Disclosures

• None
Learning Objectives

• How mycobacteria are grown and identified in the laboratory
• The advantages and limitations of current diagnostic tests
• Recent advances in molecular techniques to identify isolates and resistance mechanisms more quickly
What happens to the patient’s specimen when it is sent to the lab?

- **Specimen**: Respiratory, tissue, body fluid, urine, stool
- **Stain**: Acid-Fast Smear – *Mycobacterium* present?
- **Culture**: Perform culture on specialized medium
- **ID**: If culture grows, identify *Mycobacterium* using molecular methods
- **DST**: Perform drug susceptibility testing on isolates
Why perform a stain?

• Inexpensive indication of whether the specimen contains mycobacteria
• A stain may take about an hour to perform and report
• A mycobacterial culture requires days to weeks
• Molecular methods (PCR) is also quick, but more expensive and limited to *M. tuberculosis*
The mycobacterial cell wall is unique

- Composed of >60% lipid
  - Gram-positive organisms ~5% lipid
  - Gram-negative organisms ~20% lipid
- Long chain (C_{60}-C_{90}) fatty acids called mycolic acids
- Mycolic acids make the cell surface extremely hydrophobic and resistant to staining with basic aniline dyes or penetration by drugs.
**Mycobacterium tuberculosis** does not stain well with the Gram stain.
Acid-fast stains for mycobacteria

- Mycobacteria are referred to as “acid-fast” bacilli (AFB)
- A complex is formed between mycolic acid and dye used in the stain (e.g. carbol-fuchsin or auramine O)
- Complex is resistant to destaining by mineral acids
- Mycobacteria retain the carbol-fuchsin or auramine O stain and other bacteria do not.
Ziehl-Neelsen stain

Heat drives fuchsin stain into waxy cell walls; phenol mordant fixes stain.

AFB’s stain in red; non-AFB’s stain in blue
Auramine-rhodamine stain

400X

1000X, oil
Acid-fast stains - Limitations

• Acid-fast stains are not very specific
  • Indicates whether a mycobacterium is present in the specimen
  • Cannot be used to differentiate between mycobacteria species
    • *M. tuberculosis* looks like all the other mycobacterial species on an acid-fast stain

• Acid-fast stains are not very sensitive
  • Need 1,000-10,000 CFU/ml for a positive AFB smear
  • Positive sputum smear suggests higher likelihood of infectivity for patients with pulmonary tuberculosis.
Tuberculosis Cases with Pulmonary Involvement by Sputum AFB Smear Result, Minnesota, 2009-2013

- Not done*: 12%
- Positive: 39%
- Negative: 49%

N = 459

Source: www.health.state.mn.us/tb
2-3 AFB smears are more sensitive than 1 smear
Yield of Serial AFB Smears

<table>
<thead>
<tr>
<th>Study</th>
<th>% of Total Positives Detected by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1\textsuperscript{st} Smear</td>
</tr>
<tr>
<td>Walker et al. (2000), <em>Int J Tuberc Lung Dis</em>, 4:246.</td>
<td>77.1%</td>
</tr>
<tr>
<td>Mathew et al. (2002) <em>J Clin Microbiol</em>, 40:3482-4 (low prevalence pop.)</td>
<td>89.4%</td>
</tr>
</tbody>
</table>
Acid-fast smears prepared from early morning sputum specimens have better sensitivity

<table>
<thead>
<tr>
<th>Study</th>
<th>Random Specimen Positive (%)</th>
<th>Early Morning Specimen Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abraham et al, 2012, <em>Indian J Med Res</em>, 135: 249-51 (smear is positive)</td>
<td>21/49 (43%)</td>
<td>32/49 (65%)</td>
</tr>
</tbody>
</table>
Mycobacterial testing workflow:
Mycobacteria Culture

Specimen → Stain → Culture → ID → DST → PCR
Culture of *M. tuberculosis* complex

- Sensitivity of culture is much better than smear
  - A positive AFB smear requires 1,000-10,000 CFU/ml of specimen
  - A positive mycobacteria culture requires only 10-100 CFU/mL of specimen

