Mycobacteriology Laboratory

Max Salfinger, MD
Mycobacteriology & Pharmacokinetics Laboratory
salfingerm@njhealth.org

October 19, 2017
Introduction
Tool Box 1 & 2
Growth Detection
Identification of NTM
Antimicrobial Susceptibility Testing (AST)
Therapeutic Drug Monitoring
Strain Characterization
• National Cystic Fibrosis Foundation NTM Biorepository
• Laboratory open 7 days per week
• Specimens and cultures from all 50 states and abroad
• Specialized testing for TB and NTM

Pulmonary NTM

TB

*Updated as of July 1, 2010.*
Four integrated health care delivery systems*, 1991-2007

- **M. avium complex** 1,495 (80.1%)
- **M. chelonae/abscessus** 225 (12.1%)
- **M. fortuitum** 106 (5.6%)
- **M. kansasii** 102 (5.5%)
- **M. simiae** 53 (2.8%)
- **M. xenopi** 33 (1.7%)

*KP Southern California, KP Southern Colorado, Group Health, Geisinger
> 8,800 isolates were analyzed using *rpoB* gene sequencing

Seven *Mycobacterium* species and subspecies accounted for ~80% of all isolates tested

- **24.4%**  *M. abscessus*, incl. all 3 subspecies
- **19.9%**  *M. avium*
- **16.4%**  *M. intracellulare*
- **6.0%**   *M. chimaera*
- **5.1%**   *M. fortuitum*
- **3.8%**   *M. gordonae*
- **3.7%**   *M. chelonae*
NTM in CF – Age Related

M. scrofulaceum replaced by M. avium

Shift took place between 1975 and 1985

Municipal water treated now with chloramine instead of chlorine; Legionella pneumophila disappeared, NTM increased…
Topics

- Introduction
- Tool Box 1 & 2
- Growth Detection
- Identification of NTM
- Antimicrobial Susceptibility Testing (AST)
- Therapeutic Drug Monitoring
- Strain Characterization
✓ Specimen – sputum, bronchoscopy, formalin-fixed tissue

- NALC-NaOH versus Oxalic acid (CF w/history of *Pseudomonas aeruginosa*)
- AFB microscopy
- Solid (LJ, Middlebrook bi-plate, NTM plate) & broth-based media
- NAAT-D (TB complex, NTM –mostly MAC)
- NAAT-R (RIF, INH and more)
- Direct AST

Ideally, molecular TB Testing 7 Days a Week
✓ AFB positive culture (broth-, solid-based media)
   – NAAT-D (TB complex)
   – NAAT-R (RIF, INH and more; NTM- macrolide & aminoglycoside)
   – Identification (Sequencing-\textit{rpoB} or 16S; MALDI-TOF; Nucleic acid probe kits; Line Probes)
   – High Performance Liquid Chromatography (HPLC); PCR Restriction Analysis (PRA); Biochemicals
   – Minimal Inhibitory Concentration (MIC) (rapidly and slowly growing NTM)
   – Combination MIC
Introduction
Tool Box 1 & 2
Growth Detection
Identification of NTM
Antimicrobial Susceptibility Testing (AST)
Therapeutic Drug Monitoring
Strain Characterization
Demanding Instant Results!

20 Min

20 Hours
• Procedures kill all but 10-20% of the mycobacteria
• Contamination
  2-5% of sputum specimens on Loewenstein-Jensen medium (LJ)
NTM are an increasing problem in patients with cystic fibrosis (CF), in large part because recovery of these organisms is hampered by the presence of *Pseudomonas aeruginosa* in the respiratory tract of these patients, which rapidly overgrows mycobacteria in culture.

**Specific decontamination methods are used**

- oxalic acid method
- two-step NALC-NaOH-oxalic method. However, this method may affect the mycobacteria viability, and its effect on the recovery of *M. abscessus* in pediatric CF patients is unknown.

