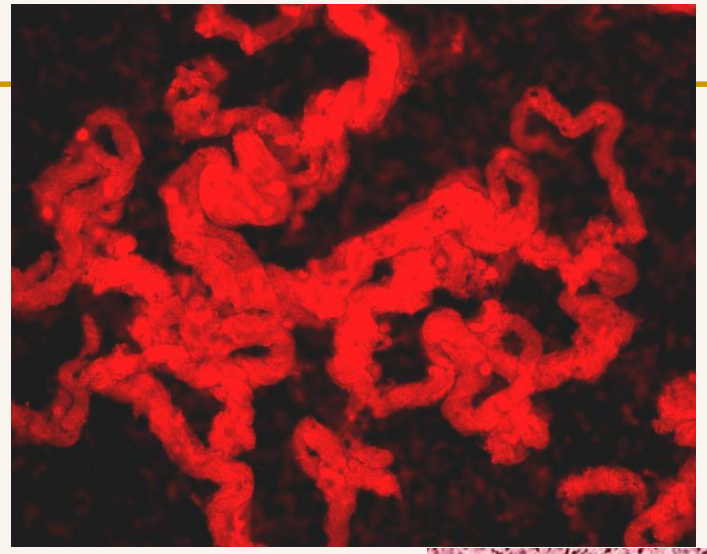
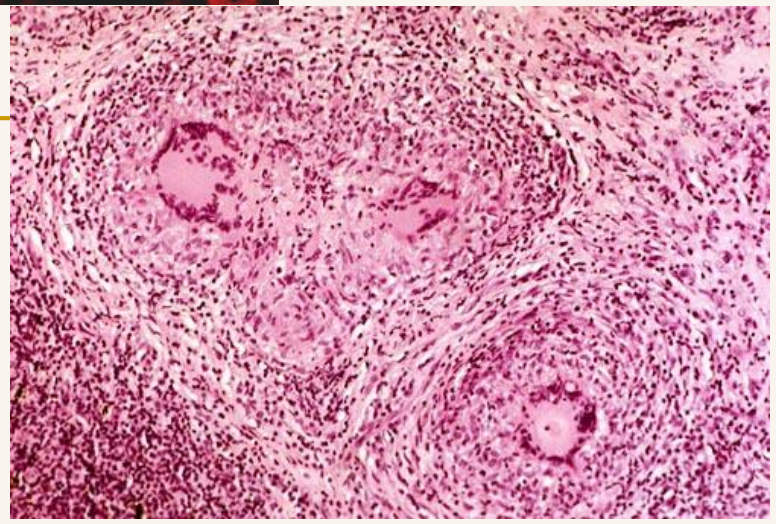
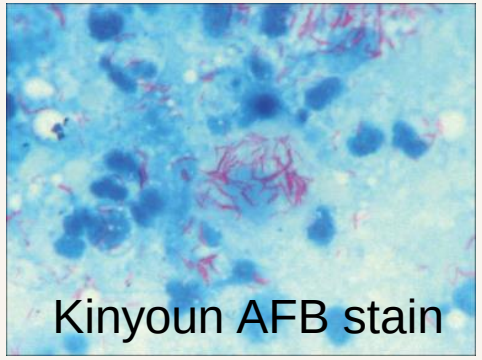
Mycobacteriology





Margie Morgan, PhD, D(ABMM)

Mycobacteria


Kinyoun AFB stain


Gram stain

- Acid Fast Bacilli (AFB)
  - Thick outer cell wall made of complex mycolic acids (mycolates) and free lipids which contribute to the hardiness of the genus
  - Acid Fast for once stained, AFB resist de-colorization with acid alcohol (HCl)
  - AFB stain vs. modified or partial acid fast (PAF) stain
    - AFB stain uses HCl to decolorize Mycobacteria (+) Nocardia (-)
    - PAF stain uses H_2SO_4 to decolorize Mycobacteria (+) Nocardia (+)
- AFB stain poorly with Gram stain / beaded Gram positive rods
- Aerobic, no spores produced, and rarely branch

Identification of the Mycobacteria

- For decades, identification started by determining the ability of a mycobacteria species to form yellow cartenoid pigment in the light or dark; followed by performing biochemical reactions, growth rate, and optimum temperature for growth. Obsolete
- With expanding taxonomy, biochemical reactions were unable to identify newly recognized species, so High Performance Liquid Chromatography (HPLC) became useful. Now also obsolete
- Current methods for identification:
  - **Genetic probes (DNA/RNA hybridization)**
  - **MALDI-TOF** Mass Spectrometry to analyze cellular proteins
  - **Sequencing 16 sRNA** for genetic sequence information

Mycobacteria Taxonomy currently >170 species

- **Group 1 - TB complex organisms**
  - *Mycobacterium tuberculosis*
  - *M. bovis*
    - Bacillus Calmette-Guerin (BCG) strain
      - Attenuated strain of M. bovis used for vaccination
  - *M. africanum*
  - Rare species of mycobacteria
    - *Mycobacterium microti*
    - *Mycobacterium canetti*
    - *Mycobacterium caprae*
    - *Mycobacterium pinnipedii*
    - *Mycobacterium suricattae*
    - *Mycobacterium mungi*
- **Group 2 - M**ycobacteria **o**ther **t**han **T**B complex ("MOTT") also known as the Non-Tuberculous Mycobacteria

Disease causing Non-tuberculous mycobacteria

(1) Slowly growing non tuberculosis mycobacteria

- *M. avium-intracullare complex*
- *M. genavense*
- *M. haemophilum*
- *M. kansasii*
- *M. malmoense*
- *M. marinum*
- *M. simiae*
- *M. szulgai*
- *M. ulcerans*
- *M. xenopi*
- *M. smegmatis*

(2) Rapid growing mycobacteria (growth in <= 7 days)

- *M. fortuitum group*
- *M. abscessus*
- *M. chelonae*
- *M. mucogenicum*

(3) Mycobacterium leprae

Mycobacteria that rarely if ever cause disease! If so, only in the immunocompromised!

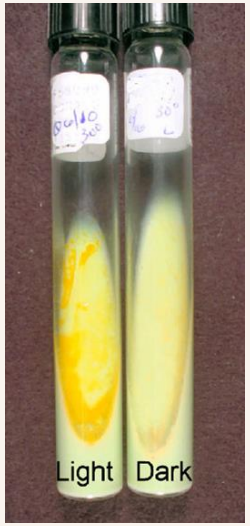
Slowly growing non-tuberculous mycobacteria

- *Mycobacterium gordonae*
- *M. gastri*
- *M. celatum*
- *M. scrofulaceum*
- *M. terrae complex*

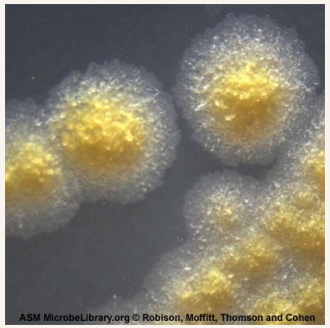
Identification of MOTT! The older way…..

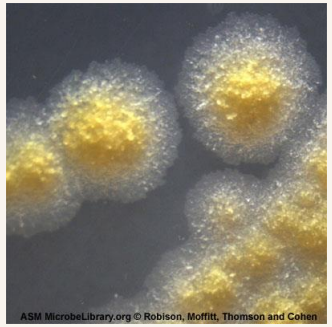
- **Based on the "Runyon System"**
  - Classification system for the non-tuberculous mycobacteria
  - Is a yellow carotenoid pigment is produced when exposed to incandescent light bulb plus the growth rate of mycobacteria species
- **Four Runyon groups**
  - **Photochromogen** = Pigment produced only when exposed to light
  - **Scotochromogen** = Pigment produced in both light and dark
  - **Non-photochromogen** = No pigment produced in either light or dark
  - **Rapid Grower** = Growth rate <= 7 days

The Light Test – Determine if mycobacteria can produce a yellow carotenoid pigment after being exposed to 8 hours of light/After light exposure, incubate for 24 hours and observe for presence of pigment.



