LABORATORY DIAGNOSIS OF ATYPICAL MYCOBACTERIAL INFECTIONS

YF Ngeow
National Public Health Laboratory
Ministry of Health Malaysia
Objectives:

1) To present overview of the microbiological diagnosis of infections by atypical mycobacteria with special reference to rapid molecular methods.

2) To discuss methodological problems with detection, identification, and differentiation.
Atypical mycobacteria

Also **NTM** (Non-tuberculous mycobacteria)
**MOTT** (Mycobacteria other than tuberculosis)

- >50 species
- Saprophytes, mostly from water and soil
- Animal pathogens
- Opportunistic pathogens in humans
- Person-person transmission rare
Atypical mycobacteria

Clinical syndromes:
- Pulmonary
- Lymphadenitis
- Cutaneous
- Disseminated

Community-acquired
- Immuno compromised
- Immuno competent

Healthcare-associated — Wounds, Procedure, Device
- Dialysis equipment
- Bronchoscopes
- Injection vials
Diagnosis based on clinical, radiographic, bacteriologic criteria

Isolation —> Disease

Exclude TB and other diseases

Diagnostic criteria for NTM lung disease
For symptomatic patients with suggestive radiography
(Mostly for MAC, *M. kansasii*, *M. abscessus*)

- **Sputum/ bronchial wash**
  - At least 3 samples within a year
  - 3 +ve cultures with –ve AFB
  - 2 +ve cultures and 1 +ve AFB

- **1 bronchial wash and unable to get sputum**
  - +ve cult with 2-4+ growth
  - +ve cult with 2-4+ AFB

- **Biopsy**
  - +ve culture
  - histology and growth in sputum or bronchial washing

(American Thoracic Society 1997)
To treat or not to treat?

- Early effective therapy prevents dissemination and treatment failure
- Species differentiation for most appropriate choice of antimicrobial agent
<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Common causes</th>
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<tbody>
<tr>
<td><strong>Pulmonary</strong></td>
<td>MAC, <em>M kansasii</em>, <em>M xenopi</em>, <em>M malmoense</em>, <em>M abscessus</em></td>
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<td></td>
<td><em>M szulgai</em>, <em>M smegmatis</em>, <em>M celatum</em>, <em>M simiae</em>, <em>M scrofulaceum</em></td>
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<td><strong>Lymphadenitis</strong></td>
<td><strong>MAC</strong>, <em>M fortuitum</em>, <em>M scrofulaceum</em>, <em>M abscessus</em>, <em>M malmoense</em></td>
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<td><strong>Skin/soft tissues</strong></td>
<td><em>M chelonea/abscessus</em>, <em>M marinum</em>, <em>M terrae</em></td>
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<td></td>
<td><em>M ulcerans</em> (West Africa, Australia) <em>M fortuitum</em>, <em>M smegmatis</em></td>
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<td></td>
<td><em>M kansasii</em>, <em>M haemophilum</em></td>
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<td><strong>Disseminated</strong></td>
<td><strong>HIV+</strong> <em>M avium</em>, <em>M kansasii</em>, <em>M genavense</em>, <em>M haemophilum</em>, others</td>
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<td><strong>HIV-</strong> <em>M abscessus/chelonea</em>, <em>M kansasii</em>, <em>M haemophilum</em></td>
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<td><strong>Health-care associated</strong></td>
<td><em>M fortuitum</em>, <em>M chelonea/abscessus</em>, <em>M mucogenicum</em></td>
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<td><strong>Contaminants</strong></td>
<td><em>M gordonae</em>, <em>M phlei</em>, <em>M fortuitum</em></td>
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</table>
National Tuberculosis Reference Laboratory

Jan-Dec 2006

Identification by Accuprobe, growth characteristics and biochemical tests

6661 mycobacterial isolates
11% NTM
83% of NTM were Runyon IV

91.4% from respiratory specimens
Others from urine, tissue biopsies, gastric lavage, peritoneal fluid, cerebrospinal fluid
Microscopy, culture, biochemistry as for MTBC

- AFB microscopy less sensitive for some species
- Some species are fastidious, difficult to grow
  \((M\text{\ haemophilum}, M\text{\ genavense}, M\text{\ conspicuum})\)
- Lower temperature incubation \((28-30^\circ\text{C}, 35^\circ\text{C})\)
  for skin/soft tissue; \(M.\text{\ marinum/chelonae/haemophilum}\)
- Some species are susceptible to specimen decontamination methods \((M.\text{\ ulcerans})\)