The ability of a **chlorhexidine decontamination method** vs. the NALC-NAOH-oxalic acid method to recover NTM from patients with cystic fibrosis has been compared. - The chlorhexidine method recovered twice as many NTM, despite a higher contamination rate. (Ferroni et al. J Clin Microbiol. 2006;44:2237-2239).
• **M. leprae** – in armadillo for research; NOT in clinical laboratories

• **Suspicion for M. ulcerans, M. marinum, M. haemophilum:** additional media incubated at 30° to 32° Celsius

• **Suspicion for fastidious organisms** (M. haemophilum, M. genavense, M. paratuberculosis) require supplements – hemin, mycobactin, etc.
Introduction
Tool Box 1 & 2
Growth Detection
Identification of NTM
Antimicrobial Susceptibility Testing (AST)
Therapeutic Drug Monitoring
Strain Characterization
M. abscessus (Rough)
M. abscessus [rough + smooth] & M. avium [translucent]
Source: [http://www.bacterio.net/mycobacterium.html](http://www.bacterio.net/mycobacterium.html)

Number of species cited in this file: **186**
Number of subspecies cited in this file: **13**
(As of September 24, 2017)
• Nucleic acid probe kits
• High Performance Liquid Chromatography (HPLC) – cell wall
• PCR Restriction Analysis (PRA)
• Line Probes
• DNA sequencing
• MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry – protein
DNA probe hybridization results
All the *M. chimaera* strains presented identical reactivity with the DNA probes tested. In the AccuProbe system, they hybridized with probes for MAC and *M. intracellulare* but not with the probe for *M. avium*.

False-Positive Results for *M. celatum* with the AccuProbe *M. tuberculosis* Complex Assay

*M. celatum* type 1 was found to cross-react in the AccuProbe *M. tuberculosis* complex assay. Subsequently, we found a statistically significant increase in the relative light units with lower temperatures, suggesting that it is necessary to perform this AccuProbe assay at between 60 and 61°C.
Laboratory Aspects of "Mycobacterium genavense," a Proposed Species Isolated from AIDS Patients

The mycolic acid pattern of patients' isolates closely resembled that of the type strain of *M. simiae* when analyzed by one- and two-dimensional thin-layer chromatography and by high-performance liquid chromatography. Whole-cell fatty acid analyses by gas-liquid chromatography distinguished the isolates from *M. simiae* but misidentified them as *M. fortuitum*. Sequence determinations of the hypervariable regions of the 16S rRNA gene indicate that these organisms belong to the recently proposed new species "*M. genavense.*"
Evaluation of the GenoType Mycobacterium Assay for Identification of Mycobacterial Species from Cultures

The DNA strip assay was evaluated for the ability to differentiate mycobacterial species. The test is based on a PCR technique targeting a 23S rRNA gene region, followed by reverse hybridization and line probe technology. Concordant results were obtained for 137 (92.6%) of 148 mycobacterial strains with the CM assay and 133 (89.9%) of 148 mycobacterial strains with the AS assay.

GenoType NTM-DR for Identifying *Mycobacterium abscessus* Subspecies and Determining Molecular Resistance

Performance of a new line probe assay for identifying the subspecies and determining the macrolide and aminoglycoside resistance levels of 50 *Mycobacterium abscessus* isolates. Agreement of GenoType NTM-DR results with sequencing and phenotypic resistance results was 92% for subspecies identification and 98% for determining molecular and phenotypic resistance.

Currently we use *rpoB* and 16S sequencing technology for molecular identification

– **Pros**
  - Sensitive
  - Accurate
  - Specific
  - Gold Standard

– **Cons**
  - Costly
  - Labor intensive, takes a few days for ID results
Treatment response rates to combination antibiotic therapy including clarithromycin were much higher in patients with *M. massiliense* lung disease than those with *M. abscessus* lung disease.

