluorochrome stain)
  - Smear Negative, Culture Negative for TB
    - Limitation of method, rapid growers (e.g. *M. abscessus*) may not stain with fluorochrome

Direct detection of *M. tuberculosis* in clinical specimens: Nucleic Acid Amplification tests (NAAT)

- Tests amplify and detect DNA or rRNA (nucleic acid) specific for *M. tuberculosis* complex in clinical specimens
- Do not distinguish live and dead bacilli
  - Can confirm diagnosis of “culture negative TB”
  - Cannot determine latent infection vs active disease
  - Has NO utility for following therapy

GeneXpert MTB/Rif-Resistance Test (Cepheid)

Automated Sample Preparation, Real Time PCR Amplification, and Detection in Cartridge: 90 minutes
**TB PCR**

<table>
<thead>
<tr>
<th>Lab</th>
<th>HMC Microbiology</th>
<th>UWMC Microbiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method (test code)</td>
<td>GeneXpert® MTB/RIF (MTBP)</td>
<td>own PCR assays for MTB complex, M. A. L, and NTM request separately (TBPCR, MAVPCR, NTMPCR)</td>
</tr>
<tr>
<td>Days performed</td>
<td>Daily</td>
<td>Mon-Fri</td>
</tr>
<tr>
<td>TAT</td>
<td>Results same day as AFB smear if ordered with AFB culture, next day if added</td>
<td>Tested day after AFB processing, results in 1-3 days</td>
</tr>
<tr>
<td>Specimens</td>
<td>AFB processed sputa, resp. samples</td>
<td>tissue, body fluid AFB proc. sput, resp. samples</td>
</tr>
<tr>
<td>Cost</td>
<td>UW Lab Med Reference Lab Services 206 685 6066</td>
<td></td>
</tr>
</tbody>
</table>

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**A Comparison of Xpert MTB/RIF® with Standard of Care for Inpatients with Suspected Pulmonary Tuberculosis**

- Xpert MTB/RIF is less expensive than three AFB smears and more accurate, particularly with regard to false-positives from non-tuberculous mycobacteria.
- The reduced time in isolation as a result of Xpert will result in substantial cost savings

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**GeneXpert MTB/RIF® at HMC**

- 2014 HMC data
  - 15 cases of pulmonary TB, 21 total cases TB
  - 40% (6 of 15) pulmonary cases were AFB smear positive, 3 of which had 4+ AFB on sputum smears
- 03/2012 – 02/2014 HMC data
  - 20 inpatient cases of pulmonary TB
  - 80% (16 of 20) were AFB smear positive
**GeneXpert MTB/RIF® at HMC**

- Sputum specimens (expectorated, induced) tested only
- BAL, bronch wash specimens tested starting July 2015
- First HMC patient sputum with AFB culture & smear order is automatically tested by Xpert MTB/RIF
- Subsequent samples are tested on request
- Other specimen types (fluid, tissue): TB PCR at UW Micro Molecular Diagnostics by request

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**GeneXpert MTB/RIF® at HMC**

- Xpert MTB results are reported the same day as AFB smear results, generally 7 days/week
- Sputum specimens (expectorated, induced) tested only
- BAL, bronch wash testing starts July 2015
- First HMC patient sputum for AFB culture & smear is automatically tested by Xpert MTB/RIF
- Subsequent samples are tested on request
- Other specimen types (fluid, tissue): TB PCR at UW Micro Molecular Diagnostics by request

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**GeneXpert MTB/RIF® at HMC**

- Positive Xpert Rifampin (“Rifampin resistant”) results are reported verbally only
- Results are confirmed by molecular testing, Drug Resistance Screening by Sequencing (DRSS), at WA State TB Laboratory
  - plus phenotypic testing once MTB has grown in culture
  - California State Dept. of Health TB Laboratory has found 15% of Xpert “Rifampin resistant” results to be silent mutations
GeneXpert MTB/RIF® at HMC

- There is no value in repeating an Xpert assay after the first positive result, so subsequent requests will be cancelled.
  - 84% of patients treated for TB are still Xpert-positive after 8 weeks, and 27% are still positive after 26 weeks, even though smear and culture positivity fall off more rapidly (Friedrich et al. Lancet Respir Med 1:462-70, 2013)

