Mycobacteriology Service Implementation

Module 4
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STAINING & MICROSCOPY

Gram Stain
Kinyoun Carbofushin Stain
Modified Kinyoun Carbofushin Stain
Acid Fast Fluorochrome Stain

Understanding Mycobacteria Staining

GLYCOLIPIDS (*mycolic acid, arabinoglactin, lipoarabinomann)*

PEPTIDOGLYCAN

CYTOPLASMIC MEMBRANE

Mycobacteria are acid fast bacilli. They resist stain decolorization with acid alcohol due to the high amount of lipid rich mycolic acids in the cell wall

Acid Fast Smear Types

- **Direct Smear** – Prepared directly from an unprocessed patient specimen. Low sensitivity unless the organism burden is very, very high – **Concentrated Smear Follow-Up Required**

- **Concentrated Smear** –**HCH laboratory method** Prepared from concentrated sediments (by centrifugation) after specimen digestion/decontamination has been completed or centrifugation of clear sterile body fluids

Utility of Acid Fast Smears

- Smear examination is a rapid and inexpensive test
- Allows diagnostic evaluation of all types of specimens
- Positive smear sometimes is the first indication of disease, but does not distinguish between dead and viable organisms
- Sensitivity is limited – Requires high organism burden (at least 5,000 AFB/mL of specimen)
- Specificity for *M. tuberculosis* is limited. All mycobacteria are AFB – Prevalence of nontuberculous mycobacteria (NTM) is on the rise
- Smear results can be used to direct therapy but are not definitive for diagnosis of active infection – ***CULTURE REQUIRED***

Basic Staining Procedure

- Prepared smear should be no larger than the size of a nickel, placed in the center of the microscope slide
- Include a control slide (positive and negative control) with each batch of smears stained and whenever stain reagents are changed
  - Assess reagent and stain procedure performance quality
  - Detect environmental contaminants
  - Determine if the microscope is functioning properly
  - Help find the plane of focus for examining the smear
- Do not place slides close together; separate by at least 2 centimeters distance
- Flood the fixed smear with the primary stain and let sit to allow uptake of the stain by the cell wall
- Rinse the smear with water until it runs clear of the primary stain
- Decolorize the smear with an acid alcohol reagent mixture
- Rinse the smear with water until it runs clear of the primary stain
- Flood the fixed smear with the counterstain and let sit to allow uptake of the stain by the cell wall
- Rinse the smear with water until it runs clear of the counterstain
- Drain excess water from the slide, air dry, and DO NOT BLOT.

Types of Acid Fast Stains

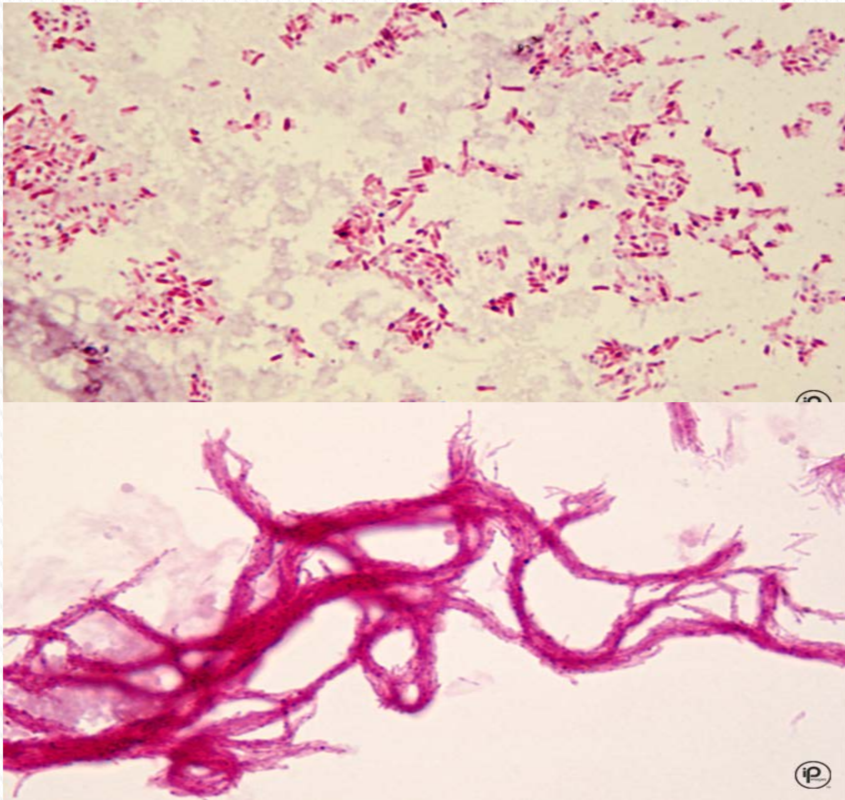
- Light microscopy used for examination
- Stain is specific but sensitivity affected by specimen quality and bioburden
- Cold stain – does not require heating to enhance uptake of primary stain
- Requires strong acid alcohol decolorizer
- Examine using 100x oil immersion objective