Group I
Photochromogen
Turns yellow after light exposure





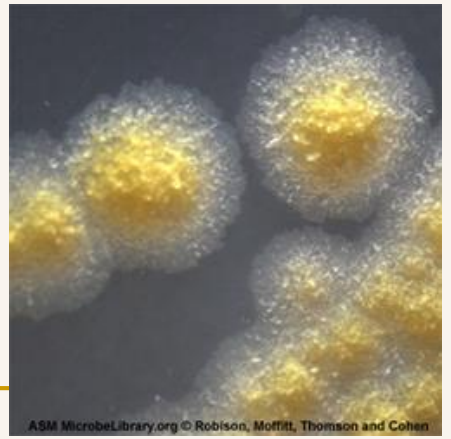
Group II Scotochromogen
Yellow pigment present in dark or with light exposure

Group III
Non-photochromogen
No pigment produced in dark or after light exposure

Runyon Classification System

- Group I - **Photochromogen** – turns yellow when exposed to light, no color in the dark. Growth in 2 -3 weeks
  - *M. kansasii*
  - *M. simiae*
  - ***M. szulgai* photochromagen when incubated at 25˚C***
  - *M. marinum*

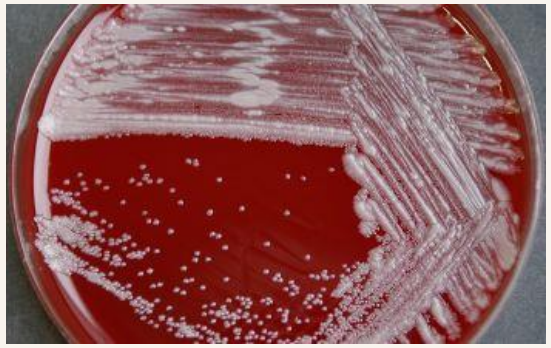
- Group II - **Scotochromogen** – yellow pigment in dark or exposure to light. Growth 2 – 3 weeks
  - *M. gordonae*
  - *M. scrofulaceum*
  - *M. xenopi (most strains)*
  - ***M. szulgai* scotochromogen when incubated at 37°C***

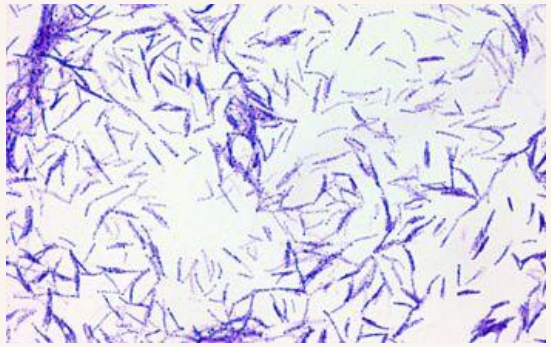


Runyon Classification continued

- Group III – **Non-photochromogen –** No pigment produced in the light or dark, growth in 2-3 weeks
  - *M. avium-intracellulare complex*
  - *M. haemophilum*
  - *M. xenopi (some strains)*

- Group IV – **Rapid growers,** grow in <=7 days
  - *M. fortuitum group*
  - *M. abscessus*
  - *M. chelonae*
  - *M. mucogenicum*
  - *M. smegmatis*





AFB Laboratory Safety

N95 Mask


PAPR

Biosafety Cabinet

Vented to outside

Level 3 biosafety precautions required in AFB laboratories that process, identify and perform susceptibility testing on mycobacteria species

- Level 2 Hepa filter biosafety cabinet with return air vented to the outside
- Biosafety certified at least yearly per to insure proper operations

- Laboratory requires negative air flow
- Anteroom for safety gear donning
- Contiguous autoclave
- N95 respirator mask (yearly mask fit) or PAPR (powered air purifying respiratory mask)
- Gloves and disposable surgical gowns for culture work

Specimen collection

- Sputum – 3 early morning collections, or
  1 early morning, plus 2 collected at least 8 hours apart
- Bronchial lavage fluid
- Tissues or Lesions
- CSF / sterile body fluids
- Urine – 3 to 5 early morning collections
- Stool – primarily for M. avium complex
- Gastric – Children unable to produce sputum,
  must neutralize gastric to pH 7.0 after for AFB to survive
- Blood /Bone marrow in disseminated disease
  - Automated systems with culture bottles
    manufactured specifically for AFB detection

AFB Specimen Processing for potentially contaminated specimens (Sputum)

- 5 ml of specimen pipetted into conical Falcon tube
- Decontaminate and liquify sputum specimen for 15 minutes with:
  - 5 ml of 4% NaOH, increases the pH to 9, and kills contaminating bacteria
  - N-acetyl-L-Cysteine to liquify mucus
- Neutralize with phosphate to pH 7.0
- Centrifuge for 30 minutes using safety cups with tight lids
  - 3000 X g to pellet the AFB
- Pour off the supernatant
- Pellet used to prepare slide for AFB staining
- Dilute the pellet with small amount of sterile saline for culture
- Incubate culture media @ 37˚C, 5-10% CO2 for 6–8 weeks

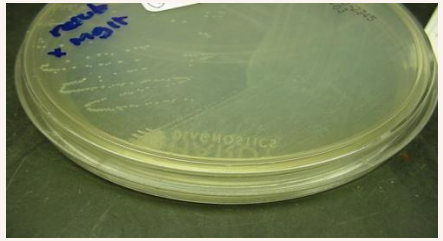
Specimen Decontamination/Digestion of potentially contaminated specimen types/ special circumstances

- Processing specimens from cystic fibrosis patients
  - Decontaminate sputum with oxalic acid to eliminate mucoid strains of *Pseudomonas aeruginosa*
    - *4% NaOH* will not kill mucoid strains
    - Oxalic acid should not be used routinely for processing all specimens, will decrease yield of AFB in culture
    - Both 4% NaOH and Oxalic acid can kill AFB if left on specimen > 15 minutes.

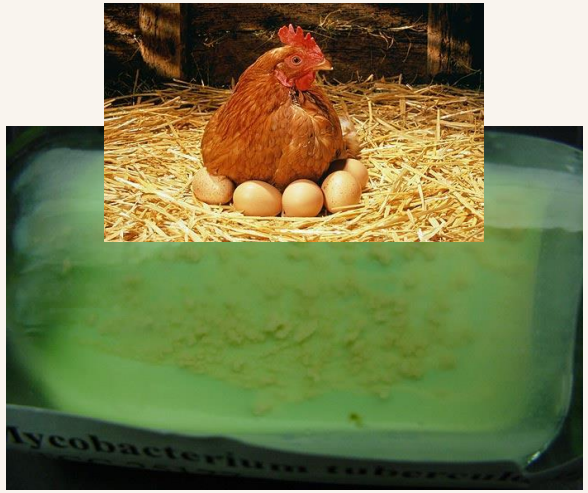
Specimen Centrifugation

- Centrifugation at 3000 X g (high speed) with safety cups
- Speed of centrifugation is important
  - AFB are lipid laden and they will float if not rapidly centrifuged
  - Pellet AFB so they are not decanted with supernatant
  - Sensitivity of the AFB stain and culture dependent upon proper speed of centrifugation

Manual Plating/Culture Media



- **Middlebrook** – Synthetic media with optimal chemical ingredients
  - Clear colored solid and liquid media
  - Used for both culture and susceptibility testing
  - Autoclave to sterilize the media
- **Lowenstein-Jensen** – Egg based with glycerol and flour
  - Solid agar, green due to addition of malachite green
  - Culture media only
  - Sterilize by inspissation – drying



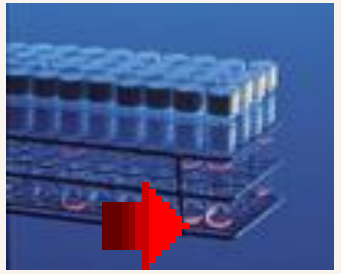
- Cultures incubated at 37˚C , 5-10% $C0_2$ for 8 weeks

Automated Detection of AFB

BD BACTEC MGIT 960 automated instrument for AFB

- Middlebrook 12B liquid media with growth indicator in the culture tube
- BACTEC detection method
  - As AFB grow in the 12B media, the AFB respire CO_2 and the amount of O_2 is decreased over time. The lower level of O_2 causes fluorescence of the indicator at the bottom of the tube and indicates organism growth in the tube.
- Incubation at 37˚C for 6 weeks

Fluorescence



MGIT 960

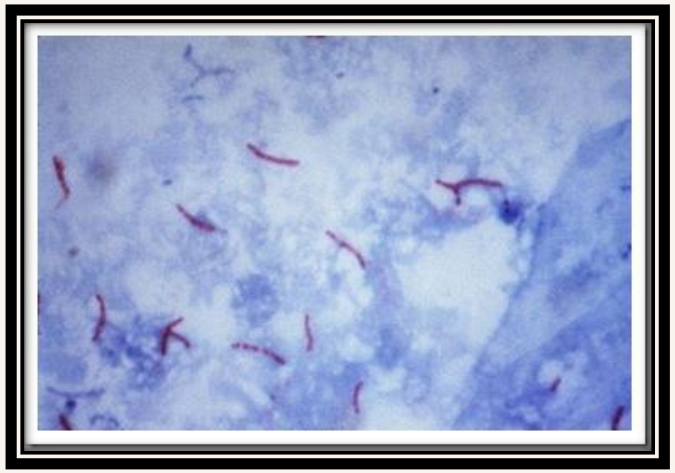
- **NAP** test for identification of TB complex organisms using MGIT

  NAP = chemical (p-nitro-α-acetylamino-B-hydroxypropiophenone)

