Disclosures

• None
Overview

- Stains for Mycobacteria
- Culture of Mycobacteria
- Molecular methods for identification of *M. tuberculosis*
  - from culture
  - directly from specimen
- *M. tuberculosis* drug resistance testing
  - rapid broth-based methods
  - molecular markers of resistance
Stains for mycobacteria
Mycobacterium tuberculosis does not stain well with the Gram stain

*M. tuberculosis* ghosting on Gram stain
Mycobacteria Cell Wall

- contain >60% lipid
  - mycolic acids ($C_{60}$-$C_{90}$ fatty acids)
  - waxes
  - Gram positive organism contains ~5% lipid
  - Gram negative organism contains ~20% lipid

- mycolic acid makes the cell surface extremely hydrophobic and resistant to staining with basic aniline dyes or penetration by drugs
Mycobacterial Stains

- mycobacteria are “acid-fast” bacilli (AFB)
- a complex is formed between mycolic acid and dye (carbol-fuchsin or auramine O)
- the complex is resistant to destaining by mineral acids (ie., acid-fast)
- so mycobacteria retain the carbol-fuchsin or auramine O stain and other bacteria do not
Ziehl-Neelsen stain
uses heat to help drive fuchsin stain into waxy cell wall; phenol as mordant to fix stain;
(Kinyoun stain method – no heat, instead uses higher concentration of phenol and fuchsin dye to aid uptake; less effective as direct stain)

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

1000X, oil

400X
Other tidbits about AFB smears/stains

- Fite stain
  - Modification of ZN; often used in Pathology
  - uses a more mild decolorizing agent that supposedly works better for “delicate” mycobacteria like *M. leprae*;
  - tissue processing in Pathology can damage the mycolic acid, sometimes making it difficult to find the AFB regardless of the stain used

- LED microscopy
  - WHO study indicated it was superior to ZN and equivalent to fluorescence microscopy and recommended replacement of fluorescence and ZN with LED microscopy
  - gaining traction in developing countries where fluorescent microscopes scarce/expensive; can run on batteries

- Cannot reliably speciate using microscopy – *Mtb* looks like *MAC* which looks like *M. abscessus*, etc.

- Positive smear suggests higher likelihood of infectivity if the patient has pulmonary *Mtb*
What % of culture-confirmed TB cases in the U.S. have positive smears?
Tuberculosis Cases with Pulmonary Involvement by Sputum AFB Smear Result, Minnesota, 2008-2012

- Positive: 40%
- Negative: 47%
- Not done/unknown*: 13%

*67% of cases without sputum smear results were under 15 years of age

N = 491

Source: www.health.state.mn.us/tb
Are Two AFB Smears Better than One? 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&lt;sup&gt;st&lt;/sup&gt; Smear</td>
</tr>
<tr>
<td>Walker et al. (2000), Int J Tuberc Lung Dis, 4:246.</td>
<td>77.1%</td>
</tr>
<tr>
<td>Ipuge et al. (1996), Trans R Soc Trop Med Hyg, 90:258.</td>
<td>83.4%</td>
</tr>
<tr>
<td>Mathew et al. (2002) J Clin Microbiol, 40:3482-4 (low prevalence pop.)</td>
<td>89.4%</td>
</tr>
</tbody>
</table>
Are Early Morning Sputum Specimens Still Preferred?

<table>
<thead>
<tr>
<th>Study</th>
<th>Spot (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>
Culture of *M. tuberculosis* complex

Sensitivity of culture is much better than smear; only 10-100 viable organisms/mL required for positive culture

**Culture**

- **Solid Medium**
  - Egg-based – Lowenstein-Jensen (LJ); TTP ~ 30 days
  - Agar-based - Middlebrook
- **Rapid Broth (Liquid) Medium (FDA-cleared systems)**
  - Reduces TTP to ~ 10 days
  - BACTEC MGIT (fluorimetric, BD)
  - VersaTREK (pressure, TREK)
Note the “rough and buff” morphology typical of \textit{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 which are detected by the instrument; sponges in bottle are supposed to provide increased surface area for growth

Tuberculosis Cases by Mycobacterial Culture Result, Minnesota, 2008-2012

- Negative: 21%
- Positive: 75%
- Not done/unknown: 4%

Source: www.health.state.mn.us/tb
Identification of *M. tuberculosis* complex from culture
Traditional Methods of Identification

