LABORATORY DIAGNOSIS OF ATYPICAL MYCOBACTERIAL INFECTIONS

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National Public Health Laboratory

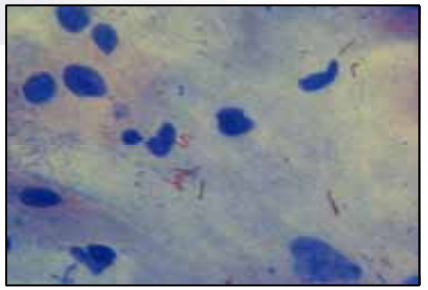
Ministry of Health Malaysia

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
   differentiation

Atypical mycobacteria

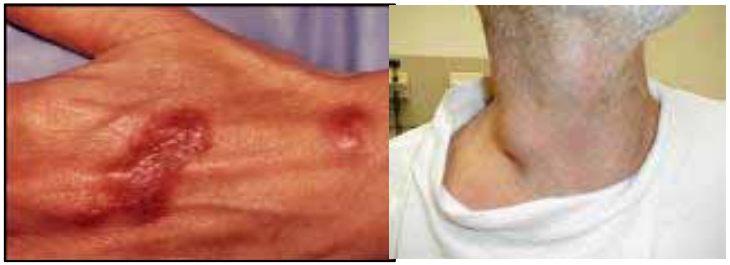


Also **NTM** (Non-tuberculous mycobacteria)
MOTT (Mycobacteria other than tuberculosis)

- >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 — *Immuocompromised immuocompetent*

Healthcare-associated – ***Wounds, Procedure, Device***

dialysis equipment

bronchoscopes

injection vials

Isolation ⟶ *Disease*

Diagnosis based on clinical, radiographic, bacteriologic criteria

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	1 bronchial wash and unable to get sputum	Biopsy
At least 3 samples within a year 3 +ve cultures with –ve AFB 2 +ve cultures and 1 +ve AFB	+ve cult with 2-4+ growth +ve cult with 2-4+ AFB	+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

Syndrome	Common causes
Pulmonary	**MAC, *M kansasii, M xenopi*, *M malmoense, M abscessus*** *M szulgai, M smegmatis, M celatum, M simiae,M scrofulaceum*
Lymphadenitis	**MAC**, *M fortuitum, M scrofulaceum, M abscessus, M malmoense*
Skin/soft tissues	***M chelonae/abscessus*, *M marinum***, *M terrae* *M ulcerans* (West Africa, Australia) *M fortuitum*, *M smegmatis* *M kansasii, M haemophilum*
Disseminated	
HIV+	***M avium, M kansasii*, *M genavense, M haemophilum , others***
HIV-	***M abscessus/chelonae*, *M kansasii, M haemophilum***
Health-care associated	***M fortuitum, M chelonae/abscessus***, *M mucogenicum*
Contaminants	*M gordonae , M phlei, M fortuitum*

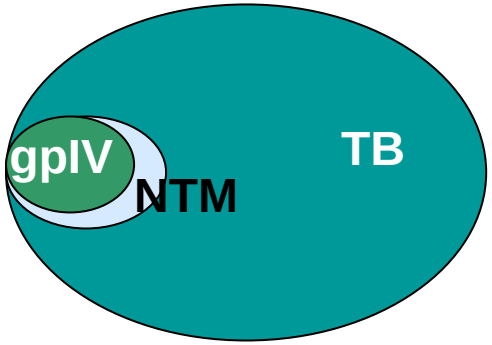
National Tuberculosis Reference Laboratory

Jan-Dec 2006

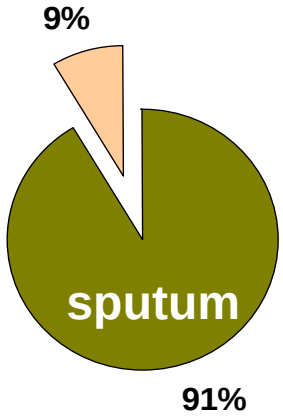
Identification by Accuprobe, growth characteristics and biochemical tests

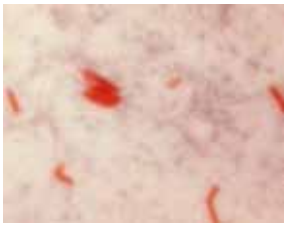
NAPHL Malaysia

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





Laboratory Diagnosis
(detection, speciation)

Microscopy, culture, biochemistry as for MTBC

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

Biochemical tests are time-consuming and results may be difficult to interpret

(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 +

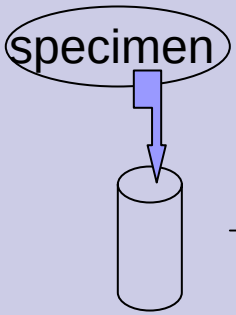
M smegmatis
M. flavescens
M phlei,

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)