  TB complex does not grow in the tube containing NAP

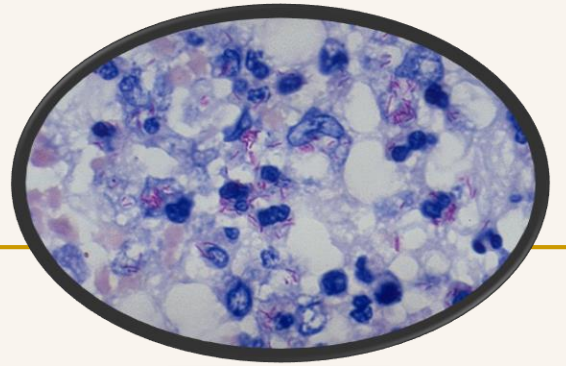
  Non-tuberculous species grow in the NAP chemical

Acid Fast Stains for Mycobacteria



- Carbol Fuchsin stains
  - Carbol Fuchsin is red colored primary stain
  - Potassium permanganate is the blue counterstain

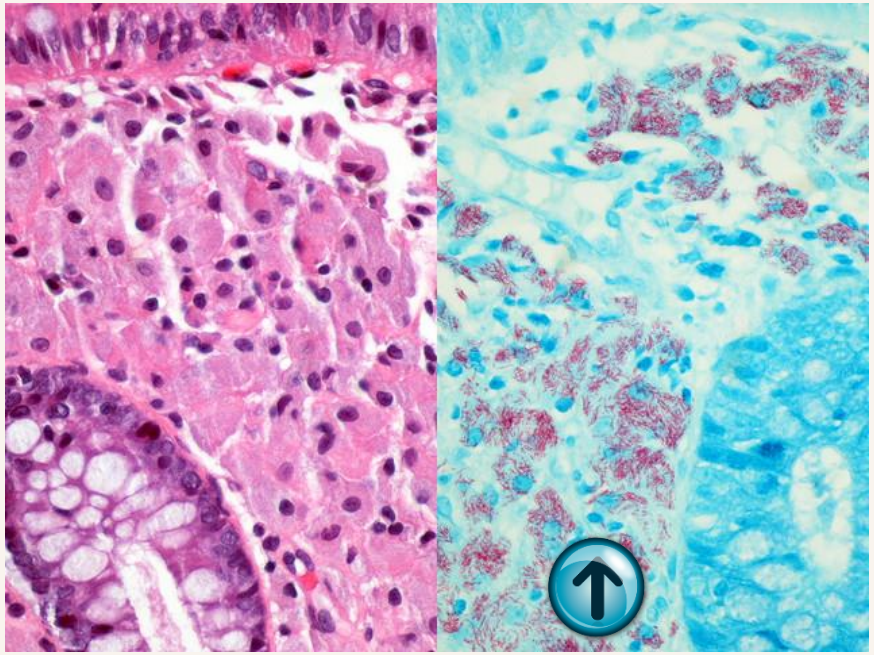
- Two methods:
  - Ziehl-Neelsen (ZN) – heat used to drive stain into lipid laden AFB
  - Kinyoun – High % of phenol in stain drives stain into AFB
- Read numerous microscopic fields for 5 minutes, using light microscopy and 100x oil objective



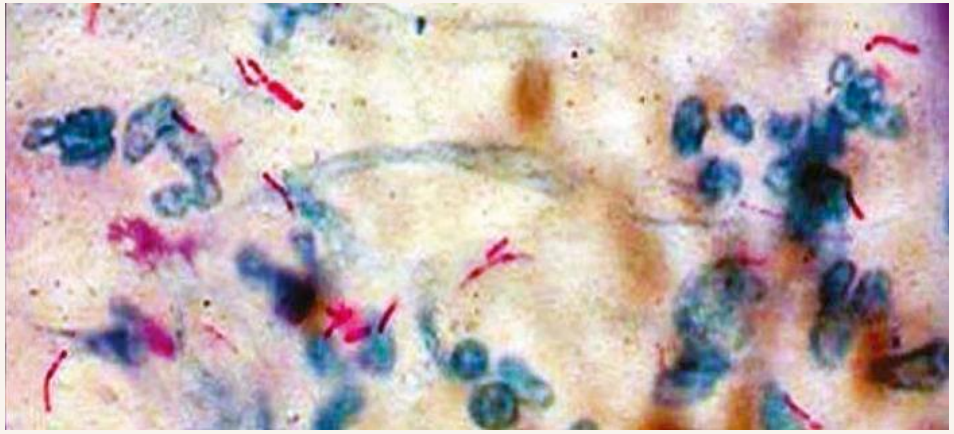
Fluorochrome stain

- Auramine Rhodamine
  - Fluorescent stain
  - Rods stain fluorescent yellow with black background
  - Read on 25X or 40X for 2 min, viewing numerous fields using a fluorescence microscope
  - Nonspecific fluorochrome binds to the mycolic acids present in the mycobacteria cell wall
- Considered more sensitive than ZN or Kinyoun for concentrated patient specimen slide examination

Acid Fast Mycobacteria morphology



Mycobacterium avium complex
Organisms are short rods without cording. The organisms tend to randomly clump.



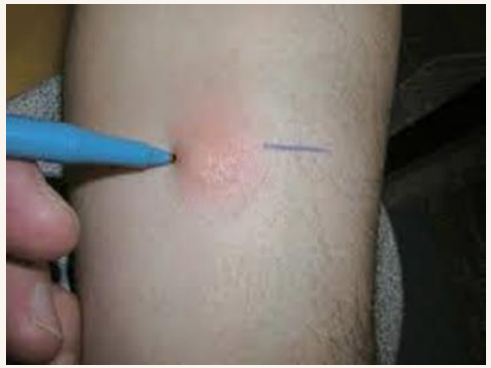
M. tuberculosis - Organisms are long rods and can appear as if they are sticking together [due to cord factor]

In broth cultures - ropes of AFB can form due to cord factor

Direct Detection of TB complex from Respiratory Specimens using Molecular Amplification

- FDA cleared assay that detects TB **complex** organism DNA in sputum
  - Cepheid Xpert-TB RIF Assay (rtPCR)
  - Detects TB complex organism gene sequence and rifampin resistance gene (rpoB)
- Sensitivity of assays
  - 99% for AFB concentrated smear (+) specimens
  - 75% for AFB concentrated smear (-) specimens
- Test of diagnosis, not cure
  - Residual rRNA and DNA can be present for up to 6 months after initial start of therapy
- AFB culture and sensitivity must always be performed

PPD (Purified Protein Derivative) Mantoux test or TB skin test

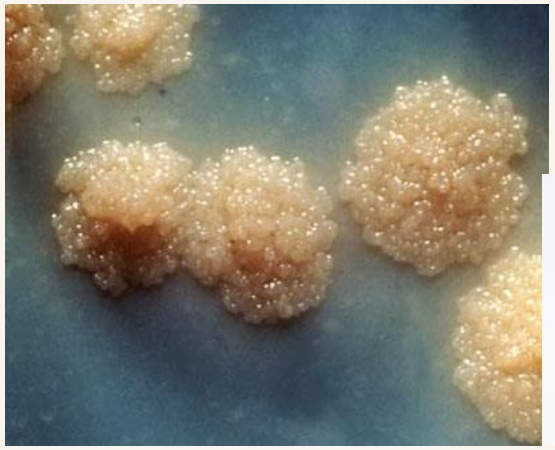


- Detects latent or current TB complex exposure
- False positive reactions can occur in patients immunized with BCG
  - BCG = Bacillus of Calmette-Guerin (M. bovis related strain)
- 25% false negative reactions
  - Usually patients with low T cell numbers or T cell reactivity
  - Technical problems with PPD administration and subjective test interpretation can lead to false negative or false positive reactions
- Measures delayed hypersensitivity (T cell response) to TB complex antigens (not specific for TB)
  - Measure (mm) area of induration at injection site
    - >=15mm positive (check for history of BCG)
    - >=10mm positive in immune suppressed or just exposed to TB and part of an outbreak investigation

Cell Mitogen assays – Interferon Gamma Release Assays (IGRAs)