- Historically, positive mycobacterial cultures were identified on the basis of
  - colonial morphology
  - growth characteristics
  - biochemical testing (niacin, nitrate, pyrazinamidase)
  - slow process taking up to 8 weeks
- Sometimes, HPLC or GLC for cell wall constituents – generally at CDC or State Public Health Labs
Molecular Methods Allow For Rapid Identification

Identification Methods for Culture Isolates
1. Nucleic Acid Hybridization Probes

- From culture only
  - no amplification step
  - need lots of target nucleic acid!
  - add probe with unique, complementary sequence to known species; chemiluminescent detection
  - identification within 2-3 hours after growth in culture

- Hologic Gen-Probe AccuProbes® (nucleic acid hybridization probes) available for:
  - *M. tuberculosis* complex
  - *M. avium* complex
  - *M. gordonae*
  - *M. kansasii*
2. Line Probe Hybridization Assays for Mycobacteria (Hain Lifesciences or Innogenetics)

- Genus- and species-specific probes bound to nitrocellulose membrane
- DNA from lysed culture extract hybridizes to the probe for identification.
  - GenoType Mycobacterium CM and AS
    - *M. tuberculosis* complex and 29 nontuberculous mycobacteria on 2 strips
  - GenoType MTBC
    - Differentiation of *M. tuberculosis* complex
  - GenoType MTBDR plus
    - *M. tuberculosis* complex plus wt and mutant *rpoB, katG, inhA*

Not approved for diagnostic use in U.S. at this time
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.)
  - uses broad range primers that will amplify all mycobacteria species
  - hypervariable region between primers used to distinguish species

Sequencing workflow in the lab

1. Lyse/Heat
2. PCR Product clean-up (removes dNTPs and primers)
3. Target amplification
4. Cycle sequencing reaction
5. Electrophoresis
6. Clean-up (removes excess dye terminators and primers)
7. Sequence analysis
Sequence analysis

• Compare the isolate sequence to known mycobacterial sequence libraries
  • MicroSeq library (AB)
  • Lab-specific custom library
  • GenBank BLAST (NCBI)
  • Curated, web-based database tools
    • SmartGene or iSentio

• TAT can be as fast as 8hrs after growth of the organism in culture; in our lab we run in batches of ~96 isolates:
  • select colonies to be sequenced in am
  • PCRs in afternoon
  • electrophorese overnight
  • read/report next am
Advantages and Limitations of Sequencing for Identification of Mycobacteria

**Advantages**

- Allows for objective identification of a wide variety of mycobacteria
- Next day identification after growth in culture

**Limitations**

- Labor-intensive, requires skilled, trained (dedicated) technologists
- Equipment and reagent costs drive total test cost up
- Results are highly dependent upon the quality of your sequence library database
4. MALDI-TOF MS - a paradigm shift in Microbiology

• Matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry is changing the way we identify microbes

• Already becoming the main technique used in many laboratories for bacterial and yeast identification

• Mycobacteria and mold identification by MALDI-TOF MS is not far behind
Two examples of MALDI-TOF MS Instruments for Identification of Microorganisms

Bruker Biotyper

bioMérieux Vitek MS
Bruker Biotyper MALDI-TOF MS
MALDI-TOF MS

Theel ES, Clinical Microbiology Newsletter, 2013, 35:155-161
Laboratory Workflow for MALDI-TOF MS ID of *M. tuberculosis* complex after growth in culture

**BSL3 Activities**
- 10ul loop-ful of organism
- Beads+500µl 70% Ethanol
- Incubate room temp 10 min
- Bead Beat 2 minutes

**BSL2 Activities**
- Centrifuge 5 min
- Speed Vac 10 min
- Speed Vac 10 min
- Decant supernatant
- 70% Formic Acid & Acetonitrile

**MALDI-TOF**
- Spot 1ul sample + 2ul of Matrix
- 70% Formic Acid & Acetonitrile

Start to finish takes ~2 hrs for 24 samples
Advantages of MALDI-TOF MS for Mycobacteria Identification