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

GI>50 → Transfer 1 ml to NAP vial → Growth=**NTM**

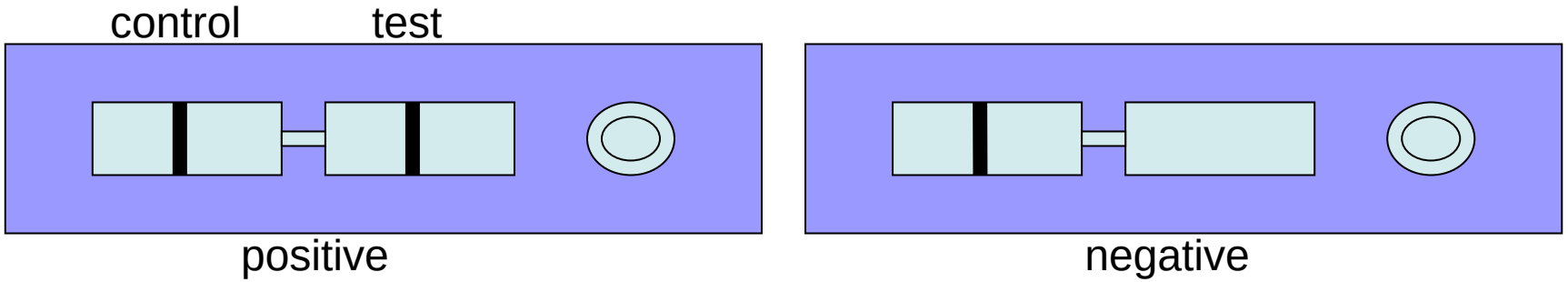
→ No growth=MTB

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

Capilia TB

Ag capture test

Identification of NTM by exclusion of MTBC



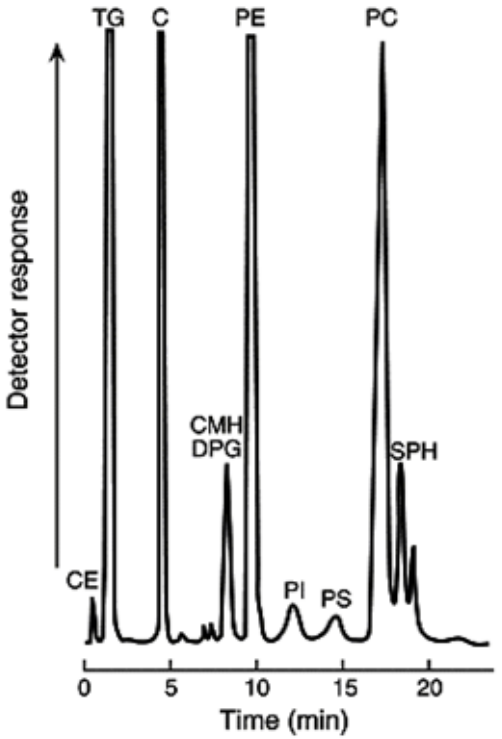
Rapid immunochromatographic assay
using monoclonal antibodies against MPB64
to differentiate TB from NTM

Isolate Identification

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 processinig

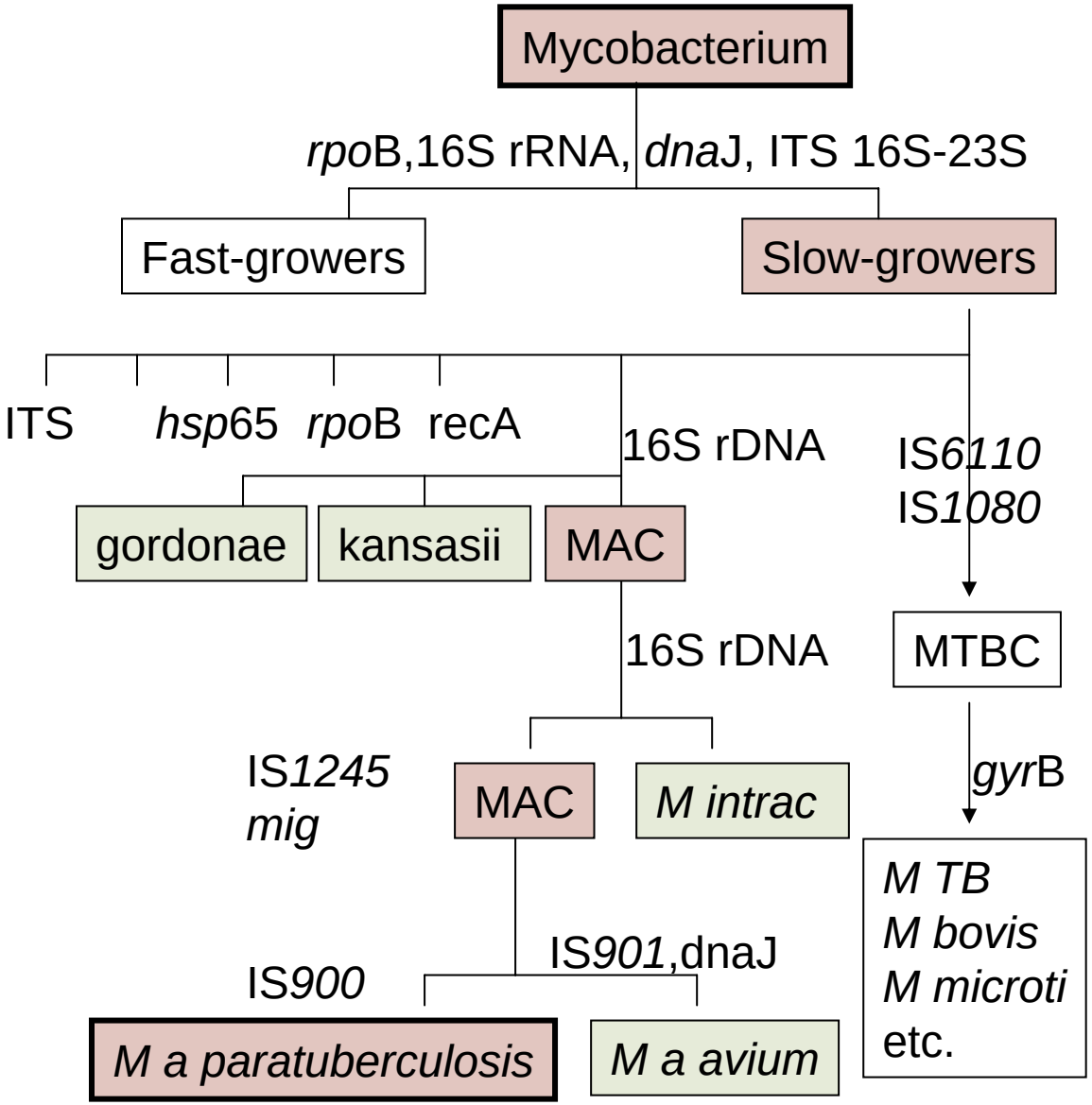
Molecular Differentiation of Mycobacterial Species

