LABORATORY METHODS:
Tuberculosis Diagnosis

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Specimen collection and transport

- Specimens (sputum, bronchial washings, urine, etc.) should be collected in a laboratory-approved sterile, leak-proof, non-breakable container
- Containers must be labeled with patient's name and date collected
- Begin collecting specimens prior to initiation of therapy

Collection and transport (2)

- Sputum is the most common specimen
  - Collect 5-10 ml of an early morning specimen, prior to eating
  - Usually 3 specimens on 3 different days are recommended for diagnosis
Collection and transport (3)

- Contaminated specimens can be minimized by
  - Instructing the patient to rinse mouth with preferably sterile water before collecting the specimen
  - Returning the specimen to the lab as soon as feasible after collection

Collection and transport (4)

- Indicate type of specimen on laboratory requisition form
- Keep all specimens refrigerated and transport as soon as possible to the lab

How many specimens to collect?

- The greater the number of specimens, the higher the probability of a positive
- Law of diminishing returns: 4 specimens doesn’t give many more positives than 3, so 3 is usual guideline
Sputum smear microscopy results according to the specimen collection

<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 PANDE et al., Indian J Tuberc 21:1974, 192

POSITIVITY ON SMEAR RELATED TO QUALITY OF SPUTUM SPECIMENS

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of specimens in each group</th>
<th>Per cent of specimens</th>
<th>Number positive</th>
<th>Per cent positive</th>
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<tbody>
<tr>
<td>Saliva</td>
<td>1125</td>
<td>32.9</td>
<td>21</td>
<td>1.8</td>
</tr>
<tr>
<td>Mucous</td>
<td>1707</td>
<td>49.9</td>
<td>68</td>
<td>3.5</td>
</tr>
<tr>
<td>Mucopurulent</td>
<td>583</td>
<td>17.2</td>
<td>220</td>
<td>37.7</td>
</tr>
</tbody>
</table>

Note: mucopurulent specimens are positive more often than saliva or mucous.

L.POLLAK & R.URBANCZIK, Bol Inform Inst Nac Tuberc (Caracas) 2:1969,5 - 8

Processing pulmonary specimens

- Digestion and decontamination
  - Pulmonary specimens are exposed to a mucolytic agent to dissolve mucin and liquefy the specimen
  - N-acetyl-L-cysteine (NALC) is the most common mucolytic agent used
Processing pulmonary specimens (2)

- Digestion and decontamination
  - Specimens are also treated with a liquid decontaminant, generally sodium hydroxide, a strong alkali which is more toxic to oral flora than AFB
  - Material is concentrated by centrifugation

Staining concentrated smear

- Fluorochrome stains
  - Fluorochrome stained smears require a fluorescent microscope
  - Generally read at 250X-450X magnification which allows rapid scanning of the smear
  - Auramine-rhodamine is an example of such a stain where the AFB appear yellow against a black background
Staining concentrated smear (2)

- Carbol fuchsin-based stains
  - Utilize a regular light microscope
  - Must be read at a higher magnification
  - Two types: Ziehl-Neelsen and Kinyoun. Both use carbol fuchsin/phenol as the primary dye (NOTE: ZN is more sensitive)
  - Smear is then decolorized with acid-(HCl) alcohol and counter-stained with methylene blue

REPORTING AFB SMEAR RESULTS*

<table>
<thead>
<tr>
<th>Number of AFB found:</th>
<th>Report:</th>
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<tbody>
<tr>
<td>0</td>
<td>— or Neg</td>
</tr>
<tr>
<td>1-2 / 300 fields</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>

*CDC System (WHO system goes up to 3+ only)
Inoculating growth media for culture

Solid media

- Two types most commonly used are
  - Lowenstein-Jensen which is egg-based
  - Middlebrook 7H 10 or 7H11 which are agar-based

Inoculating growth media for culture (2)

- Solid media have the advantage that organisms (colonies) can be seen on the surface of the medium
- If there is mixed growth or contamination, picking individual colonies can allow you to obtain a pure culture.
M. tb on egg medium

From CDC Lab Manual

Inoculating growth media for culture (3)

Liquid media
- Liquid or broth medium has the advantage of allowing detection of AFB more quickly
- Drug susceptibility testing using growth in liquid media leads to more rapid reporting of results
- Examples of liquid media are Trek and MGIT systems