Biochemical tests are time-consuming and results may be difficult to interpret
\((\text{Differentiated from MTBC by negative niacin test})\)
Serology mainly for animal seroprevalence studies

Skin testing - many false negatives, cross-reactions

Drug susceptibility testing

- Undergoing standardization and evaluation
- Uncertain prediction of clinical efficacy

Drugs for testing
- *M. kansasii* (rifampicin)
- MAC (clarithromycin)
- Rapid growers (aminog, IMP, quin, clar, cefoxitin, sulph)

Methods: Disk elution, broth microdilution, E test, BACTEC MGIT
Culture

Solid medium - growth characteristics, pigmentation
- semi-quantitative (0-4+)
(Middlebrook 7 H10 and 7 H11 or Lowenstein-Jensen)

Runyon classification:

**Photochromogens**
- pigmentation in light

**Schotochromogens**
- pigmentation in light or darkness

**Non-photochromogens**
- no pigmentation in light/darkness

**Rapid growers**
- colonies within 1 week

**Fast growers**

Pigment -
- *M. fortuitum*
- *M. chelonae/abscessus*

Pigment +
- *M. smegmatis*
- *M. flavescens*
- *M. phlei*

usually no clinical significance
**Liquid culture systems**
- more rapid
- greater range of species
- contamination/mixed growth not apparent
- positives to be confirmed with microscopy, subculture and further identification

BACTEC 460 TB, BACTEC 9000 MB, MGIT 960 (Becton-Dickinson)
VersaTREK (Trek Diagnostics)
ESP Culture System II (Trek Diagnostics)
MB/BacT system (Organon Teknika)

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The NAP test in BACTEC 460 TB (Becton-Dickinson)

- GI > 50 → Transfer 1 ml to NAP vial
- Growth = **NTM**
- No growth = **MTB**

Para-nitro-alpha-acetylamino-beta-hydroxypropio phenone (NAP)
Capilia TB

Ag capture test

Identification of NTM by exclusion of MTBC

Rapid immunochromatographic assay using monoclonal antibodies against MPB64 to differentiate TB from NTM
HPLC, GLC, GC/mass spectrometry

Based on lipid composition analysis

*Mycobacterium* sp. have species-specific long-chain 3-hydroxy fatty acids and specific fatty alcohol’s

- Saponification of mycobacterial cells
- Derivatisation of mycolic acids to p-bromophenacyl esters
- Separation in column
- Identification of mycolic acid patterns

Automated systems for HPLC patterns recognition

Highly sensitive and specific

Limitations: Requires considerable biomass
Time-consuming sample processing
Molecular Differentiation of Mycobacterial Species

**Genes**
- 16s rRNA (Ribosomal)
- ITS 16s-23S rDNA (Internal transcribed spacer)
- dnaJ (Cold-shock protein)
- Hsp 65 (Heat-shock protein)
- rpoB (RNA polymerase)
- recA
- IS 1245 (Insertion sequence)
- IS 901
- IS 900

**Common methods**
- PCR
- PCR-probe hybridisation
- PCR-RFLP
- PCR-DNA sequence analysis

**Diagram:**
- Fast-growers
  - rpoB, 16S rRNA, dnaJ, ITS 16S-23S
  - ITS
  - hsp65
  - rpoB
  - recA
- Slow-growers
  - MTBC
  - IS6110
  - IS1080
  - gyrB
  - M tuberculosis
  - M bovis
  - M microti etc.
- Fast-growers
  - gordonae
  - kansasii
  - MAC
  - IS 1245
  - mig
  - MAC
  - M intrac
  - IS900
  - IS901, dnaJ
- Slow-growers
  - M a paratuberculosis
  - M a avium
  - IS900

**Species**
- Mycobacterium tuberculosis (MTBC)
- Mycobacterium bovis
- Mycobacterium microti
The universal tree of life
16S rRNA phylogenetic tree

Bacteria

Archaea

Eukarya

16S rRNA phylogenetic tree

fungi

animals

plants

16S rRNA gene with alternating conserved and hypervariable regions

probe hybridisation

DNA-sequencing

With DNA sequencing, novel species can be identified
Limitations of species identification by rRNA gene sequencing

Dependent on gene bank information and criteria used for search
Interpretation can be difficult
Species in mycobacterial complexes are genetically closely related

Examples;
16S rRNA sequence cannot differentiate between *M kansasii* and *M gastri*
23S rRNA sequence is identical for *M kansasii* and *M celatum*

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*M marinum* and *M ulcerans* (16S rRNA sequence >99.8% identical)

