Preparing and Reading Gram Stains: I'm Not Afraid

3/23/21

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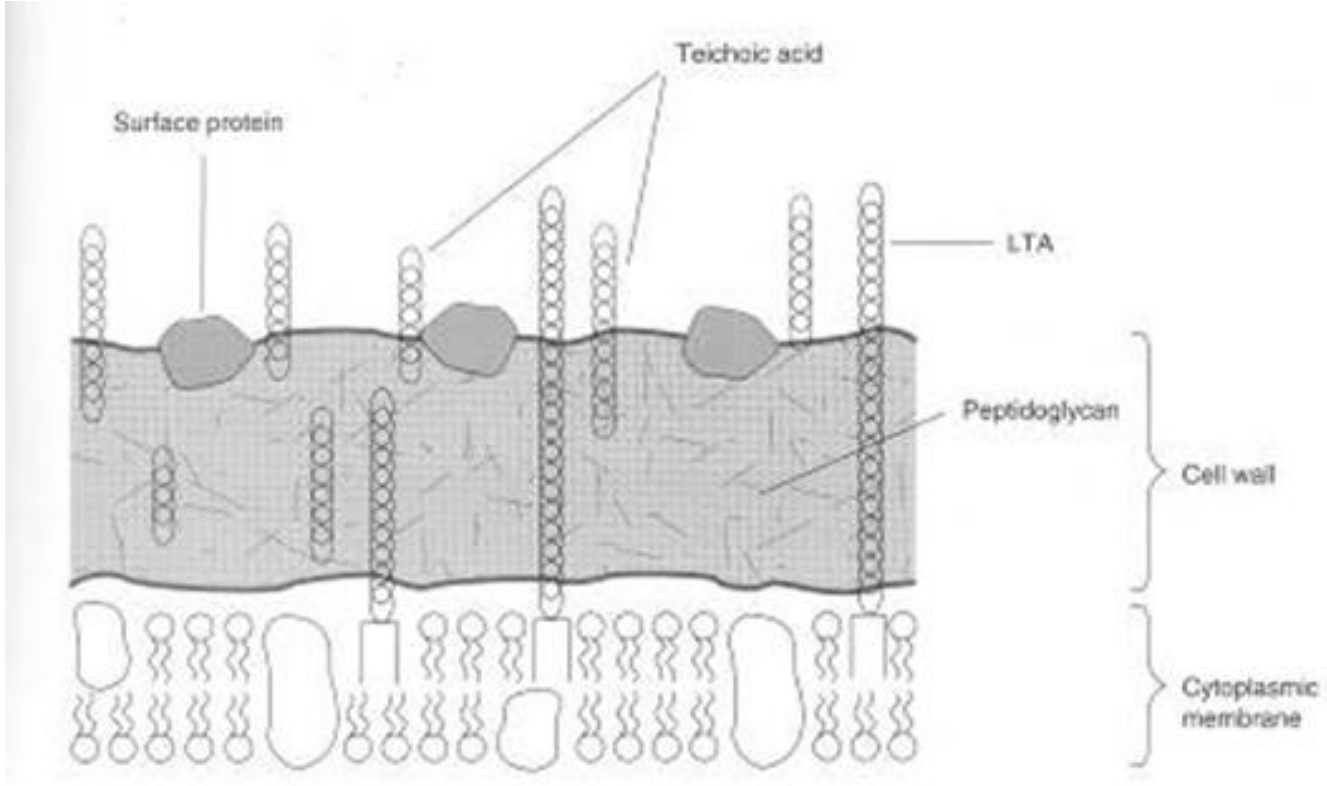
Objectives

1. Explain how to prepare, stain, and assess the quality of a Gram stain.
2. Describe the value a good quality Gram Stain provides to the clinician.
3. Discuss how the use of specific reporting terminology can determine patient care and when the terminology should be used.

Gram Stain Principle

- Hans Christian Gram discovery in the late 19th century
- Bacteria stain either **Gram positive** or **Gram negative**
- Exceptions include:
  - *Chlamydia*
  - *Mycoplasma*
  - *Ureaplasma*
  - Spirochetes

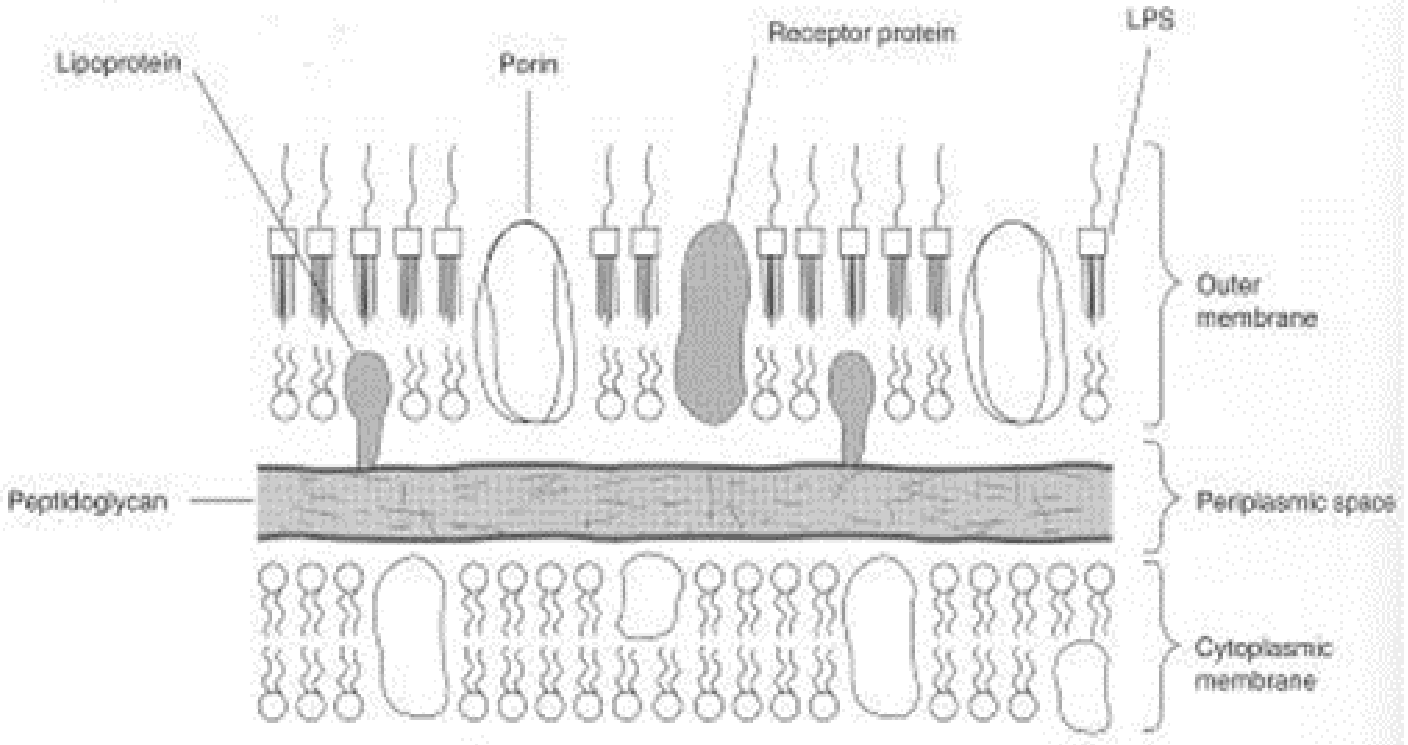
Gram Positive Organisms



Gram Positive

- **Gram positive organisms** have thickly layered peptidoglycan cell walls
- **Gram positive organisms** resist decolorization by acetone or alcohol

Gram Negative Organisms



Gram Negative

- **Gram negative organisms** have thin peptidoglycan cell walls surrounded by an additional lipopolysaccharide and protein outer membrane
- **Gram negative organisms** are readily decolorized with acetone or alcohol

Purpose

- Assess the quality of a specimen
  - Presence of cellular material
  - Accept and reject sputum
- Provide rapid presumptive diagnosis of infectious agents
  - Classify bacteria by form, size and cellular morphology
  - Determine presence of yeast and hyphae
- Determine course of treatment
- Direct the bench work-up of a specimen

Laboratory Responsibilities

- To ensure that the Gram stain result has value for the physician
- To ensure the quality of the Gram stain result

Preparing Gram Stains

Preparation of Gram Stain Smear

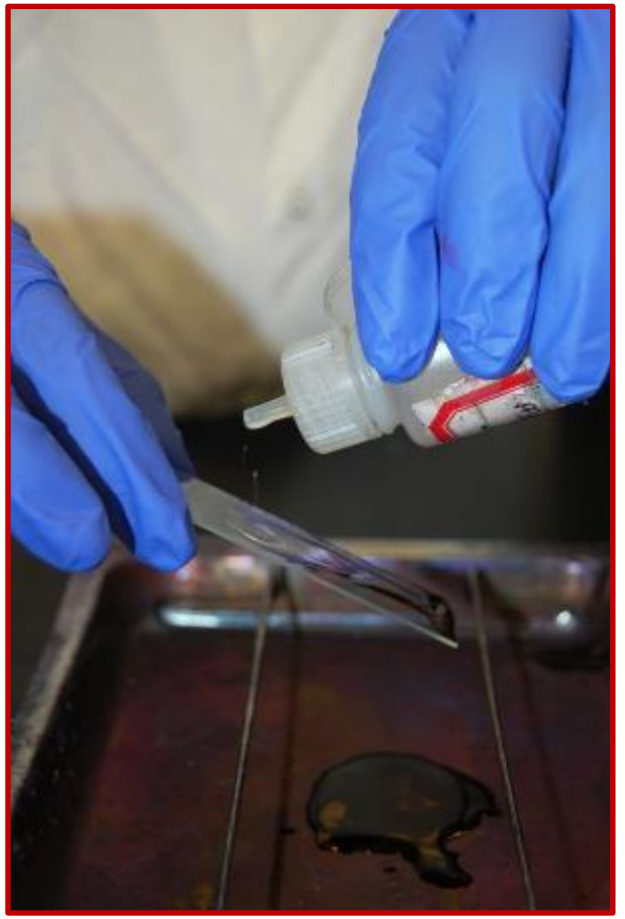
- Select the best portion of the specimen for preparing the smear
  - Blood
  - Mucous
  - Pus
- Prepare a monolayer of cells
  - Roll swab vs. pressed slides
  - Cytospin fluids

Gram Stain Procedure

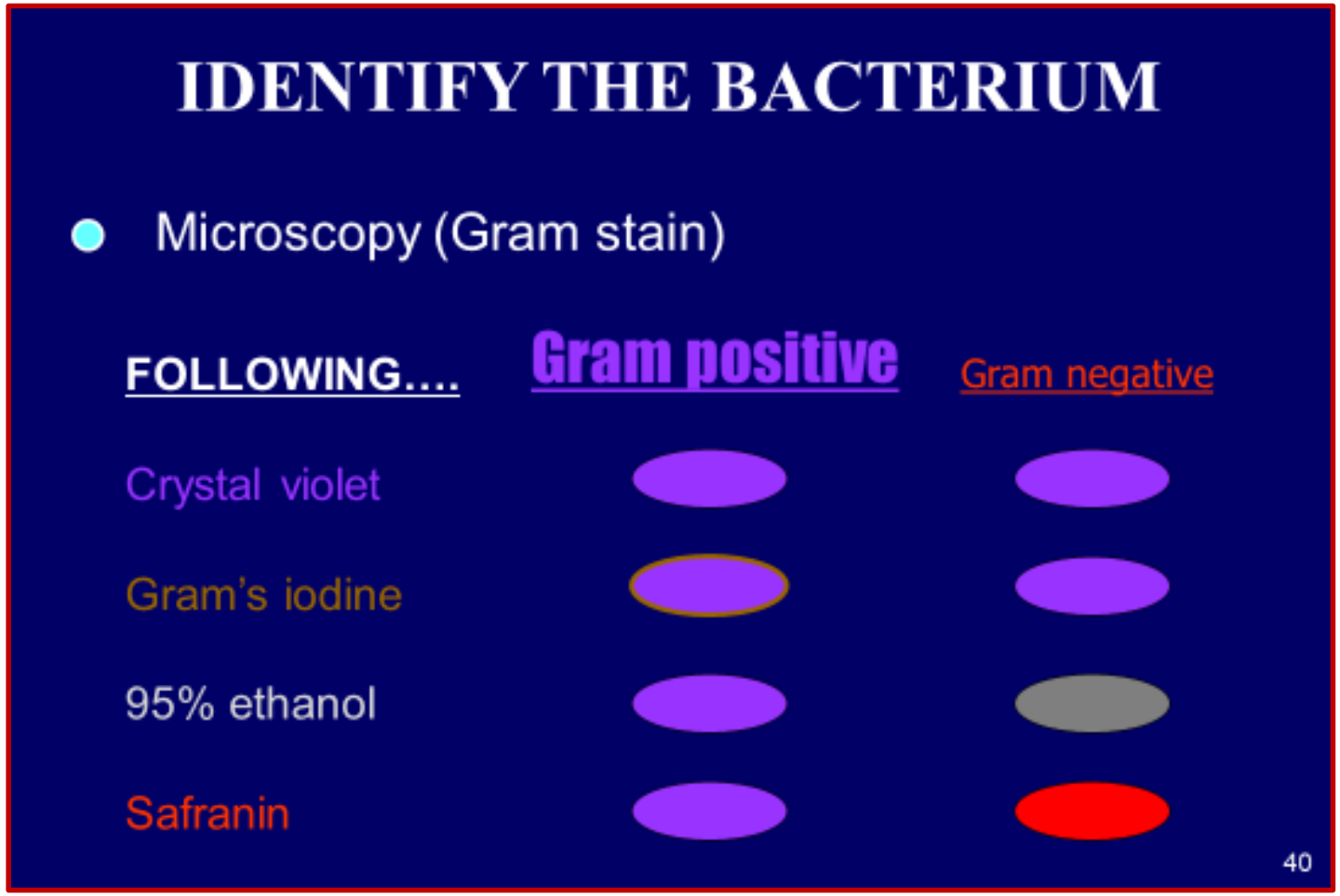
- Heat fix or methanol fix the air dried slide
- Flood the slide with Crystal Violet
- Stain for 15-30 seconds
- Flood slide with Gram's Iodine
- Stain for 15-30 seconds
- Rinse slide gently with tap water
- Decolorize the slide until the run off is clear by allowing the decolorizer to flow over the slide held at an angle. (Time is subject to the thickness of the smear and type of decolorizer being used.)

Gram Stain Procedure (continued)

- Rinse slide gently with tap water
- Flood slide with Safranin (counterstain)
- Stain slide for 15-30 seconds
- Rinse slide gently with tap water
- Drain excess water
- Air dry slide in an upright position or gently blot with bibulous paper.