- Culture media, 2 types
  - Solid medium (Lowenstein-Jensen (LJ) or Middlebrook)
  - Broth medium
    - In general, mycobacteria grow faster in broth
      - Average 10 days vs. 15-30 days
    - FDA-cleared systems: Bactec MGIT and VersaTREK systems
*M. tuberculosis* colony morphology on solid medium

Note the “rough and buff” morphology typical of *M. tuberculosis*
BACTEC MGIT 960 Culture System

MGIT - Mycobacterial Growth Indicator Tubes (Becton Dickinson)

- Fluorescent indicator in bottom of tube quenched by $O_2$
- $\uparrow$ mycobacterial growth $\Rightarrow$ $\downarrow$ $O_2$ and $\uparrow$ fluorescence
VersaTREK System

- Mycobacterial growth causes changes in bottle headspace pressure detected by the instrument.

Tuberculosis Cases by Mycobacterial Culture Result, Minnesota, 2009-2013

- Positive: 77%
- Negative: 20%
- Not done: 3%

N = 746

Source: www.health.state.mn.us/tb
Mycobacterial testing workflow:
Mycobacteria species identification

- Specimen
- Stain
- Culture
- ID
- DST

- PCR
Methods of identification from culture

- Traditional identification characteristics:
  - colonial morphology
  - growth characteristics
  - biochemical testing (niacin, nitrate, pyrazinamidase)
  - slow process taking up to 4-8 weeks

- Molecular methods allow for rapid identification
  - 1.) Nucleic Acid Hybridization Probes
  - 2.) Line Probe Hybridization Assays
  - 3.) Sequencing
  - 4.) MALDI-TOF MS
1.) Nucleic Acid Hybridization Probes

MTB or an NTM?

Hybridization Probes

Step 1

Microbiology culture plate
1.) Nucleic Acid Hybridization Probes

- Hologic Gen-Probe AccuProbes® (nucleic acid hybridization probes) available for:
  - *M. tuberculosis* complex
  - *M. avium* complex
  - *M. gordonae*
  - *M. kansasii*

- No DNA amplification step, so large amount of target nucleic acid required
- FDA-approved for identification of culture isolates
- Identification within 2-3 hrs after growth in culture

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> complex</td>
<td>99.2%</td>
<td>99.0%</td>
</tr>
</tbody>
</table>
2.) Line Probe Hybridization Assays
(Hain Lifesciences or Innogenetics)

- Genus- and species-specific probes bound to nitrocellulose membrane
- DNA from lysed culture isolate hybridizes to the probe for identification.
- Not approved by FDA for use in clinical diagnostic labs

Source: http://www.hain-lifescience.de
3.) *M. tuberculosis* Identification by DNA Sequencing

- Sanger dideoxy sequencing is the current gold standard for mycobacteria identification
  - Various targets are useful (*rpoB*, *hsp65*, 16S rDNA gene, etc.)
  - Broad range primers amplify all mycobacteria species
  - Hypervariable region between primers used to distinguish species
  - 8-24hr TAT

Hall L *et al.*, (2003) JCM 41:1447-53
4.) MALDI-TOF Mass Spectrometry

- **Matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry (MS)**
- Rapidly changing the way we identify microbes in microbiology laboratories
- Heavily utilized in many laboratories as central method for bacterial and yeast identification
- We also use MALDI-TOF MS as the method of choice for identification of mycobacteria
What happens inside the Mass Spectrometer?

Compare patient’s isolate spectrum to library of mycobacterial spectra to determine the species.

Theel ES, Clinical Microbiology Newsletter, 2013, 35:155-161
4.) MALDI-TOF Mass Spectrometry

• Advantages
  • Similar work-flow regardless of organism (bacteria, yeast, mycobacteria, mold)
  • Low consumable costs
  • Rapid turn around time, high throughput, small footprint
  • Adaptable – RUO versions allow databases expansion by user

• Limitations
  • Need pure isolate grown in culture (not a direct method)
  • Databases need expansion for less common organisms
Mycobacterial testing workflow: Direct identification from patient specimen

- Specimen
- Stain
- Culture
- ID
- DST

Perform direct PCR for *M. tuberculosis*

Identification from patient specimen *without waiting* for growth in culture
NAA tests for direct detection of MTB from patient specimens