Culture Media

- Conventional Solid Culture Media
  - Quantitation: one colony vs. 4+ growth?, NTM or following TB therapy
  - Recovery of AFB away from contaminant organisms
  - Detection >1 mycobacterial species, MTB + NTM
  - Presumptive ID based on colony morphology, pigment, growth rate

Culture Media

- Automated System with Liquid Media
  - Faster detection
“Presumptive” (means “This is a guess”) Identification by AFB Microscopic Morphology in Liquid Culture Media

- "Cording" exhibited by M. tuberculosis
- Limitation: also seen with any species that produces rough colonies, e.g. M. kansasii, M. abscessus

Kinyoun's stain

M. tuberculosis
M. haemophilum

MTB Load vs Test Results

- TB "infection without disease"
  - Positive PPD: skin test or IGRA
- Active Tuberculosis
  - “Culture-negative” TB
  - Smear-negative/Culture-positive TB
  - Smear-positive TB

Time-to-detection (TTD) in culture predicts risk of Mycobacterium tuberculosis transmission: a cohort study


- TTD < 9 days identifies patients at high risk of transmitting tuberculosis and is superior to sputum smear.
Mycobacterial Species Identification for growth in culture

- Nucleic acid probes: DNA Probe Tests
- DNA Sequence Analysis
  - also direct specimen testing: PCR and DNA sequencing
  - UWMC Microbiology Molecular Diagnostics
  - http://depts.washington.edu/molmicdx/

Gen-Probe® AccuProbe® DNA Probe: Identification of growth in culture

- DNA probe for rRNA target
- Specificity approaches 100%. Results in 2-3 hours
- Can perform for positive culture on solid or in liquid media
- Separate probe tests: MTB complex, MAC, M. gordonae, M. kansasii
- HYBRIDIZATION ONLY - NO AMPLIFICATION
- No direct specimen testing

Acid-fast smear Positive / Culture Negative

- Dead acid-fast bacilli
  - Patient on antituberculosis treatment
  - Specimen overprocessed
  - Culture method inadequate
    - M. tuberculosis
    - Mycobacterium sp. with special growth requirements
- False positive smear: AFB not from patient
  - Environmental mycobacteria
    - Specimen collection
    - Processing or stain reagents
  - Cross contamination between specimens
TB Case Continued …
- Sputum AFB smears were strongly positive
- The patient was asked to remain home and stop attending school
- Sputum TB PCR test and sputum AFB cultures confirmed *Mycobacterium tuberculosis*
- Treatment with HRZE was begun using DOT

TB Case one month later …
- Despite HRZE using DOT, the patient failed to improve
- He continued to experience fever, productive cough, and weight loss, and he was now dyspeic
- Sputum remained strongly smear positive
- A CXR was repeated and was unimproved

Now what would you do?
- Stop all TB treatment
- Switch to an all injectable TB treatment regimen
- Check antimicrobial susceptibility results on the initial isolate
- Obtain chest CT scan
Susceptibility testing of *M. tuberculosis*

- **Initial testing:** rapid automated broth method
  - Drugs each tested at a single concentration
  - Streptomycin, Isoniazid, Rifampin, Ethambutol, Pyrazinamide
  - results in 15-30 days from date specimen processed
- **Limit of detection:**
  - > 1% of inoculating population is resistant
- Test subsequent isolate if clinical or microbiological evidence of treatment failure

Conventional Agar Proportion Method

Results reported as % resistant compared with control (no drug)

Minimum time to results: 5 - 6 WEEKS
Report of Plate Sensitivities Results
- Provide
  - Drugs tested and their concentrations
  - Interpretation of results – Sensitive vs Resistant
  - Percentage of resistance
  - Colony counts at two dilution $10^{-3}$ and $10^{-5}$
  - Satisfactory growth in the control quadrant (no drug)

TB laboratory objectives/Tests used
- Determination of infection
  - Acid-fast stain
  - Culture $\rightarrow$ Organism Identification
  - Nucleic Acid Amplification (NAA) test
- Determination of susceptibility
  - Culture $\rightarrow$ Org. ID $\rightarrow$ Phenotypic tests
  - Nucleic Acid Amplification (NAA) test
- Follow treatment
  - Acid-fast stain
  - Culture

Current turnaround times for *M. tuberculosis* drug susceptibility testing
- From receipt of specimen to 1st drug susceptibilities by culture method ~4 weeks
- 2nd line drugs $\rightarrow$ additional month by agar proportion or ~2 weeks by MGIT
- Molecular methods (nucleic acid amplification & detection of mutations) can be done within a day or 2
Suspicion of drug resistance → request for molecular (rapid) testing

- History of previous treatment
- Foreign born from country with increased drug resistance
- Patients not responding well to treatment
- Patients known to have been exposed to a person with MDR-TB

When to perform molecular tests for drug resistance?