What's Happening In the Gram Stain Process



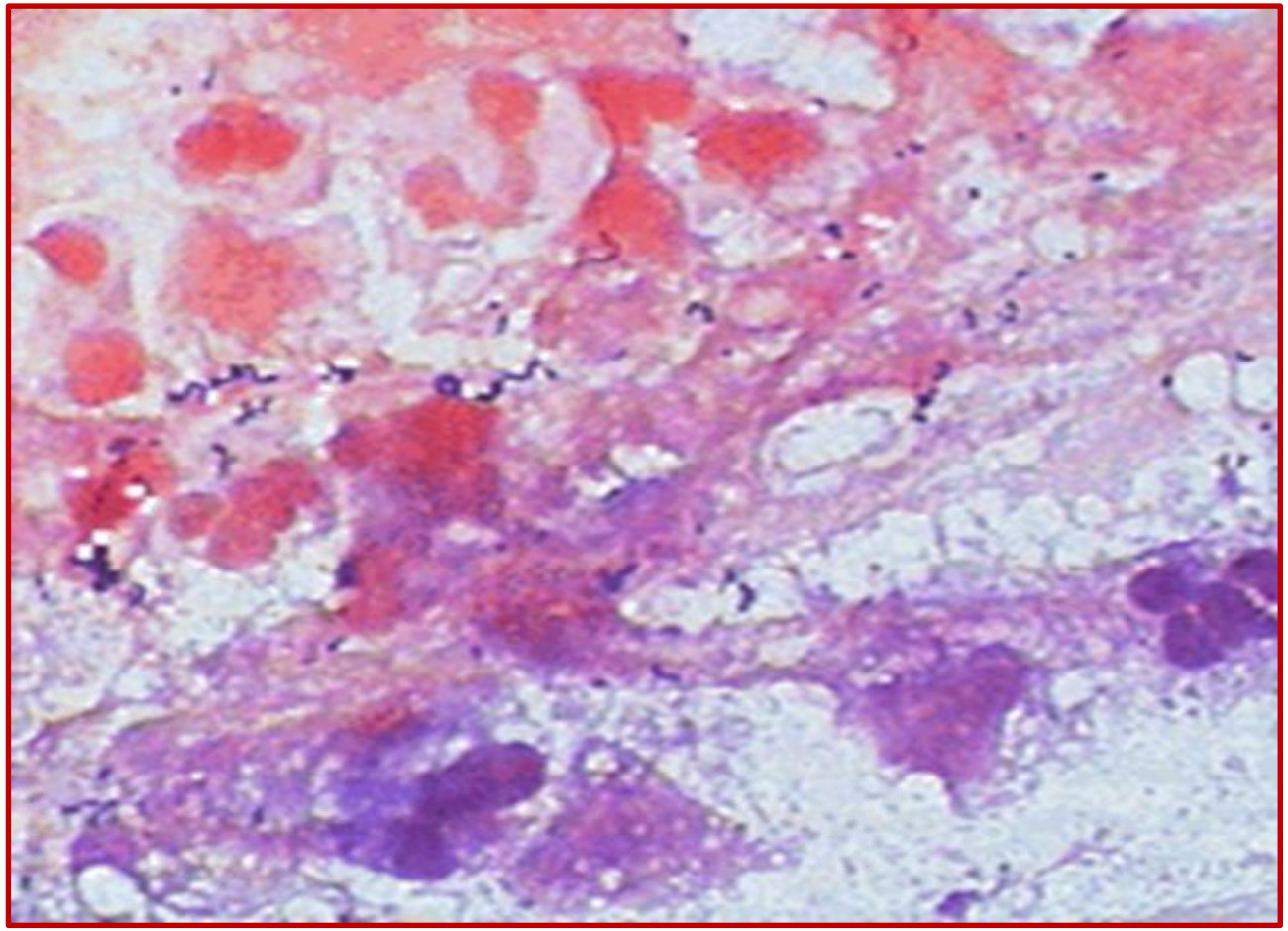
Quality Control/Assurance

- Stain a QC smear with:
  - Each new lot of stain
  - Weekly thereafter
- Inexperienced techs may want to:
  - Stain a QC smear every time they stain a Gram stain slide
- Slide Review:
  - Automatic review of a designated % of slides
  - Questionable slides set aside for review

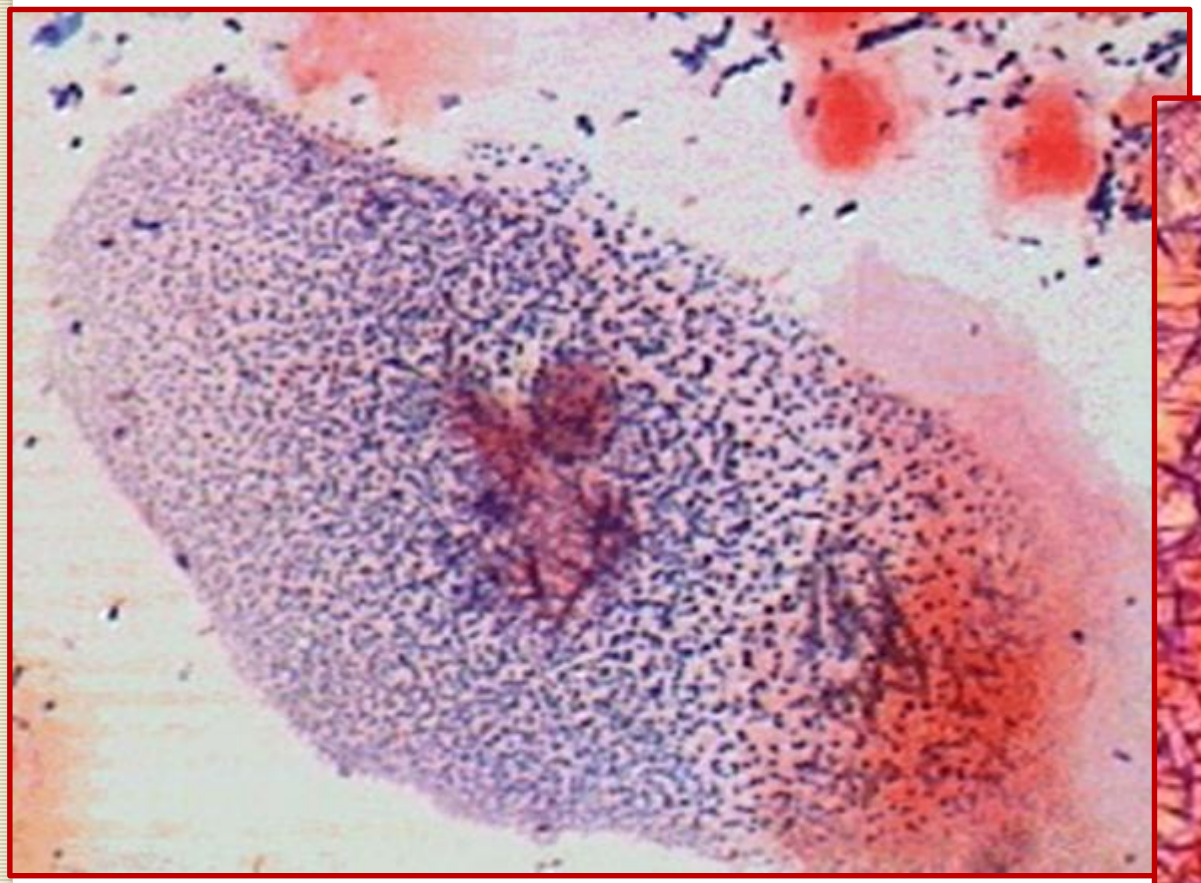
Trouble - shooting Considerations

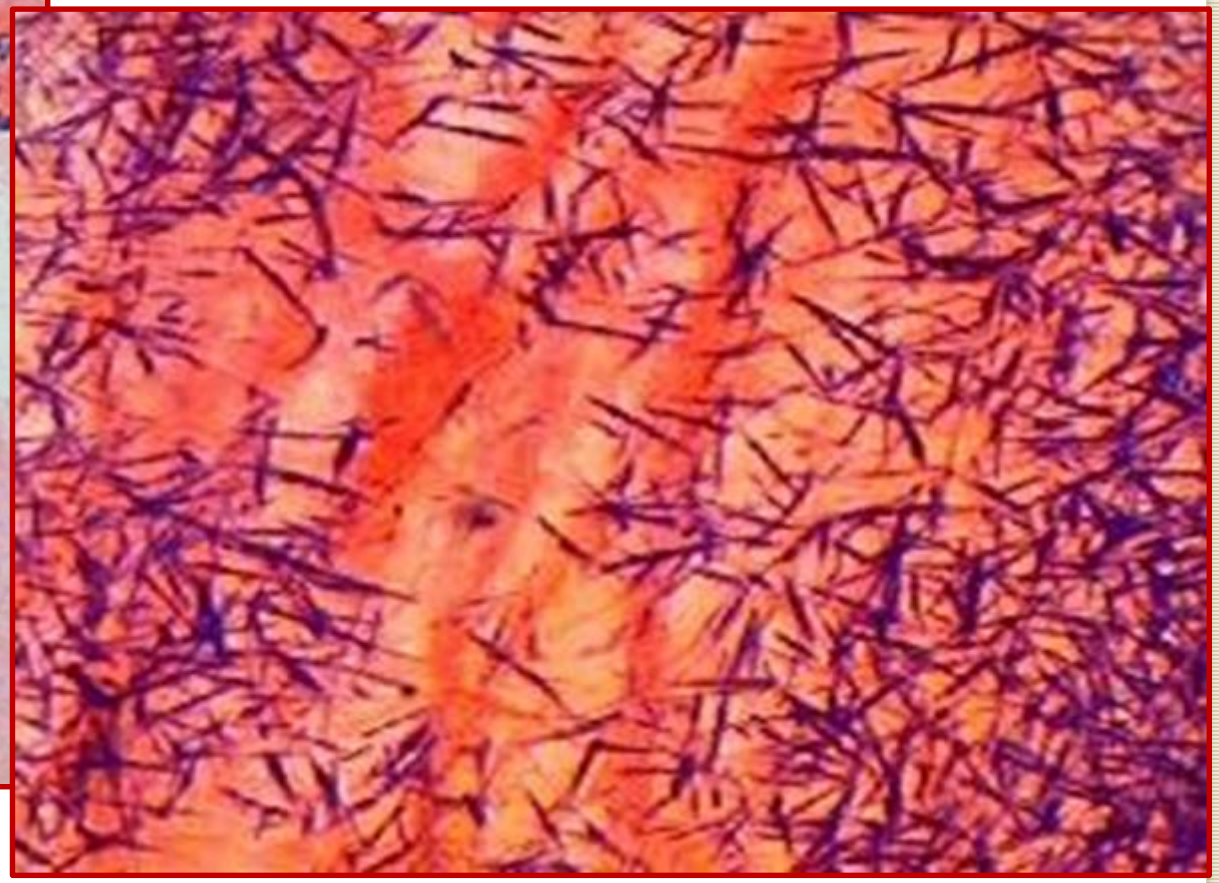
- Smear preparation
  - Thickness of smear
  - Concentration of organisms
- Smear fixing
  - Excessive heat fixing
  - Inadequate fixing
- Smear staining
  - Improper decolorization
- Antibiotic therapy
- Artifacts
- Experience

Improper Decolorization



Artifacts

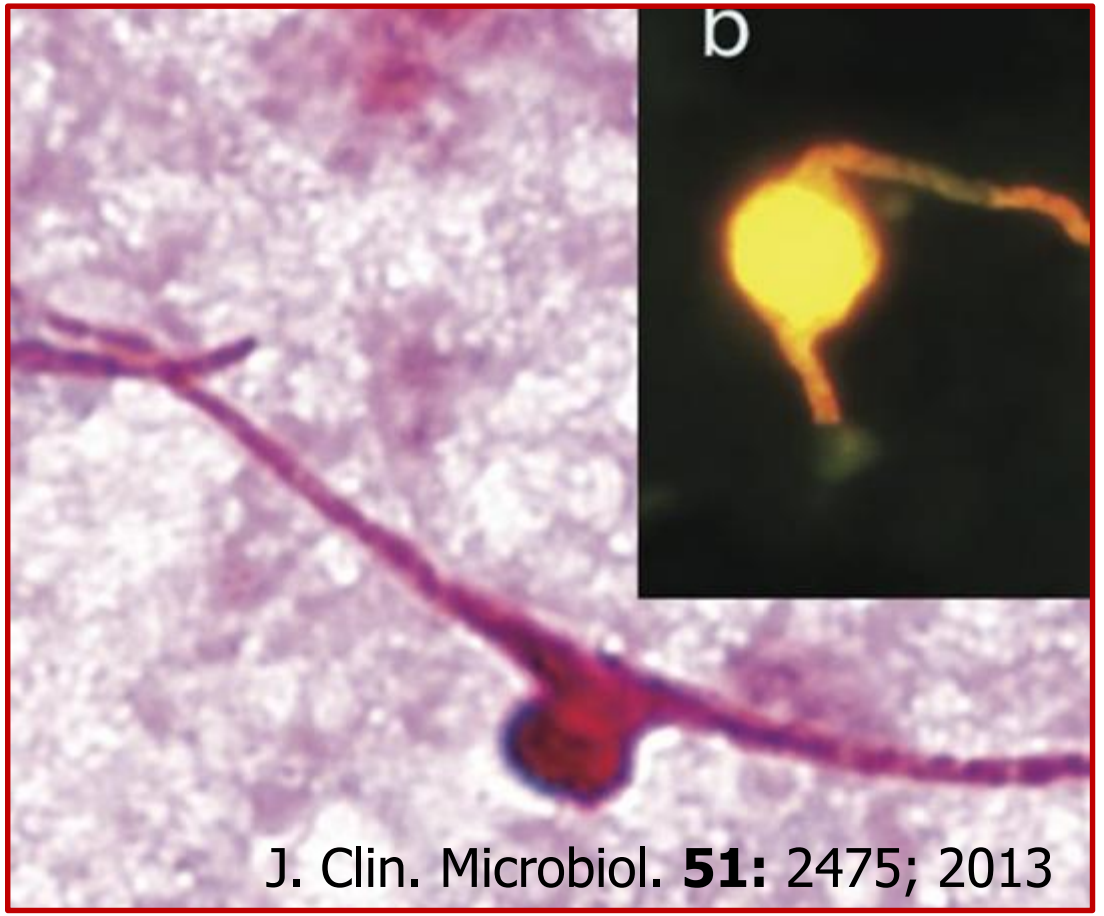




Effects of Antibiotic Therapy

Other Useful Stains

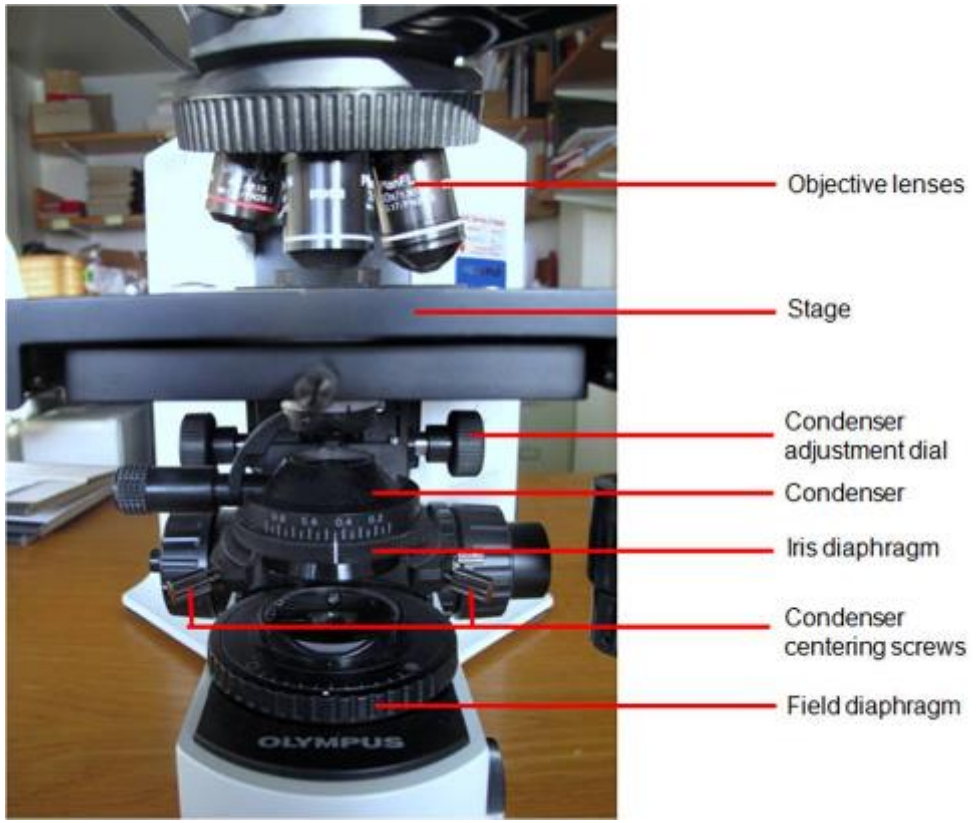
- Carbol Fuchsin, Methylene Blue, Acridine Orange
  - Simple stains
  - Used to visualize the presence of bacteria
  - Helpful with positive blood cultures that show negative gram stains
  - Not to be confused with gram stain