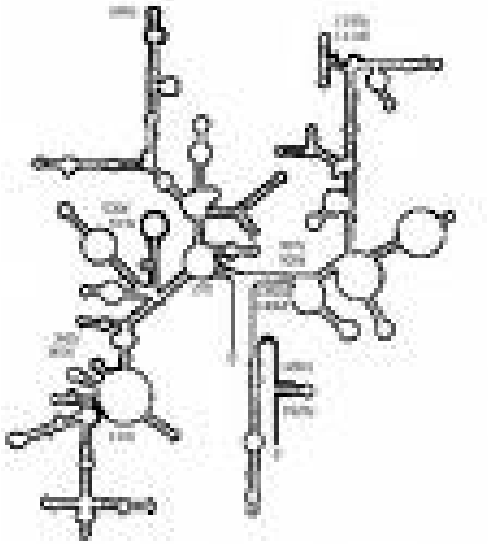
Genes

16s rRNA (Ribosomal)
ITS 16s-23S rDNA
(Internal transcribed spacer)
*dna*J (*Cold-shock protein)*
Hsp 65 (*Heat-shock protein)*
*rpo*B (*RNA polymerase)*
*rec*A
IS *1245 (*Insertion sequence)
IS *901*
IS *900*

Common methods

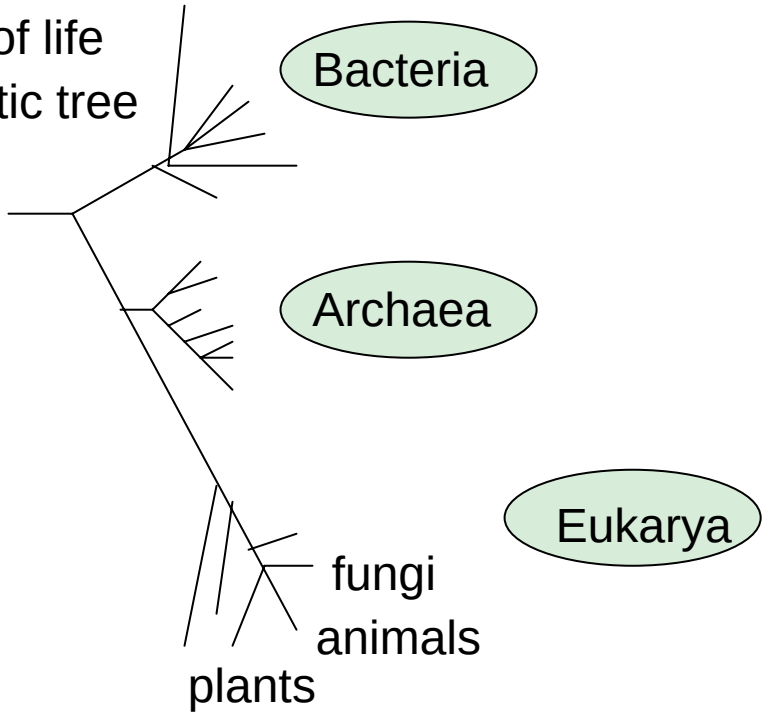
PCR
PCR-probe hybridisation
PCR-RFLP
PCR-DNA sequence analysis