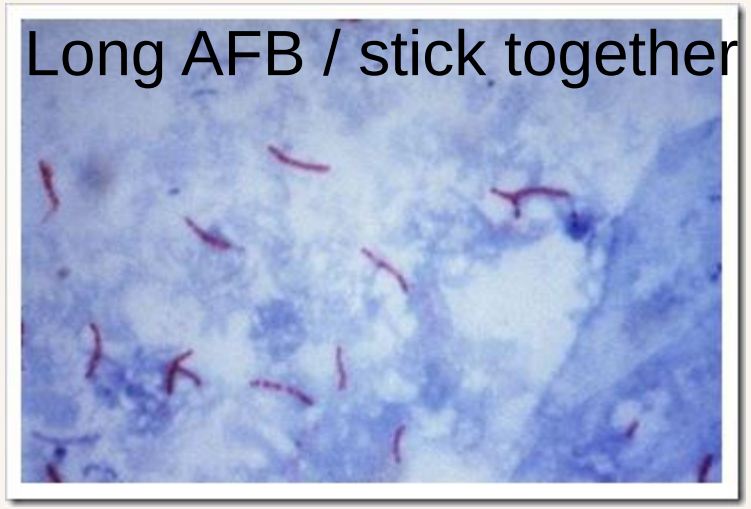
- QuantiFERON-TB-Gold Plus (QFTP)
  - CD4 and CD8 T cells in patient whole blood samples are stimulated with TB specific antigens
  - If patient has active or latent exposure to TB, the stimulated T cells will produce gamma interferon
  - Automated EIA assay measures gamma interferon produced
  - Quantitative endpoint determines a positive or negative reaction
  - Indeterminate reactions can occur if T cells are absent or inactive due to medications
- No false positive reactions from immunization with BCG
- Sensitivity >=80%, does NOT replace culture for disease diagnosis
- Sensitivity similar to PPD/ but improved specificity

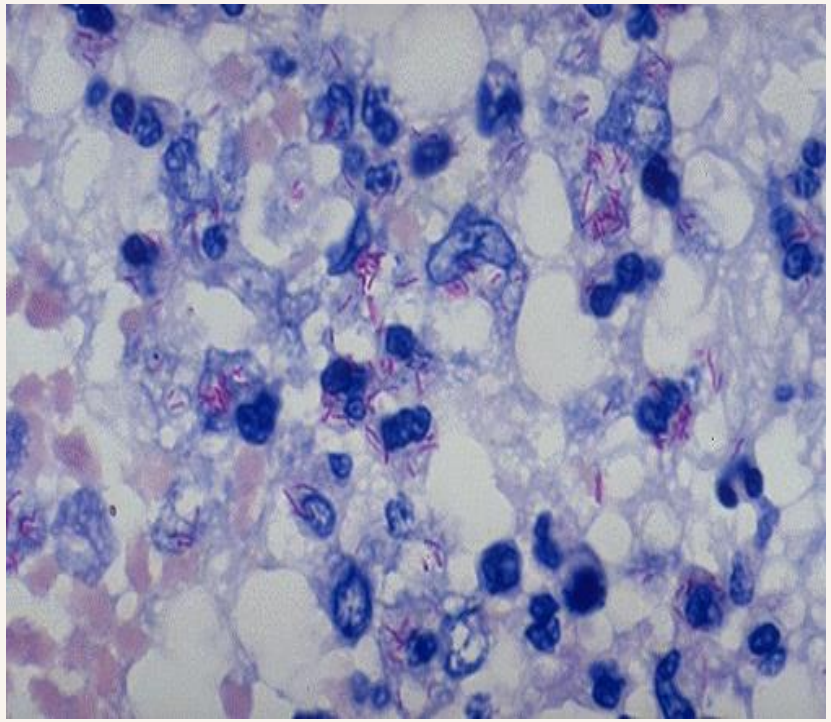
Mycobacterium tuberculosis



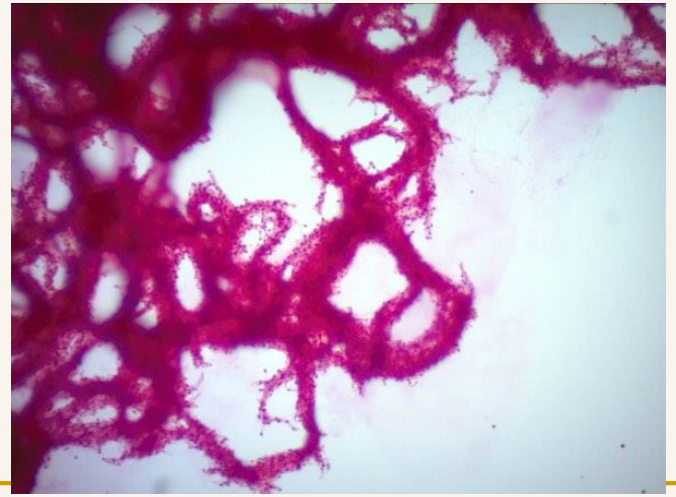
- Optimal Temp 37˚ C, Growth in 12 –25 days
- Buff colored, dry cauliflower-like colony
- Manual tests for identification – old school
  - Niacin accumulation test positive
    - Niacin produced from growth of TB on egg containing medium (LJ)
  - Nitrate reduction test positive
- **Current identification methods:**
  - Molecular DNA/RNA hybridization probe for MTB complex
  - MALDI-TOF mass spectrometry for M. tuberculosis
  - 16 sRNA sequencing for M. tuberculosis

Mycobacterium tuberculosis



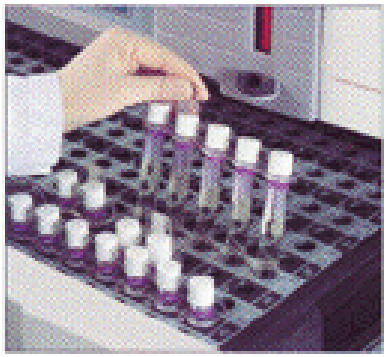


Cord factor – Due to high lipid content in cell wall, rods stick together in direct patient smears and develop long ropes when grown in broth media – this feature is unique to *M. tuberculosis*



Susceptibility testing of TB

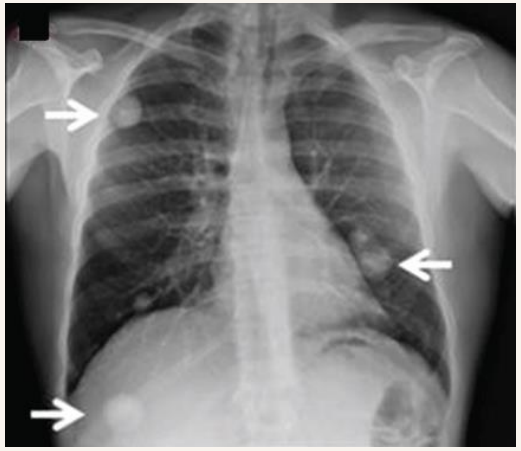
- Liquid 7H12 medium containing anti-TB antibiotic solutions
  - Tested on automated BACTEC MGIT 960 system
- Primary TB drug panel / 5 drugs
  - Isoniazid
  - Pyrazinamide
  - Rifampin
  - Ethambutol
  - Streptomycin



- If resistant to primary drugs, second line drugs tested
  - Fluoroquinolones, Kanamycin, Amikacin, and Capreomycin
- If patient remains culture positive after four months of treatment, the isolate should be retested for possible drug resistance

