

Mycobacteria

Part I

Introduction to Mycobacteria

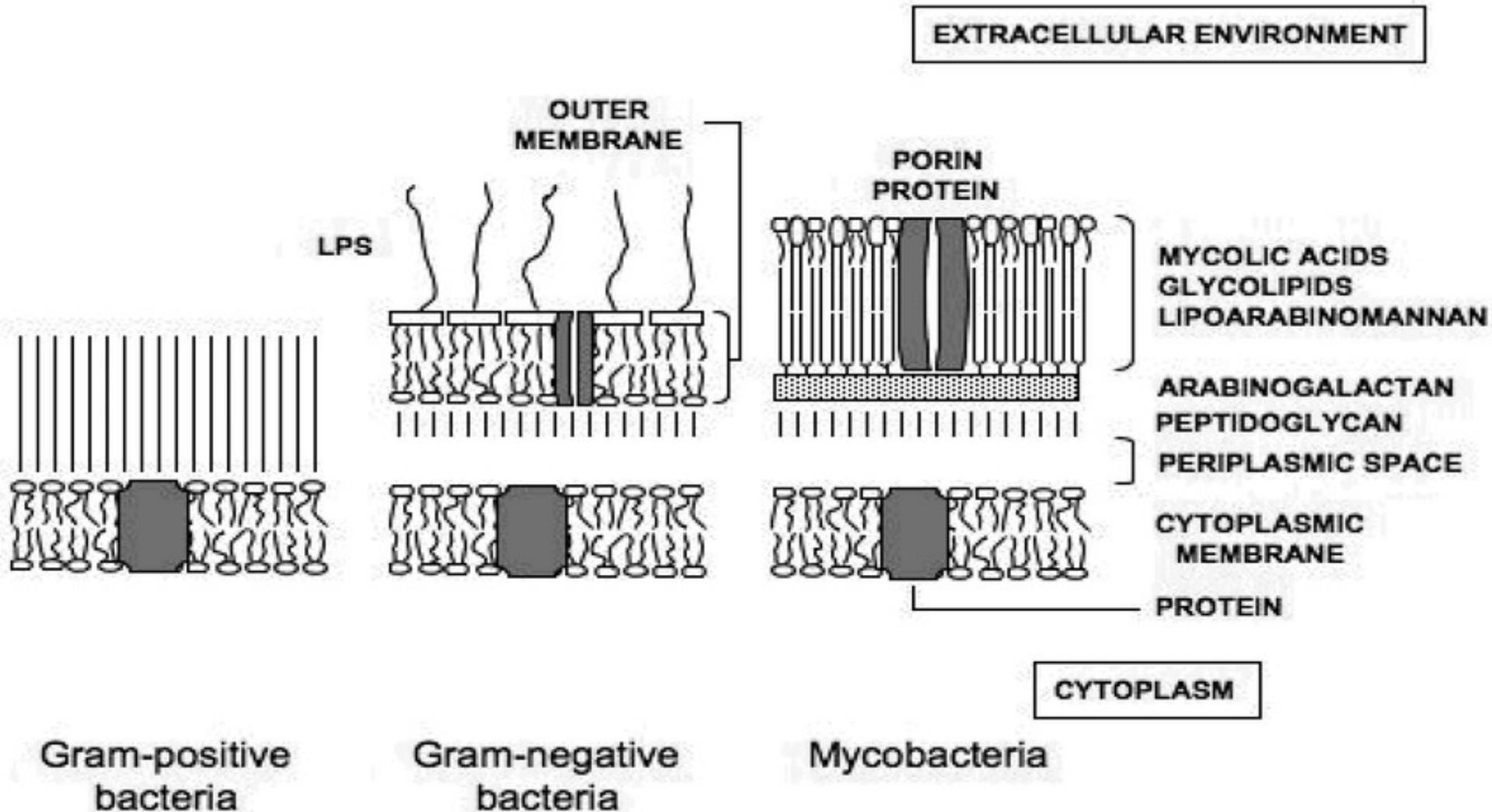
General characteristics

Healthcare safety and lab testing

Mycobacteria

- Over 190 species of mycobacteria
- Myco = fungus; so bacteria that grow in a mold like fashion
- Difficult to grow
 - May be slow or rapid growing mycobacteria
- Requires specialized media/conditions
- Acid fast staining due to mycolic acid in cell wall
 - Hydrophobic
 - Resistant to alcohols
 - Referred to as acid fast bacillus
- Some species *may* be able to form endospore

Differences between bacterial cell walls



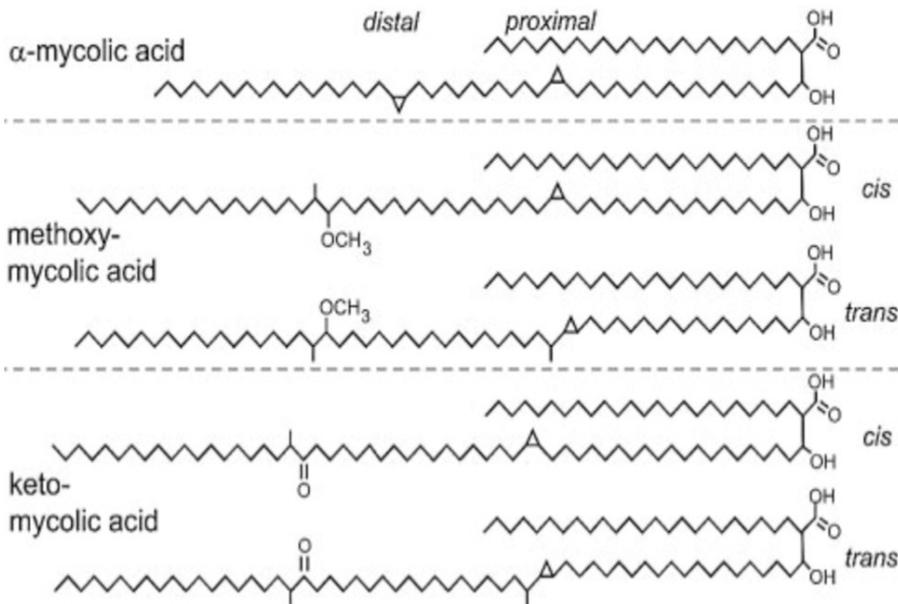
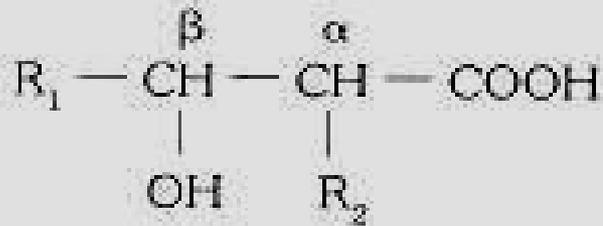
Mycobacterium Species

- Pathogens
 - *M. tuberculosis* – tuberculosis
 - *M. bovis*
 - *M. ulcerans*
 - *M. leprae* – leprosy = aka Hansen's disease
- Often Pathogen
 - *M. avium* complex
 - *M. kansasii*
 - *M. marinum*
 - *M. haemophilum*
 - *M. xenopi*
 - *M. genavense*
- Potential Pathogen
 - *M. abscessus*
 - *M. chelonae*
 - *M. fortuitum*
 - *M. malmoense*
 - *M. scrofulaceum*
 - *M. simiae*
 - *M. szulgai*

