## THE ROLE OF THE LABORATORY

### LEARNING OBJECTIVES

Upon completion of this session, participants will be able to:

1. Describe three laboratory methods used in the diagnosis and control of TB resulting in a better understanding of laboratory results and improved communication between the clinician, the laboratory, and the patient

### INDEX OF MATERIALS

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<thead>
<tr>
<th>INDEX OF MATERIALS</th>
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<tr>
<td>1. The Role of the Laboratory– slide outline</td>
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<td>Presented by: Mark Pandori, Ph.D., H.C.L.D. (A.B.B.)</td>
<td></td>
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### SUPPLEMENTAL MATERIAL

None
ADDITIONAL REFERENCES


• Barnes, P., Cave, D. Molecular epidemiology of tuberculosis. NEJM. 2003;349(12):1149-1155. Review article.


LABORATORY METHODS:
Mycobacterium tuberculosis

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Presentation Goals

- Discuss specimen collection
- Microscopy & Culture (growth): “traditional” identification
  - Including susceptibility testing
- Nucleic Acid based detection (aka “molecular” testing
Slides:

- ACPHL, M.W.P.
- Dr. Ed Desmond, CDPHL
- The United States Agency for International Development (USAID) TB Response (TBCARE 1)

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

- Collect specimens prior to initiation of therapy
Specimen 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

Specimen 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
Specimen 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
Processing pulmonary specimens

- Digestion and decontamination
  - Pulmonary specimens are exposed to a mucolytic agent to dissolve mucin and to 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
MTB are “Acid Fast Bacilli” (AFB)

- Once they are stained (with any of a variety of stains)
  - they resist de-colorization by acid-alcohol treatment
  - they have waxy, tough outer membranes and walls

Staining concentrated smear

- Fluorescent stains
  - Fluorochrome stained smears require a fluorescent microscope
  - 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. (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>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>Negative</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)*
Growth media for culture: 2 kinds of media

Solid media

- Two types most commonly used are:
  - Lowenstein-Jensen (egg-based)
  - Middlebrook 7H10 or 7H11

Liquid Media

- Essentially one:
  - 7H9
  - The "MGIT" broth
  - It is: Liquid 7H10 minus the Vit. B6 and malachite green

Advantages of solid media:

- Organisms (colonies) can be seen on the surface of the medium; morphology is visible
- If there is mixed growth or contamination, picking individual colonies can allow you to obtain a pure culture
Solid culture media

7H10 7H11

Solid culture media: Lowenstein-Jensen
Inoculating growth media for culture

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
Role of the Laboratory

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Recovery of mycobacteria, BACTEC vs. solid media

<table>
<thead>
<tr>
<th>Study</th>
<th>BACTEC % recovered</th>
<th>Solid medium % recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anargyros</td>
<td>91%</td>
<td>52% (2 LJs)</td>
</tr>
<tr>
<td>Isenberg</td>
<td>87%</td>
<td>71% (1 agar plate)</td>
</tr>
<tr>
<td>Kirihara</td>
<td>88%</td>
<td>77% (1 LJ)</td>
</tr>
<tr>
<td>Morgan</td>
<td>72%</td>
<td>62% (1 LJ)</td>
</tr>
<tr>
<td>Park</td>
<td>93%</td>
<td>56% (1 agar plate)</td>
</tr>
<tr>
<td>Roberts</td>
<td>95%</td>
<td>42% (1 LJ, 1 LJ Gruft)</td>
</tr>
<tr>
<td>Stager</td>
<td>89%</td>
<td>73% (2 LJs)</td>
</tr>
<tr>
<td>Wilson</td>
<td>92%</td>
<td>80% (1 agar plate)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37% (1 LJ)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>86% (7H11 biplate)</td>
</tr>
</tbody>
</table>

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)

Identification of acid-fast bacilli (AFB)

Growth characteristics (preliminary ID)

- Preliminary indication of \( M. \ tb \) can be made from physical parameters (in addition to microscopic observation)
  - Rate of growth; temperature
  - Colonial morphology
  - Pigmentation
Rate of growth

- Rapid-grower: isolated in less than a week
  - not TB

- Slow-grower: usually 3 weeks, up to 6 weeks
  - could be TB

Growth temperature

- Incubation: 36 ± 1 °C

*M. tuberculosis* does not grow at lower or higher temperatures.
Pigment production

Non-chromogen → TB
Chromogens → non-TB

Colonial Morphology

Smooth
Rough
Identification of acid-fast bacilli (AFB)

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 \textit{M. tb}

Biochemical tests
- Niacin production
- Nitrate reduction
- Catalase negative at 68 ° C

Always use pure cultures, otherwise they will yield false results.