Koh et al Am J Respir Crit Care Med 183:405-410 (2011)

*M. avium* – *M. intracellulare* – *M. chimaera* and **Not** *M. avium* complex
**Erythromycin Methylase Gene (41)**

- Macrolide antibiotics activate the *erm*(41) gene
- Results in inducible (delayed) resistance to clarithromycin and/or azithromycin
- **Mutations** or deletions inactivate this gene resulting in **macrolide susceptibility**
- Presence of wildtype or a mutated sequence differs within the 3 subspecies
M. abscessus/M. bolletii isolates 491 bp *erm*(41) gene

M. *massiliense* isolates 218 bp *erm*(41) gene with deleted region

*M. massiliense* is positive for the *erm*(41) gene but contains a 273-bp deletion within the gene rendering the gene nonfunctional.
<table>
<thead>
<tr>
<th>Band No. 1 (CC): Conjugate Control</th>
<th>Band No. 2 (UC): Universal Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugate Control (CC)</td>
<td>Universal Control (UC)</td>
</tr>
<tr>
<td>SP1</td>
<td>SP1</td>
</tr>
<tr>
<td>SP2</td>
<td>SP2</td>
</tr>
<tr>
<td>SP3</td>
<td>SP3</td>
</tr>
<tr>
<td>SP4</td>
<td>SP4</td>
</tr>
<tr>
<td>SP5</td>
<td>SP5</td>
</tr>
<tr>
<td>SP6</td>
<td>SP6</td>
</tr>
<tr>
<td>SP7</td>
<td>SP7</td>
</tr>
<tr>
<td>SP8</td>
<td>SP8</td>
</tr>
<tr>
<td>SP9</td>
<td>SP9</td>
</tr>
<tr>
<td>SP10</td>
<td>SP10</td>
</tr>
<tr>
<td><strong>erm(41)</strong> Locus Control (erm(41))</td>
<td><strong>erm(41)</strong> C28</td>
</tr>
<tr>
<td>rrl Locus Control (rrl)</td>
<td>rrl wild type probe (rrl WT)</td>
</tr>
<tr>
<td>rrl mutation probe 1 (rrl MUT1)</td>
<td>rrl mutation probe 2 (rrl MUT2)</td>
</tr>
<tr>
<td>rrl mutation probe 3 (rrl MUT3)</td>
<td>rrl mutation probe 4 (rrl MUT4)</td>
</tr>
<tr>
<td>rrs Locus Control (rrs)</td>
<td>rrs wild type probe (rrs WT)</td>
</tr>
<tr>
<td>rrs mutation probe 1 (rrs MUT1)</td>
<td></td>
</tr>
</tbody>
</table>

**Legend:**
- **staining**
- **facultative staining**
- **no staining**
4,5,6 & 9,10 = abs ssp abs
MA-resistant
• Intrinsic Contamination of Heater-Cooler Devices Used in Cardiac Surgery with *Mycobacterium chimaera* – United States

• Environmental samples vs. clinical samples

• Single nucleotide polymorphisms (SNPs) were called and used to infer a maximum likelihood tree

• **Results** from pairwise comparisons among all sequences across a core genome of approximately 4Mb revealed a maximum of **3 SNPs** between any two isolates related to the outbreak investigation, versus approximately **80-300 SNPs** between the outbreak isolates and the epidemiologically unlinked isolates

• Notes from the Field: *Mycobacterium chimaera* Contamination of Heater-Cooler Devices Used in Cardiac Surgery — United States Morbidity Mortality Weekly Report. October 14, 2016. 65(40);1117–1118
MALDI-TOF MS (Matrix-Assisted Laser Desorption ionization - time of flight mass spectrometry)

MALDI-TOF MS offers a rapid, protein-profiling based technique for identification of mycobacterial isolates from liquid or solid culture media, with high analytical capabilities at a less expensive cost compared to \( rpoB \) gene sequence analyses.
MALDI-TOF MS can reliably and rapidly identify