MGIT Incubator
Accuracy problems in the TB lab

- False positive results, due to:
  - Cross-contamination during specimen processing
  - Specimen mix-up or mislabeling
- Inadequate primary culture media (some labs use only solid media)
- Inaccurate drug susceptibility results due to inadequate quality control
Identification of acid-fast bacilli (AFB)

Growth characteristics (preliminary ID)
- Preliminary indication of *M. tb* can be made from macroscopic & microscopic observation
  - Rate of growth
  - Colonial morphology
  - Pigmentation
Colonial Morphology (1)
- Smooth colonies on 7H-10 medium

Colonial Morphology (2)
- Rough colony on 7H-10 medium

Identification of acid-fast bacilli (AFB) (2)

Biochemical tests
- There is a battery of 8-12 biochemical tests used to differentiate within the mycobacterium genus
- Nitrate reduction and niacin accumulation are definitive for M. tb
Nucleic acid probe tests (non-amplified)
- DNA probe tests are species or complex specific
- Require less time than biochemical tests for identification
- Commercial probes are available for *M. tb* complex, MAC, *M. kansasii* and *M. gordonae*

Identification of acid-fast bacilli (AFB) (3)

High performance liquid chromatography (HPLC)
- HPLC uses a chromatography method to identify mycobacteria based on their mycolic acid profiles (cell wall composition)
- Instrument is expensive/usually reserved for larger laboratories
- MALDI-TOF (matrix assisted laser desorption ionization-time of flight) is now being validated
- Like HPLC, expensive instrument, but quicker

Susceptibility testing of *M. tuberculosis*

When to test
- All primary *M. tb* isolates from patients should be tested
- In addition, test isolates from relapse or re-treatment cases
- Test when drug resistance is suspected
Susceptibility testing of *M. tuberculosis* (2)

Methods for susceptibility testing

- Agar proportion method compares growth on agar media with and without one of the four primary drugs
- Broth based (MGIT, Trek)
  - Requires inoculation of the strain in broth with each of the (5) primary drugs, plus control vial
  - Growth of the strain in a vial with a drug indicates resistance to that drug
Direct detection of *M. tuberculosis* in clinical material

- Several commercial nucleic acid amplification tests (NAAT) for *M. tb* are now available, including
  - Gen-Probe Amplified Mycobacterium Tuberculosis Direct (AMTD) (for smear + or smear -)
  - Cepheid GeneXpert (now has FDA clearance)
- These tests are designed to amplify and detect DNA specific to *M. tb*
- The sensitivity of these methods allows for direct detection of *M. tb* in clinical specimens

Uses of NAAT for Direct Detection of MTBC in Respiratory Specimens

(NAAT = nucleic acid amplification test)

CDC guidelines recommend as standard of practice
- Will allow quicker diagnosis in some smear neg patients
- Only if patients are true TB suspects
- Only for untreated patients
- Not necessary to test smear positives when classic TB symptoms and history are present
- Do test smear negatives when clinical suspicion of TB is moderate or high

More encouragement to use NAAT

  - For smear neg patients, health care providers often/typically don’t start Rx until culture is +
  - Results in a delay in initiation of Rx, typically 3 weeks
  - NAA would detect many of these patients, → earlier initiation of Rx
- MMWR guidelines: Jan. 16, 2009 58(1):7-10
Genotyping methods

- Spoligotyping (spacer oligonucleotide typing)
- MIRU/VNTR (mycobacterial interspersed repetitive units/ variable number of tandem repeats)
- RFLP fingerprinting (restriction fragment length polymorphism)
- Whole genome sequencing (WGS)

Spoligotyping summary

- PCR-base method, so you can start with a small amount of DNA from a TB culture
- Gives a result as a number, so to tell if 2 strains are different, just see if they have different numbers
- Not too powerful at discriminating different strains. Sometimes strains that are not part of the same outbreak will have the same spoligotype—e.g., Manila strain & Beijing strain
- Is now performed at CDC, using DNA sequencer

MIRU summary

- A PCR-based method, like spoligotyping
- Like spoligotyping, the result is a number (24 digits)
- Uses a DNA sequencer instrument to analyze the PCR products
- Like spoligotyping, MIRU sometimes doesn’t discriminate between unrelated strains
- Since April 2009, a new 24 locus MIRU protocol is in use, making it more powerfully discriminatory
- RFLP will be required less often
Using MIRU and spoligotyping together