- Acid-fast smear-positive specimen
- Some of the specimen sediment is available for sending to the reference lab, and:
- Drug-resistant TB is suspected.
- TB suspects have wide contacts
- Smear AFB positive but culture negative for undiagnosed TB.
- MTB cultures are mixed with other bacteria.

Drug Resistance Screening by Sequencing (DRSS)

- Method developed, validated, performed by Washington State Public Health Tuberculosis Laboratory since 6/18/12.
- Screens for mutations on MTB complex DNA that could indicate resistance
  - rpoB for Rifampin, katG and inhA for Isoniazid, and pncA for Pyrazinamide
- MTB complex isolates (cultures) tested, or concentrated respiratory samples if sufficient DNA available, i.e. if > 2+ AFB on smear
Drug Resistance Screening by Sequencing (DRSS)

- Results of prelim. drug resistance within 1 week
- Limitations: screens only for common mutations, at least 30% of culture population must carry mutation to be detected
- Requires approval by TB Control Officer: Dr. Narita, Dr. Spitters, or Dr. Lindquist
- Contact WA State TB Lab for more information: 206 418 5473, Alla Ostash, Lead Mycobacteriologist
  alla.ostash@doh.wa.gov

Results for Molecular Detection of Drug Resistance; Conventional Drug Susceptibility Test in Progress

<table>
<thead>
<tr>
<th>Locus (region) examined</th>
<th>Result</th>
<th>Interpretation (based on in house evaluation of 254 clinical isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB (RRDR)</td>
<td>MUTATION</td>
<td>Rifampin resistant</td>
</tr>
<tr>
<td>inhA (promoter)</td>
<td>MUTATION</td>
<td>Isoniazid resistant</td>
</tr>
<tr>
<td>katG (ser315 codon)</td>
<td>MUTATION</td>
<td>Ethambutol resistant</td>
</tr>
<tr>
<td>embB (Met306,Gly306)</td>
<td>MUTATION</td>
<td>Ethambutol resistant</td>
</tr>
<tr>
<td>pncA (promoter, coding region)</td>
<td>MUTATION</td>
<td>Probably Pyrazinamide resistant</td>
</tr>
<tr>
<td>gyrA (QRDR)</td>
<td>No mutation</td>
<td>Cannot rule out fluoroquinolone resistance</td>
</tr>
<tr>
<td>rrs (1400 region)</td>
<td>No mutation</td>
<td>Cannot rule out resistance to injectable drugs (kanamycin, capreomycin, amikacin)</td>
</tr>
<tr>
<td>eis (promoter)</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td>tlyA (entire ORF)</td>
<td>No mutation</td>
<td></td>
</tr>
</tbody>
</table>

http://www.cdc.gov/tb/topic/Laboratory/mddr.htm

Summary

- The laboratory is a critical partner in the TB control system
- Rapid, reliable results are essential for early detection and management of TB cases and to guide interventions to prevent ongoing transmission
- Even in the molecular era, conventional AFB smear and culture remain core assays for clinical diagnosis and treatment of TB

Ken Jost, Tuberculosis Applications Scientist, Texas Dept. of State Health Services, Laboratory Services, Austin, TX
NAA Testing Resources

- CDC—Updated Guidelines for the Use of Nucleic Acid Amplification Tests in the Diagnosis of Tuberculosis, 2009
  http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5801a3.htm
  http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6241a1.htm
- APHL—Laboratory Considerations for Use of Cepheid GeneXpert MTB/RIF Assay

Thank you!

Acknowledgements:
- Ed Desmond, Director, California State Dept. of Health TB Laboratory
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- Charles Nolan, Medicine, Univ. of Washington
- David Park, Pulmonary, Harborview Medical Center, Seattle
- Ken Jost, Tuberculosis Applications Scientist, Laboratory Services, Texas Dept. of State Health Services, Austin, TX
- Christopher Spitters, Seattle-King Co. TB Control