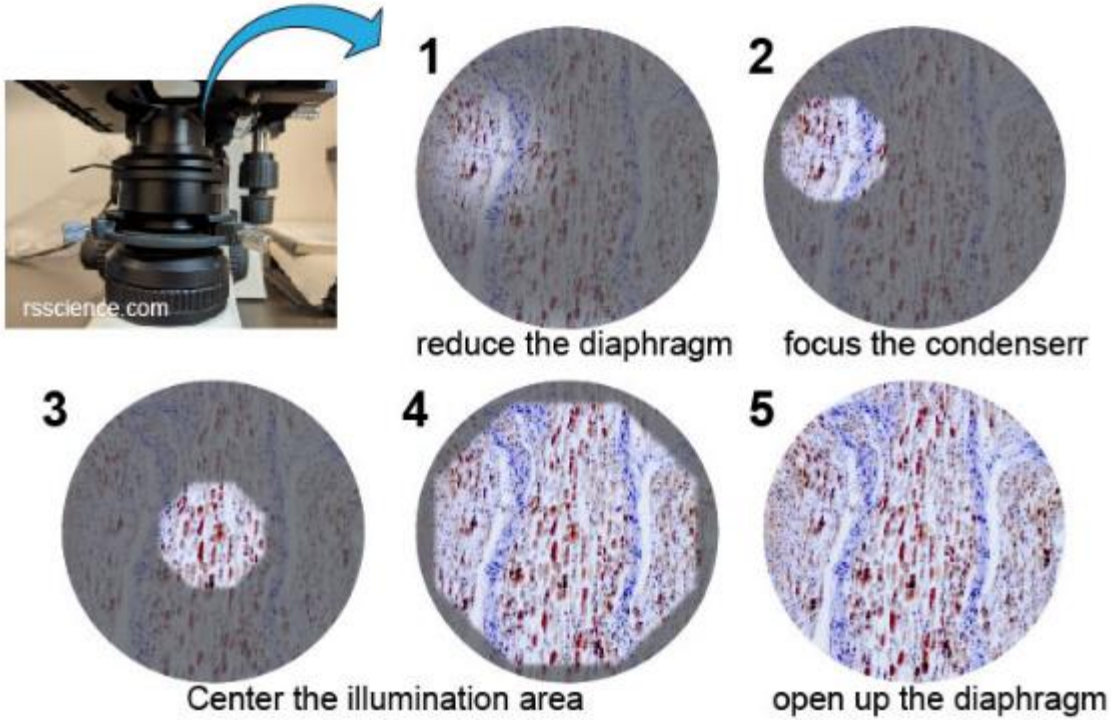
Reading and Reporting Gram Stains

Step 1: Proper Use of Your Microscope

Koehler illumination



Steps of Koehler Illumination



Consider the Source

- Sterile sites:
  - Sources such as blood, CSF, and other fluids are sterile and the presence of organisms is significant
- Sources containing normal flora:
  - Sources such as sputum, throat, reproductive normally have bacteria present
  - Normal flora is represented by the presence of both gram negative and positive organisms in similar amounts
- Possible BT Agents:
  - Found in blood, lower respiratory, and wound cultures

Consider the Age of the Culture

Always consider the possibility that you may be working with a high risk bioterrorism select agent and perform all work-up in a biosafety cabinet whenever you see any of these key indicators:

- Blood culture becomes positive ≥36 hours and
  - Gram stain shows small GNR or GNCB/GPCB
  - Or Gram stain shows boxcar shaped GPR with or without spores
- Slow growing tiny colonies at 24-48 hours and
  - Gram stain shows small GNR or GNCB
- Isolate only grows, or grows better, on chocolate agar and
  - Gram stain shows small GNR or GNCB
- Growth of flat non-pigmented irregular colonies with comma projections and ground glass appearance and
  - Gram stain shows boxcar shaped GPR with or without spores

Gram Stain Quantitation

- Gram stain is at best a semi-quantitative estimation
  - No ability to standardize the inoculum
- Different methods for reporting quantitation
- Scan 10 – 20 fields at 10X to quantitate cellular material
- Scan 20 – 40 fields at 100X (oil immersion) to quantitate organisms

Example of Reporting Quantitation Criteria

Reported Quantity	Per low power field (10X)	Per oil immersion field (100X)
Rare/(1+)	<10 cellular elements	<10 organisms
Moderate/ (2+)	>10 but <25 cellular elements	>10 but <25 organisms
Many/ (3+)	≥ 25 cellular elements	≥ 25 organisms

Method suggested by Dr. Tom Thomson, Jr.

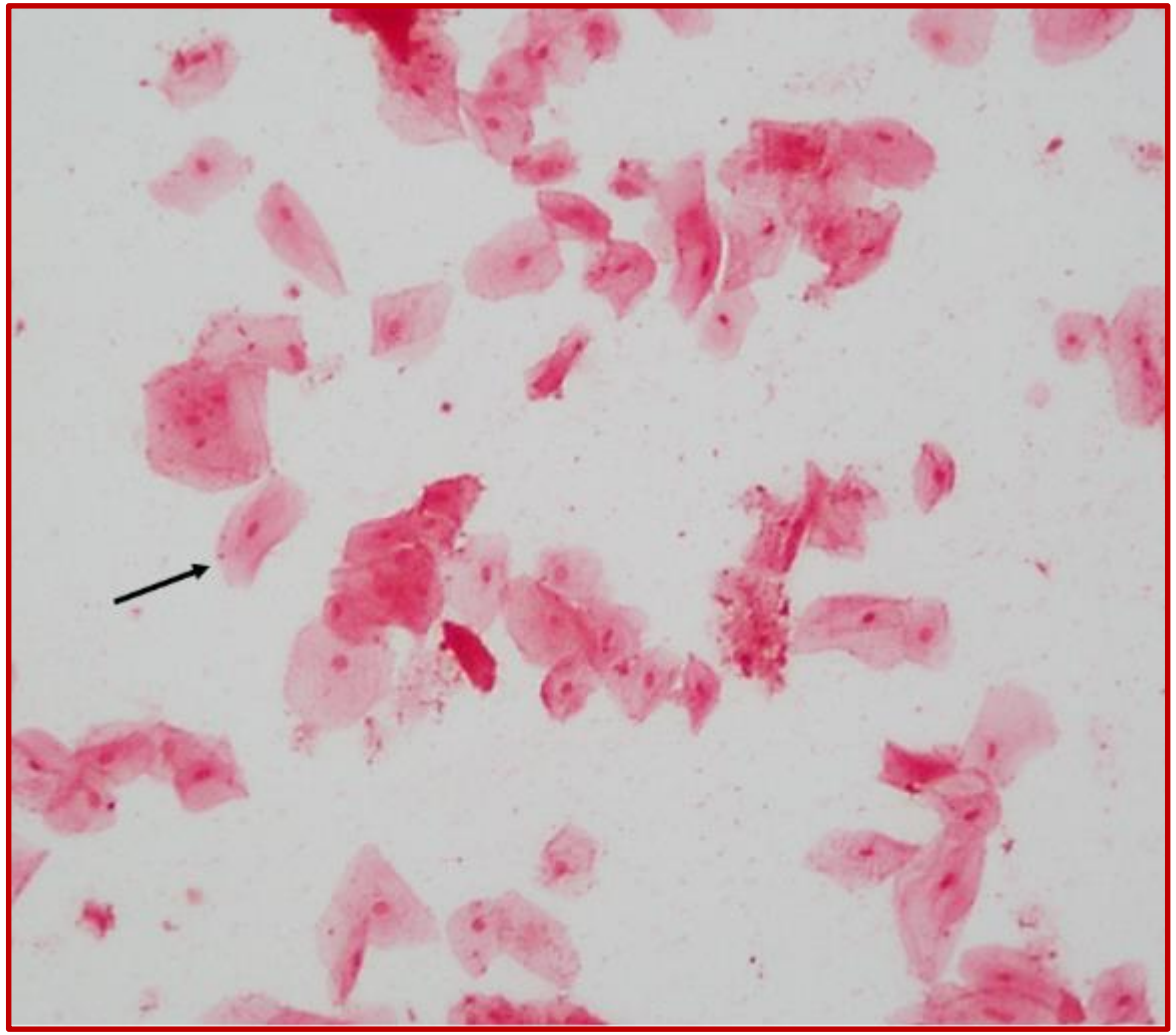
Assess the Quality of the Smear

- Even monolayer of cells with no thick and thin portions
- Evenly decolorized with no sections that are over or under decolorized
- Assess the cellular material present
  - PMNs indicate inflammation or infection
  - RBCs indicate deep culture or traumatic injury
- Use quantitation to assess the quality of a sputum specimen and decide whether to accept or reject the specimen

Rejected Sputum

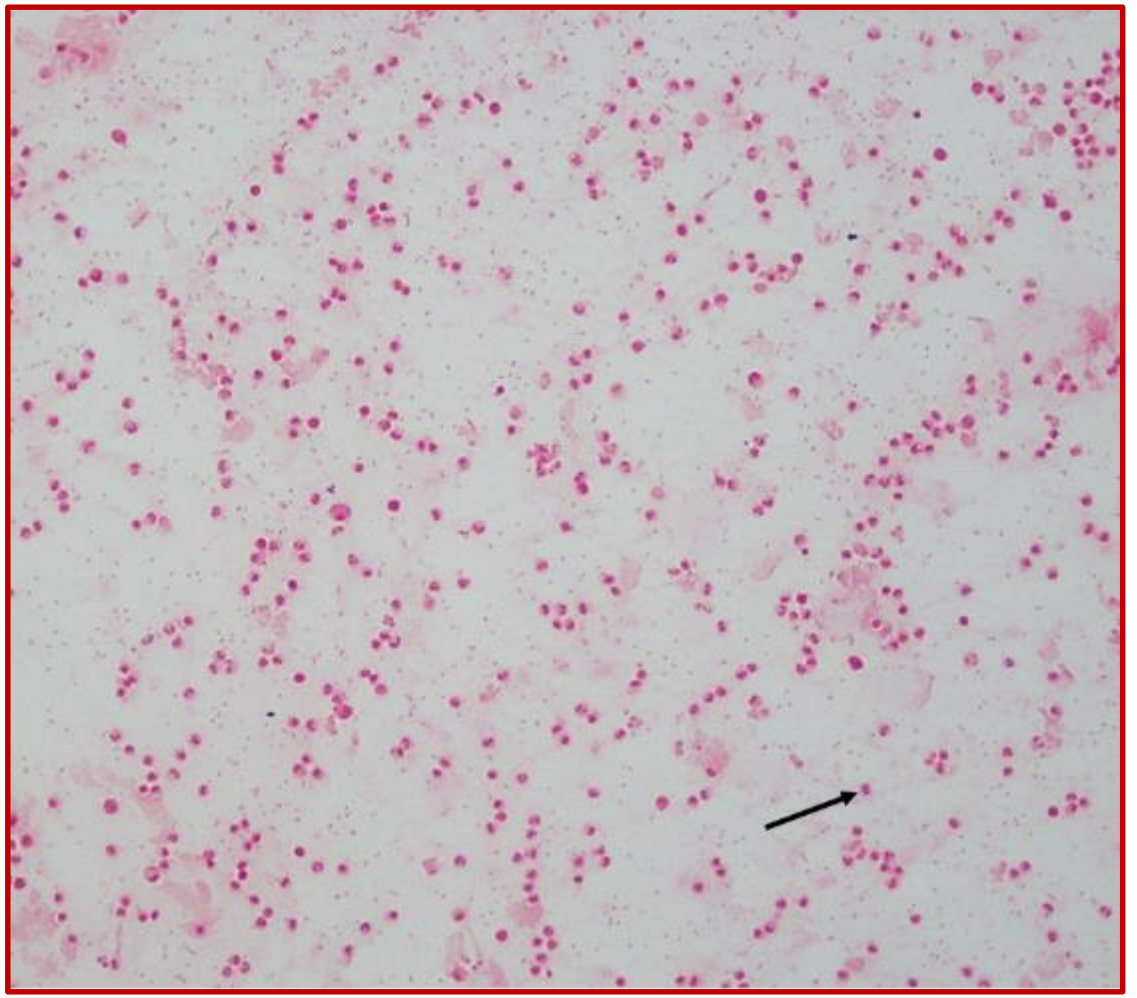
Rejection Criteria:

- >25 Squamous Epis/10X LPF and
- < 25 PMNs/10X LPF



Acceptable Sputum

Many PMNs and few Epis
10X /LPF



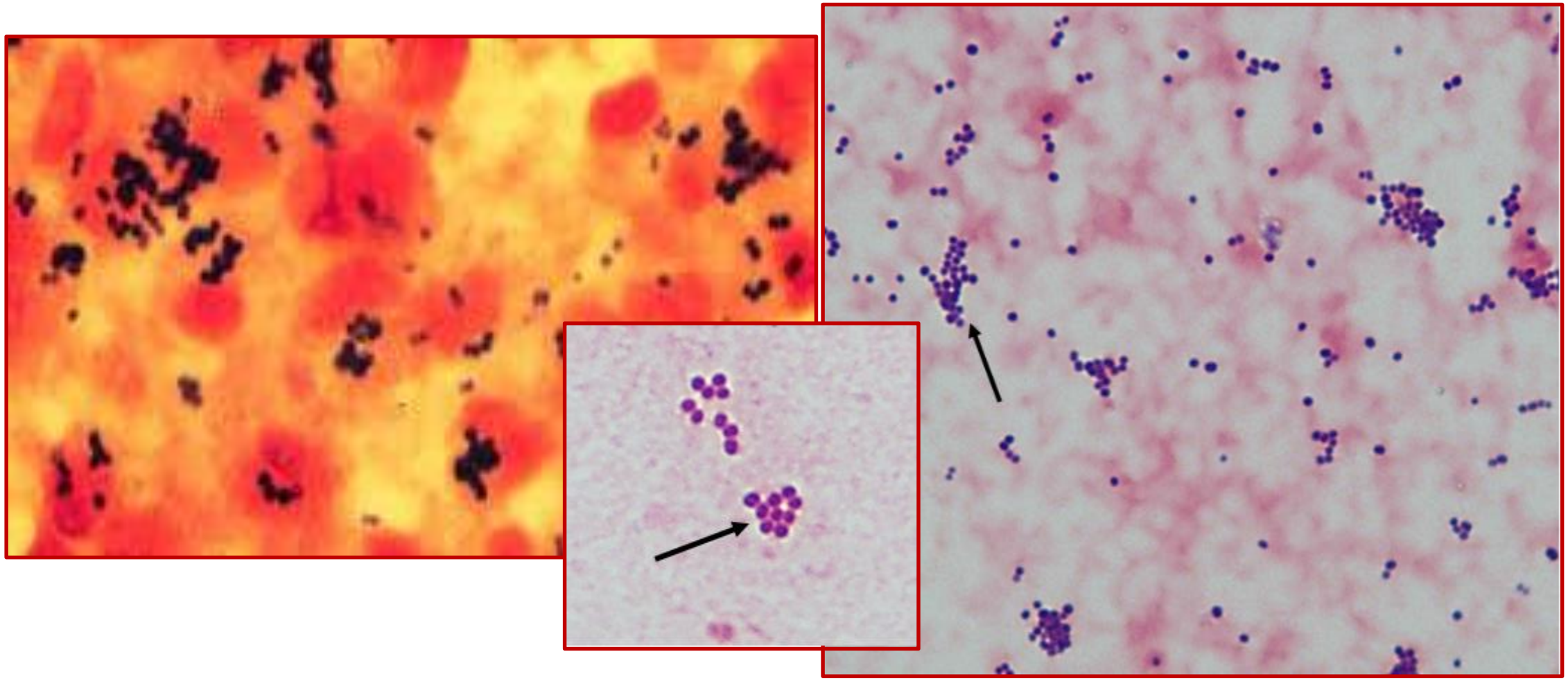
Useful Morphology Terminology

- **Cocci** – large or tiny
  - Clusters
  - Pairs/Chains
- **Diplococci**
- **Coccobacilli**
- **Rods (bacilli)** – thick or thin
  - Fusiform
  - Filamentous
  - Branching
  - Diphtheroid
  - Boxcar
- **Yeast** – budding or Pseudohyphae
- **Mold -** septate or nonseptate hyphae