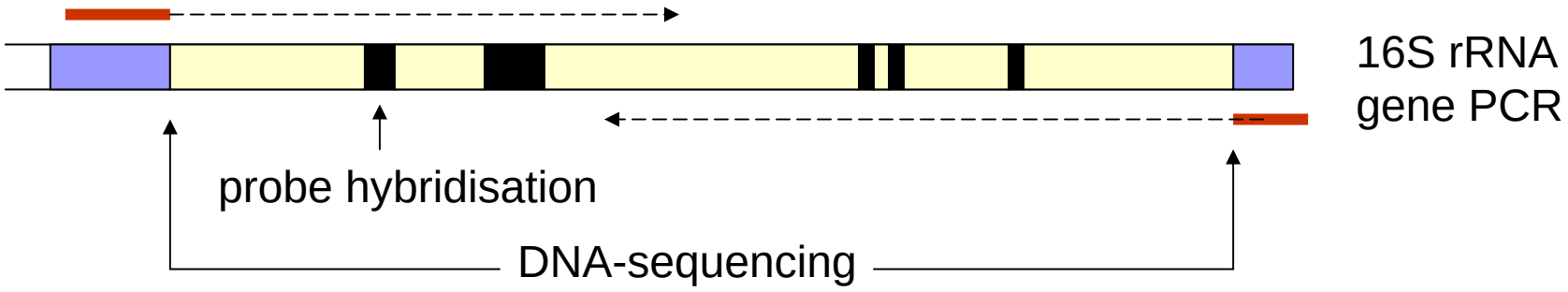




16s rRNA gene with alternating conserved and hypervariable regions

The universal tree of life
16S rRNA phylogenetic tree





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

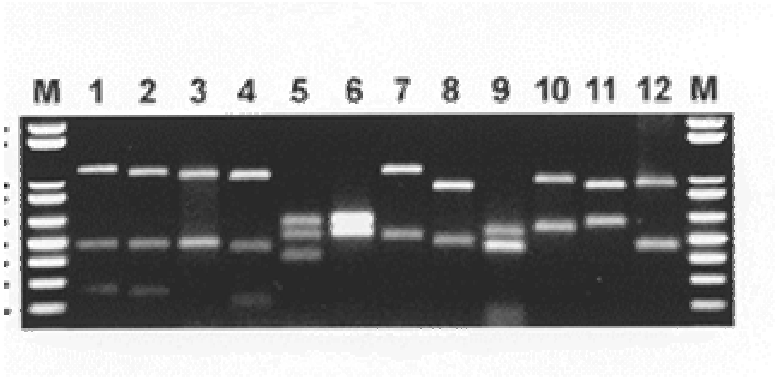
Examples;
16S rRNA sequence cannot differentiate between *M kansasii* and *M gastri*
23S rRNA sequence is identical for *M kansasii* and *M celatum*

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

	Score
gi/46251175/gb/AY513243.1/ *M marinum* SCCTB	2755
gi/44894449/gb/AY509248.1/ *M marinum* SCCSH	2748
gi/311935/emb/X58954.1/MU16SRRNA *M ulcerans* 16S rRNA	2742
gi/2832580/emb/X88926.1/MUR16SRNA *M ulcerans* 16S rRNA	2742
gi/46255176/gb/AY513244.1/ *M marinum* SCCTB	2740
gi/44894447/gb/AY509246.1/ *M marinum* SCCSH	2740

M marinum: pigment +, IS *2606* –, *Hae*111 on *rpo*B 200-80
M ulcerans: pigment -, IS *2606* +, *Hae*111 on *rpo*B 210-60-85

PCR-RFLP Analysis (PRA) for Speciation



Electrophorogram

Fragment size (bp) Species

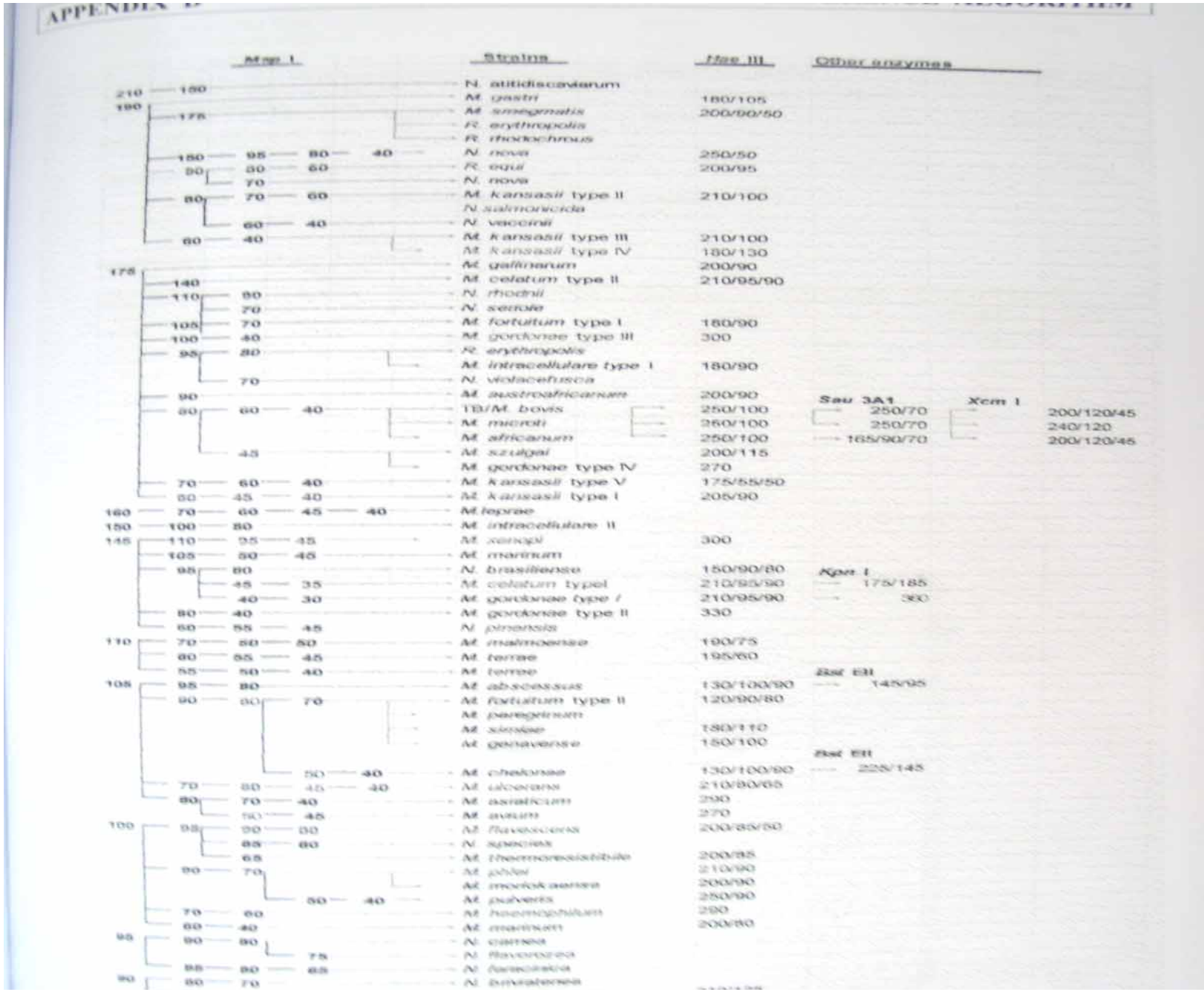
175—100—80---------- M intracellulare
---70---------- M fortuitum
--- 80---60—40-------- MTB complex
--- 60---40---------- M kansasii