Mycobacterium Species

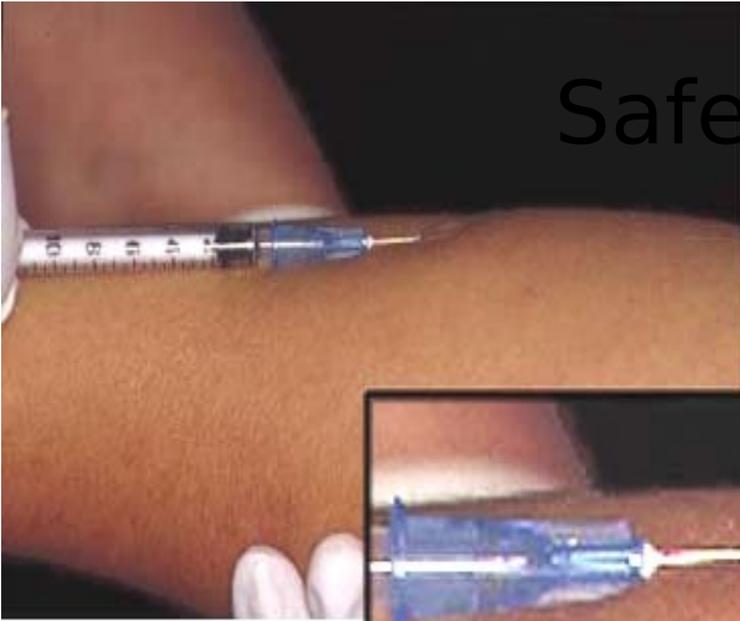
- Rare Pathogen (Saprophyte)
 - *M. gordonae*
 - *M. flavescens*
 - *M. gastri*
 - *M. nonchromogenicum*
 - *M. terrae*
 - *M. phlei*
 - *M. smegmatis*
 - *M. vaccae*
 - *M. thermoresistibile*

General Characteristics Acid Fast Bacilli



- Non-motile
- Non-spore forming rods
- High lipid content in cell wall – mycolic acids
 - Resists staining and decolorization
 - Acid Fast
- Slow growing aerobes

Safety



- Purified Protein Derivative (PPD) or tuberculin skin test (TST)
 - Monitor employees
 - Mantoux Test
 - Antigen injected intradermally
 - Read at 48 hours for a raised firm area 10mm or more.



Quantiferon Gold-TB Test

- Measures cell-mediated immune response to antigen
 - CD4 and CD8 activation
- Blood specimen
 - Pre-packaged kit
 - QFT-Plus = lithium heparin
 - Rapid transport is essential for testing
 - Room temperature



	Mitogen – Positive Control Low response may indicate inability to generate IFN- γ
	Nil – Negative Control Adjusts for background IFN- γ
	TB1 – Primarily detects CD4 T cell response
	TB2 – Optimized for detection of CD4 and CD8 T cell responses



Safety



- Requirements:
 1. Separate room from rest of the lab
 2. Negative pressure room
 - Nonrecirculating air
 - 6-12 room air changes/hour
 3. Face mask/respirators
 - Must be N-95 rated
 4. Gloves and gowns
 5. Caps and shoe covers
 6. Specialized centrifuges and hoods
 - All work is done under hoods
 - Non-aerosolized centrifuges
 7. Stronger disinfectants
 8. Electrical incinerators

Safety



Safety



- Splash proof discard containers
- Tuberculocidal disinfectants
 - Sodium hypochlorite (0.1-0.5%)
 - Phenol (5%)
 - Phenol-soap mix
 - Formaldehyde (3-8%)

Introduction to Mycobacteria

Sample requirements and Testing

General Processes

1. Receive sample
2. Emulsify the sample using NaOH
3. Centrifugate the sample to make a pellet
 - Discard supernatant
4. Neutralize the sample
5. Centrifuge
6. Add to liquid culture and solid slant media
 - Liquid – MGIT
 - Modified middlebrook and antibiotics that contain fluorophore
 - Takes 10-22 days
 - Solid – LJ agar
 - Incubated for 10 weeks
 - Takes about 30 days if positive
7. Stain for screening:
 - Acid fast and or Auramine phenol fluorescent stain

Positive Screening or Culture

- Prompt treatment
- Contact and respiratory isolations to protect others
- Contact tracing

Digestion and Decontamination

- Liquefy sample and kill non-mycobacterial organisms while sparing the mycobacteria
- Maintain a balance between recovery rate and contamination rate
 - 2-5% Contamination rate
- Things to consider
 - Agent and its concentration
 - Length of decontamination period
 - Centrifuge speed
 - Temperature

Digestion and Decontamination

- Sodium Hydroxide (NaOH) – more commonly used
 - Digests and decontaminates
 - Added to sample prior to testing
 - 2%, 3% or 4%
 - Slightly less harmful to mycobacteria than contaminants
- N-acetyl-L-cysteine (NALC) or Dithiothreitol
 - Liquefying agent only
 - Combine with the NaOH procedure
 - Use lower concentration of NaOH

Digestion and Decontamination

- Benzalkonium chloride (Zephiran) with trisodium phosphate (Z-TSP)
 - Digestion (TSP) and decontamination (Zephiran)
- Oxalic Acid (5%)
 - Specimens contaminated with *P. aeruginosa*

Neutralization, Centrifugation

- Phosphate buffer
- Use a pH indicator to see reaction is neutralized
- Spin at 3000xg to lower centrifugation time and lessen exposure to higher temperatures
- Centrifuge to concentrate
- Inoculate directly

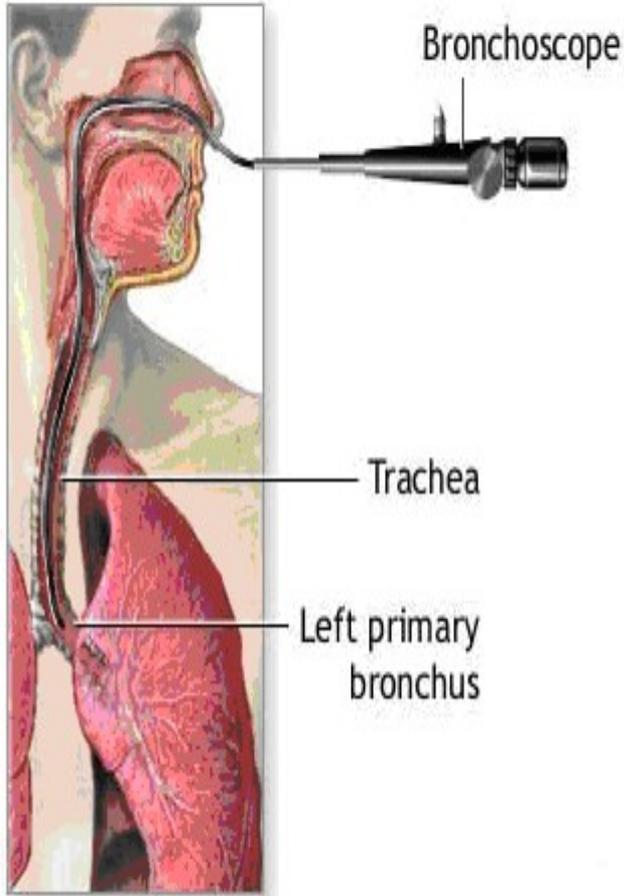


Specimens



- Collect before initiation of therapy
- Process immediately or refrigerate if delayed up to 24 hours

Respiratory Specimens



- Sputum
 - Deep cough
 - First morning
 - Set of three cultures collected 3 days (or every 8 hours)
- Bronchial Washing
- Bronchial Brushing
- Bronchoalveolar lavage (BAL)
- Leukens trap