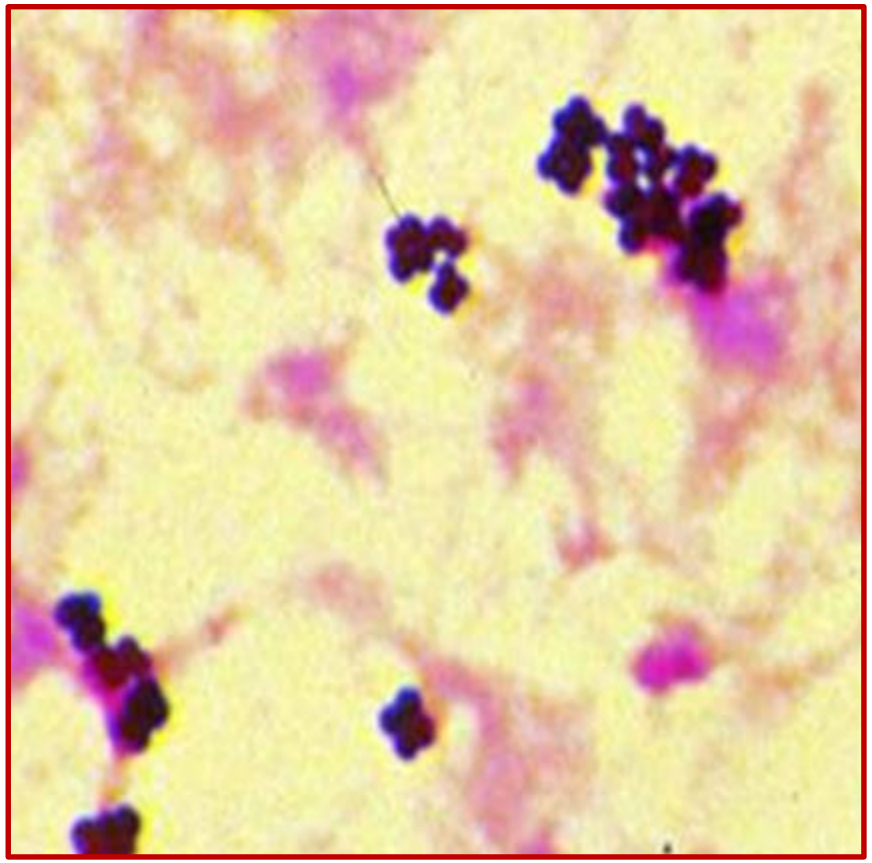
Let's Talk Cocci

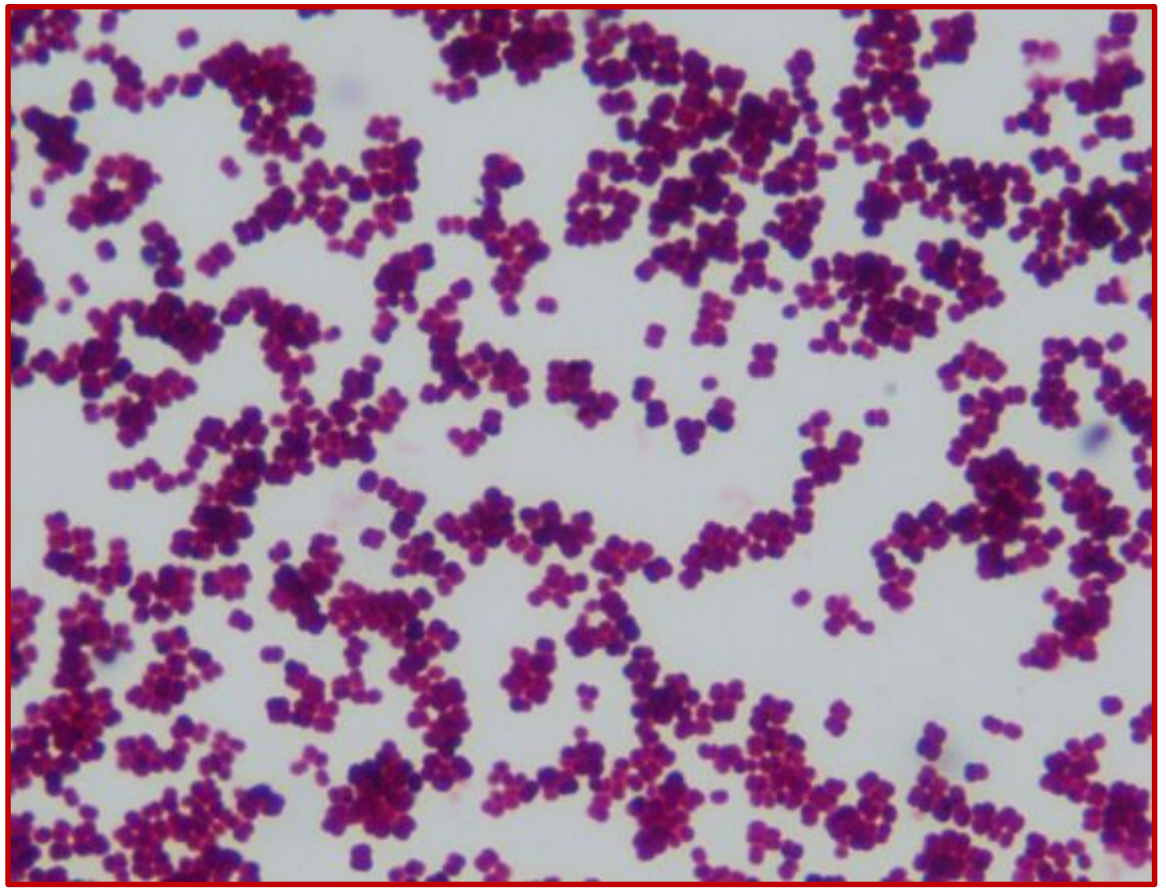
- Clusters/Tetrads
- Pairs/Chains

Gram Positive Cocci in Clusters

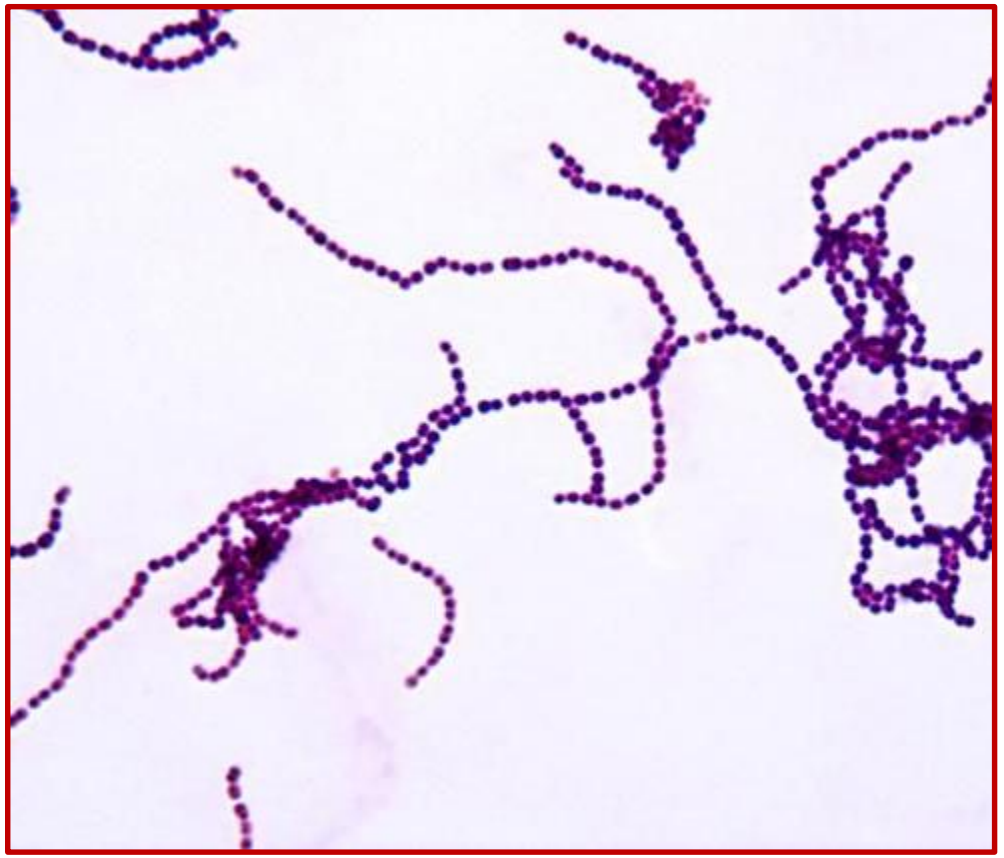


Gram Positive Cocci in Tetrads



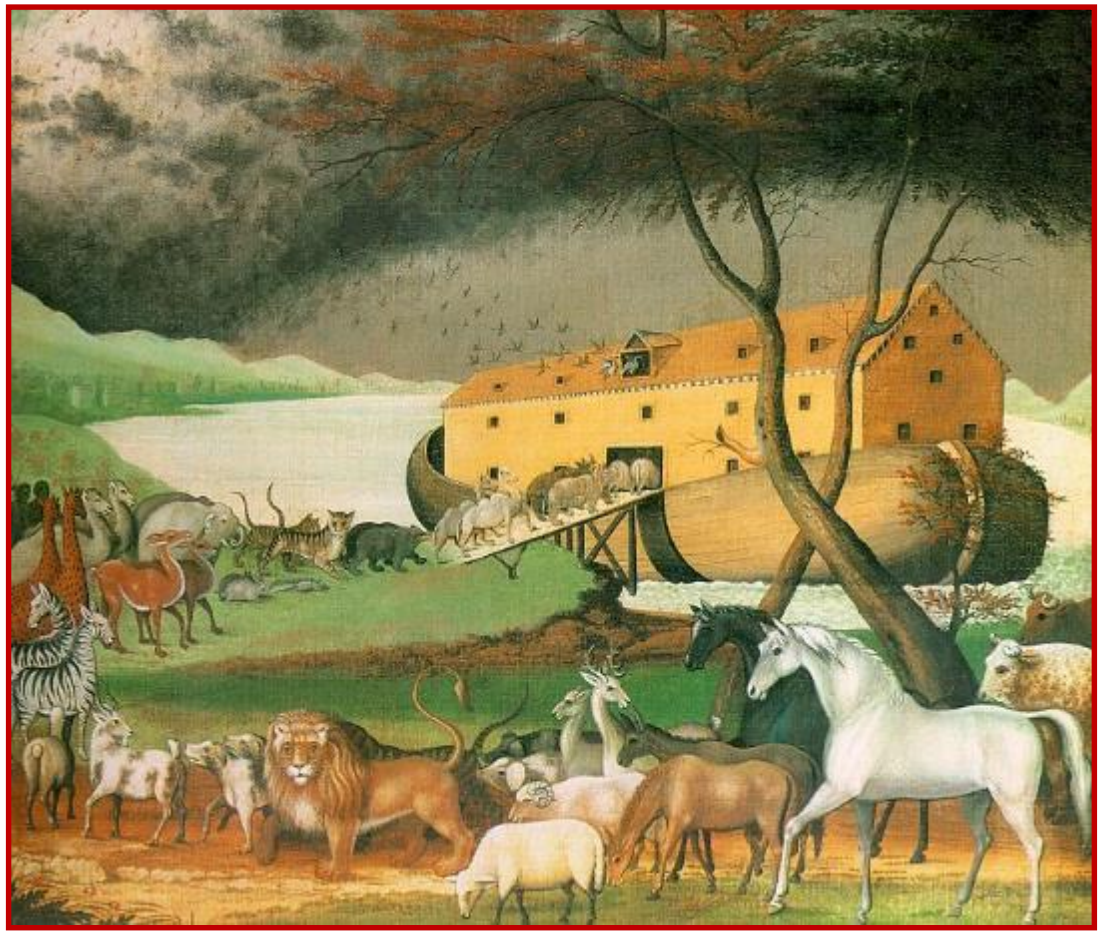


Gram Positive Cocci in Chains



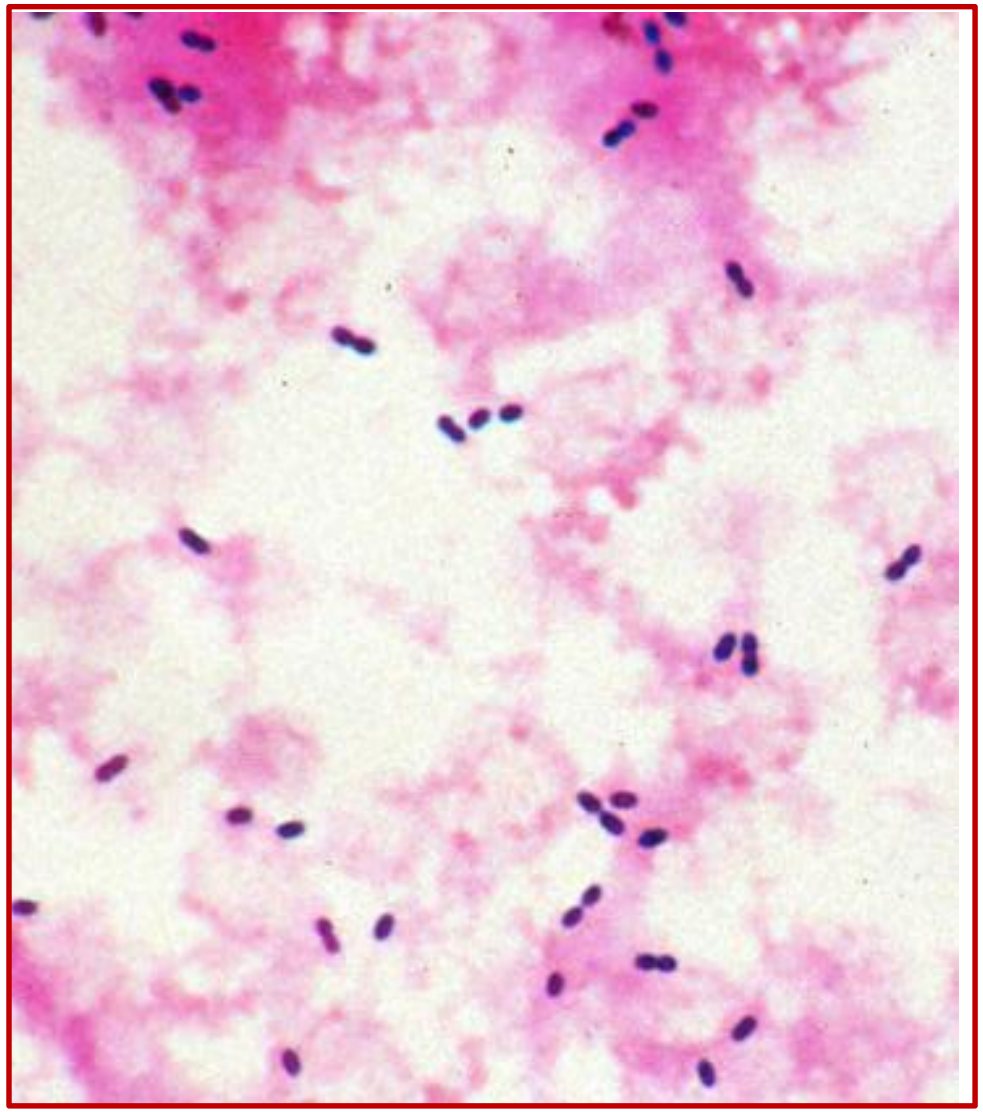
Lets Talk Diplococci!

