Identification of Mycobacteria
Identification of Mycobacteria

• Accurate and prompt identification is important for patient management and public health response

• Culture results are used for
  – Diagnosis of clinically significant disease
  – Initiating or altering antimicrobial therapy
  – Respiratory isolation decisions
  – Initiating contact investigations
Identification of Mycobacteria

BACKGROUND
National TB Laboratory Services Survey

- APHL and CDC developed and launched the National TB Laboratory Services Survey in 2010
- Purpose—to assess the overall capability and capacity of laboratories in the United States providing TB services
- 580 of the 656 respondents perform some level of TB testing
580 (100%) labs perform AFB smear microscopy

474 (81.7%) perform culture

215 (37.1%) perform MTBC identification

94 (16.2%) perform first-line DST

- 93% of Public Health Laboratories
- 26% of Clinical Laboratories
National TB Laboratory Services Survey (3)

Primary Method for Identification of MTBC from Culture (n=213)

- GenProbe Accuprobe: 83.1%
- HPLC: 1.9%
- Other Molecular Method: 9.9%
- Biochemicals: 4.2%
- Line Probe Assay: 0.5%
- Other: 0.5%

APHL. National TB Laboratory Services Survey Report. 2012
Biosafety Recommendations for Manipulations of Mycobacterial Cultures

• All procedures for tuberculosis (TB) culture propagation and subsequent manipulation of the cultures are performed in BSL-3 facilities

• The use of containment equipment (e.g., biosafety cabinet, centrifuge safety cups), practices to minimize aerosol production, and respiratory protection are essential
Identification Methods

• Growth Characteristics
  – Growth rate
  – Optimal temperature
  – Colony morphology
  – Pigment production

• Conventional biochemical reactions

• High Performance Liquid Chromatography (HPLC)

• GenProbe® Accuprobe ®

• Line Probe assays

• MALDI-TOF

• DNA Sequencing
Identification of Mycobacteria

CLASSICAL METHODS
Growth Characteristics

- Growth Rate
  - Can be observed on the primary solid media, but dependent on appropriate incubation temperature and number of organisms in the primary specimen
  - To perform a standardized growth test from subculture
    - Inoculate a defined suspension of mycobacteria on solid media
    - Incubate at 30°C and 35–37°C
    - Observe for growth at 5–7 days and weekly thereafter
  - Rapidly Growing Mycobacteria: form visible colonies within 7 days of incubation (usually 3–4 days)
  - Slowly Growing Mycobacteria: require more than 7 days for visible colonies to form
Growth Characteristics (2)

- Colony Morphology
  
  Observe on solid media, use stereoscope to visualize young and small colonies
  
  - Colony texture and consistency
    
    • Rough, smooth, butyrous (butter-like), dry, granular, mucoid, translucent
  
  - Colony pigment
    
    • Color range: Non-pigmented, buff, yellow, orange, pink
      
      – Photochromogen: require light to form pigment
      – Scotochromogen: form pigment in either light or dark
      – Non-photochromogens: no pigment
Growth Characteristics (3)

*M. tuberculosis* complex

*M. gordonae*

Conventional Biochemical Testing

• Classical approach to identification
• Requires sufficient amount of bacterial cells and several weeks of incubation
• New mycobacterial species cannot be reliably identified by biochemical and other phenotypic tests
• Current recommendations are for rapid methods
High Performance Liquid Chromatography (HPLC)

• Cell wall mycolic acids are extracted and derivatized to fluorescent or UV-adsorbing esters and then separated by chromatography.

• A pattern of peaks (chromatogram) is generated as mycolic acids are detected.

• Identification is based on comparison of isolate’s pattern to a database or library of chromatograms.
Considerations for HPLC

• Advantages
  – Some methods can identify *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM) from broth culture and directly from clinical specimens
  – Cost of individual sample testing is relatively inexpensive
  – FDA-cleared system commercially available

• Limitations
  – Initial equipment costs are high
  – Some methods require mature solid medium growth
  – Problematic for identification of rapidly-growing mycobacteria; limited ability to resolve some NTM groups/complexes
  – Extraction uses hazardous chemicals; hazardous waste production
Fluorescence-HPLC Patterns

$\text{M. tuberculosis}$

$\text{M. kansasii}$

$\text{M. avium cx.}$
Identification of Mycobacteria

MOLECULAR AND EMERGING METHODS
GenProbe Accuprobe

- In-solution hybridization assay for identification of growth on solid or liquid media
  - Nucleic acids are extracted after organisms are lysed and made non-viable
  - Single-stranded labeled DNA probes (in tubes) are allowed to anneal to target RNA
  - If present, RNA:DNA hybrids are detected by chemiluminescence
- Commercially available kits for identification of MTBC, *M. avium* complex, *M. gordonae*, *M. kansasii*
Considerations for Accuprobe