Algorithm for identification of species

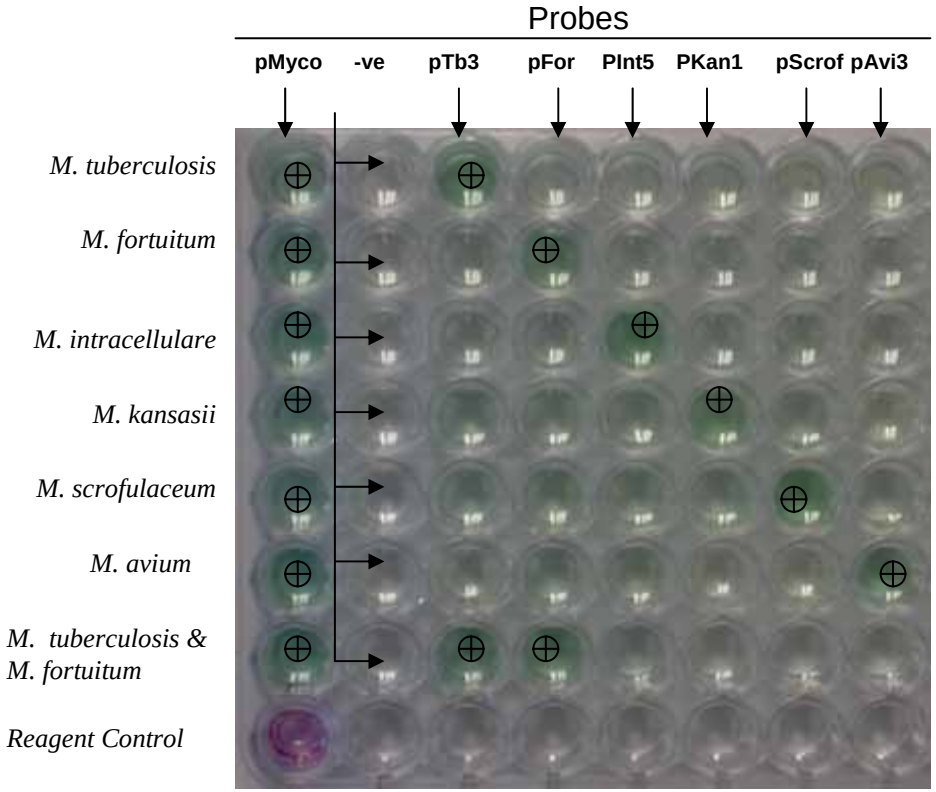
Lee, Pak, Cho, Bai, Kim. J Clin Microbiol 2000
*rpo*B gene PCR and *Msp*1 digestion

PCR-RFLP with *hsp* 65 or *rpo*B genes

- time consuming; more than 1 RE may be required
- requires extensive in-house validation
- difficult to detect small differences among bands produced


APPENDIX
Mse I
Strains
Hae III
Other enzymes
N. otitidiscaviarum
M. gastri 180/105
M. smegmatis 200/90/50
R. erythropolis
R. rhodochrous
N. nova 250/50
R. equi 200/95
N. nova
M. kansasii type II 210/100
N. salmonicida
N. veccinii
M. kansasii type III 210/100
M. kansasii type IV 180/130
M. gallinarum 200/90
M. celatum type II 210/95/90
N. rhodnii
N. senegalensis
M. fortuitum type I 180/90
M. gordonae type III 300
N. erythropolis
M. intracellulare type I 180/90
N. violaceofusca
M. austroafricanum 200/90
TB/M. bovis 250/100
M. microti 260/100
M. africanum 250/100
M. szulgai 200/115
M. gordonae type IV 270
M. kansasii type V 175/55/50
M. kansasii type I 205/90
M. leprae
M. intracellulare II
M. xenopi 300
M. marinum
N. brasiliensis 150/90/80
M. celatum typeI 210/95/90
M. gordonae type I 210/95/90
M. gordonae type II 330
N. pinensis
M. malmoense 190/75
M. terrae 195/60
M. terrae
M. abscessus 130/100/90
M. fortuitum type II 120/90/80
M. peregrinum
M. simiae 180/110
M. genavense 150/100
M. chelonae 130/100/90
M. ulcerans 210/80/65
M. asiaticum 250
M. avium 270
M. flavescens 200/80/50
N. asteroides
M. thermoresistibile 200/85
M. gilvum 210/90
M. scrofulaceum 200/90
M. paluens 250/90
M. haemophilum 250
M. marinum 200/80
N. carnea
N. flavorubra
N. farcinica
N. brevibrachia
Sau 3A1
250/70
250/70
165/90/70
Xcm I
200/120/45
240/120
200/120/45
Xpn I
175/185
330
Rsr BII
145/95
Rsr BII
225/145