- Similar work-flow regardless of organism (bacteria, yeast, mycobacteria, mold)
- Cost effective and “Green” – low consumable costs
- Rapid turn around time, high throughput
- Automated, robust, interlaboratory reproducibility
- Single colony requirement
- Small footprint
- Low exposure risk – sample inactivation
- Adaptable – can be an open system w/ databases expandable by user
Limitations of MALDI-TOF MS for Identification of Mycobacteria

- Need growth in culture
- Requires pure isolate
- Phase of growth, media, timing all factors
  - best performance, your spectral library needs to be composed of spectra produced under comparable conditions to your everyday working practices
- Databases need expansion for less common organisms
- Instrument maintenance downtime (if using a single instrument)
- Regulatory issues
- May not be a bit slower than sequencing for slowly growing mycobacteria
Mass Spectrometry Equipment Costs

- Purchase cost: ~$200,000
- Steel plates (10): ~$5,000
- Service contract (year): ~20,000
- Maintenance cost (year): ~$5,000

Remember – Mass spectrometry can also be used for identification of bacteria, mycobacteria, moulds on the same platform; next generation instruments will likely be linked with susceptibility platforms too.
New Workflow for Mycobacteria with MALDI-TOF MS

Culture to media; wait for growth

MALDI-TOF MS (same day ID)

if no ID

Sequencing (next day ID)
Direct Identification of *M. tuberculosis* complex *without waiting* for growth in culture
Nucleic Acid Amplification-based (NAA) tests

• CDC recommends:
  • NAA testing be performed on at least one (preferably the first) respiratory specimen from each patient with suspected pulmonary TB
    • if it would alter case management
    • If it would alter TB control activities
  • NAA testing does not replace the need for culture
1. *Mycobacterium tuberculosis* Direct Test (MTD) from Hologic Gen-Probe

- people frequently refer to this as the “TB probe” assay but that is not correct; this is a PCR-like amplification method
- transcription-mediated amplification of *M. tuberculosis* complex rRNA directly from respiratory specimens
- clinical specificity: 99-100%
- clinical sensitivity:
  - smear positive: 91-95%
  - smear negative: 83-100%
Limitations of MTD test

• technically “fussy” test
  • inhibition from specimen components a concern;
  • open PCR system so false positives due to contamination are possible.
• negative does not rule out \textit{M. tuberculosis} infection (still need to do a culture)
• detects presence of nucleic acid but doesn’t indicate if the organism is still viable
• cross-reactions occur w/ some rare mycobacteria: \textit{M. celatum}, \textit{M. terrae}-like organisms, \textit{M. holsiaticum}
• can be costly
2. Laboratory-developed PCR Tests (LDTs)

- closed PCR system – reduced opportunity for false-positives
- good sensitivity and specificity but it can vary since each test developed/verified independently
- often less expensive than MTD
- some can be used on a wider variety of specimen types included smear negative specimens and formalin-fixed, paraffin-embedded tissue blocks
Example of Real-time PCR Workflow in our Laboratory

Approximate turn-around time = 4h
Direct comparison of Mayo LDT PCR assay with the GenProbe MTD test

<table>
<thead>
<tr>
<th>Assay</th>
<th>MTD</th>
<th>Agreement (%)</th>
<th>kappa coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>LightCycler PCR</td>
<td>+</td>
<td>49</td>
<td>538/542 (99.3%)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3</td>
<td>489</td>
</tr>
<tr>
<td></td>
<td>-</td>
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</tbody>
</table>
3. Cepheid Xpert MTB/RIF Test

- WHO-endorsed
- Runs on the Cepheid GeneXpert system
- Recently FDA-approved for respiratory specimens
- Detects *M. tuberculosis* complex and provides information about RIF resistance

Source: www.finddiagnostics.org
Xpert accuracy for detection of *M. tuberculosis* complex

- Chang et al, 2012, J Infect 64:580-8:
  - Meta-analysis of 18 studies with 10,224 patients total
  - Pulmonary TB:
    - Sensitivity, Smear positive disease – 90.8%
    - Sensitivity, Smear negative disease – 74.3%
    - Specificity - 98.4%
  - Extrapulmonary TB:
    - Sensitivity - 80.4%
    - Specificity – 86.1%