• Advantages
  – Identifies four frequently-isolated mycobacteria; three clinically significant
  – Performed routinely by many laboratories
  – Relatively easy to use
  – FDA-cleared

• Limitations
  – No nucleic acid amplification occurs during this assay; sufficient culture growth is necessary for identification
  – Beware of relative light units (RLU) values that are near the cutoff; “high negative” values could indicate that the target organism is present in low numbers
Line Probe Assay

• Platform is nitrocellulose strip used for identification of MTBC and commonly-isolated NTM by reverse hybridization
  – Hybridization of denatured DNA to probes on the membrane strip

• Commercially developed assays
  – HAIN GenoType Mycobacterium CM (MTBC + 24 NTM) and GenoType Mycobacterium AS (19 NTM)
  – Innogenetics INNO-LiPA Mycobacteria v2 (MTBC + 7 NTM)
Sample preparation

Hybridization and visualization

Considerations for Line Probe Assay

• Advantages
  – Nucleic acid amplification for increase sensitivity
  – Some assays detect mutations associated with MTBC drug resistance
  – Relatively low implementation costs

• Limitations
  – Not FDA approved
  – Can be difficult to differentiate bands with visual inspection
  – Sometimes difficult to identify species within *Mycobacterium fortuitum* complex, *M. chelonae/abscessus* group
MALDI-TOF

- “Matrix-Assisted Laser Desorption Ionization Time-of-Flight”
- A form of mass spectrometry
- Used to analyze proteins and other macromolecules
- Recent advances have allowed application to the clinical realm
- Charged particles are accelerated by a laser. Time of flight is proportional to the ion’s mass. Chromatograms are compared to a library for identification.

http://www.bruker.com/
Considerations for MALDI-TOF

• Advantages
  – Small sample size
  – Rapid identification
  – Can also be used for identification of many bacteria and fungi in the laboratory

• Limitations
  – Difficult heat inactivation/cell disruption for Mycobacteria
  – Database limitations
  – Initial cost investment high
  – Cannot identify to species within the MTBC
DNA Sequencing

- Determining the precise order of nucleotides
- DNA sequence is compared to a database of sequences from known/characterized organisms
- 16S rRNA, rpoB and hsp65 genes are commonly sequenced for identification

http://seqcore.brcf.med.umich.edu/doc/dnaseq/interpret.html
Real-time PCR

- Detection of amplified target in real time by measuring fluorescence
- Primarily used for direct detection in clinical specimens, but can be used for identification from culture as well
  - Some assays detect mutations associated with MTBC drug resistance
Considerations for Molecular and Emerging Methods

• Advantages
  – Quicker turnaround time (TAT)
  – Uses liquid or solid media cultures, possibly smear-positive clinical specimens
  – Ability to recognize new strains

• Limitations
  – Cost, in particular the initial setup cost
  – Database management
  – Specialized equipment
  – Requires new expertise and training
  – New methods do not always replace standard methods
Identification of Mycobacteria

CLINICAL SIGNIFICANCE
Clinical Significance of MTBC

• Identification of MTBC is the most important finding in the laboratory and has serious clinical and public health consequences
  – Isolation almost always signifies disease
    • Exception: A single patient specimen in the absence of clinical indications may be a false positive
  – MTBC is not found in the environment
MTBC Culture Results for Patient Management

• In culture-positive TB cases, clinical response is evaluated by culture conversion
  – Recommend 2 negative cultures by the end of 2 months (initial treatment phase) to document culture conversion
    • 80% of drug-susceptible TB patients have negative cultures with 2 months of treatment initiation
  – If cultures are still positive after 4 months, the patient is deemed to have treatment failure and patient management must be re-assessed
Clinical Significance of NTM

• Not all NTM isolation is clinically significant as sources of diseases in humans
• Considerations for clinical relevance
  – Clinical setting and host
  – Organism species and its pathogenic potential
  – Source of the culture isolate & likelihood for contamination/colonization
  – Quantification of organisms detected both in culture and AFB smears
  – Number of positive cultures
Laboratory Considerations for Determining Significance of NTM

- Isolates from aseptically or surgically collected specimens
  - NTM is abundant on primary culture (3+ to 4+) or was the only organism isolated

- Isolates from sputum
  - NTM is from two or more sputum cultures (collected on different days) and
  - the only mycobacterial species present and
  - abundant growth on primary culture (3+ to 4+)

Clinical Relevance of Mycobacteria Isolated from Respiratory Specimens

<table>
<thead>
<tr>
<th>Species pathogenicity</th>
<th>Patient risk factors</th>
<th>Test indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis</td>
<td>Severe</td>
<td>NTM associated syndrome</td>
</tr>
<tr>
<td>M. kansasii</td>
<td>immunocompromised HIV</td>
<td></td>
</tr>
<tr>
<td>M. avium-intracellulare</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. abscessus</td>
<td>Immunosuppressive drugs</td>
<td></td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>Bronchiectasis and other underlying lung disease</td>
<td></td>
</tr>
<tr>
<td>M. xenopi</td>
<td>Older age</td>
<td></td>
</tr>
<tr>
<td>M. gordonae</td>
<td>Cystic fibrosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetes, system illness</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Otherwise healthy</td>
<td></td>
</tr>
</tbody>
</table>