Gastric Aspirate

- Children under 3 with pulmonary TB
- Neutralize immediately; how / why do we do this?
- First morning collection after fast
- Set of 3 cultures collected on 3 days

Specimens

- Urine
 - Entire volume of first morning midstream (15 ml minimum)
- Stool
 - Patients with HIV or at risk for HIV infection
 - Usually looking for MAC to determine risk of developing disseminated disease
- Tissue and Body Fluids
 - No swabs

Specimens



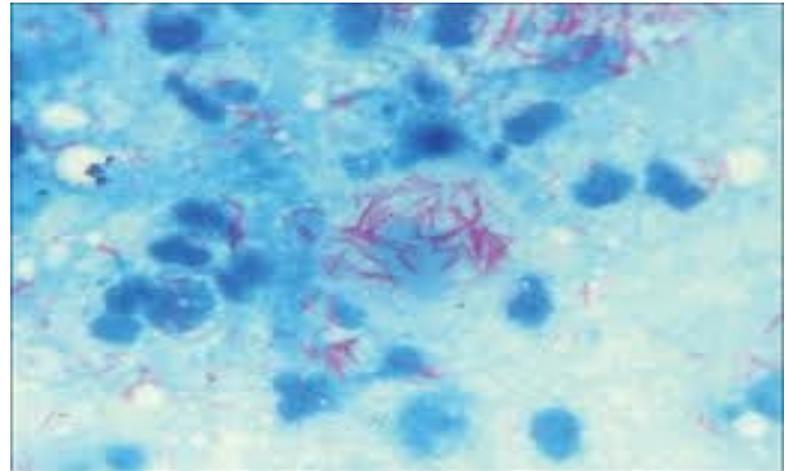
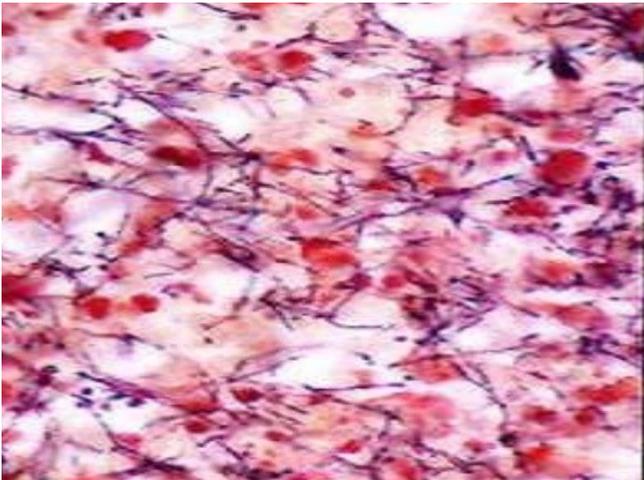
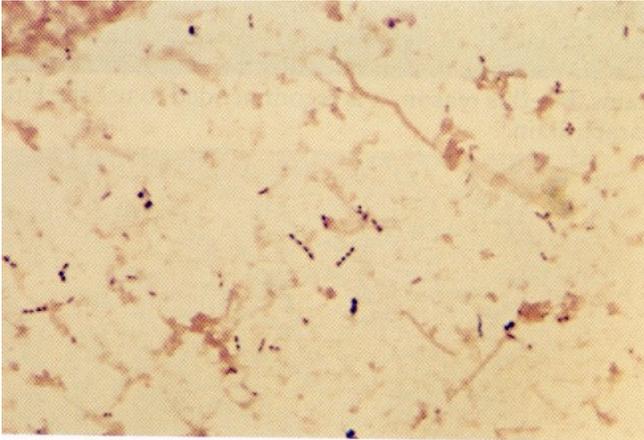
- Blood cultures
 - Usually MAC in HIV and immunocompromised patients
 - Isolator lysis centrifugation system
 - BACTEC

Smear Preparation

- Heat fix the slide
- Run QC with each batch of patient slides
- Common Testing:
 - Gram stain
 - Acid fast stain

Stains

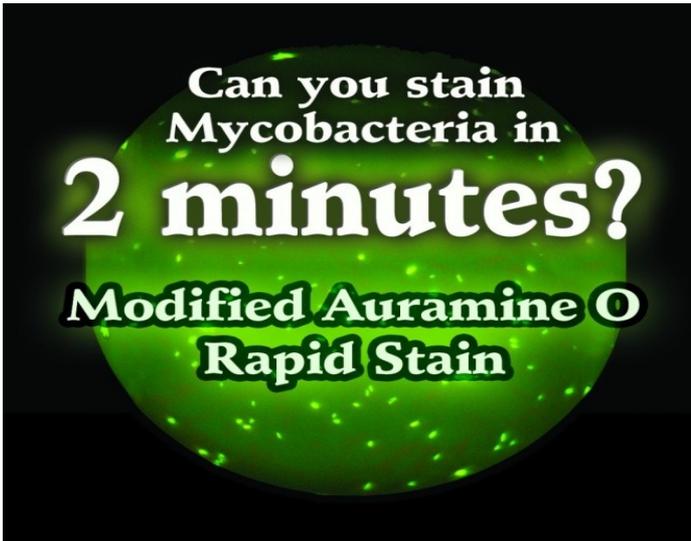
- Gram
 - Faint beaded rods
 - May be gram variable
 - May not stain
- Acid Fast Staining
 - Mycolic acid stains red
 - Everything else is blue



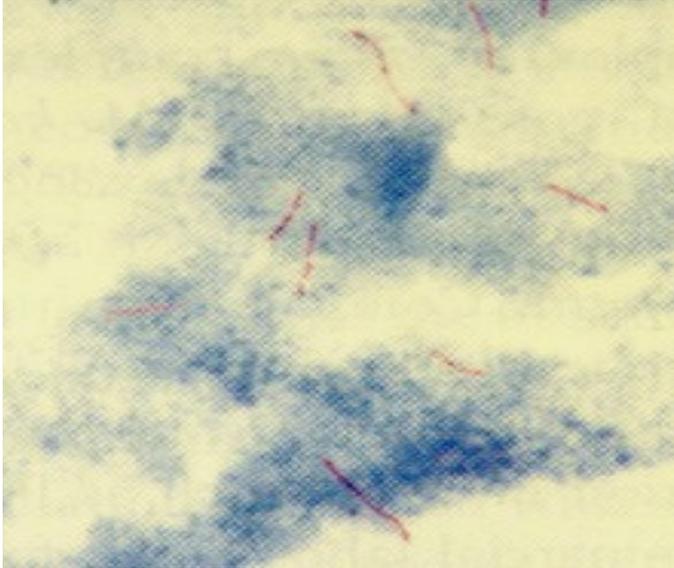
Stains



- Auramine-Rhodamine fluorochrome
 - Sensitive
 - Read under 250X-400X
 - Bright yellow-orange
 - Fluorochrome dye complexes with the mycolic acids in cell wall
- Useful screening test only, cannot make an ID