- **Time to diagnosis comparison:**
  - Smear microscopy = 1 day (non-specific)
  - Broth culture took an average of 16 days
  - Solid media plate cultures took an average of 20 days
  - Xpert – same day diagnosis
Xpert MTB/RIF and Rifampin resistance

- \textit{rpoB}: gene encoding beta subunit of bacterial RNA polymerase

- Mutations in an 81bp region of the \textit{rpoB} gene are responsible for ~96% of RIF resistance in \textit{Mtb}; also predicts MDR TB since the majority of RIF-resistant isolates will also be INH-resistant

- Some false positive RIF resistance with Xpert
  - PPV is lower in low prevalence settings
  - CDC recommends reporting Xpert RIF-R as a preliminary result pending confirmation with sequencing; growth-base DST is still required
Strengths of Xpert MTB/RIF assay

• Good sensitivity and specificity for respiratory specimens
• Rapid 2hr TAT
• Detect MTB and RIF resistance
• Closed PCR system with low risk of cross-contamination
• GeneXpert platform is multi-functional and can be used for other tests (e.g., *C. difficile*, HIV viral load)
• Simple for operators to perform
• No advanced biosafety equipment needed
Weaknesses of Xpert MTB/RIF assay

• Xpert has better sensitivity than smear with respiratory specimens but a culture is still necessary
• False-positive RIF resistance is possible; need to confirm RIF-resistance with sequencing
• Not as sensitive or specific for extrapulmonary specimens
• Expensive – need to purchase GeneXpert platform; cartridges are $65 each in E.U. and U.S.; $10 discounted price for high burden and developing countries
• Need continuous electrical power and air conditioning (challenge in developing countries)
• Sample storage limited to 3 days at RT, 7 days at refrigerated temps
• Can’t differentiate between live and dead *M. tuberculosis* (can’t use to monitor treatment)
4. Line Probe Assays for Mycobacteria (Hain Lifesciences or Innogenetics)

**M. tuberculosis** complex speciation

Not approved for diagnostic use in the U.S.

Source: [http://www.hain-lifescience.de](http://www.hain-lifescience.de)
Interferon Gamma (IFN-γ) Release Assays for the Diagnosis of Latent TB Infection
Quantiferon-TB Gold In-Tube (GIT)

• FDA approved in 2005

• Based on the release of IFN-γ in response to in vitro stimulation of T cells to:
  • ESAT6, CFP-10, and TB 7.7

• Uses ELISA technology for detection of released IFN-γ

• A positive result can support the diagnosis of active or latent TB, but other medical and diagnostic tests should be used to confirm or rule out disease
Quantiferon-TB GIT: Strengths

• Rapid TAT: 24 hours
• Objective interpretation
• Not boosted by BCG and NTM
• Serial testing capability
• High Specificity: Not affected by most NTM or BCG
Quantiferon-TB GIT: Weaknesses

• Not well studied in immunocompromised hosts, pregnant women or children

• Pre-analytical processing steps must be strictly adhered to

• Cross reactivity is possible between NTM
  • *M. marinum*, *M. kansasii*, *M. szulgi*, *M. flavescens*

• How do you handle low positives in low risk patients in low prevalence areas?
T-spot TB Test Overview

- FDA approved in 2008
- Uses ELISpot technology
- PBMCs added to wells coated with antibodies to IFN-γ and stimulate with TB specific antigens
- Detection of IFN-γ using conjugated secondary antibodies and substrate.

Source: http://oxfordimmunotec.com/
Drug Susceptibility Testing of *M. tuberculosis* complex
Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard – Second Edition

Clinical and Laboratory Standards Institute (CLSI)

CLSI Document M24-A2, published 2011
Provides guidance for resistance testing of:

- *M. tuberculosis* complex
- *M. avium* complex (clarithromycin)
- other slowly growing mycobacteria (limited guidelines)
- rapidly growing mycobacteria
- *Nocardia* spp. and other aerobic actinomycetes
M. tuberculosis complex DST

- each drug is tested at a “critical concentration”
  - lowest concentration of anti-tuberculous drug that inhibits 95% of “wild strains” that have never been exposed to drugs but does not inhibit strains isolated from patients who fail to respond to therapy and are considered resistant.
  - exact drug concentration tested depends on medium/platform used
M. tuberculosis complex DST