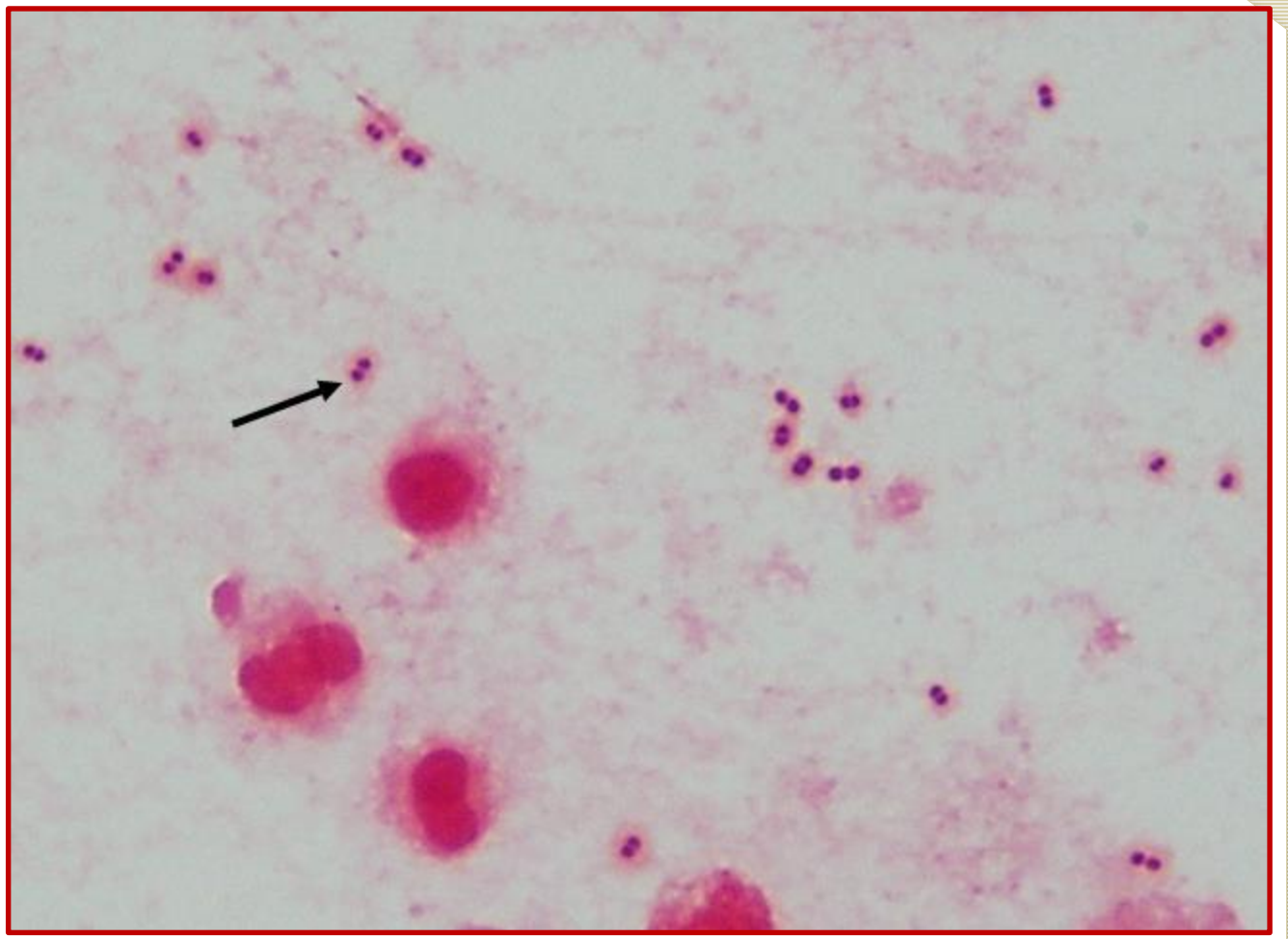
- Lancet shaped
- Kidney bean



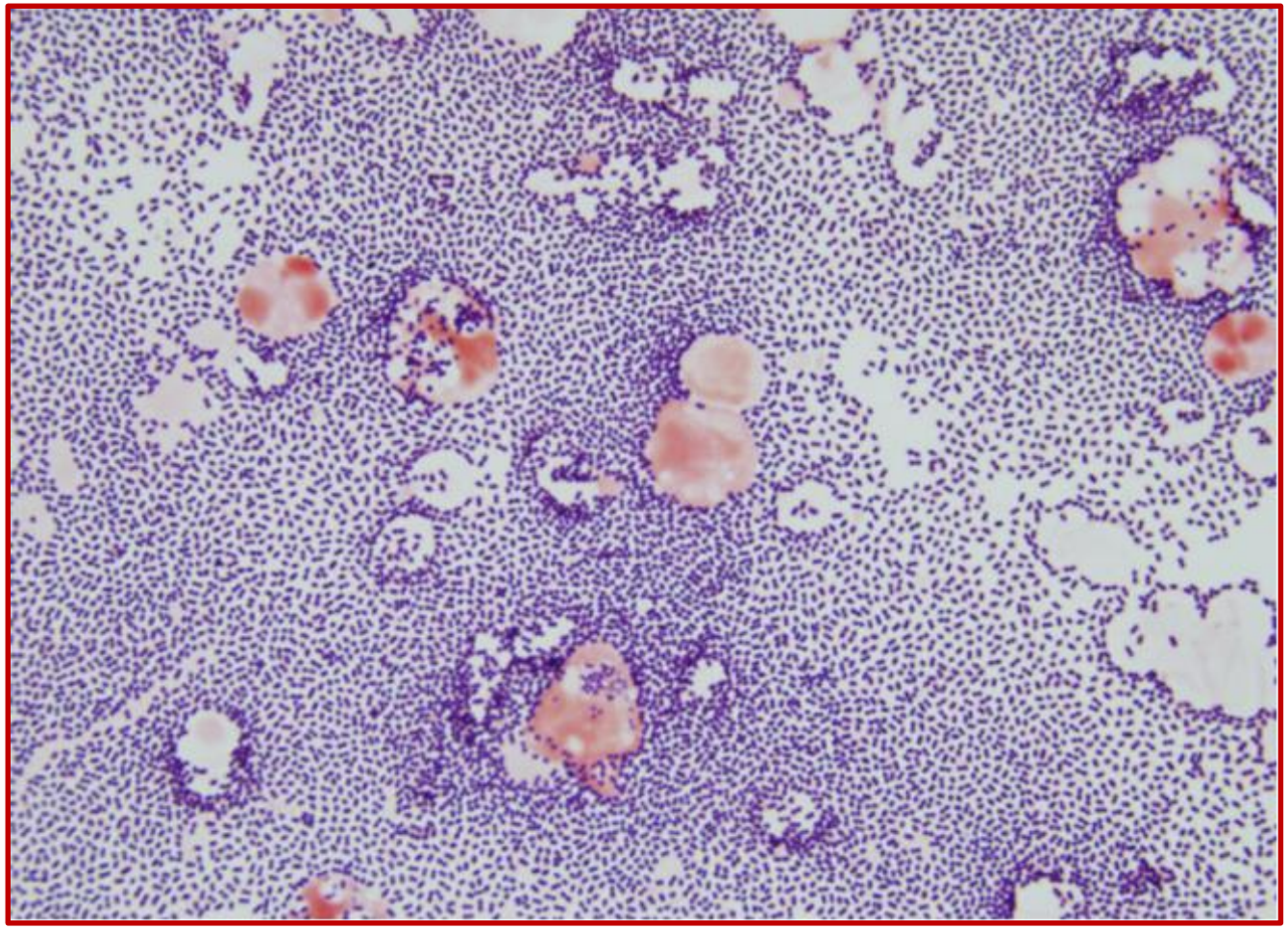
A painting by the American Edward Hicks (1780–1849), showing the animals boarding Noah's Ark two by two.