- approximately 88% of *Mycobacterium* species, 90% of *Nocardia* species, and 51% of other aerobic actinomycetes encountered in routine clinical practice at a tertiary medical center/reference laboratory.
  - Using a custom, enhanced library and a streamlined extraction procedure
- Described the ability of the manufacturer’s library to identify these groups of organisms and described the effects of lowering the accepted cutoff score from 2.0 to 1.7
  - As the manufacturer continues to expand its database, many laboratories will have the ability to identify many of the isolates they routinely encounter using MALDI-TOF MS.
  - An expanded custom library may ultimately be the most useful tool for identification of the uncommon species encountered most often in a reference laboratory setting.

*Buckwalter et al. J Clin Microbiol. 2016 Feb;54(2):376-84*
Topics

- Introduction
- Tool Box 1 & 2
- Growth Detection
- Identification of NTM
- Antimicrobial Susceptibility Testing (AST)
- Therapeutic Drug Monitoring
- Strain Characterization
Mycobacterium tuberculosis complex
Mycobacterium avium complex
Mycobacterium kansasii
Mycobacterium marinum
Miscellaneous slowly growing NTM
Rapidly growing mycobacteria (RGM)

CLSI M24-A2 (2011) Susceptibility testing of mycobacteria, nocardia, and other aerobic actinomycetes – Approved standard, second edition (in revision)
Rapidly growing NTM
- NTM4: 15-Drug MIC, including Clofazimine/Amikacin Combo
- NTM6: 20-Drug MIC, including Clofazimine/Amikacin Combo

Slowly growing NTM
- NTM10: 10-Drug MIC, incl. RIF-EMB Combo
- NTM9: RIF-EMB Combo, including RIF and EMB single drug MIC
<table>
<thead>
<tr>
<th>N=2,318</th>
<th>%</th>
<th>EFFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,642</td>
<td>71</td>
<td>SYNERGISTIC</td>
</tr>
<tr>
<td>584</td>
<td>25</td>
<td>ADDITIVE</td>
</tr>
<tr>
<td>91</td>
<td>4</td>
<td>NO EFFECT</td>
</tr>
</tbody>
</table>

National Jewish Health – unpublished data
Topics

- Introduction
- Tool Box 1 & 2
- Growth Detection
- Identification of NTM
- Antimicrobial Susceptibility Testing (AST)
- Therapeutic Drug Monitoring
- Strain Characterization
Heysell et al. *Therapeutic Drug Monitoring for Slow Response to Tuberculosis Treatment in a State Control Program, Virginia, USA*
Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 16, No. 10, October 2010
Topics

- Introduction
- Tool Box 1 & 2
- Growth Detection
- Identification of NTM
- Antimicrobial Susceptibility Testing (AST)
- Therapeutic Drug Monitoring
- Strain Characterization
identification of genotypes which can be used to predict drug-resistant phenotype

the determination of genetic relatedness which may point to a single source
• Intrinsic Contamination of Heater-Cooler Devices Used in Cardiac Surgery with *Mycobacterium chimaera* – United States

• Environmental samples vs. clinical samples

• Single nucleotide polymorphisms (SNPs) were called and used to infer a maximum likelihood tree

• **Results** from pairwise comparisons among all sequences across a core genome of approximately 4Mb revealed a maximum of 3 SNPs between any two isolates related to the outbreak investigation, versus approximately 80-300 SNPs between the outbreak isolates and the epidemiologically unlinked isolates