Isolate Identification



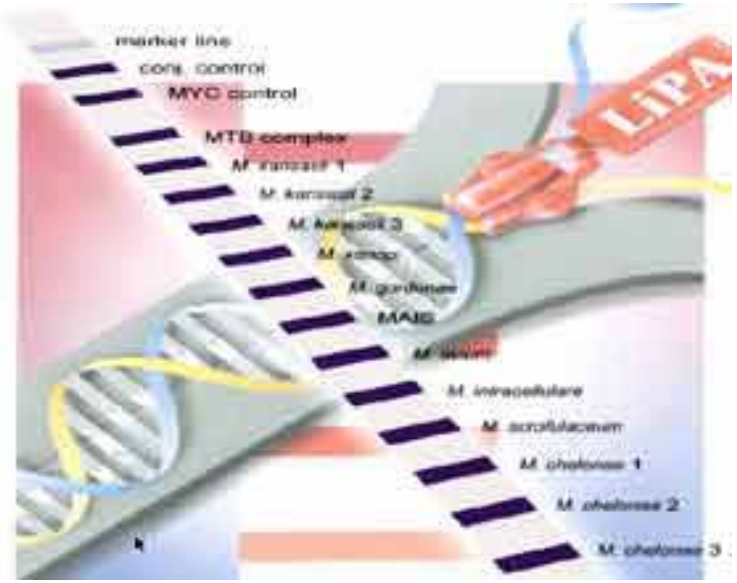
Oligonucleotide-Specific Capture Probe Hybridisation (OSCPH)

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

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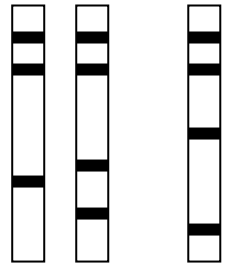
HAIN Lifescience DNA strips

GenoType **Mycobacterium CM**
GenoType **Mycobacterium AS**



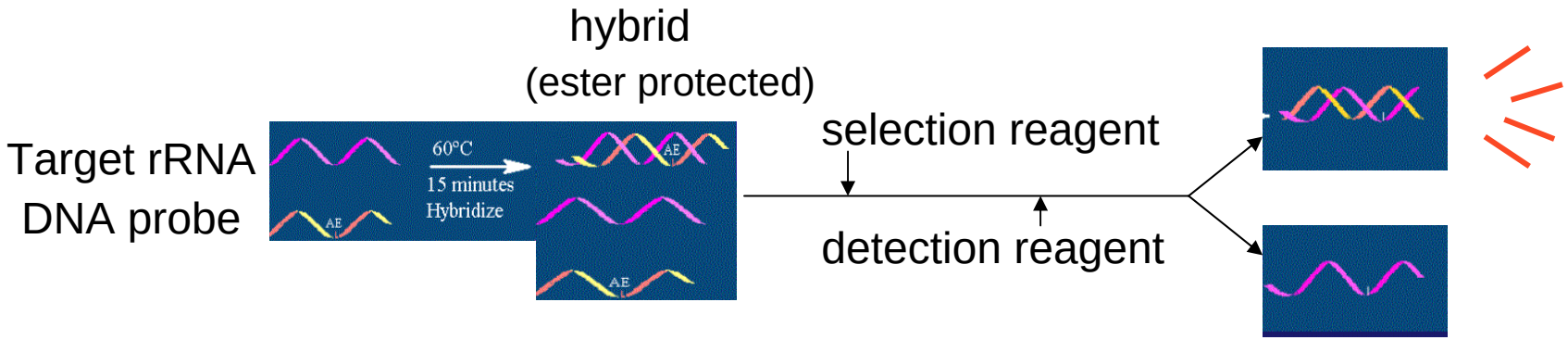
Rapid differentiation of >30 mycobacterial species

Isolate Identification

AccuProbe

GEN-PROBE
Pioneering Advances In Healthcare Through DNA Probes

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*



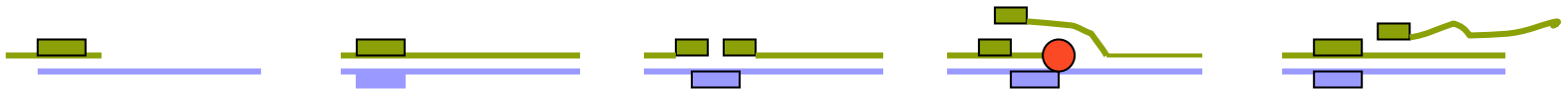
- Requires large amounts of cultured bacteria
- “Trial and error" testing, one probe at a time
- Mixed infections not detected unless isolates routinely tested with all probes
- Limited species identification