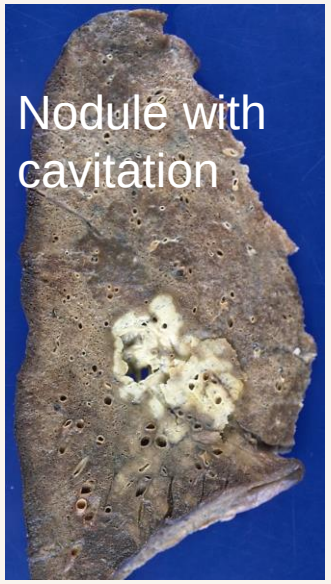
Tuberculosis

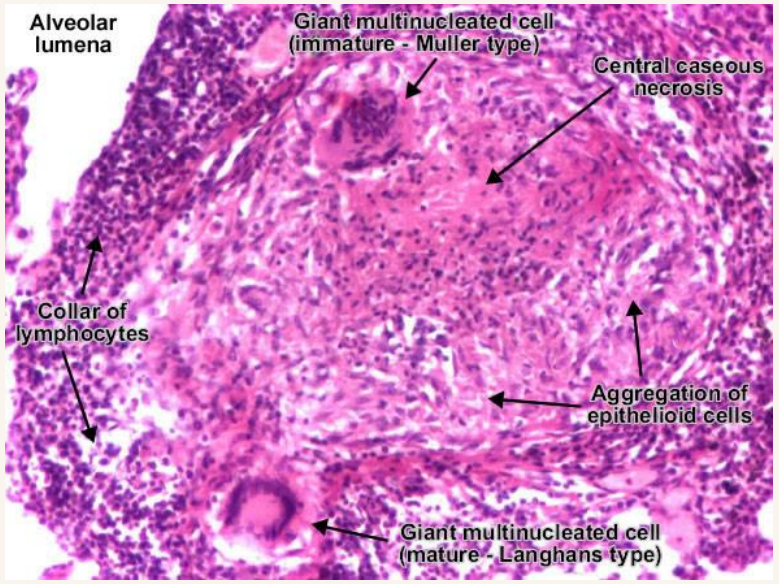
TB infection
Lung nodules

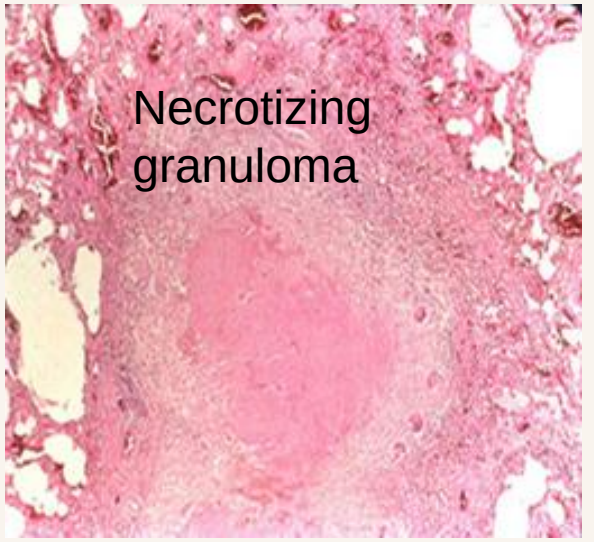


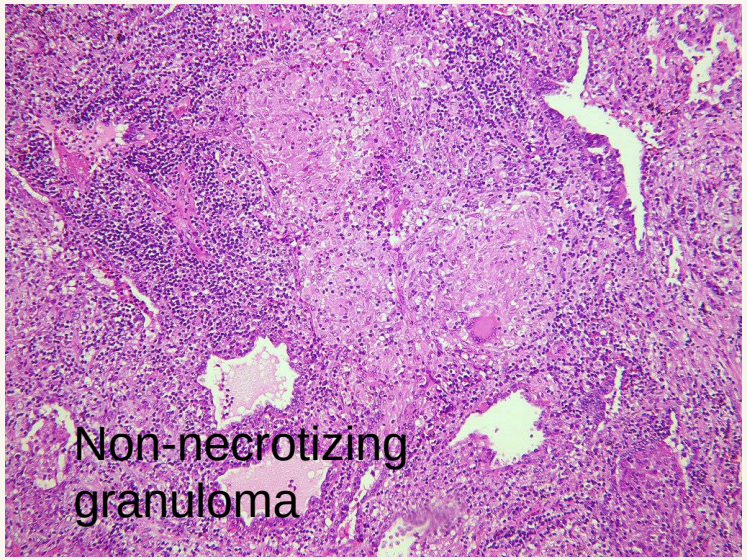
- Classic primary disease is slowly progressive pulmonary infection with cough, weight loss, and low-grade fever, presentation can vary depending on degree of immune suppression:
  - Ghon's complex: Primary lesion in lung and an associated lymph node
  - Miliary TB: Wide-spread dissemination of infection via the bloodstream, occurring most often in AIDS, elderly, children, immunosuppression and with some medications (Remicade-infliximab)
- Secondary tuberculosis: occurs mostly in adults as reactivation infection
  - Granulomatous inflammation much more florid and widespread than in primary disease.
  - Upper lung lobes are most affected, and cavitation can occur
- TB is spread by respiratory droplets
  - All patients suspicious for TB require respiratory isolation precautions

Pathology of *Mycobacterium tuberculosis*







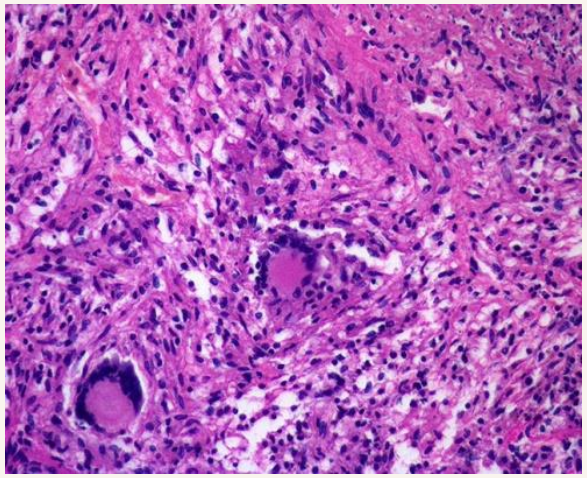


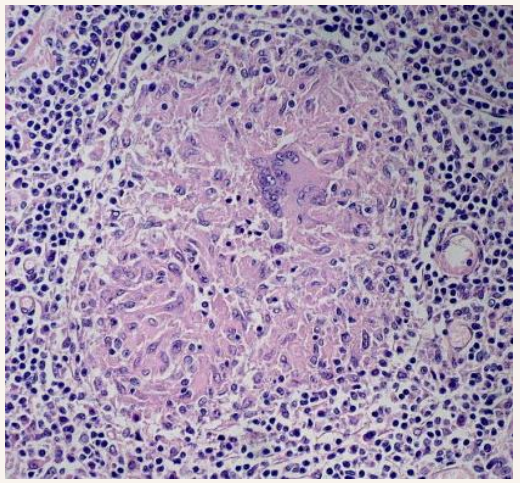
Hallmark of *Mycobacterium tuberculosis* infected tissue is necrotizing granulomatous inflammation, but non-necrotizing granulomas can also be present

- Composed of epithelioid histiocytes surrounding a central necrotic zone
- Variable number of multinucleated giant cells and lymphocytes.

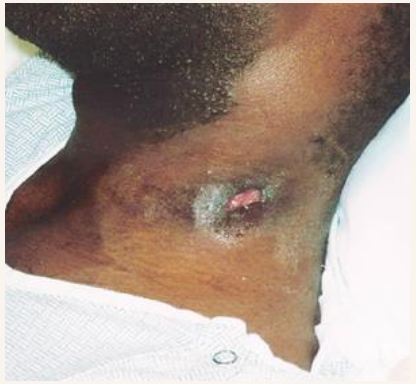
TB in HIV/AIDS patients

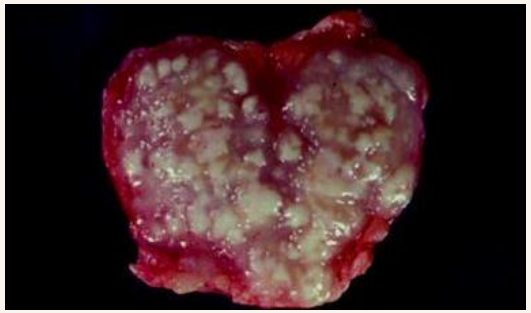
- TB is a common opportunistic infection affecting HIV/AIDS patients
- Trend toward being multi-drug resistant (at least INH and Rifampin)
- Progressive decline of cell mediated immunity (low CD4 count) makes for a greater risk of extra pulmonary (miliary) dissemination
  - Granulomas with and without caseation can occur

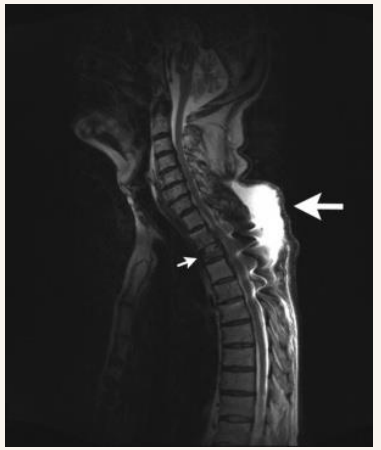




M. tuberculosis outside the lung







- **Scrofula** –Unilateral lymphadenitis (cervical lymph node) most often seen in immunocompromised adult or children
  - Caseous necrosis often present
  - Fine needle aspiration to obtain diagnostic specimen
    - *M. tuberculosis* most cause common in adults
    - *M. avium complex* and other MOTT (2-10%) in children

- **Pott's disease** - TB infection of the spine
  - Usually secondary to primary pulmonary infection
  - Manifests as a combination of osteomyelitis and arthritis that usually involves more than 1 vertebra.