Figure adapted from Clinical and Laboratory Standards Institute (CLSI), *Laboratory Detection and Identification of Mycobacteria; M48–A, 2008*
Mixed Cultures

- Mixed mycobacterial cultures may indicate significant mixed infection
- Mixed mycobacteria species?
  - Visual inspection of solid media for different colony types
  - HPLC or MALDI-TOF may show “extra peaks” (confusing for identification prediction software)
  - Molecular results may be “heterogeneous” (un-interpretable)
- Multiple specimens from a patient can help clarify whether a mixed culture is clinically significant
- Need pure culture growth for susceptibility testing
Mixed Cultures (2)

Picture courtesy of Wisconsin State Public Health Laboratory
Identification of Mycobacteria

ADDITIONAL RECOMMENDATIONS AND PRACTICES
Recommended Turnaround Time

• Identification of MTBC ≤ 21 days from specimen receipt
  – Molecular methodologies have dramatically decreased the TAT for identification once culture has grown
  – Laboratory workflow and testing practices affect TAT
  – Referral of testing can lead to increased TAT

Tenover, et. Al, 1993; Healthy People 2010 goals
Identification and Reporting Considerations

- For patient management and public health purposes, report preliminary identification (MTBC or not MTBC) as soon as possible.
- Use a multi-faceted approach:
  - Ensure that identification result matches phenotypic results (e.g., colony morphology and growth rate) before issuing final report.
  - Discordant results should be investigated.
Differentiating Within the Complex

- MTBC includes *M. tuberculosis*, *M. bovis*, *M. bovis BCG*, *M. caprae*, *M. microti*, *M. africanum*, *M. canettii*, *M. pinnipedii*, and *M. mungi*

- Laboratory should consult with healthcare providers and TB program to decide the necessity of separating members of MTBC

- Due to unique epidemiology and inherent PZA drug resistance, *M. bovis* and BCG should always be differentiated from other members of MTBC
How Far to Go with Identification of NTM

- Clinically significant NTM isolates should be routinely identified to the species level
  - Exception *M. avium* complex (MAC)
    - Patient management and treatment are not different between *M. avium* and *M. intracellulare*

- The level of service and the choice of methods applied in the laboratory should be determined by the patient population served and by the resources available

- Communication between the clinician and laboratorian is essential for determining the importance and extent of analysis for a clinical NTM isolate
Possible Algorithm for Identification with Accuprobe

Positive Culture containing AFB

Accuprobe for MTBC

Positive

Report MTBC

Negative

Other Accuprobe testing

Negative

Report MAC, M. kansasii, or M. gordonae

Positive

Accuprobe-negative organisms: Depending on laboratory policy, perform further in-house identification or send to reference laboratory for ID
Possible Algorithm for Identification with HPLC or MALDI-TOF

- Positive Culture containing AFB
  - HPLC or MALDI-TOF
    - Inconclusive results
      - Perform further testing based on initial results and growth characteristics
    - Conclusive results
      - Report Results
  - Slow-grower: Accuprobe and 16S sequencing (biochemicals as last resort)
  - Rapid-grower: rpoB sequencing

- Conclusive results
Referral of Isolates for Identification

• Reference facilities should be utilized by laboratories lacking appropriate technologies and resources

• Any AFB isolate that is not identified in-house should be sent on the original media within one working day

• Laboratories should monitor the TAT of the reference facility to which their specimens are sent
Considerations for Referral of Isolates

• **Advantages**
  – Provides testing and expertise that may not be otherwise available in cases of
    • Limited resources or staffing in-house
    • Low volume or few positive cultures (difficulty maintaining proficiency)
  – Cost effectiveness
  – May increase TAT due to shipment of isolate

• **Limitations**
  – Additional shipping costs
  – Need trained and certified personnel for shipping
  – Loss of internal knowledge on status of testing
Transport of Category A and Category B Substances

- Isolates of MTBC (including broths known to be positive for MTBC) are considered Category A (Infectious Substances)
- Patient specimens (e.g., sputum) are considered Category B (Biological Substances)
- Transport of both isolates and patient specimens is regulated by the Department of Transportation (DOT) and the International Air Transport Association (IATA) rules
Transport of Category A and Category B Substances

• Persons involved in shipping Category A and Category B substances must be trained and certified

• Packaging, shipping, and transport is complex and all regulations must be followed completely
  – Infractions may result in fines by regulatory agencies