Isolate 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*

HAIN Lifescience

AMTD (Gen-Probe)
rRNA amplification by TMA

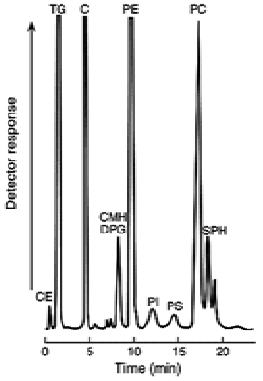
↓

HPA

BD ProbeTec ET
SDA

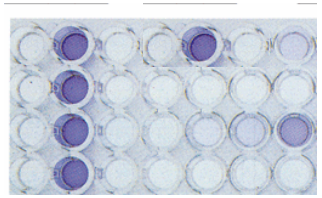
Roche **COBAS AMPLICOR** System

PCR-MEIA



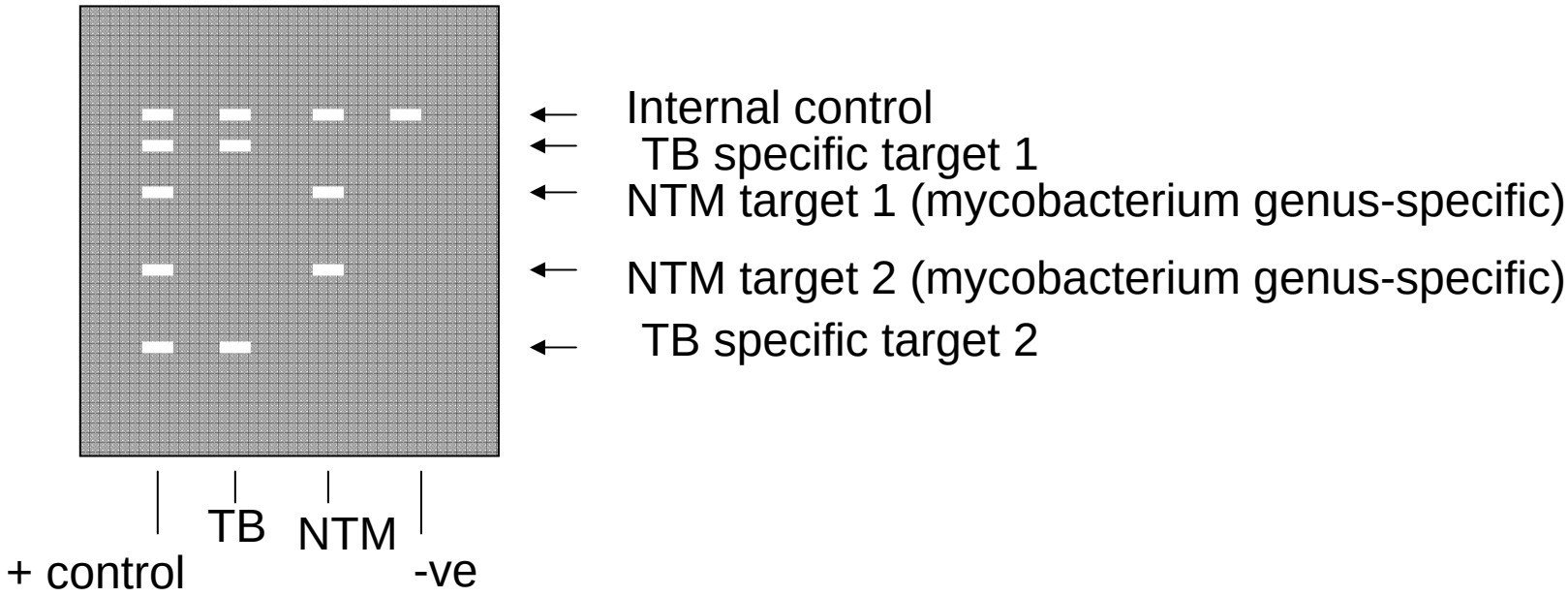
HPLC with fluorescence detection used directly on smear+ve sputum
Sensitivity of 56.8% (*M. tuberculosis)*
33.3% (*M. avium* complex)

OSCPH directly performed on clinical specimens
“Sensitivity equal to culture”



For clinical specimens

Multiplex PCR for TB/NTM

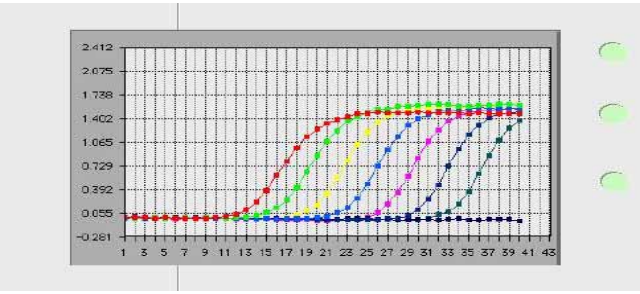


Mycobacterium genus-specific sequences in 16s rRNA, *hsp* 65 genes
MTBC –specific sequences in insertion sites e.g. IS*6110*