• CDC recommendation:
  • NAA testing be performed on at least one (preferably the first) respiratory specimen from each patient with suspected pulmonary TB
  • NAA testing does not replace the need for culture

• FDA-cleared
  • 1.) Hologic/Gen-Probe MTD
  • 2.) Cepheid GeneXpert MTB/RIF

• RUO in U.S.
  • 3.) Hain LineProbe

• Laboratory Develop Tests (LDTs)
  • 4.) Rapid cycle/real-time PCR
1.) *M. tuberculosis* Direct (MTD) Test
(Hologic Gen-Probe)

- FDA-approved for direct detection of *M. tuberculosis* complex from respiratory specimens
- Transcription-mediated amplification of *M. tuberculosis* complex rRNA
- Clinical sensitivity:
  - Smear positive: 91-95%
  - Smear negative: 83-100%
- Clinical specificity: 99-100%
- Technically challenging test
  - Open PCR system so false positives due to cross-contamination of specimens are possible.
  - Cross-reactions occur w/ some rare mycobacteria: *M. celatum, M. terrae*-like organisms, *M. holsiaticum*
  - Culture still required for drug susceptibility testing
2.) Cepheid GeneXpert® MTB/RIF Test

- FDA-approved for respiratory specimens
- Results in about 2 hrs; minimal hands-on needed
- Detects *M. tuberculosis* complex and mutations associated with rifampin resistance using molecular beacon technology

- Pulmonary TB:
  - Sensitivity, Smear positive disease – 90%
  - Sensitivity, Smear negative disease – 74%
  - Specificity - 98%

Chang *et al*, 2012, J Infect 64:580-8
3.) Line Probe Assays (Hain Lifesciences)

*M. tuberculosis* complex

direct detection

Not approved for diagnostic use in the U.S.

Source: http://www.hain-lifescience.de
4.) Laboratory-developed PCR Tests (LDTs)

Advantages

• Closed PCR system – reduced opportunity for false-positives
• Good sensitivity and specificity (but can vary between labs)
• Often less expensive
• Wider variety of specimen types included smear negative specimens and formalin-fixed, paraffin-embedded tissue blocks

Limitations

• Often not as well-characterized as FDA-cleared tests
• Culture still needed

<table>
<thead>
<tr>
<th>Assay</th>
<th>Hologic MTD</th>
<th>Agreement (%)</th>
<th>kappa coefficient</th>
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</thead>
<tbody>
<tr>
<td>Mayo PCR</td>
<td>+</td>
<td>49/1</td>
<td>538/542 (99.3%)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>3/489</td>
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</tr>
</tbody>
</table>
Mycobacterial testing workflow:
Drug Susceptibility Testing

Specimen → Stain → Culture → ID → DST

PCR

Phenotypic
Molecular
Phenotypic Drug Resistance Testing

1.) Indirect agar proportion method

- Gold standard for all drugs except pyrazinamide
- Not rapid (14-21 days)
- Labor-intensive, technically complex
- No FDA-cleared, commercially available kit

Organism is resistant to drug A in the upper right compartment (>1% of inoculum shown by upper left control quadrant is growing in presence of drug). Organism is susceptible to drugs B & C in the lower compartments. Control quadrant in upper left contains no drugs.
Phenotypic Drug Resistance Testing

2.) Rapid broth susceptibility testing

- BD MGIT and VersaTREK systems, FDA-cleared
- Compare growth rates in tubes +/- critical drug concentrations

CDC goal is results for first-line drugs reported within 15-30 days after receipt of the specimen
### Phenotypic Drug Resistance Testing

#### 3.) Broth microdilution MIC plate

<table>
<thead>
<tr>
<th>CFL</th>
<th>MXF</th>
<th>RIF</th>
<th>AMI</th>
<th>STR</th>
<th>RFB</th>
<th>PAS</th>
<th>ETH</th>
<th>CYC</th>
<th>INH</th>
<th>KAN</th>
<th>EMB</th>
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<td>16</td>
<td>32</td>
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<td>16</td>
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<td>8</td>
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<td>4</td>
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<td>2</td>
<td>4</td>
<td>2</td>
<td>8</td>
<td>5</td>
<td>32</td>
<td>0.5</td>
<td>5</td>
<td>4</td>
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<tr>
<td>1</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>1.2</td>
<td>0.25</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>0.5</td>
<td>0.12</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>2</td>
<td>0.12</td>
<td>1.2</td>
<td>0.12</td>
<td>0.5</td>
</tr>
<tr>
<td>0.25</td>
<td>0.06</td>
<td>0.12</td>
<td>0.12</td>
<td>0.25</td>
<td>0.12</td>
<td>0.5</td>
<td>0.3</td>
<td>2</td>
<td>0.03</td>
<td>0.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