• Stay tuned for a future Morbidity Mortality Weekly Report
<table>
<thead>
<tr>
<th>State</th>
<th>Reporting Time for NTM</th>
<th>What is Required to be Reported (websites accessed on 12-11-2016)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maryland</td>
<td>within one working day</td>
<td><em>Mycobacterium</em> spp., other than <em>Mycobacterium tuberculosis</em> complex or <em>Mycobacterium leprae</em></td>
</tr>
<tr>
<td>Mississippi</td>
<td>one week</td>
<td>Nontuberculous mycobacterial disease</td>
</tr>
<tr>
<td>Missouri</td>
<td>within 3 days</td>
<td>Nontuberculosis mycobacteria (NTM)</td>
</tr>
<tr>
<td>Nebraska</td>
<td>within 7 days</td>
<td><em>Mycobacteria</em> spp. (including <em>M. tuberculosis</em> complex organisms [for genotyping] and all “atypical” species, to include culture, nucleic acid histological evidence)</td>
</tr>
<tr>
<td>Nevada</td>
<td>not specified</td>
<td>Submission of isolates of <em>Mycobacterium</em> spp.</td>
</tr>
<tr>
<td>New Jersey</td>
<td>within 72 hours</td>
<td><em>Mycobacterium</em>, atypical</td>
</tr>
<tr>
<td>New Mexico</td>
<td>within 24 hours</td>
<td>Tuberculosis or other nontuberculous mycobacterial infections (including <em>Mycobacterium avium</em> complex or leprosy)</td>
</tr>
<tr>
<td>Oregon</td>
<td>one working day</td>
<td>Nontuberculous mycobacterial infection (nonrespiratory)</td>
</tr>
<tr>
<td>Tennessee</td>
<td>one week</td>
<td>Nontuberculosis mycobacteria (extrapulmonary only)</td>
</tr>
<tr>
<td>Virginia</td>
<td>immediate</td>
<td>Results of cultures positive for any member of the <em>Mycobacterium tuberculosis</em> complex (i.e., <em>M. tuberculosis</em>, <em>M. bovis</em>, <em>M. africanum</em>) or Results of rapid methodologies, including acid amplification, which are indicative of <em>M. tuberculosis</em> complex or any other mycobacteria.</td>
</tr>
<tr>
<td>Wisconsin</td>
<td>within 72 hours</td>
<td>Mycobacterial disease (nontuberculosis)</td>
</tr>
</tbody>
</table>

**2017: Ohio-NTM no longer notifiable; Tennessee-added extrapulmonary NTM**
• Bi-monthly newsletter
• Please feel free to sign up – it is free 😊

• https://www.nationaljewish.org/for-professionals/newsletters/ntm-tb-insights-newsletter/ntm-tb-insights-sign-up

• Or
Additional Information

- Quality Specimen
- Contamination Rate
- Media
Thank-you!
Quality testing requires a quality specimen.
Sputum, expectorated or induced:
Collection: Instruct patients on the proper method of sputum collection
- the material brought up from the lungs after a productive cough what is desired, and not nasopharyngeal discharge and saliva
- 5 - 10 mL sputum collected in a sterile container.
- Difficulty in producing sputum
  - sputum induction by inhalation of an aerosol of sterile hypertonic saline (3%) or sterile water produced by a nebulizer that causes coughing. Label as INDUCED
- Perform in areas with adequate environmental controls under supervision.
- 3 consecutive specimens in 8- to 24-hour intervals, with at least one being an early morning specimen.
- Sputum specimens should not be pooled.
CSF:
- Collection: At least 5 mL of CSF should be aseptically collected.
- Minimum volume required: 2 to 3 mL; optimal volume is 10 mL.
- A separate sample should be collected for chemistry and hematology.

Gastric Lavage:
- Collection: Specimens should be collected in early morning before patients eat and while they are still in bed. The lavage should be performed with 25 to 50 mL of chilled, sterile, distilled water. Recovered sample should be placed in a leak-proof, sterile container (e.g., 50-mL conical tube).
- Transport: Gastric wash or lavage material should be submitted in a sterile leak-proof container, such as a sterile 50-mL conical tube or sterile urine collection container.
- Transport time and temperature: Specimens should be transported at room temperature as soon as possible.
  - If transport is delayed for more than one four hour, specimens should be neutralized with 100 mg sodium carbonate within one hour of collection, and transported as soon as possible at room temperature.
Abscess:
• Tissue (at least 1 g, if possible) or fluid is preferred. Tissue should not be frozen or preserved.
• A swab is strongly discouraged unless it is the only specimen available. Swabs should be submitted in 2 to 3 mL sterile saline. Swabs submitted in transport medium or a commercial swab transport device are unacceptable.