Gram Positive Diplococci



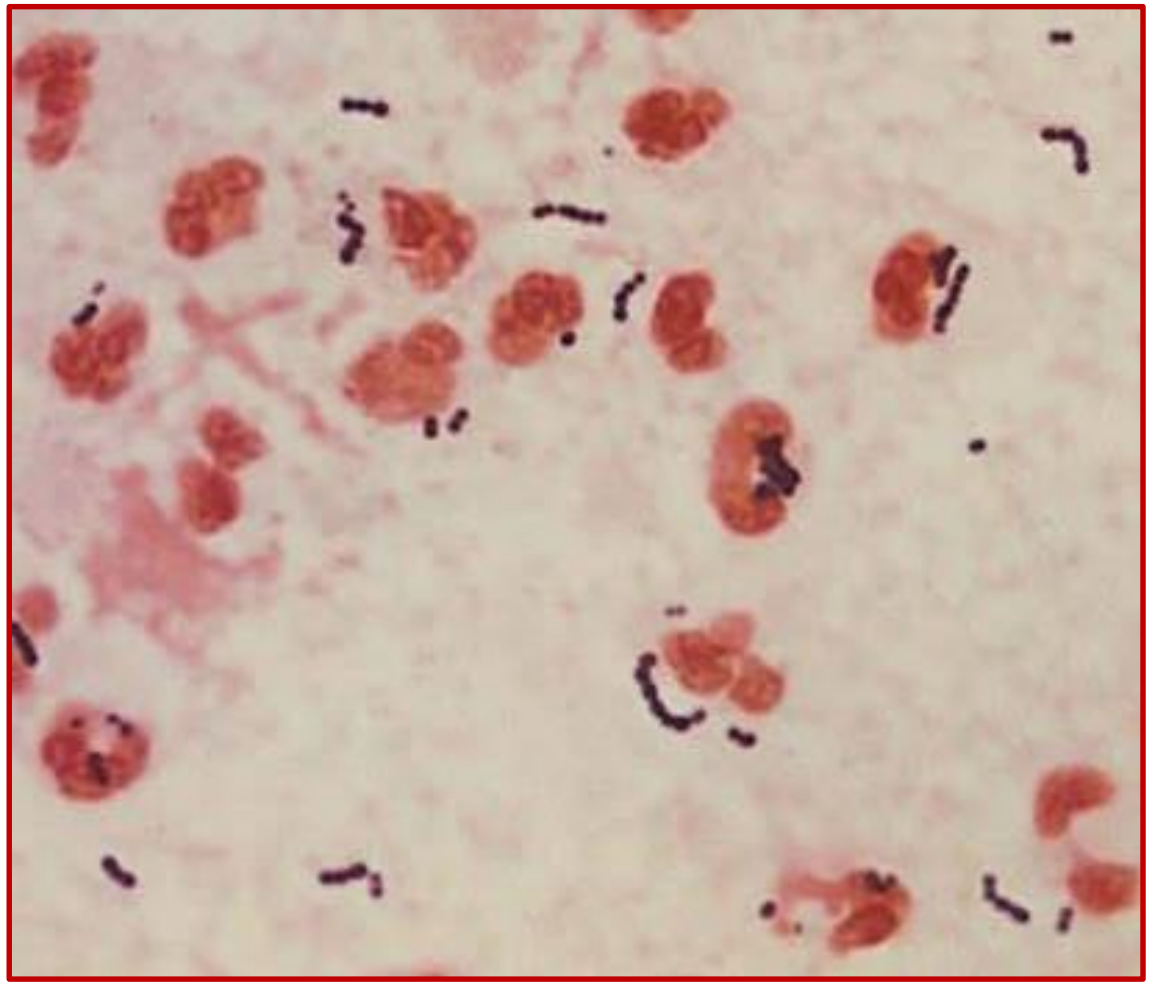


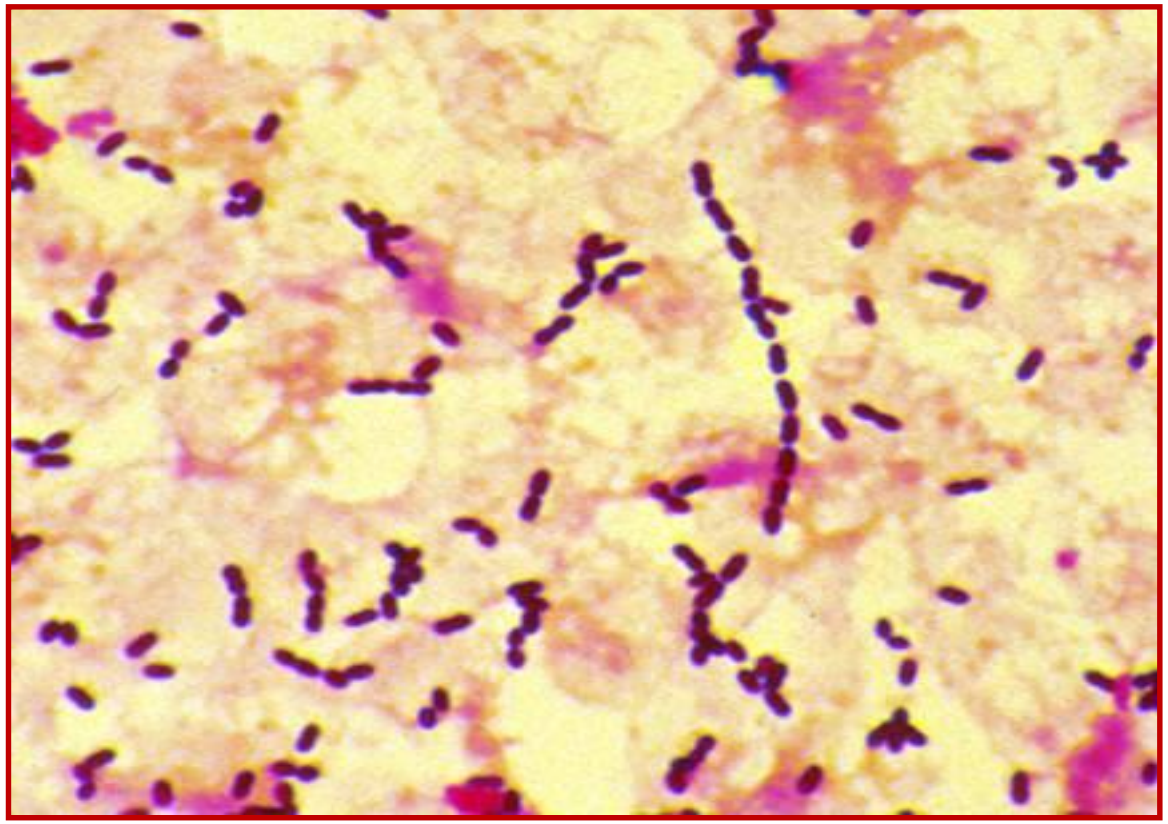
Gram Positive Diplococci in a CSF



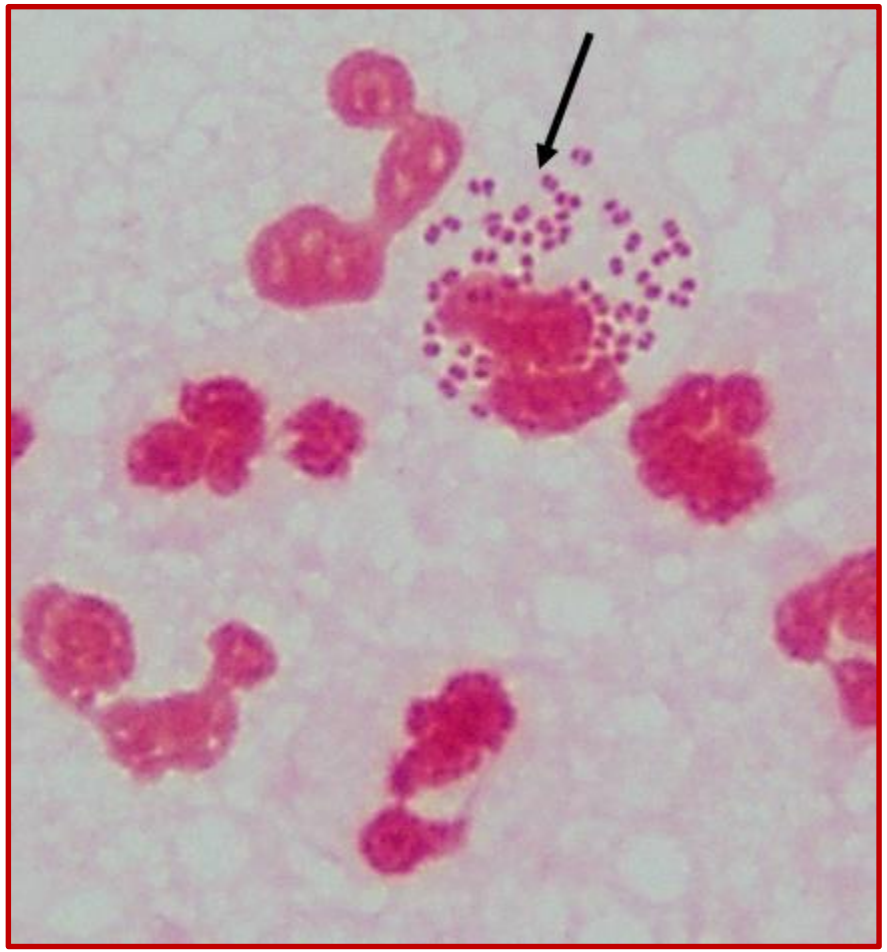
J. Clin. Microbiol. **52:** 3835; 2014

Gram Positive Cocci in Pairs and Chains

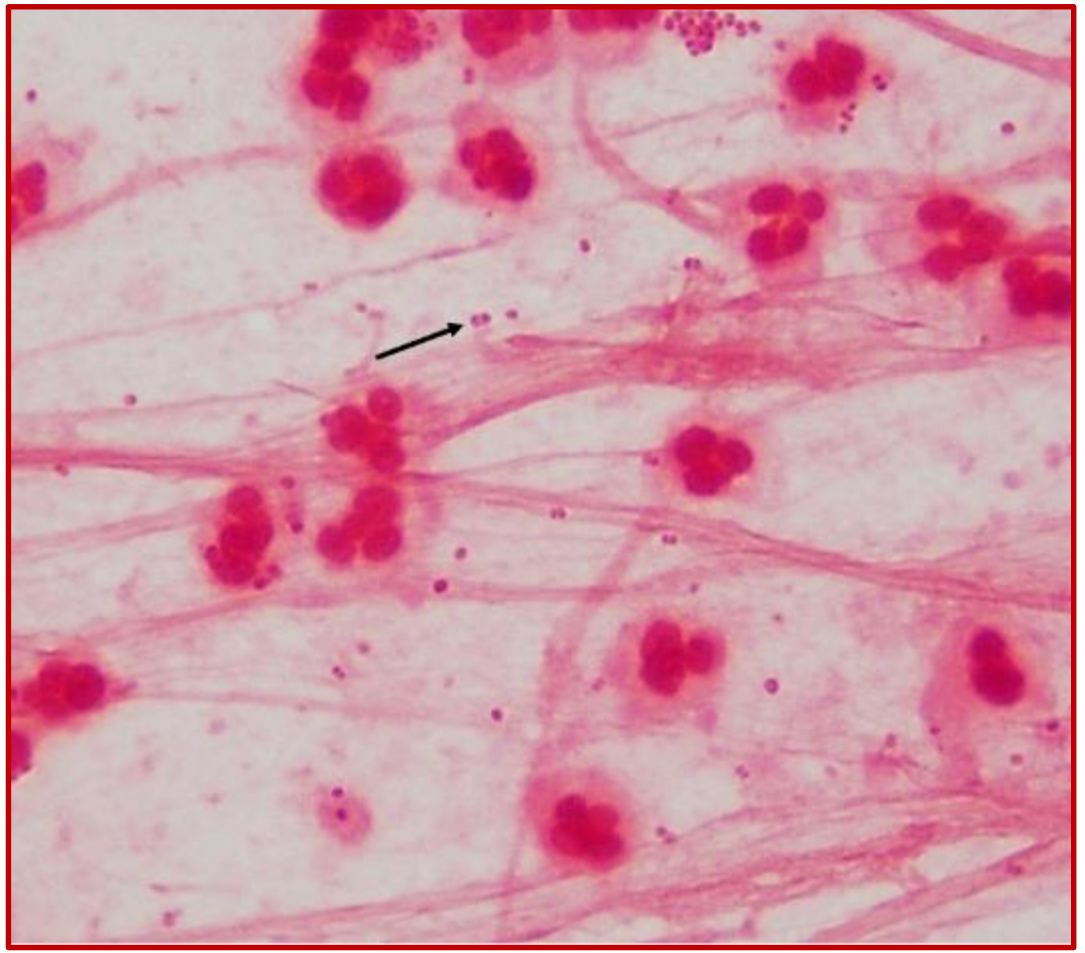




Gram Negative Diplococci



Gram Negative Cocci?



Lets Talk Coccobacilli!

- **Pleomorphic**
  - *adjective* Referring to a variable appearance of morphology

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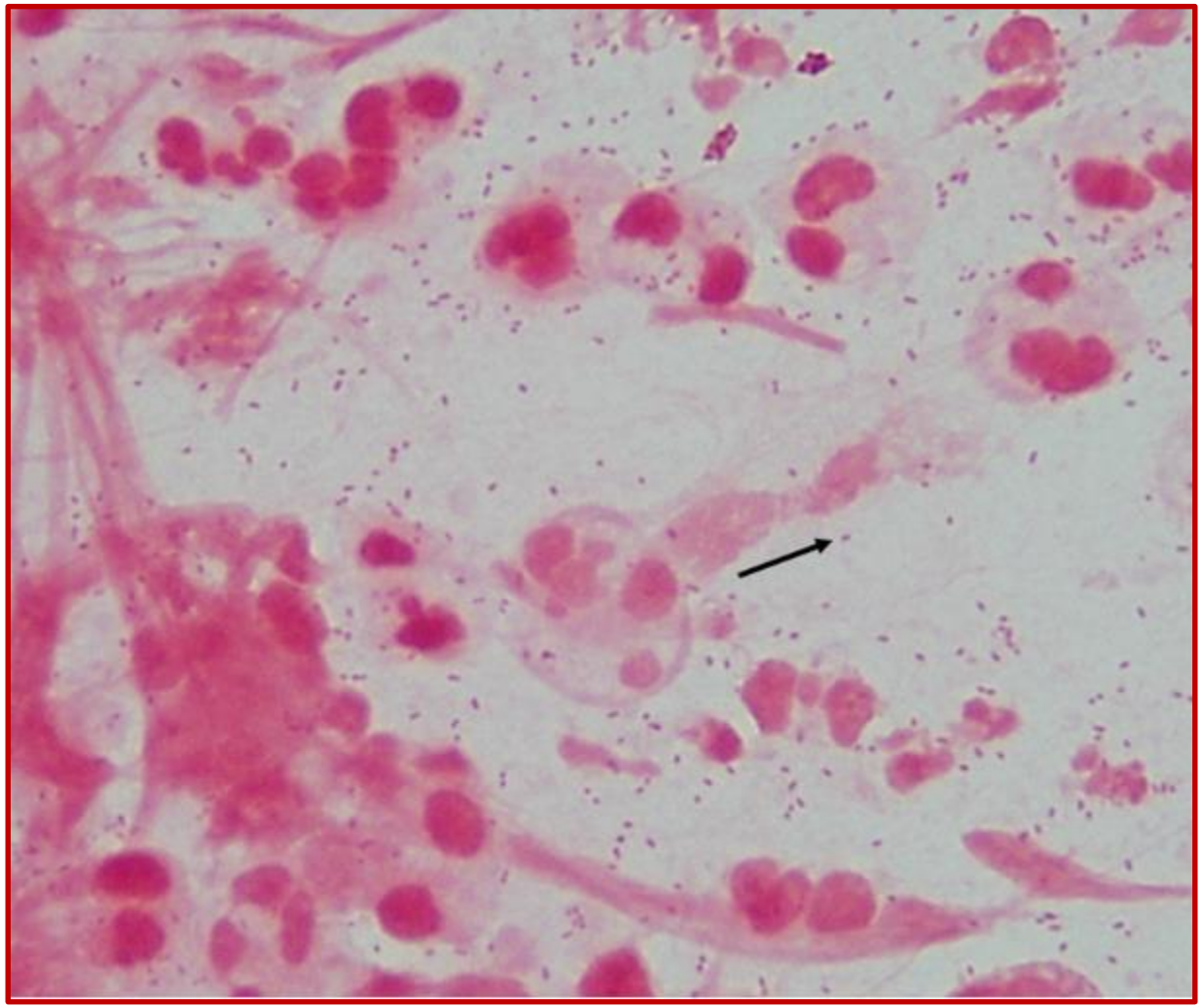
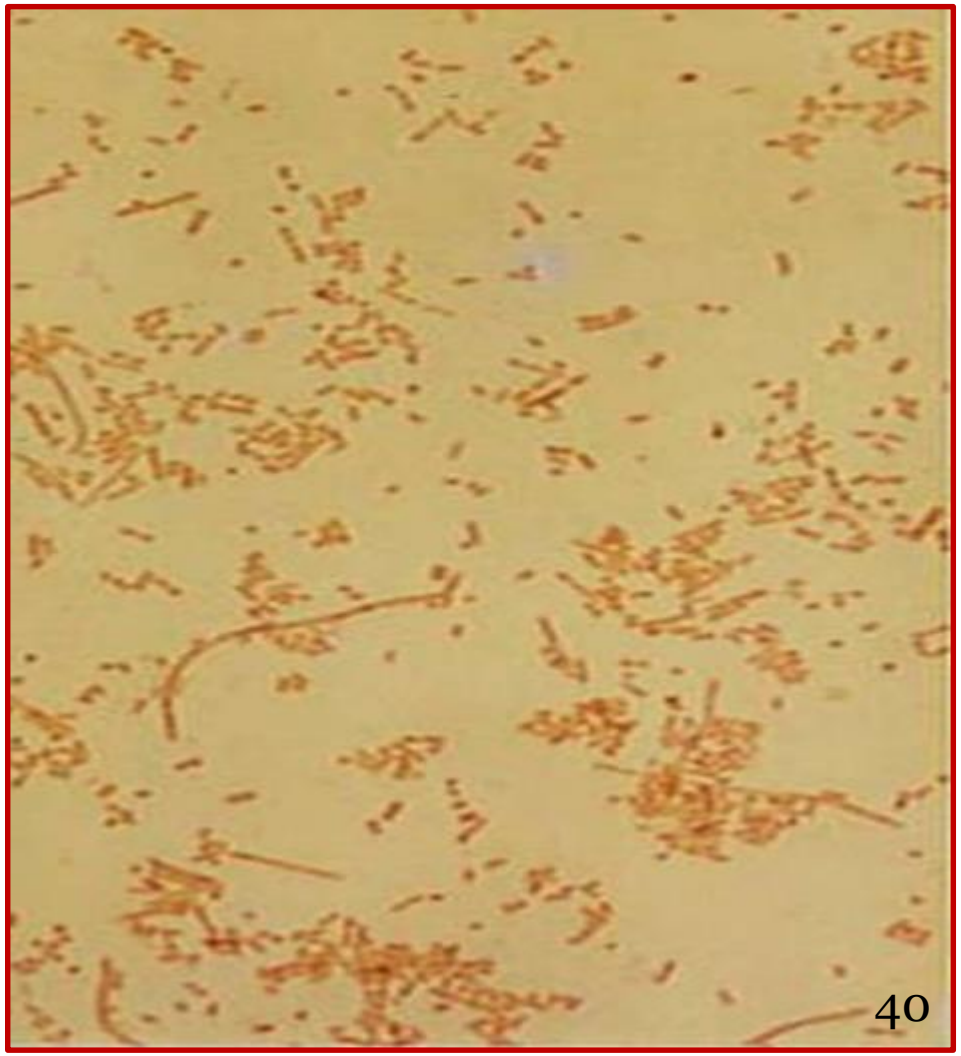
Gram Negative Coccobacilli

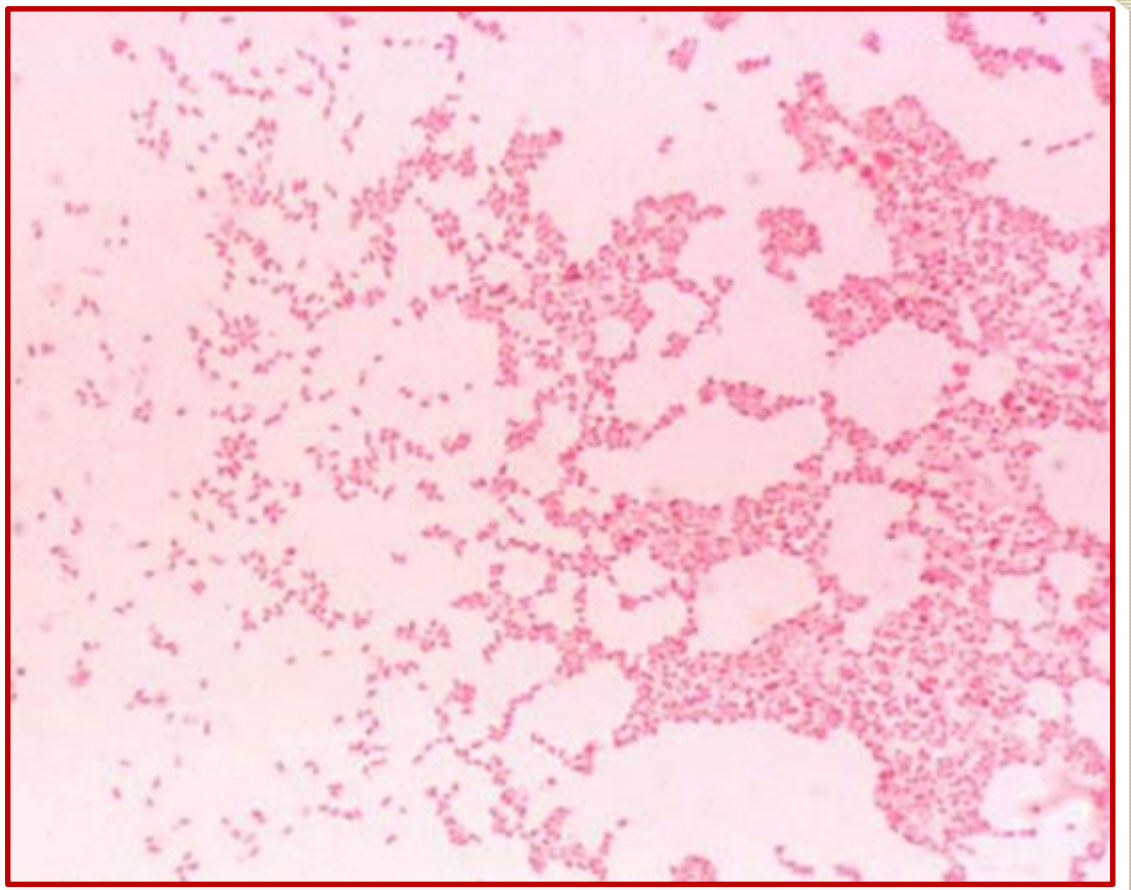




Gram Negative Coccobacilli

What is the source?