Mycobacterium bovis

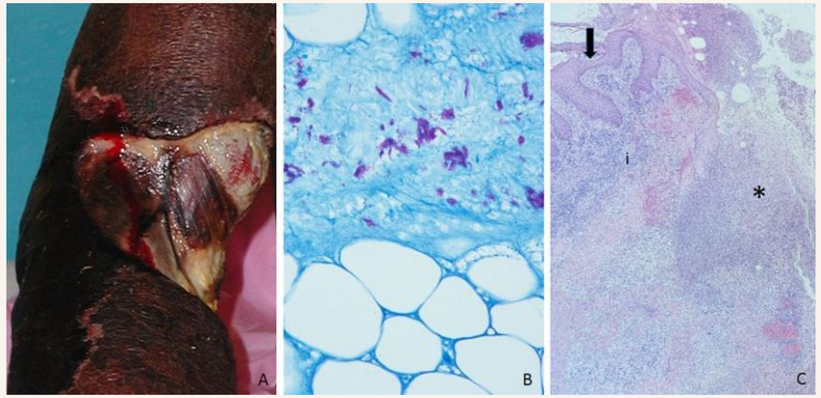
- Produces disease in warm blooded animals, cattle
  - Spread to humans by inhalation or raw milk products
  - Disease states in humans similar to that caused by TB

	M.bovis	M. tb
Nitrate	Negative	Positive
Growth in T2H*	No growth	Growth
Pyrazinamidase enzyme	Negative	Positive
Pyrazinamide susceptibility	Resistant	Susceptible

*(Thiophene-2-carboxylic hydrazide)

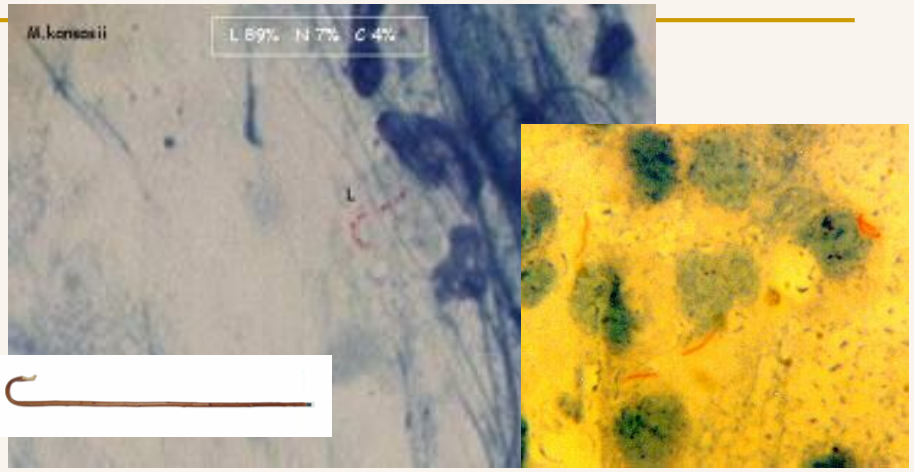
- M. bovis BCG (Bacillus Calmette-Guerin)
  - BCG is an attenuated strain of M. bovis
    - Used for TB vaccination in countries with high prevalence of TB
  - Instillation of BCG into bladder can be used to treat bladder cancer / on occasion it can cause bladder infection

Mycobacterium ulcerans



- **Buruli ulcer in Africa and known as Bairnsdale ulcer in Australia**
- Endemic in tropical areas with limited medical care,
- Starts with mild skin trauma forming nodule from exposure to contaminated stagnant waters
- Leads to progressive and destructive ulcers, peak age group 5-15 yrs
- Due to production of mycolactones (cell cytotoxin)
- **Optimum growth temp @ 30˚ C** /** Does not grow well or at all at 37*C
  - ****Skin lesions suspect for AFB should be cultured at both 30˚ and 37˚C**
  - Difficult to grow requiring 3- 4 weeks/ molecular techniques preferred

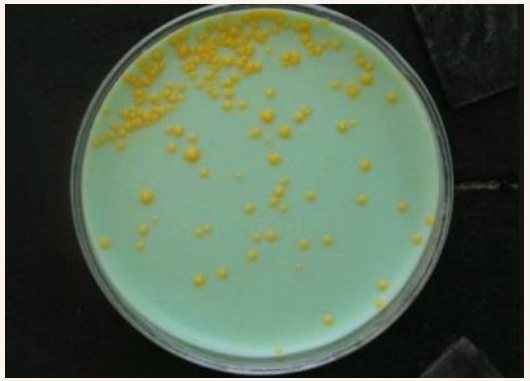
Mycobacterium kansasii



- Culture 37* C, growth in10-20 days
  - **Photochromogen**
  - Niacin accumulation test negative
  - Nitrate reduction positive
  - Tween 80 positive / tests for presence of the lipase enzyme
  - 68*C catalase positive
- AFB are larger than TB - rectangular and very beaded with **Shepherd's crook shape**

- Clinical disease mimics pulmonary TB but less likely to disseminate
  - Disease acquired from contaminated tap water
  - Predisposition for diseased lung (COPD, pneumoconiosis)
  - More likely seen in immune suppressed, alcohol abuse, and HIV
  - Produces granulomatous inflammation in lung

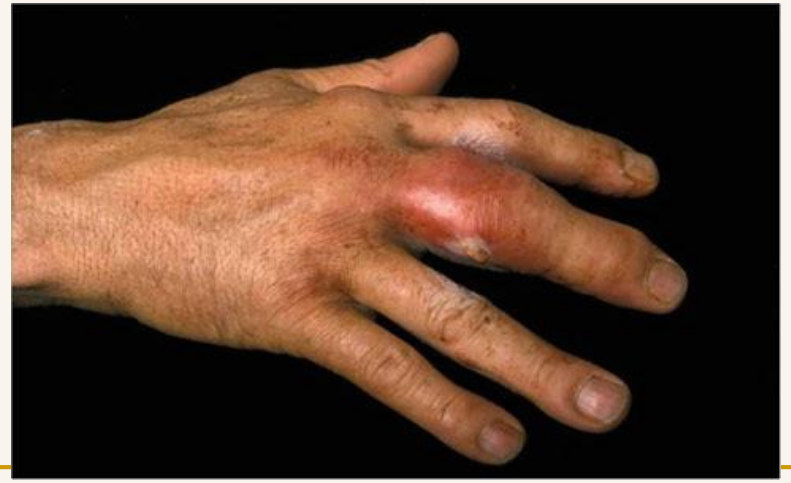
Mycobacterium marinum

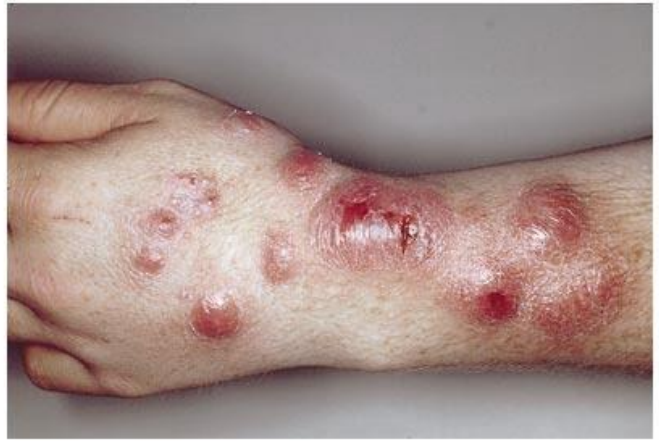


- Photochromogen
- Optimum temp for growth is **30˚** C
  - Grows poorly or not at all at 37°C
  - Grows in 5-14 days
- Normal habitat is contaminated fresh and salt water
  - Infection associated with skin trauma occurring in water
    - Swimming pools (swimming pool granuloma)
    - Cleaning fish tanks with bare hand and arm
    - Ocean (surfing)
    - Punctures from fish fins

Mycobacterium marinum

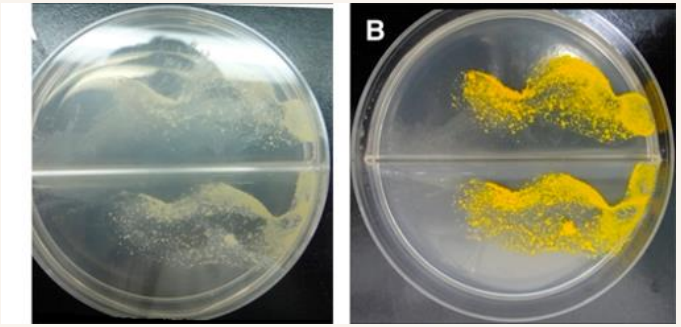
- Disease: Tender, red or blue/red subcutaneous nodules develop at the site of trauma after 2-3 weeks
- Biopsy skin nodules for culture and histopathology
- Lesions classically spread up arm along lymphatics,
  - Infection appears similar to diseases Sporotrichosis, Nocardiosis, and rapid growing Mycobacteria species



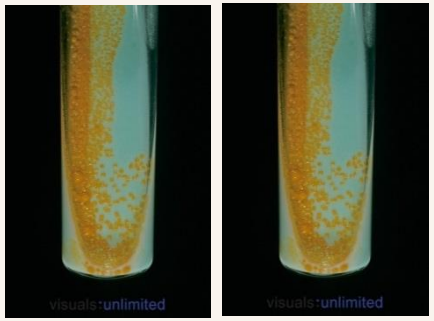


Mycobacterium szulgai

- **Scotochromogen at 37˚C / Photochromogen at 25˚C**
  - **Only AFB species that has a different light test result based on temperature of incubation**
- Growth at 37 ˚C in 12 - 25 days
- Rare cause of pulmonary disease in adults
- Associated with alcohol abuse, smoking, COPD, AIDS and immune suppressed
- Pulmonary symptoms like TB