• For details regarding these regulations, please see the information provided in the Reference section
Example of a Category A System

- Package the isolate according to current DOT and IATA regulations
- A triple packing system must be used
Conclusions

• Accurate and timely identification of mycobacteria is crucial

• Healthcare providers and TB Control Programs should be consulted to determine the level of TB laboratory services provided in your jurisdiction
Conclusions

• There are a growing number of clinically significant NTM

• For robust identification, use a multi-faceted approach that includes a rapid identification and phenotypic assessment before issuing a final report
Conclusions

• Samples should be referred if laboratories lack sufficient testing volume to maintain technical proficiency, staff expertise, or resources necessary to provide high quality results and rapid TAT

• Use appropriate biosafety measures when working with cultures of mycobacteria, especially MTBC
Identification of Mycobacteria

ADDITIONAL INFORMATION
Packing and Shipping Guidance

- IATA Infectious Substances website: http://www.iata.org/whatwedo/cargo/dgr/Pages/infectious_substances.aspx
### Examples of Mycobacterial Species and Clinical Conditions

<table>
<thead>
<tr>
<th>NTM Species</th>
<th>Clinical Setting</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. abscessus or M. avium complex (MAC)</td>
<td>Cystic fibrosis (CF) or bronchiectasis in older Caucasian women</td>
<td>respiratory</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>Esophageal achalasia and chronic regurgitation, or history of mineral oil ingestion</td>
<td>respiratory</td>
</tr>
<tr>
<td>M. marinum</td>
<td>Extremity lesion after fish tank or other marine exposure</td>
<td>Biopsy or aspirate</td>
</tr>
<tr>
<td>M. kansasii or MAC</td>
<td>Hairy-cell leukemia, advanced HIV disease</td>
<td>Blood, bone marrow</td>
</tr>
<tr>
<td>M. chelonae, M. abscessus or M. haemophilum</td>
<td>Disseminated skin lesions in patients on chronic immunosuppressive drugs, e.g. corticosteroids</td>
<td>Biopsy or aspirate</td>
</tr>
<tr>
<td>M. chelonae, M. mucogenicum, M. fortuitum or M. abscessus</td>
<td>Central venous catheter, prosthetic device, surgical wound, injection site, or other local trauma</td>
<td>Biopsy or aspirate</td>
</tr>
</tbody>
</table>

Clinical and Laboratory Standards Institute (CLSI), *Laboratory Detection and Identification of Mycobacteria; M48–A*, 2008
21st Century Algorithm

- **Process Specimen**
  - 1 day
  - **AFB Microscopy**
  - 2 to 6 weeks
  - **Culture Positive**
  - 2 to 3 weeks
  - **Species Identification**
  - **Drug Susceptibility**

- **Molecular DST**

- **Amplification-based Tests**
Mycobacteria Identification Algorithm: Accuprobies + Other Methods

Positive Culture containing AFB

Accuprobe: MTBC, MAC, gordonae, kansasii

Accuprobe Negative

Perform HPLC, MALDI-TOF, sequencing

Report Preliminary or Final Results

Inconclusive results

Send to Reference Laboratory
Niacin Test with Paper Strips

Nitrate Reduction

COLOR PLATE 15. Nitrate color standards.
Differentiation within MTBC

<table>
<thead>
<tr>
<th>Organism</th>
<th>Niacin</th>
<th>Nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M. bovis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M. bovis BCG</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
## Growth Characteristics

### Optimal Growth Temperature

<table>
<thead>
<tr>
<th>Optimal Temperature</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 ± 2°C</td>
<td>Optimum incubation temperature for most slowly-growing mycobacteria</td>
</tr>
<tr>
<td>30 ± 2°C</td>
<td>M. ulcerans, M. marinum, M. conspicuum, M. chelonae</td>
</tr>
<tr>
<td>22-25°C</td>
<td>M. haemophilum, M. stomatepiae</td>
</tr>
<tr>
<td>42°C</td>
<td>M. xenopi</td>
</tr>
</tbody>
</table>
Pigment Production Assay
# Runyoun Classification of Non-tuberculous Mycobacteria

<table>
<thead>
<tr>
<th>Runyoun Group</th>
<th>Description</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Slow growing, photochromogens</td>
<td>M. kansasii, M. marinum</td>
</tr>
<tr>
<td>II</td>
<td>Slow growing, scotochromogens</td>
<td>M. gordonae, M. scrofulaceum</td>
</tr>
<tr>
<td>III</td>
<td>Slow growing, non-photochromogens</td>
<td>M. avium, M. ulcerans, M. haemophilum, M. xenopi</td>
</tr>
<tr>
<td>IV</td>
<td>Rapid growers</td>
<td>M. chelone, M. abscessus, M. fortuitum</td>
</tr>
</tbody>
</table>
Colony Morphology Slides
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