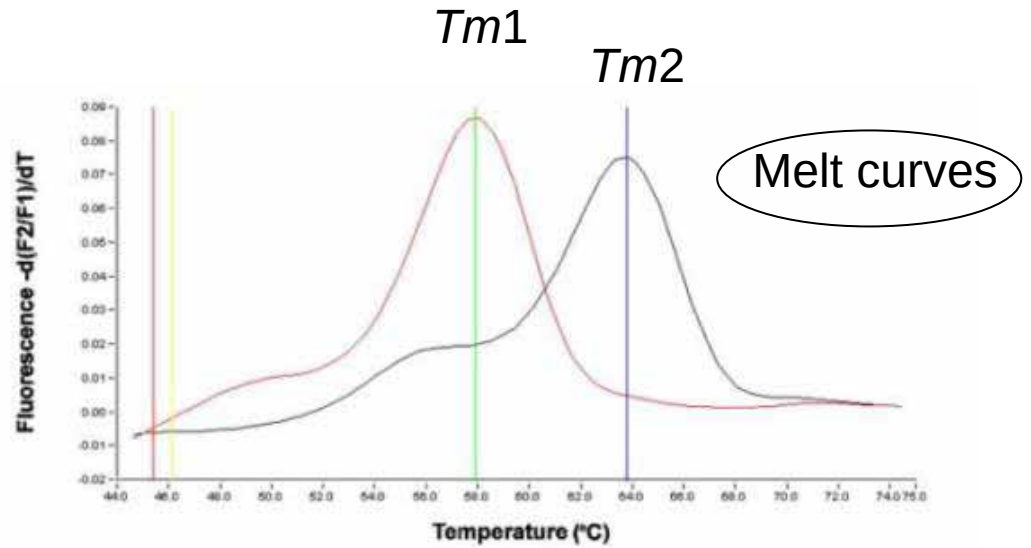
For clinical specimens

Real-time PCR for TB/NTM

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



RTmPCR amplification curves



(*Tm* = temperature at which probe dissociates from target site)

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 *rpo*B 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

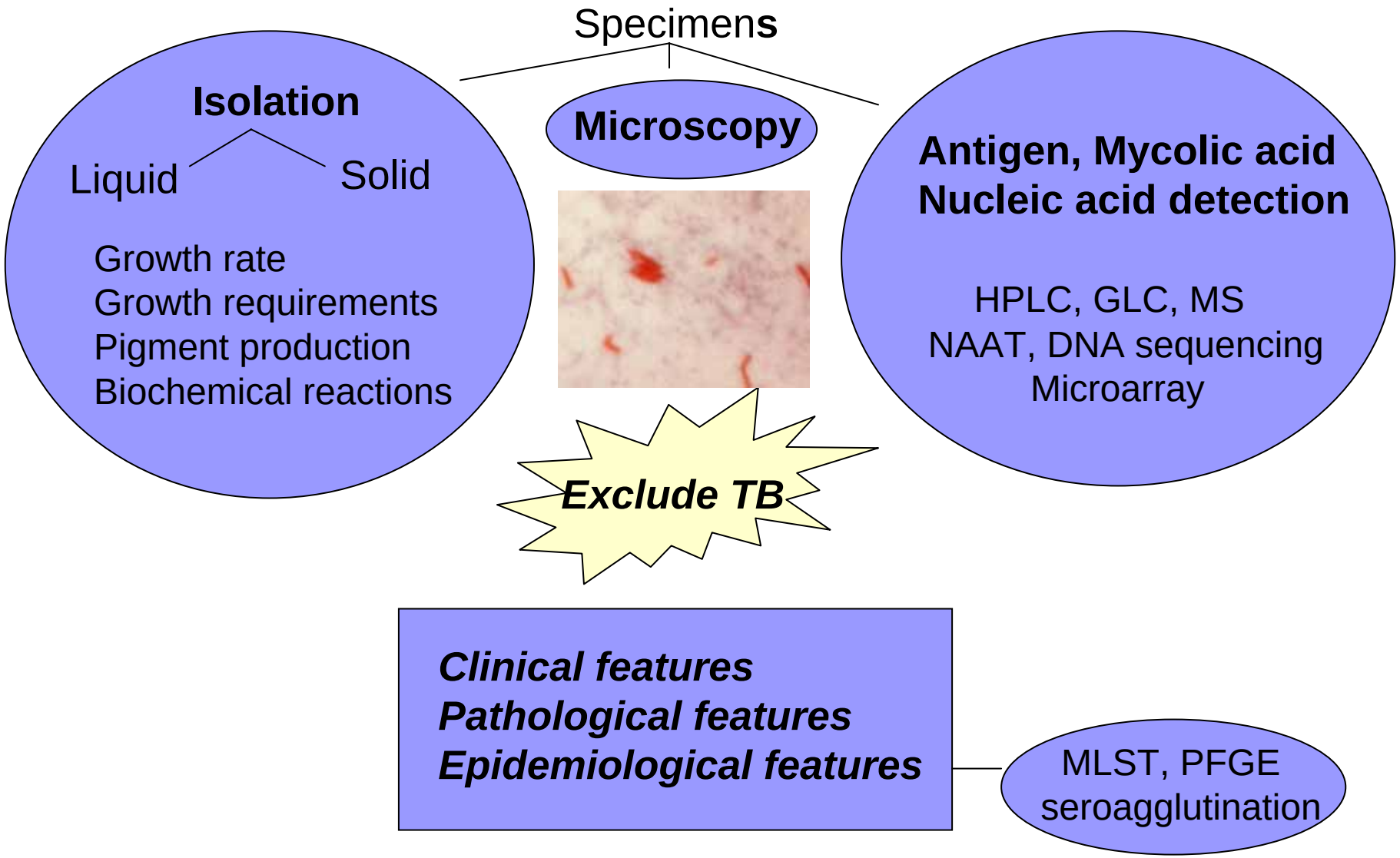
Epidemiological studies:

- 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




Thank You