25˚ C - Photochromogen



37˚ C - Scotochromogen

Mycobacterium xenopi

- Scotochromogen
- Thermophilic AFB with growth at 42˚C
  - Capable of growing in hot water systems, the usual source of infection
- Growth in 14 - 28 days
- **Egg nest** colony produced on solid media
- Rare cause of pulmonary disease
  - Clinical disease like TB
  - Occurs in patients with preexisting lung disease (chronic obstructive pulmonary disease or bronchiectasis) and HIV/AIDS

M. avium complex

- Complex includes eight species of environmental and animal AFB
  - Non-photochromogen
  - All species biochemically and genetically very similar
  - *M. avium and M. intracellular* most common species
  - Growth at 37 ˚C / 7 – 21 days
  - Smooth / creamy colony / buff colored
  - Short rods, not beaded, irregular clumps of AFB
  - Inert in biochemical reactions
  - Identify using
    - Molecular DNA/RNA hybridization probe (M. avium **complex**)
    - MALDI-TOF mass spectrometry (species within complex)
    - 16s rRNA sequencing (species within complex)

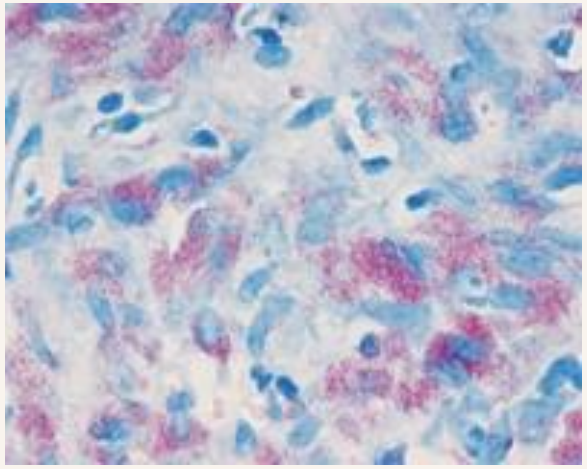
M. avium complex clinical correlation

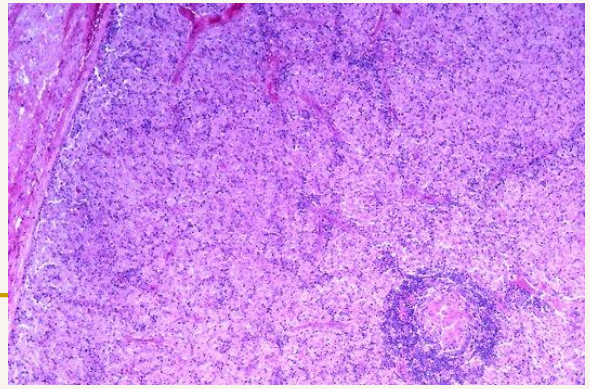
- Disease in the somewhat normal host
  - Adults mostly have chronic pulmonary disease, fibro-cavitary or nodular bronchiectasis disease presentation
    - Produces chronic cough in elderly with prior lung damage (COPD)
  - Children mostly scrofula with chronic granulomatous inflammation with lymph node involvement

- M. chimaera (a species within the M. avium complex)
  - Environmental and nosocomial pathogen
  - Reported as a contaminate in heater–cooler units used in cardiac surgery/ aerosolized water from instrument positive with M. chimaera
  - The airborne transmission of M. chimaera over an open surgical field caused post surgical infections

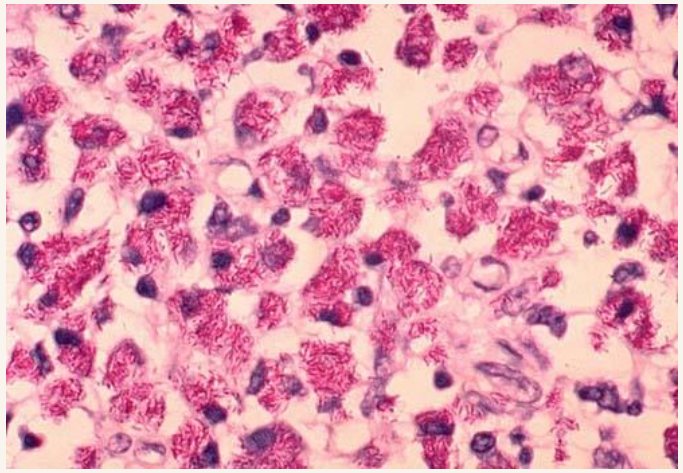
M. avium complex (MAC, MAI)

- HIV/AIDS – common opportunistic infection in AIDS
  - Nonspecific low-grade fever, weakness, weight loss, picture of fever of unknown origin rather than a pulmonary infection
    - Diagnosis: Isolation of MAC from respiratory, blood or bone marrow culture
  - Abdominal pain and/or diarrhea with malabsorption
    - Positive stool AFB smears and cultures
- In tissue:
  - Pathology: non-necrotizing granulomas
  - High organism load can be present in infected tissue
  - AFB are small (short) rods and not beaded
    - No cord factor

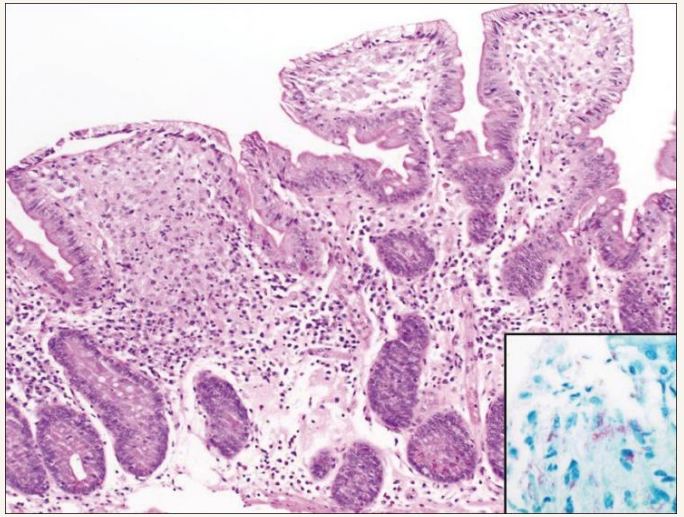




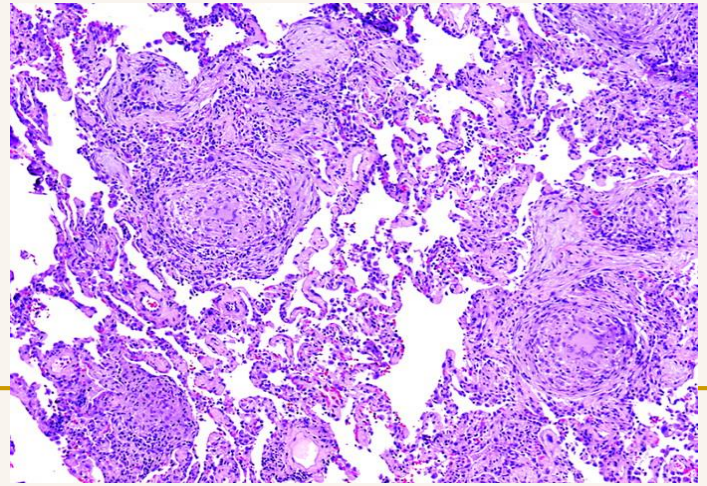
M avium-complex in lymph node tissue (AFB Kinyoun stain) – packed with AFB



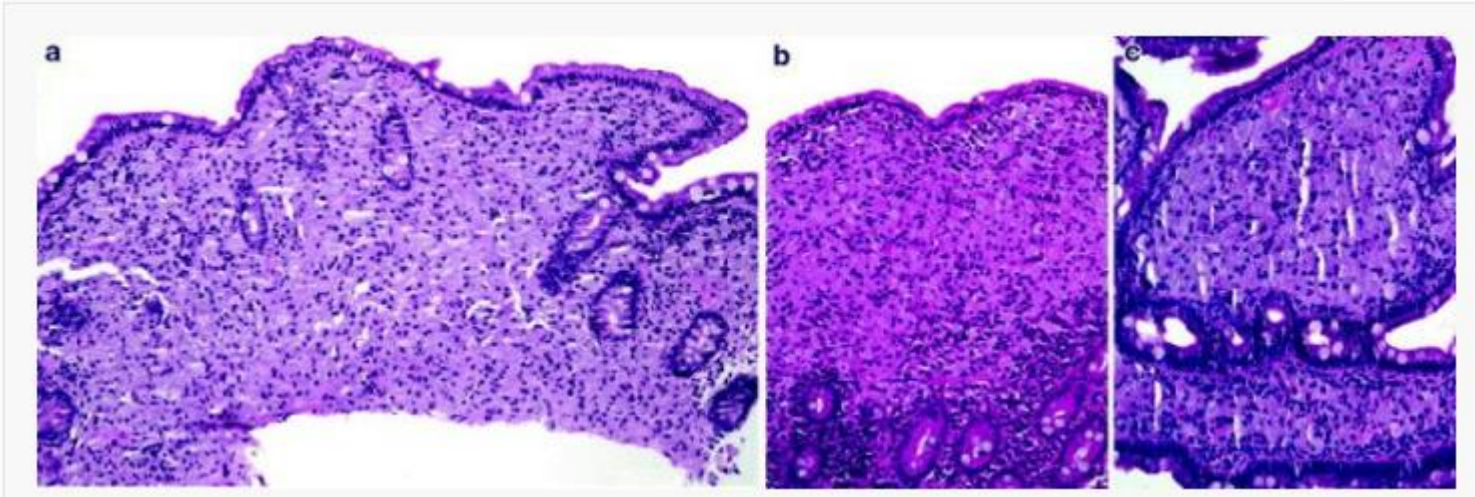
Bowel -Lamina propria expanded from predominately Lymphohistocytic infiltration