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*M marinum*: pigment +, IS 2606 −, Hae111 on rpoB 200-80
*M ulcerans*: pigment -, IS 2606 +, Hae111 on rpoB 210-60-85
PCR-RFLP Analysis (PRA) for Speciation

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<th>Fragment size (bp)</th>
<th>Species</th>
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<td>175 —100—80--------</td>
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<td>70</td>
<td>M fortuitum</td>
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<td>80—60—40</td>
<td>MTB complex</td>
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<tr>
<td>60—40</td>
<td>M kansasii</td>
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</table>

Electrophorogram

Algorithm for identification of species

*rpoB* gene PCR and *Msp1* digestion

PCR-RFLP with *hsp 65* or *rpoB* genes
- time consuming; more than 1 RE may be required
- requires extensive in-house validation
- difficult to detect small differences among bands produced
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<th>Map 3</th>
<th>Other enzymes</th>
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### Isolate Identification

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#### Oligonucleotide-Specific Capture Probe Hybridisation (OSCPH)

- **PCR** for genus-specific 16S rRNA sequence
- PCR product identified in wells with oligonucleotide-specific probes

**Identification limited by availability of probes**
INNO-LiPA line probe assay (Innogenetics)

Isolate (from solid/liquid culture)

Extract DNA

Amplify 16-23S rRNA spacer region

Reverse hybridise with species-specific probes immobilised as parallel lines on strip of membrane

One strip for detection and identification of MTBC and 17 other species

HAIN Lifescience DNA strips

GenoType Mycobacterium CM

GenoType Mycobacterium AS

Rapid differentiation of >30 mycobacterial species
Hybridisation Protection assay (HPA) with acridinium ester-labelled probes
For identification of MTBC, *M. avium*, *M. intracellulare*, *M. avium* complex, *M. kansasii*, and *M. gordonae*

- Requires large amounts of cultured bacteria
- “Trial and error" testing, one probe at a time
- Mixed infections not detected unless isolates routinely tested with all probes
- Limited species identification
BD ProbeTec ET culture identification Assay

for MTBC, *M kansasii*, (MAC)
from LJ slope or MGIT 960 culture

Isothermal Strand Displacement Amplification (SDA) technology

Amplification of target sequence by repeated nicking, strand displacement and priming of displaced strands

Real-time detection using FRET

Limited species identification
Direct tests on respiratory specimens
For detection of *M. tuberculosis*, *M. avium*, and *M. intracellulare*

**AMTD (Gen-Probe)**
- rRNA amplification by TMA
- HPA

**BD ProbeTec ET**
- SDA

**Roche COBAS AMPLICOR System**
- PCR-MEIA

HPLC with fluorescence detection used directly on smear+ve sputum
- Sensitivity of 56.8% (*M. tuberculosis*)
- 33.3% (*M. avium* complex)

**OSCPH directly performed on clinical specimens**
- “Sensitivity equal to culture”
Multiplex PCR for TB/NTM

Mycobacterium genus-specific sequences in 16s rRNA, hsp 65 genes
MTBC –specific sequences in insertion sites e.g. IS6110
Real-time PCR for TB/NTM

PCR product detection with fluorogenic probes or SYBR Green 1 dye
Post-amplification melt curve analysis

Limited range of melting temperatures but wide variety of NTM
Overlapping of $Tm$ expected
**Mycobacterium** species identification and rifampicin resistance testing with high-density DNA probe arrays

(J Clin Microbiol 1999:49-55)

82 unique 16SrRNA sequences and *rpoB* alleles
Rapid detection / discriminating systems

Particularly good for difficult-to-grow species

**BUT**

- Costly QA, technical and clinical validation
- Requires considerable skill
- Unable to distinguish living from dead bacteria
- Lack of sensitivity for AFB –ve specimens
- Commercial systems identify limited species
- Inadequately evaluated for different specimen types
- Limited gene bank information
Epidemiological studies:
- monitor trends in the occurrence of new strains
- identify possible sources of infection
- investigate outbreaks

Genotyping methods:
- PFGE (pulsed field gel electrophoresis)
- Plasmid profile analysis (50% have plasmids)
- MLEE (multi-locus enzyme electrophoresis)
NTM Detection, Identification and Strain Differentiation

**Isolation**
- Liquid
- Solid
  - Growth rate
  - Growth requirements
  - Pigment production
  - Biochemical reactions

**Microscopy**

**Antigen, Mycolic acid**
- Nucleic acid detection
  - HPLC, GLC, MS
  - NAAT, DNA sequencing
  - Microarray

**Exclude TB**

**Clinical features**
- Pathological features
- Epidemiological features

**Specimens**
Thank You