Kinyoun Carbofushin

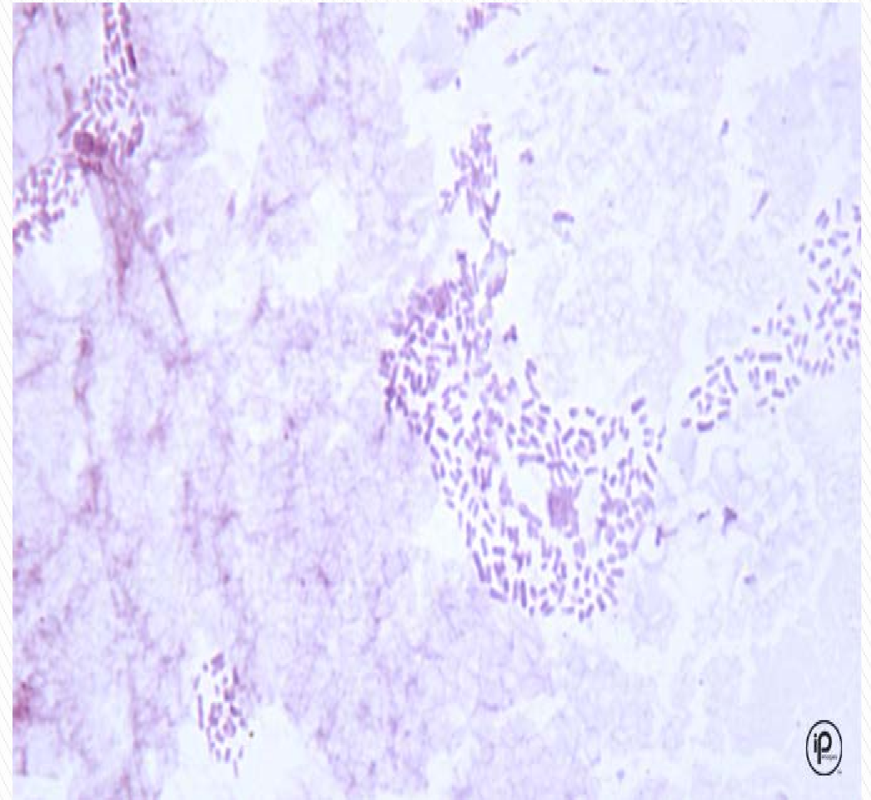
- Fluorescent microscopy used for smear examination
- Stain is sensitive but specificity affected by specimen quality and composition
- Cold stain – does not require heating to enhance uptake of primary stain
- Requires weak acid alcohol decolorizer