Stains: Acid fast

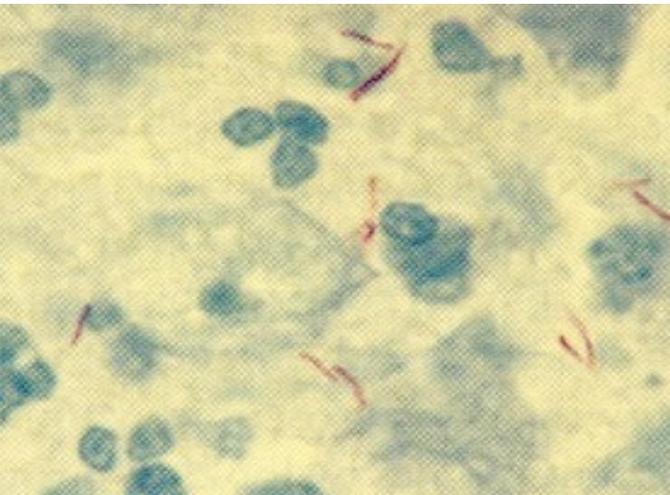


1. Ziehl-Neelson

- Carbofuchsin
- Acid Alcohol
- Methylene Blue
- Apply **heat**
- 300 fields under oil

2. Kinyoun Stain

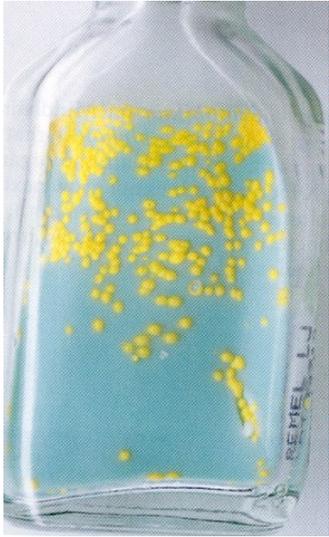
- Cold stain
- 300 fields under oil
- Carbol fuchsin
 - High concentration of phenol to fix slide



False Positive Smears

- Debris on slide
- Scratch on slide
- Nocardia spp, Rhodococcus spp
 - Gram positive filamentous rods
- *M. gordonae* from tap water
- Cross contamination

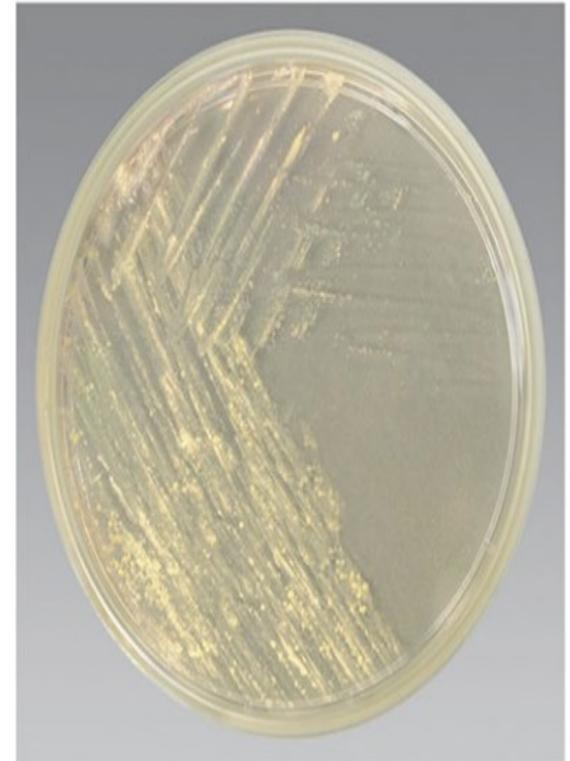
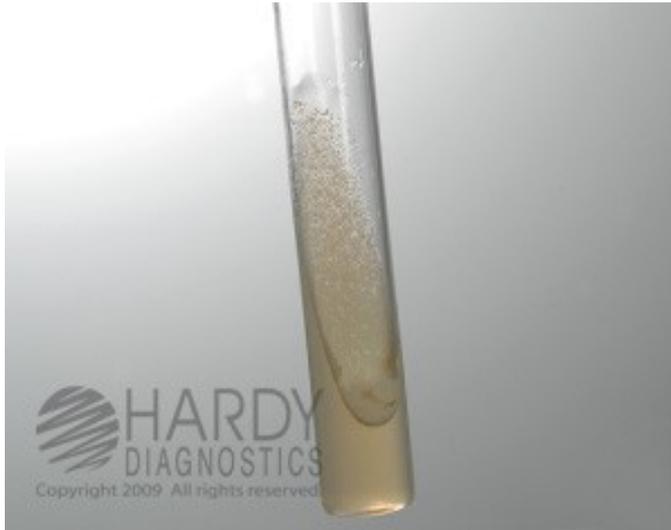
Solid Media



- Egg based:
 - Petragnani
 - American Trudeau Society Medium
 - **Lowenstein Jensen**
 - Malachite green suppresses gram positive bacteria
 - **Gruft**
 - LJ with antibiotic
- Chocolate Agar Slant
 - Incubated at RT for non-M.tb and nocardia ID

Media

- Serum albumin agar based
 - Middlebrook 7H10 and 7H11
 - Mitchison's selective 7H11



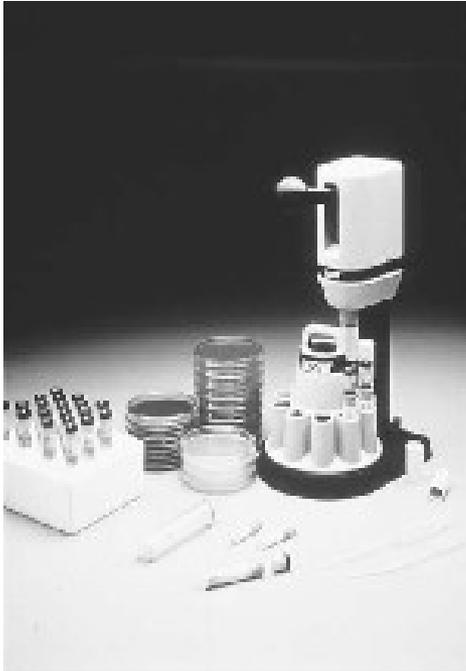
Liquid Media



- Middlebrook 7H9 and Dubos Tween Albumin
 - Mainly for subcultures
- BacT Alert, MGIT, BACTEC, etc
 - Monitored constantly for positivity by instrument
- MGIT testing
 - Lateral flow immunochromogenic assay to rapidly ID *M. tb* for drug susceptibility



Liquid Media



- Complex and time consuming
- Isolator Lysis-Centrifugation System
 - Allows quantitation in blood cultures
 - Uses saponin and SPS
 - Lyses RBCs
 - Ficoll density gradient separates cell layers
 - Extra enrichment for faster growth when subbed on plates

Media



- Biphasic
 - Middlebrook 7H9 broth and a paddle with 7H11, LJ and Chocolate
 - 5-8%CO₂
- Contains liquid and solid media for enhanced growth and detection