Granulomatous inflammation lung tissue (H&E stain)

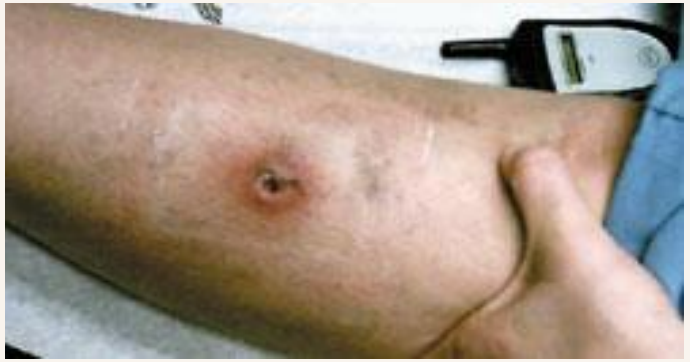


Small bowel biopsies from AIDS patients with MAI infection show the characteristic diffuse histiocytic infiltrate leading to marked villous blunting (**a**–**c**)



Rapid Grower Mycobacteria species

- Growth in <=7 days
- Low virulence organisms, environmental contaminates
- 20 species but most common:
  - M. fortuitum skin and surgical wound infections, catheter sites
  - M. chelonae skin infections in immune suppressed, catheter sites
  - M. abscessus chronic lung infection and skin infection in immune suppressed
  - Biochemical reactions:
    - All 3 species Arylsulfatase positive
    - M. fortuitum: Nitrate reduction positive
      Iron uptake positive
    - M. chelonae and M. abscessus: Nitrate negative



Rapid growing Mycobacteria

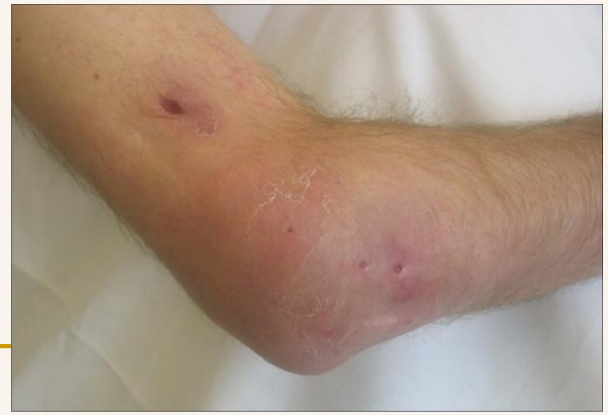
- MALDI-TOF and 16 sRNA sequencing for identification to species
- Antibiotic therapy assisted by directed susceptibility tests
  - Varying susceptibility patterns within the group, but clarithromycin is a primary therapy
  - Molecular genetic testing can assist with clarithromycin resistance and provide rapid information for therapy

Miscellaneous species

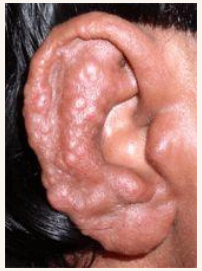
- *M. gordonae*
  - Scotochromogen
  - Rarely if ever causes infection
  - Commonly found in tap water and can be an AFB culture contaminant acquired during collection or processing
    - Use sterile/distilled water in AFB processing to prevent culture contamination

Miscellaneous pathogenic species

- *Mycobacterium haemophilum*
  - Fastidious slow growing AFB requiring the addition of hemoglobin or hemin to culture media for growth
    - Will not grow on LJ media, Middlebrook, or in automated systems (Middlebrook 12B media) without the addition of hemin supplement
    - Growth best at 30*C
  - Disease:
    - Painful subcutaneous nodules and ulcers isolated primarily in AIDS patients or immunosuppressed
    - Lymphadenitis in children

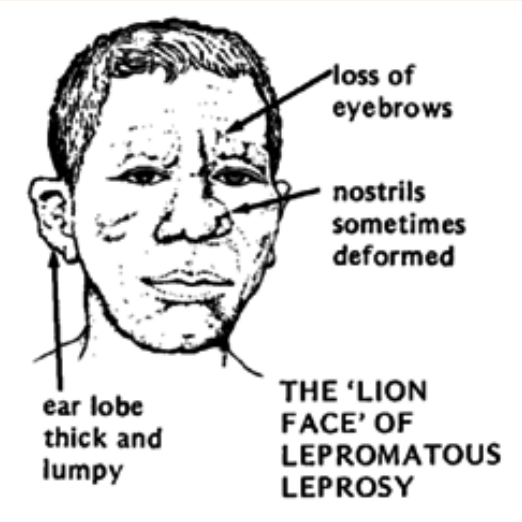


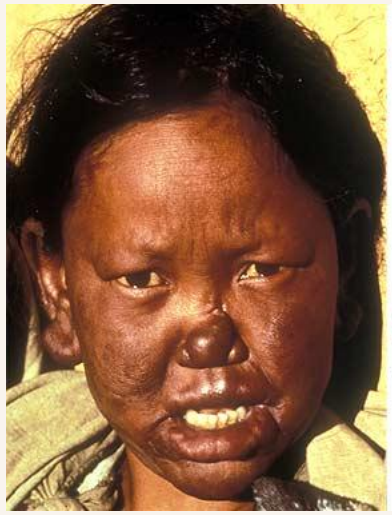
Mycobacterium leprae

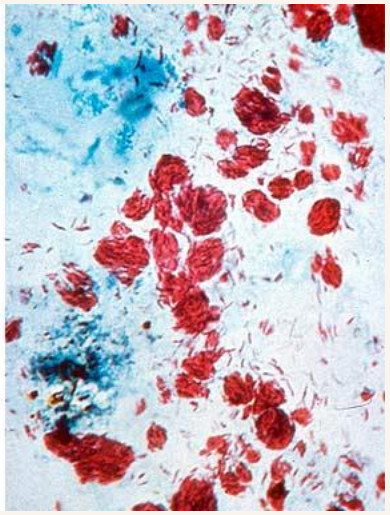


- Leprosy – Hansen's Disease
- Affects the skin, peripheral nervous system and mucous membranes
  - Clinical signs and symptoms aid in diagnosis: Peripheral neuropathy with nerve thickening, numbness in earlobes or nose, loss of eyebrows
  - Infection by person-person spread via inhalation of infectious droplets
  - Curable with early diagnosis and appropriate therapy (dapsone with rifampin or clofazimine
- Endemic in India, Brazil and Indonesia (2 types)
  - **Lepromatous** – Severe disfiguring lesions, large numbers of AFB in lesions
  - **Tuberculoid** - Less severe and fewer lesions, lower numbers of AFB in lesions
- Non-culturable on laboratory media
- PCR performed from tissue for definitive diagnosis
- Armadillo is a natural reservoir of M. leprae

Lepromatous leprosy /Multibacillary

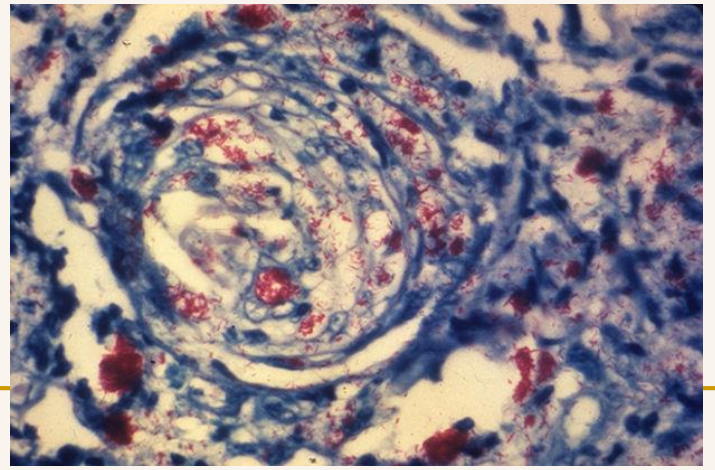






AFB in "cigar packet" arrangement

Skin biopsy - AFB seen in nerve fiber



Tuberculoid leprosy/ Paucibacillary