Fluorochrome

Types of Acid Fast Stains

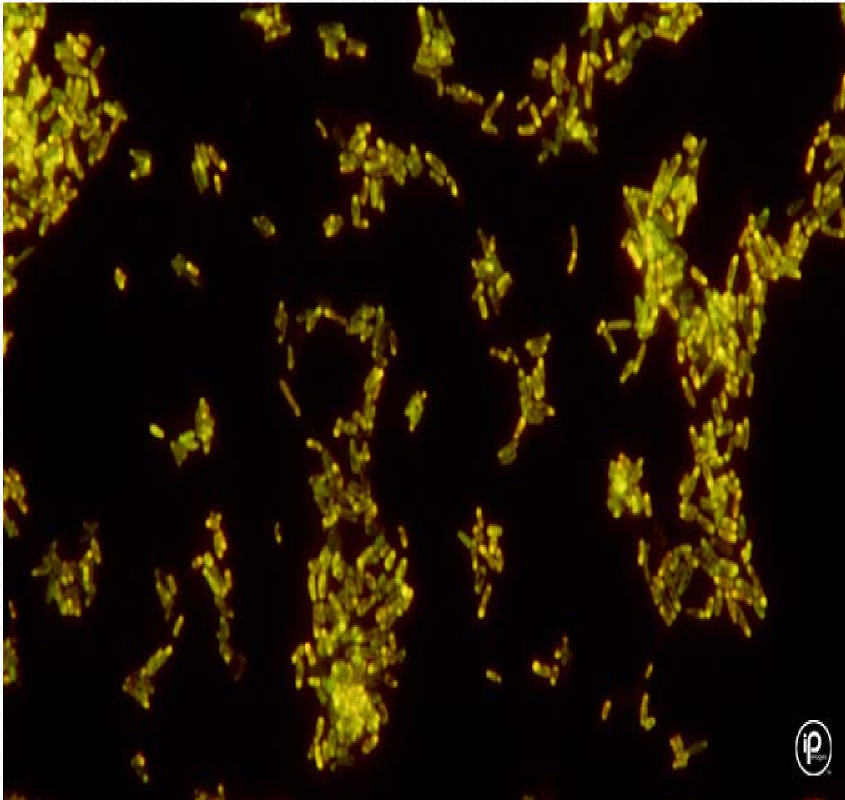


Kinyoun Positive

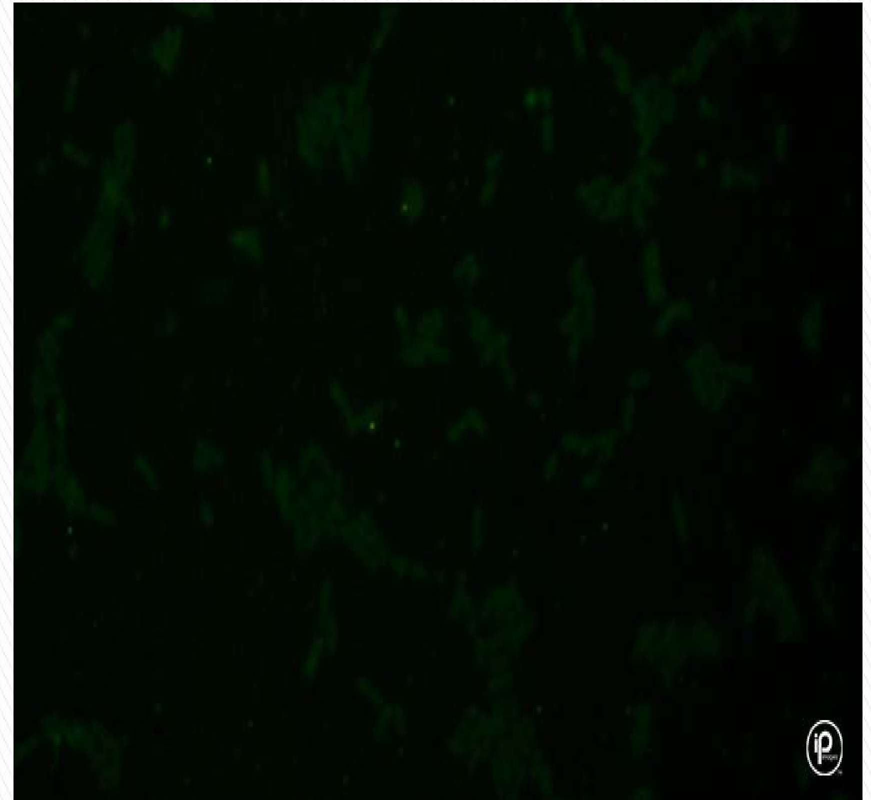


Kinyoun Negative

Types of Acid Fast Stains



Fluorochrome Positive



Fluorochrome Negative

Reading and Reporting Smears

- **Smears should be examined and the results reported within 24 hours of specimen** processing and smear preparation
- Check the settings and function of the microscope before each use
- Clean each microscope objective with a separate piece of lens paper before each use
- Clean the objective used with a separate piece of lens paper between the review of each slide to limit carryover between microscope slides