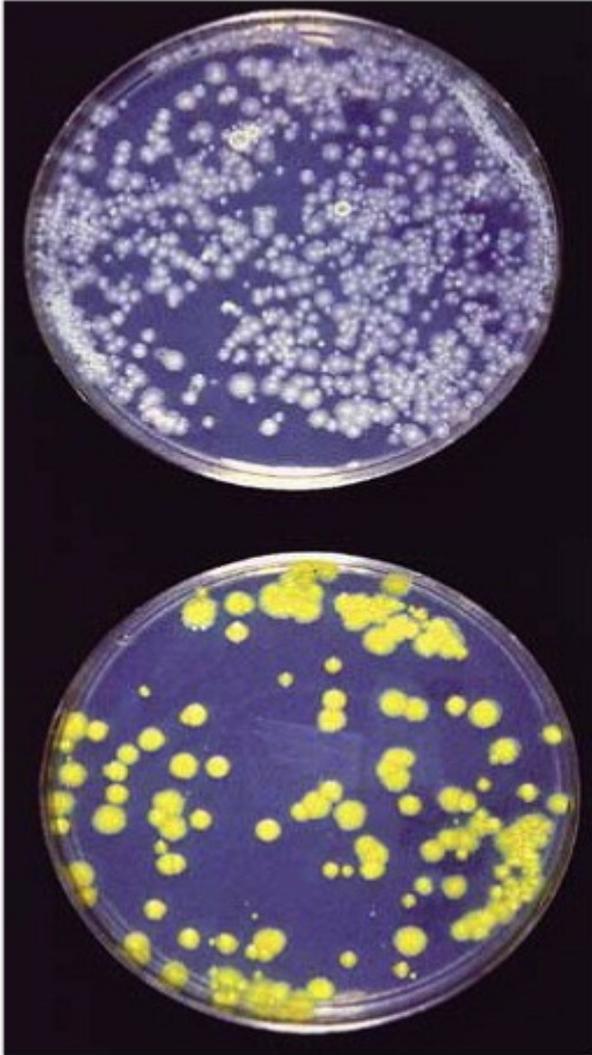
Identification

- Stain
 - Confirm organism is acid fast
 - Look at size
- Colony Morphology
 - Smooth or rough (friable), mucoid
 - Cording
 - Color (white, buff, yellow, orange)
 - Photoreactivity
 - Photochromogens
 - Non-photochromagens
 - Scotochromagens
- Growth Rate determined from time of subculture
 - From 3-60 days
 - <7 days are rapid growers
 - >7 days are slow growers

Identification

- Incubation length of time
 - 8 weeks
 - Tubes read weekly
- Temperature of best growth
 - 35-37° for most
 - *M. marinum*, *M. ulcerans*, *M. haemophilum* grow best at 30-32°C.
 - *M. zenopi* grows best at 42°C
- Biochemicals
 - Very slow- requiring several weeks
 - Use a panel of biochemical tests not just one

Photoreactivity



B

- Photochromogens
 - Produce carotene pigment after exposure to light
- Some mycobacteria are photochromogens and allows for differential ID

Photoreactivity



- Scotochromogens
 - Produce pigment in light or dark



- Nonchromogens
 - Buff in light or dark

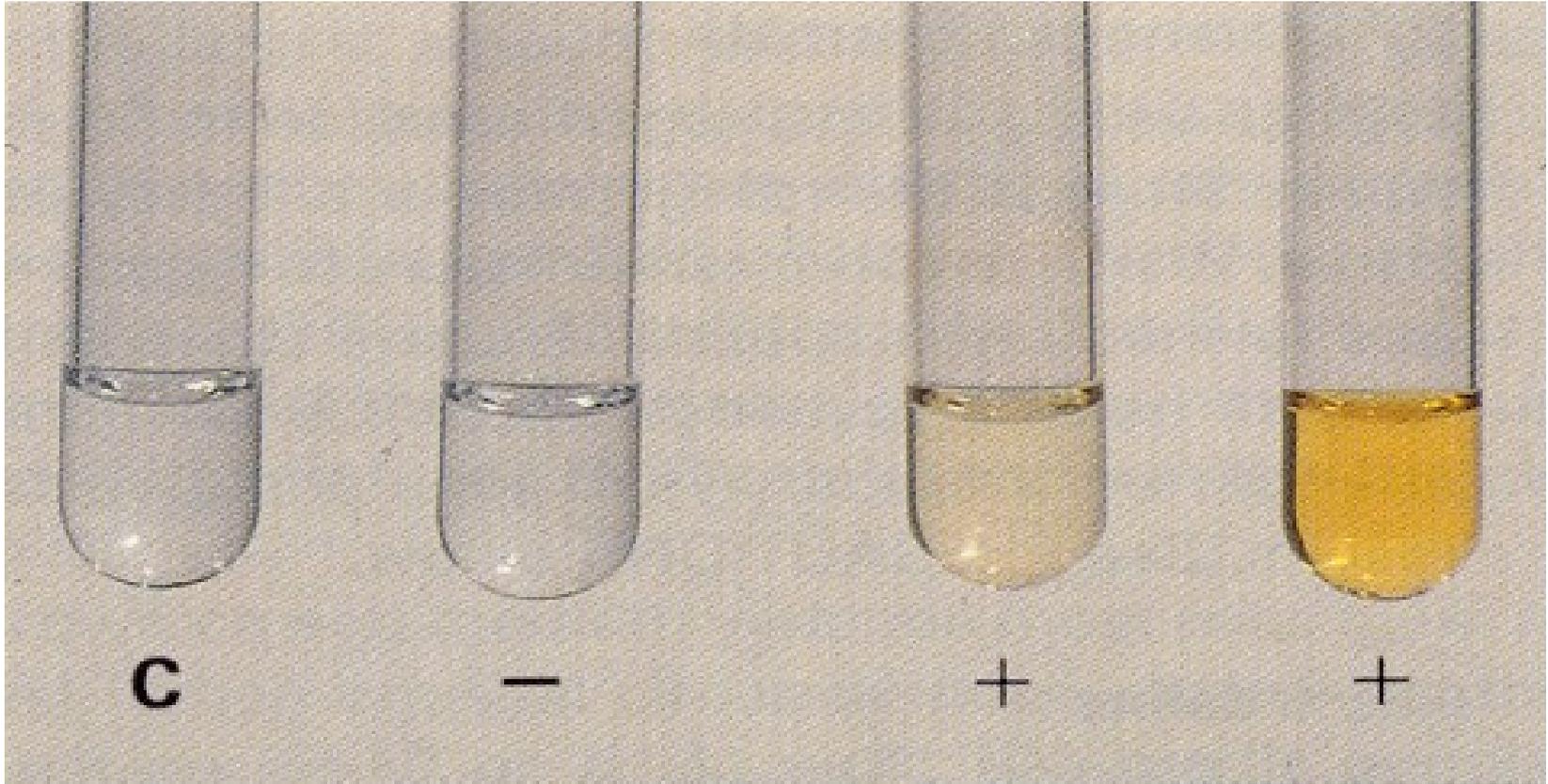
Non-tubercular Mycobacterium

Runyon Group	Species	Pigment Formation	Group/Complex
I	<i>M. kansasii</i>	Photochromogens	N/A
	<i>M. marinum</i>		
	<i>M. simiae</i>		
II	<i>M. scrofulaceum</i>	Scotochromogens	N/A
	<i>M. szulgai</i>		
	<i>M. gordonae</i>		
III	<i>M. avium</i>	Non-chromagens	MAC
	<i>M. intracellulerae</i>		
	<i>M. ulcerans</i>		
IV	<i>M. fortuitum</i>	Rapid growers	N/A
	<i>M. chelonae</i>		

Niacin Accumulation

- Most mycobacteria convert free niacin to niacin ribonucleotide
- *M. tuberculosis* does not
 - niacin (nicotinic acid) accumulates
 - Niacin positive
- Nicotinic acid reacts with cyanogen bromide in the presence of amine to form yellow color
- Use growth from egg agar 3-4 weeks old
- Helps rule in *M. tb* from other mycobacterium spp.
 - Most mycobacterium are negative for niacin testing