- Newest method
- MYCOTB plate from TREK Diagnostics
- LDT (not FDA-cleared)
- Rapid (14 days)
- Contains INH, RIF, EMB and 9 second-line drugs
- Provides MIC endpoint – helpful for isolates with MIC near critical concentration (CC) breakpoint that give fluctuating results w/CC method

Mycobacterial testing workflow: Drug Susceptibility Testing
Molecular Drug Resistance Testing

1.) Mayo LDT PCR detects isoniazid resistance

- Resistance mediated through *katG* S315T mutation.
- Method run from culture isolates or directly from specimens to give indication of potential isoniazid resistance.
- Same day TAT

**Graph:**
- **Fluorescence:** $-d(F2/F1)/dT$
- **Temperature (°C):** 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76
- **Lines:**
  - **S315T**
  - **WT**

Mayo Clinic Center for Tuberculosis
Molecular Drug Resistance Testing

2.) Sequencing of *pncA* for pyrazinamide resistance

- Broth susceptibility testing of PZA can over-call resistance
  - MGIT (up to 68% false resistance)
    - Piersimoni C et al., 2013, J Clin Microbiol. 51:291-4
    - Simons SO et al., 2012, J Clin Microbiol. 50: 428-34
  - VersaTREK (~70% false resistance)
    - Simner PJ et al., manuscript in preparation

- Sequencing of the *pncA* gene from culture isolates can help
  - Mutations associated with resistance occur throughout this 558bp gene so sequence entire gene and promoter region
  - Performed by CDC, Mayo or the NYS DOH Wadsworth Center
  - Can’t interpret mutations that have yet to be characterized
Molecular Drug Resistance Testing

3.) Next-generation sequencing for resistance mutations

- Currently under development
- NGS to examine 9 genes for resistance mutations to 1st and 2nd line drugs
- Allows for detection of resistance of low abundance variants within mixed population

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Resistance gene(s)</th>
<th>Major resistance mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampin</td>
<td>rpoB</td>
<td>D516V/G, H526Y/D/R, S531L</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>katG; inhA</td>
<td>S315T; C(-15)T</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>embB</td>
<td>M306L/V/I, M308, G406</td>
</tr>
<tr>
<td>Fluoroquinolones (moxifloxacin and ofloxacin)</td>
<td>gyrA</td>
<td>G88C, A90V, S91P, D94G/H/Y</td>
</tr>
<tr>
<td>Aminoglycosides (kanamycin and streptomycin)</td>
<td>rrs; rpsL; gidB</td>
<td>A1401G, C1402T, G1484T; K43R, K88R; R21Q</td>
</tr>
</tbody>
</table>
Summary

• AFB stains are rapid, but insensitive and nonspecific.
• Mycobacterial culture should always be ordered together with AFB stain.
• Identification after growth in culture is rapid using molecular methods.
• Direct identification of MTB using molecular methods most often uses smear-positive respiratory specimens; certain methods allow for other specimens.
• Molecular detection of some drug resistance markers is available for Mtb culture isolates and directly for smear-positive respiratory specimens.
Questions & Discussion
What is the major benefit of performing acid-fast staining from patient specimens?

A. Staining is the most sensitive detection method.

B. Staining is the most specific detection method.

C. Staining provides a rapid indication of mycobacterial presence.

D. Staining is no longer performed in the clinical lab setting.
Mycobacterial culture should always be ordered together with AFB stain?

A. True
B. False
3.) All of the following methods can currently be used to detect drug resistance, except:

- A) PCR
- B) Sequencing
- C) Broth microdilution
- D) MALDI
All of the following methods can currently be used to detect drug resistance, except:

A. PCR  
B. Sequencing 
C. Broth microdilution 
D. MALDI