Reading and Reporting Smears

Interpretation	Fluorochrome Stain	Kinyoun Stain
Negative	No fluorescence seen **or** fluorescing organisms seen that lack AFB morphology	No acid fast organisms seen against a blue or green background
POSITIVE	Acid fast organisms with the proper morphology are seen with a bright yellow fluorescence against a dark background **Smears are considered truly positive when at least 3 acid fast bacilli are seen on the entire smear.**	Acid fast organisms stain **red** against a blue or green background

Reading and Reporting Smears

- IT'S NOT JUST THE STAIN REACTION BUT ALSO THE MORPHOLOGY THAT COUNTS!
- Mycobacteria morphology may be:
  - Short or long slender bacilli, occurring singly or bent
  - Beaded bacilli
  - Long twisted bundles (Cording)
- Other organisms may stain acid fast:
  - Actinomycetes (occasionally Nocardia species may stain acid fast)
  - Corynebacterium species
  - Cryptosporidium species
  - Cyclospora species
  - Isospora belli
  - Sarcocystis

Reading and Reporting Smears

Kinyoun Carbolfushin Stain

Report	Quantity of Acid Fast Organisms
Rare	3 – 9 organisms per 100 oil immersion fields
Few	1 – 9 organisms per 10 oil immersion fields
Moderate	1 – 9 organisms per oil immersion field
Numerous	>9 organisms per oil immersion field
Number of acid fast organisms seen	1 – 2 organism seen on entire smear
No acid fast organisms seen	None

Reading and Reporting Smears

Fluorochrome Stain

Report	Quantity of Acid Fast Organisms (40x)
Rare	2 – 18 per 50 fields
Few	4 – 36 per 10 fields
Moderate	4 – 36 per field
Numerous	> 36 per field
Number of acid fast organisms seen	1 – 2 organism seen on entire smear
No acid fast organisms seen	None

CRITICAL VALUES

- Any positive AFB smear
- Detection of any reportable pathogen other than AFB as a consequence of the performance of AFB smear testing (consult with the pathologist before reporting)

CULTURE READING

- BACTEC MGIT 960
  Solid Agar Media –
  Lowenstein Jensen

Bactec MGIT 960

Bactec MGIT 960

- MGIT = Mycobacteria Growth Indicator Tube
- Middlebrook 7H9 base
- PANTA Supplement – contains Oleic acid, Albumin, Dextrose and Catalase with Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin

Bactec MGIT 960

- Instrument continuously monitors all tubes for 42 days (6 weeks)
- The tubes contain a fluorescent compound that is sensitive to oxygen. As organisms grow and use the oxygen the fluorescent compound is allowed to fluoresce and growth is detected
- Growth also can be detected visually by seeing a non-homogenous turbidity, flakes or small grains in the broth. Also **note the presence of yellow – orange pigment in the broth**.
- Positive tubes are stained using Kinyoun method and subcultured to Lowenstein Jensen (LJ) agar
- An aliquot of AFB positive broths is sent to the reference lab for identification and further testing as needed

MycoF/Lytic Bottle

- Contains a lytic agent to lyse WBCs that have phagocytized mycobacteria
- Used for blood and bone marrow cultures
- Will grow mycobacteria, fungi and routine bacteria
- Incubated for continuous monitoring in the routine Bactec blood culture instrument

Solid Agar Media

- Lowenstein Jensen Agar

- Egg based mediacontains Oleic acid, Albumin, Dextrose and Catalase with glycerol, defined salts, vitamins, cofactors
- Malachite green serves as an inhibitory agent for contaminating flora that is less inhibitory to mycobacteria

Solid Agar Media

Blood Agar

- Used to check for breakthrough bacterial contamination after specimen processing
- May be used for isolation of select mycobacteria

Chocolate Agar

- Used to isolate *Mycobacterium haemophilum* that requires hemin for growth (X-factor)

Solid Agar Culture Reading

- Check the blood agar plate for breakthrough contaminants for the first 72 hours incubation
- Check the solid agar slants for growth within the first 72 hours to 7 days = Rapid Grower
- Check solid agar slants for growth weekly for 6 – 8 weeks
- If growth observed, note:
  - Growth Rate
  - Growth Temperature
  - Colony Texture and Consistency
  - Colony Pigment