Niacin Accumulation



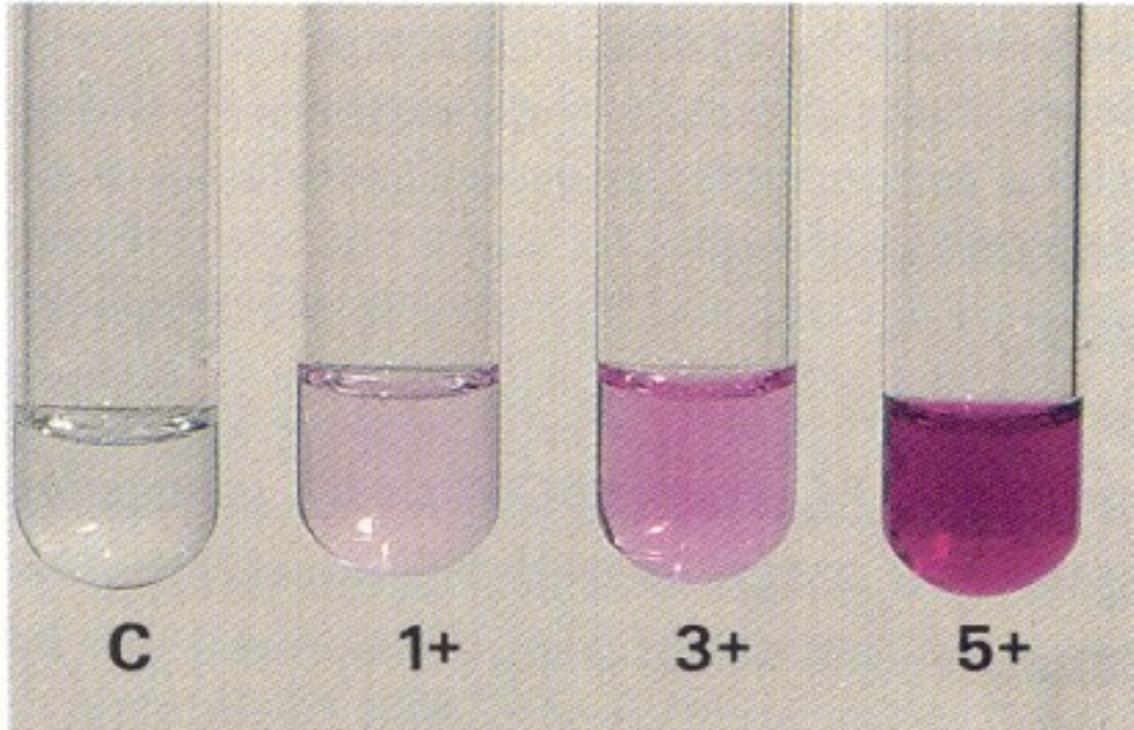
COLOR PLATE 11. Niacin chemical test.

Nitrate Reduction



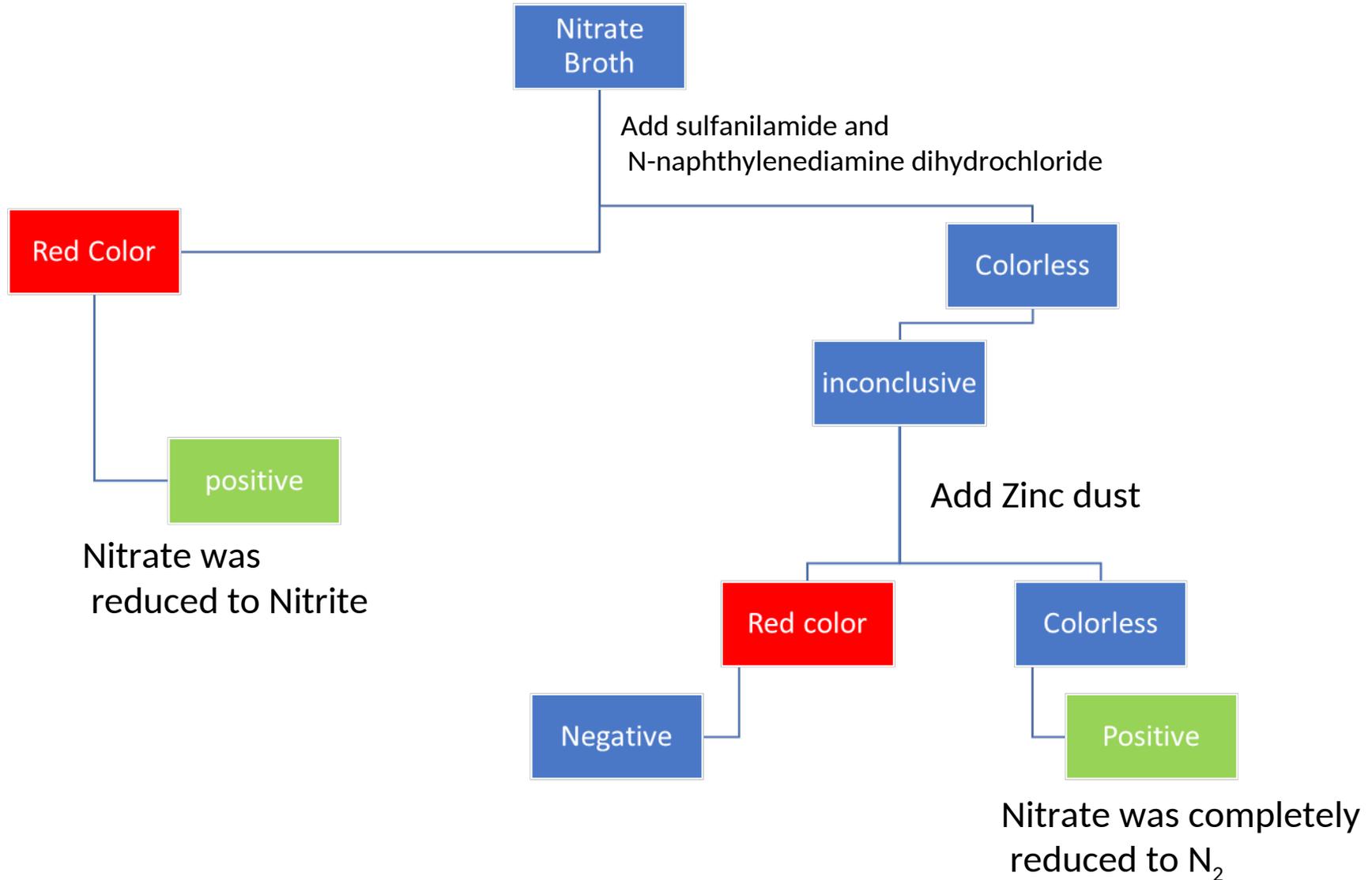
- Add sulfanilamide and N-naphthylenediamine dihydrochloride and look for color changes:
 - Red is positive = reduced
 - No color is inconclusive = inconclusive
- Add zinc dust
 - Red is negative (Nitrate is still present) = not reduced
 - No color is positive (Nitrate is not present) = reduced to N₂
- Strong positive for *M.tb*
- Negative for *M. bovis*

Nitrate Reduction



COLOR PLATE 13. Nitrate reduction test with liquid reagents.