- Agar proportion is the current gold standard for all drugs except pyrazinamide
  - not rapid (14-21 days)
  - labor-intensive, technically complex
  - no FDA-cleared, commercially-available kit
- Broth method is recommended for rapid TAT
  - CDC goal is results for first-line drugs reported within 15-30 days after receipt of the specimen
Drug susceptibility testing on solid medium
Indirect proportion method

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.
Semi-automated Mycobacterial Susceptibility Testing in Liquid Culture

Compare growth rates in bottles/tubes +/- critical concentrations of drug
**M. tuberculosis complex resistant isolates**

- If the isolate is resistant to any agent
  - preliminary report issued
  - consider confirming resistance by 2\textsuperscript{nd} method or 2\textsuperscript{nd} lab
  - consider initiating testing of secondary agents to avoid delays

- If the isolate is resistant to only PZA consider
  - speciation
    - *M. bovis* is mono-PZA-resistant
    - most isolates of *M. tuberculosis* are PZA-susceptible
New Method for Mtb DST – MIC Plate

- broth microdilution method
- multi-center studies supporting FDA-submission completed
- rapid (14 days)
- contains INH, RIF, EMB and 9 second-line drugs
- test 1\textsuperscript{st} and 2\textsuperscript{nd} line drugs simultaneously with same inoculum
- provides MIC endpoint – helpful for isolates with MIC near CC breakpoint that give fluctuating results w/CC method

Molecular detection of *Mtb* drug resistance markers
Direct Detection of INH resistance using real-time PCR

![Graph showing the relationship between temperature and fluorescence](image)

- **Fluorescence**
  - d(F2/F1)/dT

- **Temperature (°C)**

  - S315T
  - WT
Pyrazinamide resistance – Sequencing of \textit{pncA}

- Broth susceptibility testing of PZA can overcall 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 PS et al., manuscript in preparation

- Sequencing of the \textit{pncA} gene 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
RIF resistance using Cepheid GeneXpert MTB/RIF

- amplifies an 81bp region of the \textit{rpoB} gene
- contains 96% of known mutations conferring rifampin resistance
- also predicts MDR TB since most isolates resistant to rifampin are also isoniazid resistant

http://www.cepheid.com
Molecular Detection of Drug Resistance at the CDC

- offered for *M. tuberculosis* complex isolates and nucleic-acid amplification-positive (NAAT+) sputum sediments
- perform pyrosequencing and conventional sequencing
- provides rapid identification of mutations associated with resistance to many TB drugs
- limitations include
  - insufficient data to definitively associate all mutations detected with resistance;
  - not all mechanisms of resistance are known
  - not all resistance loci are sequenced
- use in conjunction with conventional DST results
Molecular resistance testing at the CDC

<table>
<thead>
<tr>
<th>Drug</th>
<th>Locus/Loci examined</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>rifampin</td>
<td>rpoB</td>
<td>97.1</td>
<td>97.4</td>
</tr>
<tr>
<td>isoniazid</td>
<td>inhA &amp; katG</td>
<td>86.0</td>
<td>99.1</td>
</tr>
<tr>
<td>fluoroquinolones</td>
<td>gyrA</td>
<td>79.0</td>
<td>99.6</td>
</tr>
<tr>
<td>kanamycin</td>
<td>rrs &amp; eis</td>
<td>86.7</td>
<td>99.6</td>
</tr>
<tr>
<td>amikacin</td>
<td>rrs</td>
<td>90.0</td>
<td>98.4</td>
</tr>
<tr>
<td>capreomycin</td>
<td>rrs &amp; tlyA</td>
<td>55.2</td>
<td>91.0</td>
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<tr>
<td>ethambutol</td>
<td>embB</td>
<td>78.8</td>
<td>94.3</td>
</tr>
<tr>
<td>pyrazinamide</td>
<td>pncA</td>
<td>86.0</td>
<td>95.9</td>
</tr>
</tbody>
</table>

Summary

• AFB stains are rapid but insensitive and nonspecific
• Culture should always be ordered together with AFB stain
• Identification after growth in culture is rapid using molecular methods
• Direct identification using molecular methods most often uses smear-positive respiratory specimens; certain methods allow for other specimens
• Detection of drug resistance markers is available for culture isolates and directly for smear-positive respiratory specimens
Questions & Discussion