J. Clin. Microbiol. **49:** 2085; 2011



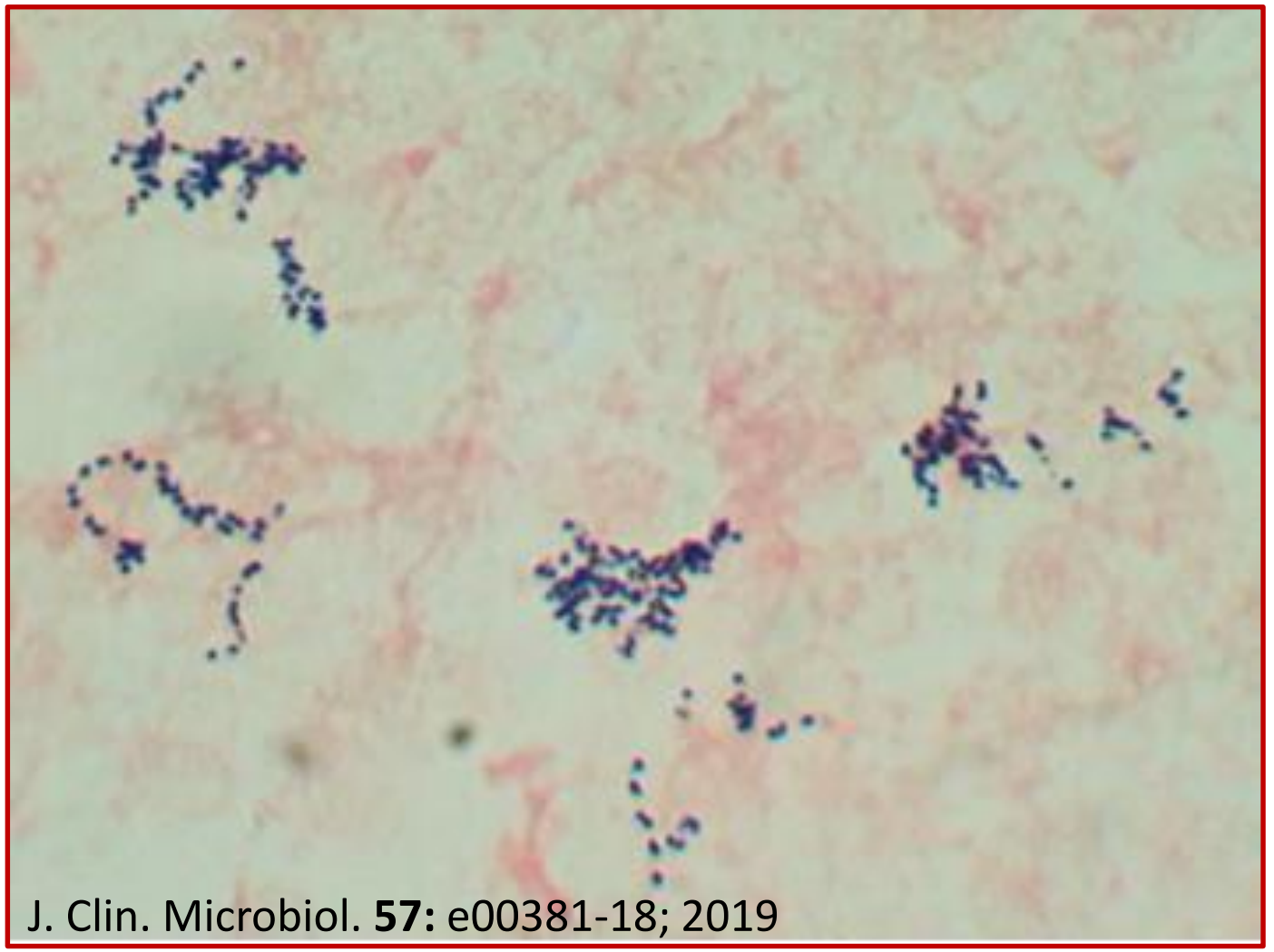
How old is this culture?

Gram Positive Coccobacilli

Huh!

What am I looking at?
Tiny GPCB?

What is the source and the age of this culture?

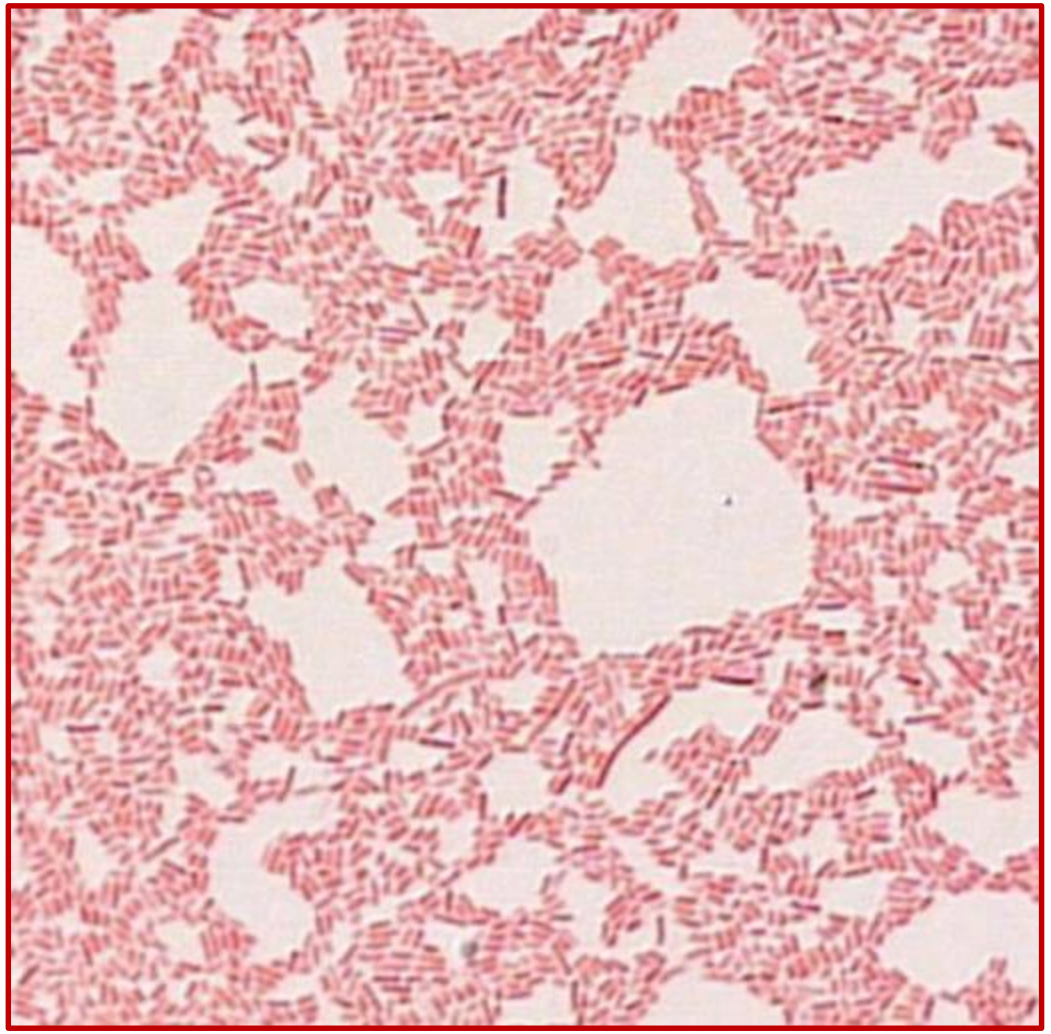


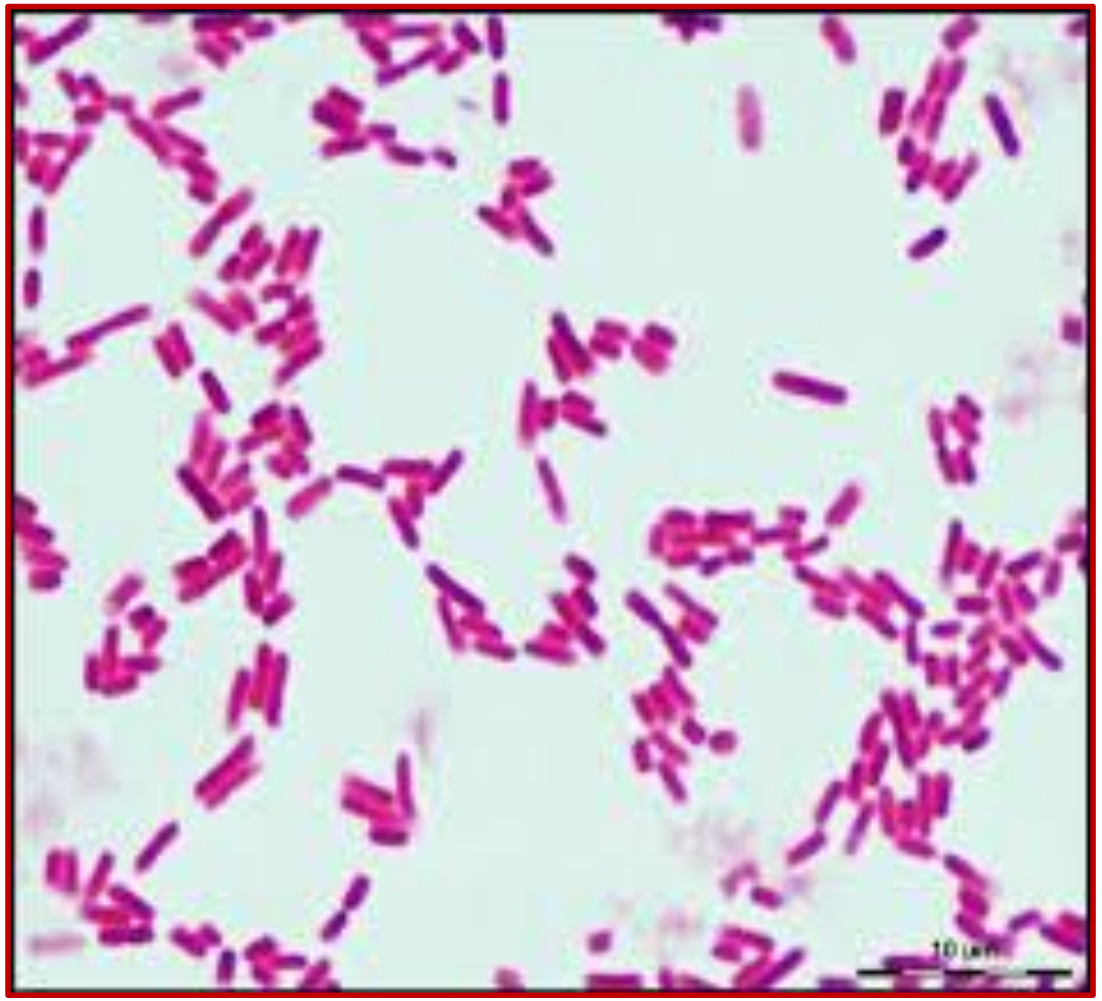
J. Clin. Microbiol. **57:** e00381-18; 2019

Lets Talk Bacilli/Rods!