Nitrate reduction flow chart

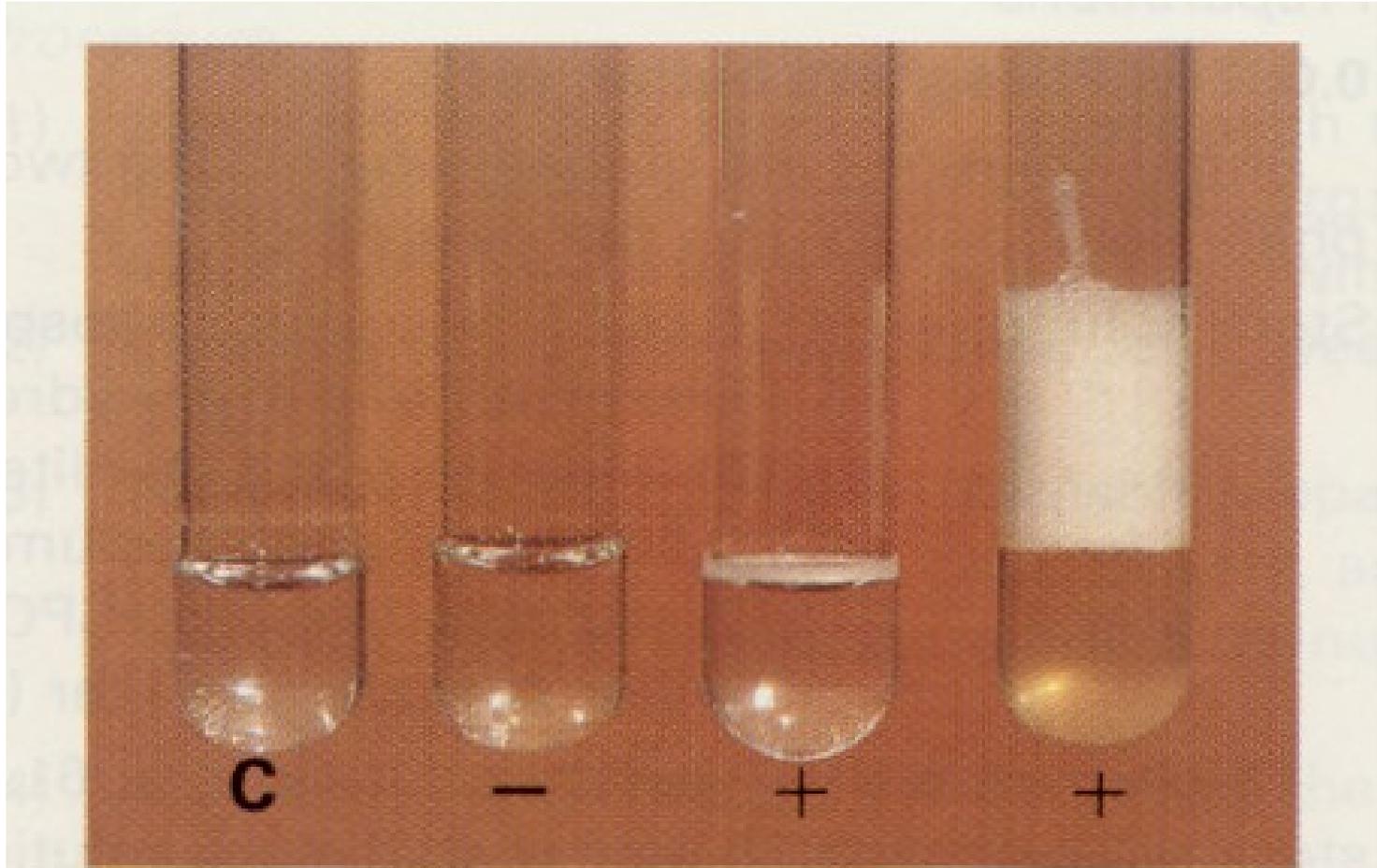


Heat Stable Catalase



- Almost all mycobacteria are catalase positive but not all are heat stable catalase positive
 - *M. tb* = negative
 - *M. kansasii* = strong positive > 45 mm
 - Other mycobacteria = variable positive < 45 mm
- Heat organism in phosphate buffer to 68°C for 20 minutes.
- Cool and add Tween 80 and Hydrogen Peroxide
- Check for 20 minutes before calling negative

Heat Stable Catalase

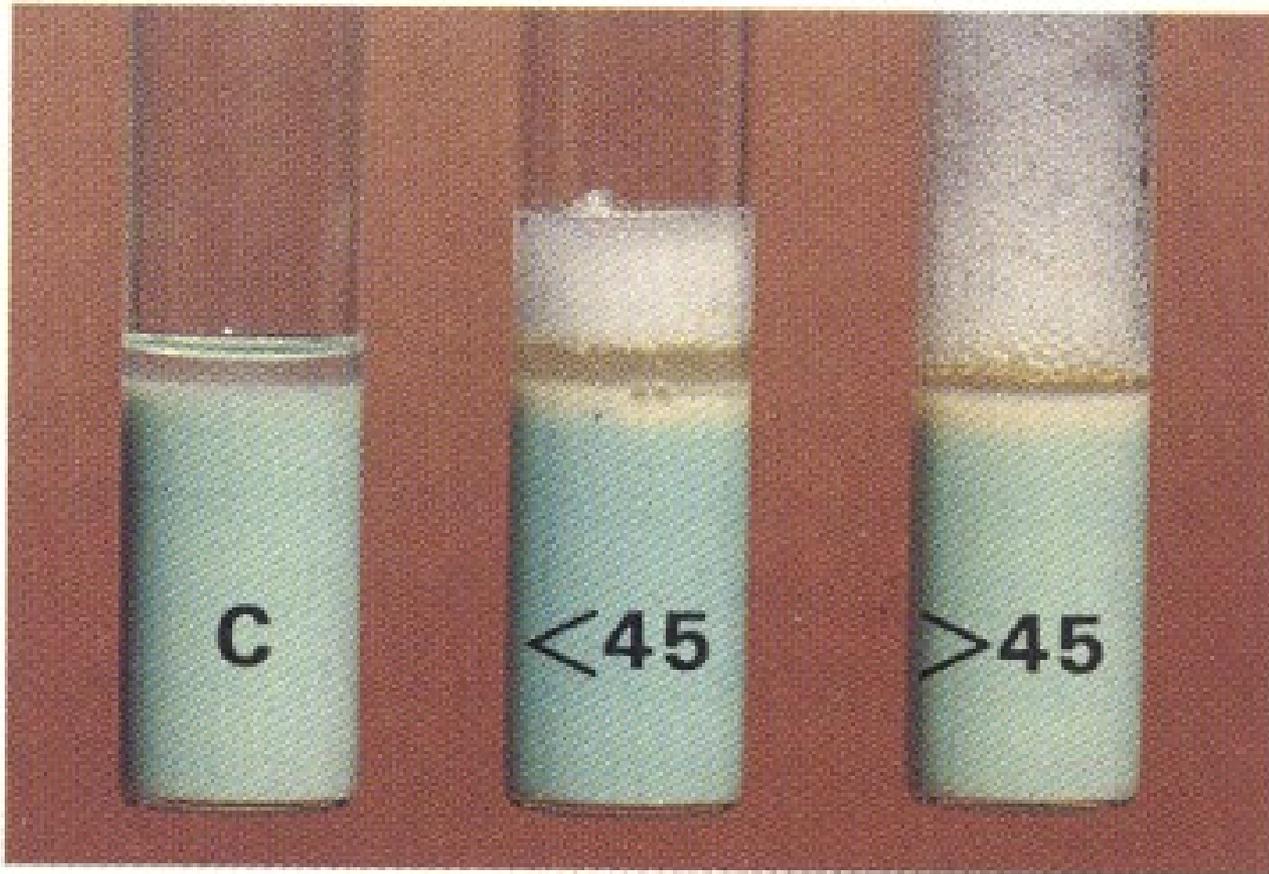


COLOR PLATE 8. Heat stable catalase test.

Semiquantitative Catalase

- Grow organism on LJ deep for 2 weeks
- Add Tween 80 and Hydrogen Peroxide
- Measure the column of bubbles after 5 minutes
- Record as > 45 mm or < 45 mm

Semiquantitative Catalase



COLOR PLATE 7. Semiquantitative catalase test.