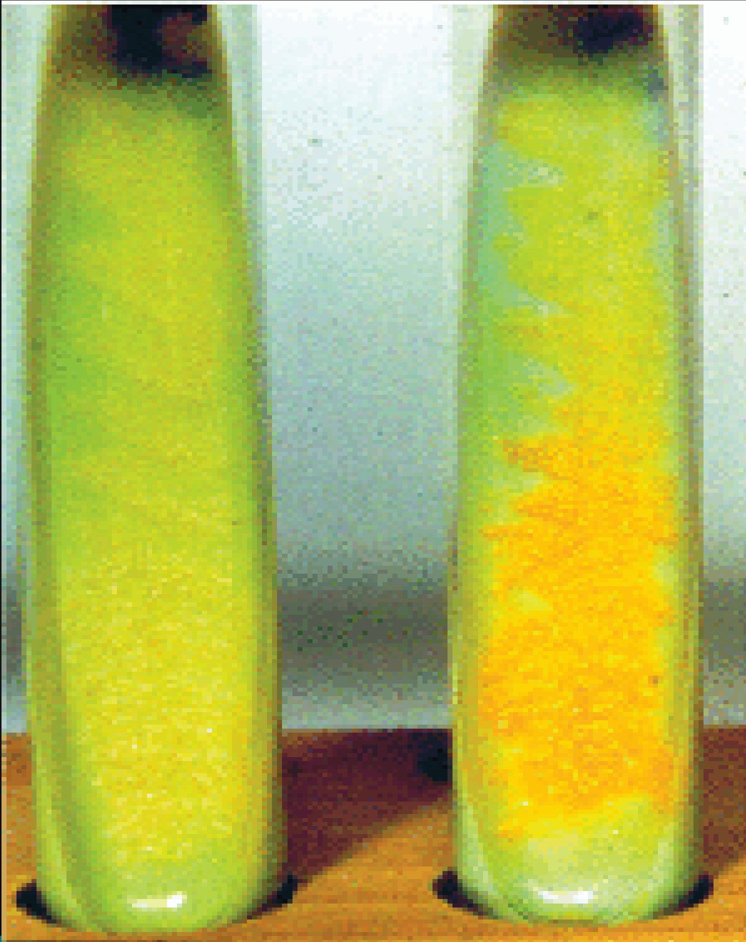
Runyon Classification for Nontuberculous Mycobacteria (NTM)

- Group I: **Photochromogens** – pigment changes after exposure to light *M. kansasii*
- Group II: **Scotochromogens** – pigmented in the dark and in the light *M.gordonae*
- Group III: **Nonphotochromogens** – No pigment *M. avium complex*
- Group IV: **Rapid Growers**
  - ***M. fortuitum group***
  - ***M. chelonae/M. abscessus group***

Mycobacterium tuberculosis complex

- Rough
- Heaped
- Buff (non-pigmented)
- Complex includes:
  - *M. tuberculosis*
  - *M. bovis – BCG vaccine*
  - *M. africanum*

Photochromogen



- Has no pigment or a lighter pigment when incubated in the dark
- Has pigment or a darker pigment after exposure to light

Mycobacterium kansasii complex

- Photochromogen
- Clinically, histopathologically and radiologically resembles tuberculosis, but is not transmitted from person to person

Scotochromogen

Has the same level of pigment in the dark and after exposure to the light

Mycobacterium gordonae

- Scotochromogen
- Most often a contaminant (water); sometimes called the “tap water bacillus”

Nonphotochromogen

- Has no pigment
- Most have smooth colonies, but rough colonies do occur

Mycobacterium avium complex

- Nonphotochromogen
- Widely distributed in water, soil, dust, mammals and poultry
- Pathogenicity common in AIDs patients

Rapid Growers

- Mature growth occurs in 3 –7 days
- Ubiquitous in nature
- Can contaminate water supplies (i.e. reagents and wash solutions)
- May be mistaken for actinomycetes

Mycobacterium fortuitum complex

- M. fortuitum most commonly associated with human disease
- Identification to species requires molecular techniques, but not necessary for treatment
- Treated with common antibiotics

to be continued