- Both are PCR-based strain typing methods, so you can do them with just a small amount of DNA
- If 2 strains of M. tuberculosis are different from one another, it is unlikely that they will have the same spoligotype and the same MIRU type
  - Possible exceptions include Manila strains and Chinese “Beijing” strains

RFLP typing of TB strains

- Involves DNA electrophoresis, and requires a lot of DNA
- Must have a pea-sized lump of TB bacteria to start
- A complicated procedure that takes ~ a week
- Result is a visual pattern—easy to compare by eye, but difficult to make a database
- The most powerful of the strain typing methods
- Performed only on strains that match by MIRU and spoligo, and only by specific follow-up request

IS6110-RFLP fingerprinting

![IS6110-RFLP fingerprinting](CDC)
Universal genotyping approach

- All isolates
  - spoligotype
  - MIRU-VNTR → 3-4 weeks
- Clustered isolates
  - IS6110-RFLP → Additional 3 weeks

Genotyping: Uses
1. Cross contamination studies
2. Outbreak investigation
3. TB Control needs, such as identifying settings where transmission occurs

Note: when genotyping links patients in a cluster, but no epi links are found, whole genome sequencing may be helpful in identifying which patients are truly linked in the same chain of transmission

Contact info for (Michigan) genotyping laboratory

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Fax: 517-335-9631

927 Terminal Drive Lansing, MI 48906

Please note that cultures for genotyping are sent through the county public health laboratory.
Molecular Detection of Drug Resistance:

Resistance Mutations

GeneXpert &
Pyrosequencing

Principles of Molecular Detection of Drug Resistance

- GeneXpert uses molecular beacon probes in an automated, user friendly (expensive) system
- With molecular beacons: if normal susceptible gene targets are present, molecular beacon will hybridize and "light up"
- Absence of molecular beacon signal suggests drug resistance
- For sequencing (e.g., pyrosequencing), the actual gene sequence is determined
- Actual sequence is more informative

Limitations of molecular detection of drug resistance

- The predictive value of a negative result for INH (no mutations found) is in the low 90s (about 93%) to date.
- Pyrosequencing or GeneXpert can be used to guide treatment until conventional drug susceptibility results are available (usually about a month later).
- Detection of a mutation in rpoB gene by GeneXpert doesn't always mean rifampin resistance
  - Some rpoB mutations don't cause rif resistance
  - When GeneXpert detects a mutation, follow-up DNA sequencing should be done to confirm rifampin resistance.
- Molecular testing should be followed by culture-based DST
Detection of Drug Resistance Mutations

- Drugs of interest:
  - For XDR screening
    - INH, RIF, KAN, AMK, CAP, IQs
  - Targeted Genes for pyrosequencing
    - \textit{katG}, \textit{inhA} promoter for INH
    - \textit{rpoB} for RIF
    - \textit{rrs} for KAN, AMK & CAP
    - \textit{gyrA} for Quinolones
  - GeneXpert detects resistance to rifampin only

Comparison of MB & PSQ

<table>
<thead>
<tr>
<th></th>
<th>GeneXpert MB</th>
<th>PSQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Report</td>
<td>Mutation present or not</td>
<td>Exact SQ</td>
</tr>
<tr>
<td>Interpretation of silent mutations or mutations not conferring R</td>
<td>Misinterpret as R</td>
<td>Does not misinterpret</td>
</tr>
<tr>
<td>Hands-on time</td>
<td>1.5 hr, simple</td>
<td>2.5 hr, more steps</td>
</tr>
<tr>
<td>Total test time</td>
<td>2.5 hr</td>
<td>5 hr</td>
</tr>
</tbody>
</table>

Advantages of PSQ over MB

- Results show sequences.
  - You would know what mutations are if present.
  - More information provided and less ambiguity.
- Silent mutations do not lead to wrong interpretation.
- Mutations not conferring resistance do not lead to wrong interpretation.
- Difficult targets may still be sequenced.
  - At present, GeneXpert detects resistance to rifampin only.
Suggestion for requesting molecular detection of drug resistance

- Acid-fast smear-positive specimen
- Some of the specimen sediment is available for sending to reference lab (State Microbial Diseases Lab or CDC)
- Drug resistance is suspected, or
- A susceptible population has been exposed, or
- The culture is mixed or non-viable, so regular drug suscept. testing can’t be done
- CDC also has Molecular Detection of Drug Resistance (MDDR) program: tests for mutations associated with resistance to additional drugs—ethambutol, pyrazinamide

Thank you