Arylsulfatase

Arylsulfatase

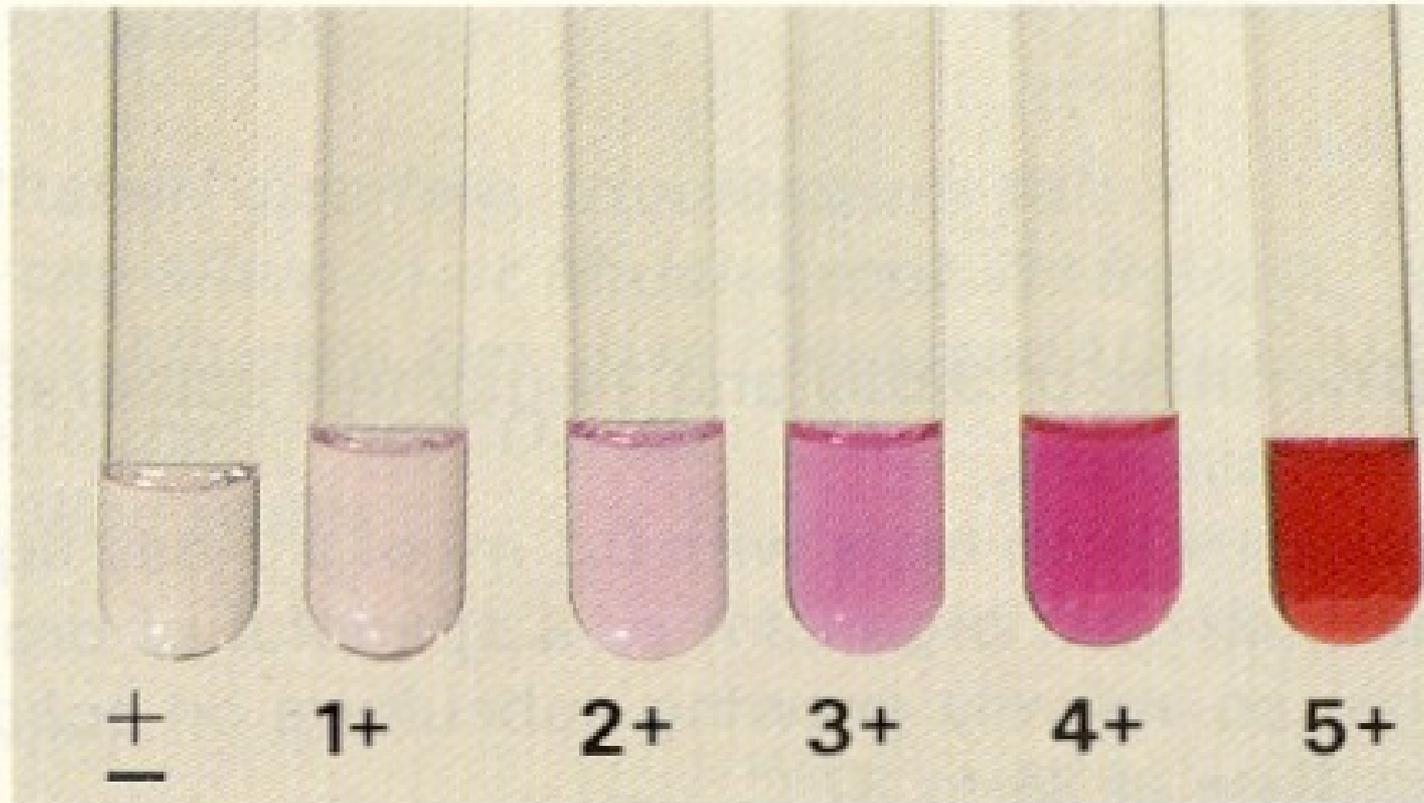
Tripotassium phenolphthalein disulfate

→

free phenolphthalein

- Add 2N sodium carbonate
- Red is positive
- Perform 3 day and 14 day tests

Arylsulfatase



COLOR PLATE 5. Color standards for arylsulfatase test.

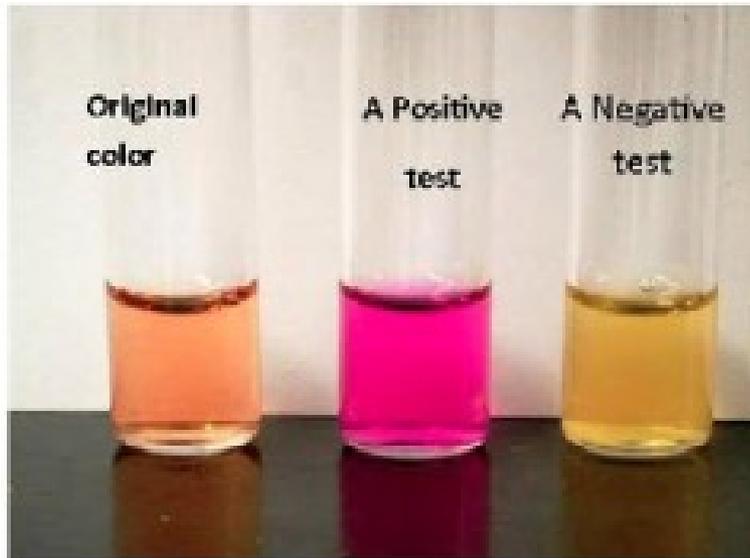
MacConkey without Crystal Violet



- Used for rapid growers

Urease

- Incubate at 37°C for 3 days



Iron Uptake

- AFB converts ferric ammonium citrate to iron oxide on egg based medium
- Add 20% aqueous ferric ammonium citrate
- Brown color is positive

Gas Liquid Chromatography (GPL) and High Pressure Liquid Chromatography (HPLC)

- The long chain fatty acids (mycolic acids) are extracted and run through a column
- Quantity and type are species specific
- Pros
 - Rapid identification
 - Highly reproducible
- Cons
 - Expensive equipment

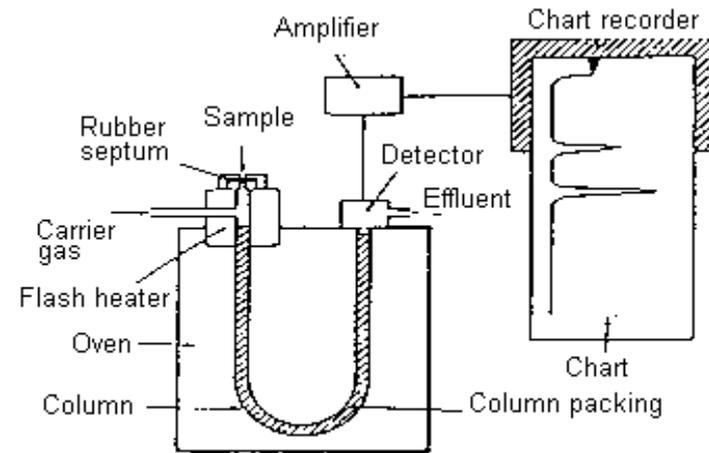


Figure 2. Gas Liquid chromatography

Identification

- DNA probes
 - Uses nucleic acid hybridization (DNA-RNA)
 - M. TB complex, *M. gordonae*, MAC, *M. kansasii*
- PCR
 - M. TB complex, *M. gordonae*, MAC, *M. kansasii*, *M. xenopi*, *M. chelonae*

Amplification tests from Direct Specimens