Blood:
• Collection: Manufacturer’s instructions for automated blood culture systems should be followed.
• Alternatively, 10 mL whole blood should be collected aseptically in a 0-mL yellow-top collector tube containing SPS, or green-top collector tube containing heparin.
• Blood must not be collected in a red-top tube, EDTA (purple top), or ACD (yellow top).
• Minimum volume is 5 mL for adults; 1 mL for children.
Specimens that may be contaminated with normal flora microorganisms, such as sputum, need to be digested and decontaminated before inoculating to culture media.

- This step helps prevent overgrowth of more rapidly growing microbes, and also digests the mucin that may bind any mycobacteria present and inhibit their recovery.
- All decontamination reagents are toxic to microorganisms. However, the goal is to inhibit the normal flora but not the hardier mycobacteria.
- The laboratory should monitor the overall specimen contamination rate.
  - The goal is not to reduce this rate to zero since that would indicate too many mycobacteria are being lost in the decontamination process.
  - Instead, it is expected that under normal circumstances, between 2% and 5% of specimens will be overgrown by normal flora for solid media and 7 to 8% for liquid media containing antimicrobials.
1980: semi-automated liquid medium for routinely detecting mycobacteria was introduced

- Liquid medium supports growth of MTBC and the majority of other mycobacteria significantly better than solid media, with an increase in the number of positive cultures.
- The time to detection of positive cultures is shorter than solid media with supplements.
- Some of the very fastidious mycobacteria, such as *M. genavense*, may only grow in liquid media.
• **Lowenstein-Jensen (LJ)** is one of the oldest and most commonly used egg-based media.
  – **Gruft** medium contains penicillin and nalidixic acid to further reduce contamination.
  – **Ogawa** is also commonly used in limited-resource countries, as it replaces asparagine with the more economical sodium glutamate.
  – Plain LJ is a selective medium, as it contains malachite green, which inhibits growth of contaminating bacteria and fungi. In limited-resource countries, this may be the only medium used, while in the majority of resource-rich countries, LJ medium may be used in conjunction with liquid media.
  – Although CO2 is not essential for growth on egg-based media, it stimulates earlier and more luxuriant growth.

• Although LJ medium may be commonly used both for detection and antimicrobial susceptibility testing, the counting of colonies is difficult due to the smaller surface of the slant.

• Egg-based media support growth of the MTBC and many other mycobacteria, but in general it has a lower yield of NTM.

• Most NTM grow slowly on LJ medium, compared to liquid media.
Middlebrook 7H10 agar and Middlebrook 7H11 agar are two types of agar media commonly used for detection of mycobacteria.

- Middlebrook 7H11 contains casein hydrolysate, which is known to help in the growth of fastidious and isoniazid-resistant MTB.
- Once prepared, the medium is stored refrigerated and the shelf life is usually four to six weeks.
- Middlebrook agar medium base, once autoclaved, should not be reheated. The prepared medium should not be exposed to direct sunlight.

Advantages of plated agar-based media:

- They are clear and thus, it is easier to observe colony morphology and enumerate colonies.
- If contaminated, the contaminating colonies sometimes are isolated and mycobacterial colonies can still be recovered.
- For isolation of mycobacteria from clinical specimens, antibacterial and antifungal antimicrobials may also be added to further inhibit contaminating bacteria and make it more selective.
- The average time to detection of growth is generally earlier than with the egg-based media, with a recommended incubation period of 6 weeks for MTB and 8 weeks for some slowly growing NTM, such as *M. haemophilum*. 