Test should be performed in the BSC – aerosols are produced.
Identification of acid-fast bacilli (AFB)

Nucleic acid probe tests (non-amplified)

- Requires a pure colony of organism
- DNA probe tests are species-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) (2)

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 aka “Mass Spec.” (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
- Isolates from relapse or re-treatment cases
- 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
MGIT System (Becton Dickinson)
Molecular Tests

-- Allow for direct detection of MTB in a clinical specimen

-- No culture required; they amplify DNA/RNA

-- Highly specific

-- Very fast
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
- Test smear negatives when clinical suspicion of TB is moderate or high

More recommendations to use Molecular/ 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
2 FDA Approved NAA Tests

- **Cepheid GeneXpert** and **Gen-Probe MTD test** – the only FDA-approved options
  - **MTD** is labor intensive and time consuming
  - **MTD** may be the most sensitive method
  - **GeneXpert** easy to do; provides drug susceptibility data

Homebrew / RUO Tests

- All are PCR-based
- Homebrew: rather inexpensive
- Performance can be excellent (See *Halse & Musser JCM 2010, NYS lab*)

But:
- They can require much more **initial set-up** / quality control
NAAT methods: challenges

- **Culture still rules:** higher sensitivity
  - higher specimen volume is tested by culture than by NAAT (MTD & GeneXpert may be exceptions)
  - TB is a bit tougher than other organisms
  - sputum often doesn’t contain many MTB organisms (compared to viral specimens (herpes, flu etc..) for example)

Gene Xpert (Cepheid)

- Single use cartridges
- Extraction and amplification: in the cartridge
- Fully Automated
Using the Cepheid Gene Xpert:

Clinical Specimen → Treat with NALC-NaOH and make concentrate → Gene Xpert, results

Nested PCR: rpoB gene

- Take product of PCR 1, use as target in reaction 2
- Increase specificity by having two sets of primers needed for amplification
- Increase sensitivity by amplifying target prior to second PCR
Target DNA sequence: \textit{rpoB} gene

- The target of rifampin: RNA polymerase subunit B

- PCR amplifies a small region relevant for rifampin resistance; uses 5 probes to assess for mutations

![Diagram of rpoB gene and probes](image)

Cepheid MTB: positive result

- five probes

- assay has an Internal PCR Control (for inhibition assessment)

Test gives semi-quantitative results: "high", "medium", "low", "very low" and "negative"
Cepheid MTB: negative result

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Summary of GXP sensitivities described in the literature:

<table>
<thead>
<tr>
<th></th>
<th>smear positive</th>
<th>smear-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moure et al (2011), JCM</td>
<td>ND</td>
<td>75.30%</td>
</tr>
<tr>
<td>Boehme et al (2010) NEJM</td>
<td>98.20%</td>
<td>72.50%</td>
</tr>
<tr>
<td>Marlowe et al (2011) JCM</td>
<td>98%</td>
<td>72%</td>
</tr>
<tr>
<td>Helb et al (2010) JCM</td>
<td>98.40%</td>
<td>71.70%</td>
</tr>
<tr>
<td>Armand et al (2011) JCM</td>
<td>100%</td>
<td>48%</td>
</tr>
</tbody>
</table>
Genotyping methods

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

Spoligotyping summary

- 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

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
Genotyping: Uses

1. Cross contamination studies

2. Outbreak investigation

3. TB Control needs, such as identifying settings where transmission occurs
Molecular Detection of Drug Resistance:

**PCR**
- Fast
- Uses “probes” to look for pre-characterized mutations; *GeneXpert can only assess Rif susceptibility*
- Can be integrated directly into testing
- Can be “fooled” by silent mutations

**DNA Sequencing**
- More time than PCR
- “Reads” entire DNA code
- Detects any and all mutations
- Cannot be “fooled”

Either can guide treatment until culture DST is completed
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 (Public Health 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 susceptible 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

Notice: Next Generation Sequencing

The ability to generate massive amounts of DNA sequence data very quickly

- Can be used to “Deep Sequence” which means to sequence everything within a given specimen
- Can be used to perform “Whole Genome Sequencing”
- Platforms becoming affordable: ~$100 per sample
- Can multiplex samples

Available now at our Laboratory

New York State PH Lab uses this for all Drug susceptibility testing
Whole Genome Sequencing:

Perhaps achievable ~24 hours

Performed on an isolate
(and possibly a specimen)

You get:

EVERYTHING
Whole Genome Sequencing:

Perhaps achievable ~24 hours

Performed on an isolate (and possibly a specimen)

You get:

Everything

That means:

- species
- genotyping; strain
- virulence factors
- Drug Susceptibility

Thank you