Disclosure

• Trek Diagnostics
Objectives

• Describe laboratory methods used for the detection, identification, genotyping, and drug susceptibility testing of *M. tuberculosis*
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 make 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](http://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), <em>Int J Tuberc Lung Dis</em>, 4:246.</td>
<td>77.1%</td>
</tr>
<tr>
<td>Ipuge et al. (1996), <em>Trans R Soc Trop Med Hyg</em>, 90:258.</td>
<td>83.4%</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>
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 *M. tuberculosis*
MGIT - Mycobacterial Growth Indicator Tubes (Becton Dickinson)

- fluorescent indicator in bottom of tube quenched by O₂
- ↑ mycobacterial growth = ↓ O₂ and ↑ 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

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

N = 806

Source: www.health.state.mn.us/tb
Identification of \textit{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

PCR Product clean-up (removes dNTPs and primers)

lyse/heat

target amplification

cycle sequencing reaction

clean-up (removes excess dye terminators and primers)

sequence analysis

electrophoresis

...CTC CTC CCT GGT GGA AGC ATA...
...Leu Leu Pro Gly Gly Ser Ile...
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 \textit{M. tuberculosis} complex after growth in culture

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

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 → 70% Formic Acid & Acetonitrile

MALDI-TOF

start to finish takes ~2 hrs for 24 samples

Spot 1ul sample + 2ul of Matrix

MALDI-TOF
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 use 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 (preferrably 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 *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: *M. celatum, M. terrae*-like organisms, *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></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>49</td>
<td>1</td>
<td>538/542 (99.3%)</td>
</tr>
<tr>
<td>-</td>
<td>3</td>
<td>489</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Agreement (%) is calculated as the proportion of samples that agree between the two methods. The kappa coefficient is a measure of the agreement between the two methods, adjusted for chance agreement.
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

- $rpoB$: gene encoding beta subunit of bacterial RNA polymerase
- Mutations in an 81bp region of the $rpoB$ gene are responsible for ~96% of RIF resistance in $Mtb$; 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
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 2nd method or 2nd 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 1st and 2nd 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 \textit{Mtb} drug resistance markers
Direct Detection of INH resistance using real-time PCR

Fluorescence $-\frac{d(F2/F1)}{dT}$

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 $rpoB$ 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>
</tr>
<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