- Fusiform
- Branching
- Diphtheroid
- Filamentous
- Boxcar
  - Spore forming

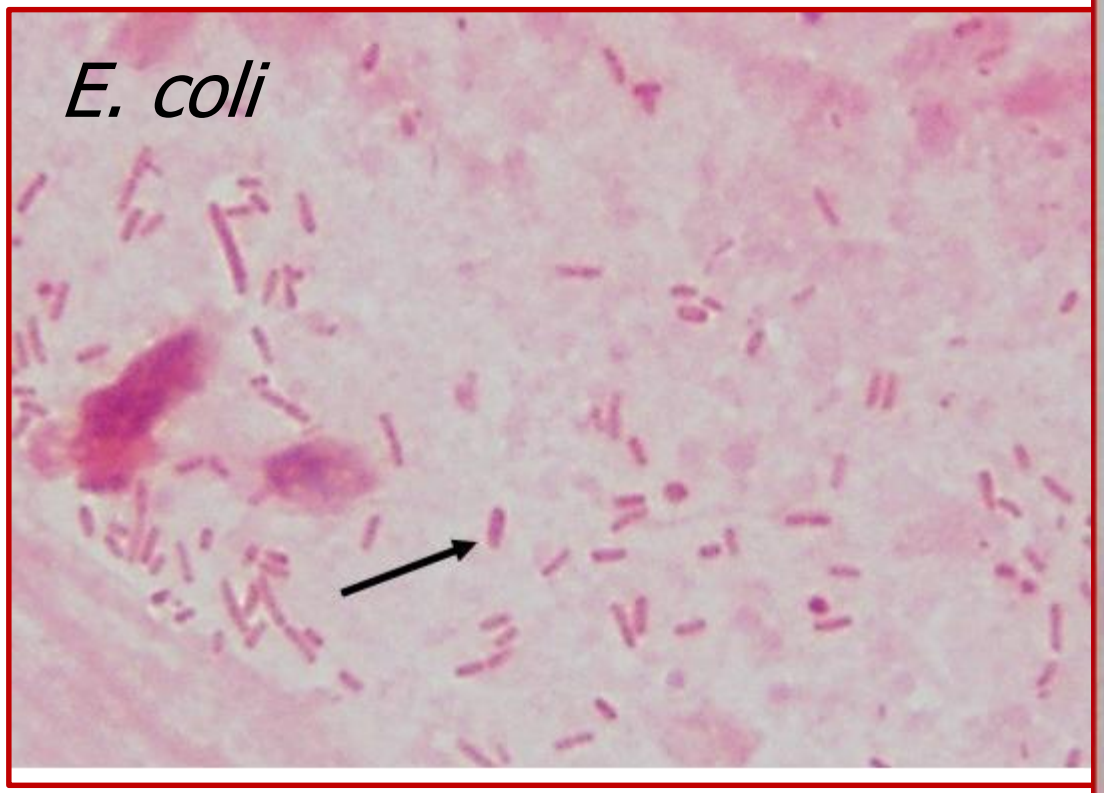
Gram Negative Bacilli

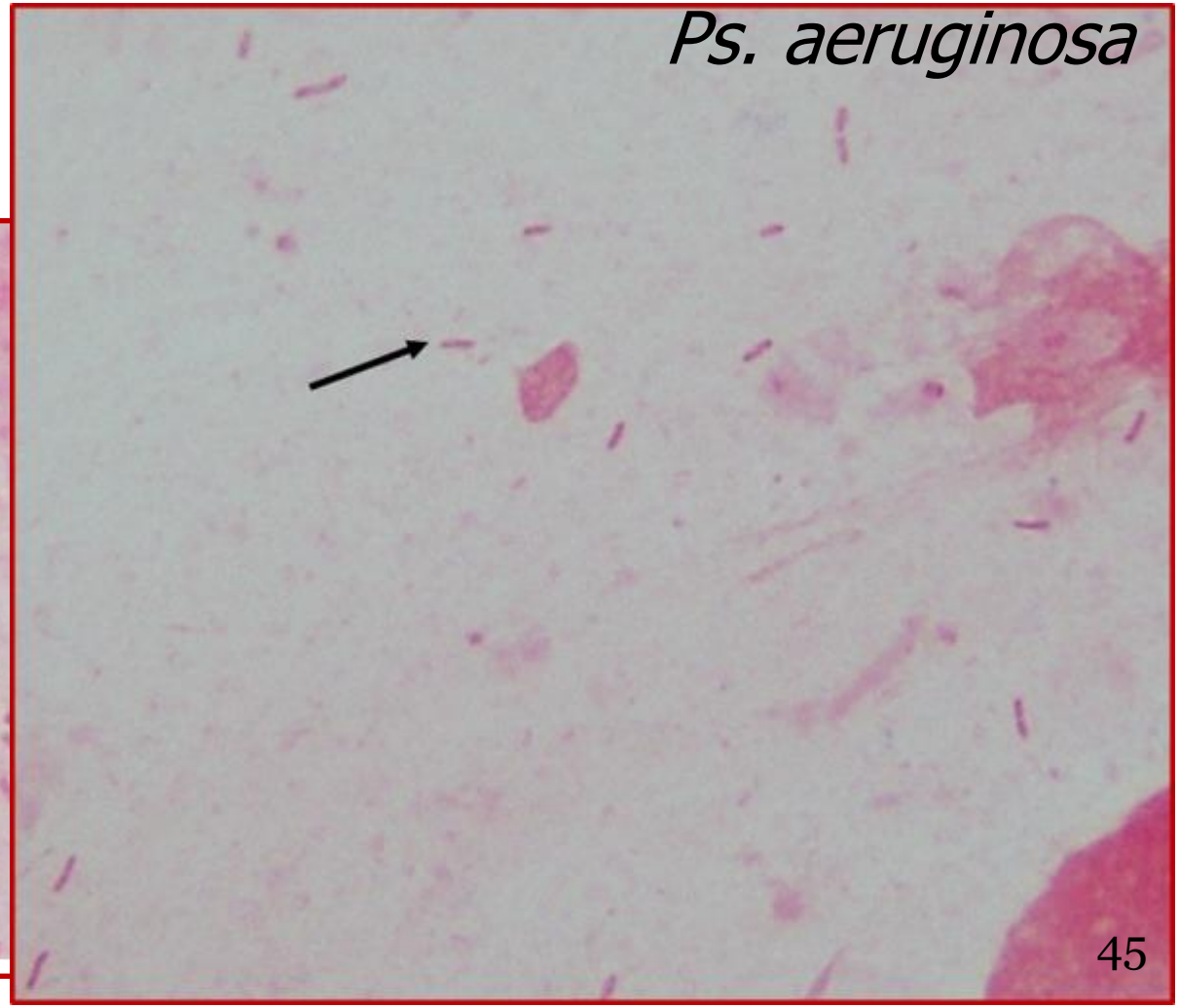




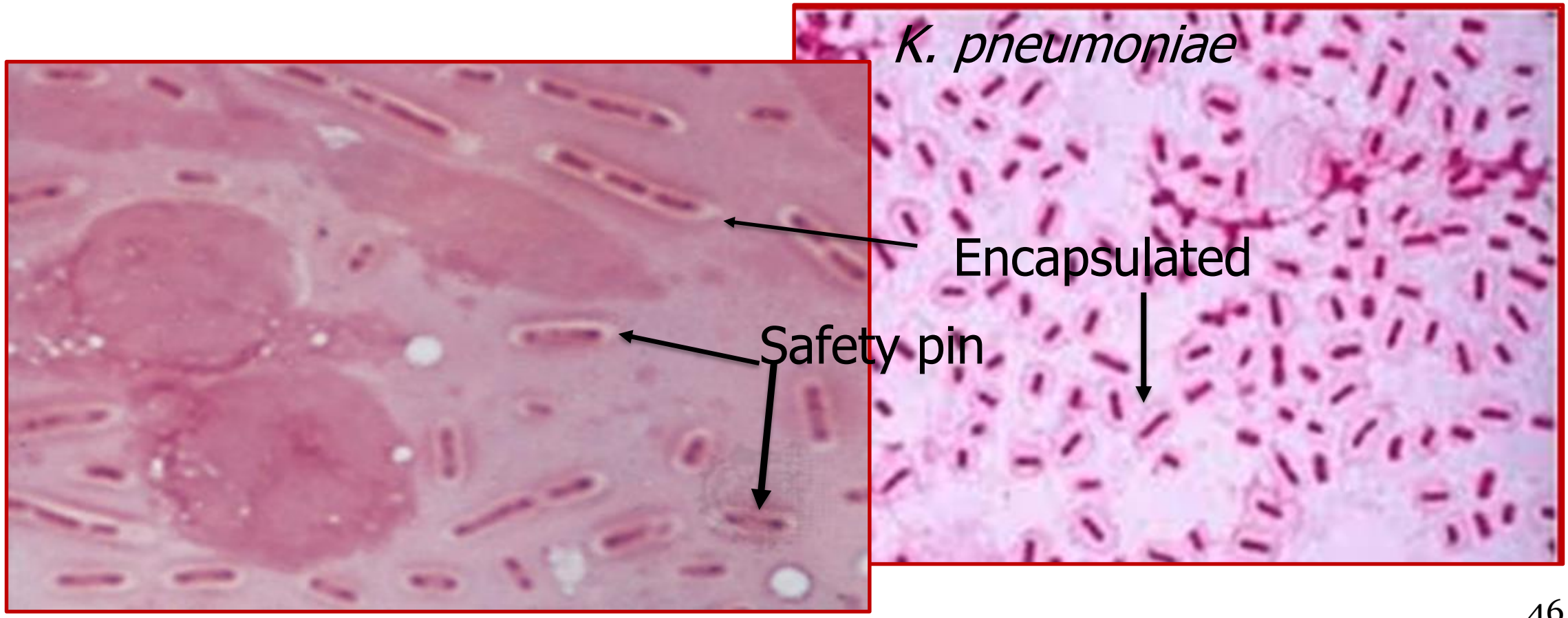
Gram Negative Bacilli

Size Comparison:





Gram Negative Bacilli (Safety Pin / Encapsulated)



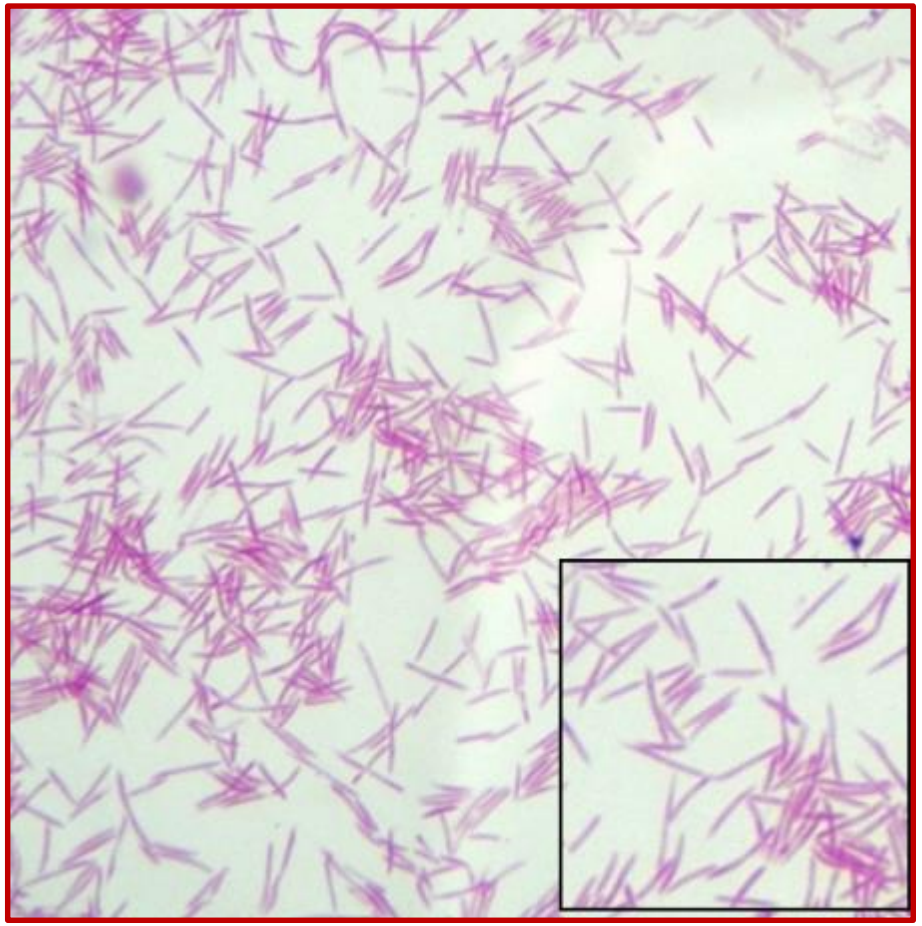
Gram Negative Bacilli (Faint and Curved)

Campylobacter spp.

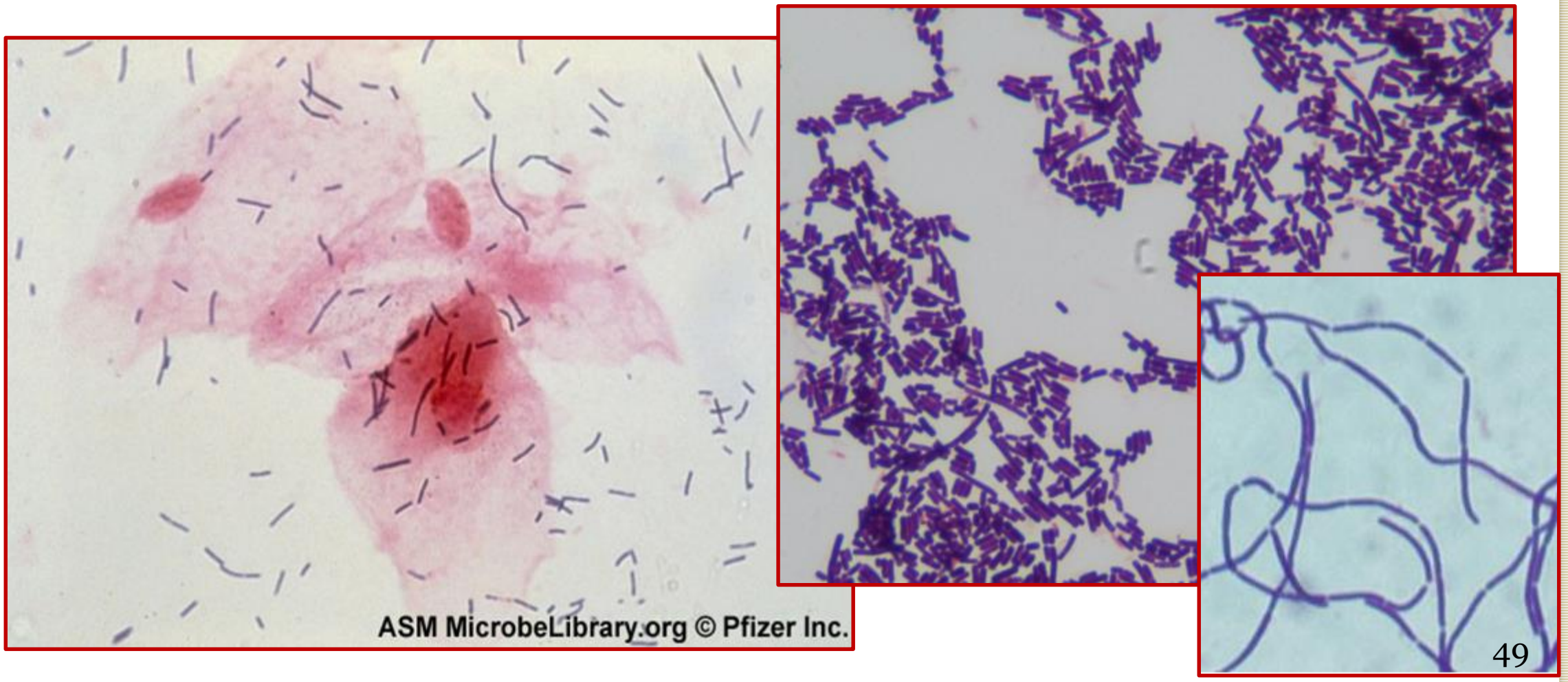
J. Clin. Microbiol. **55:** 3151-3152; 2017

Gram Negative Bacilli (Fusiform)

Fusobacterium nucleatum

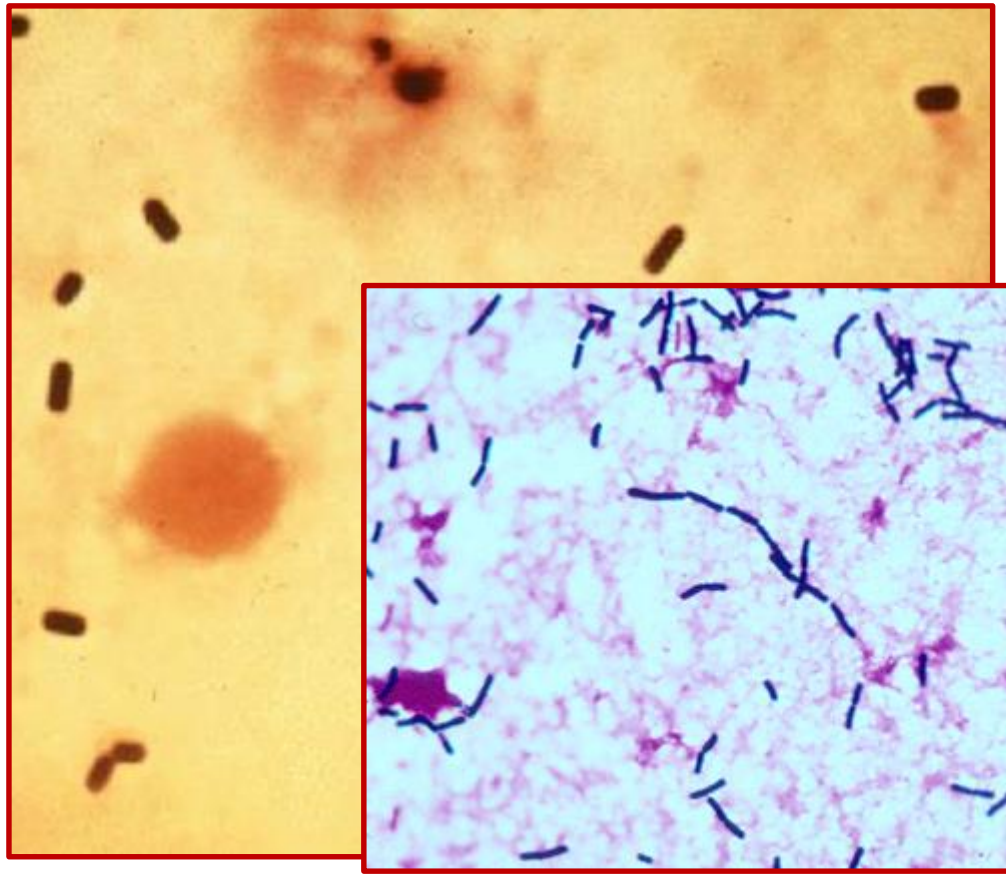


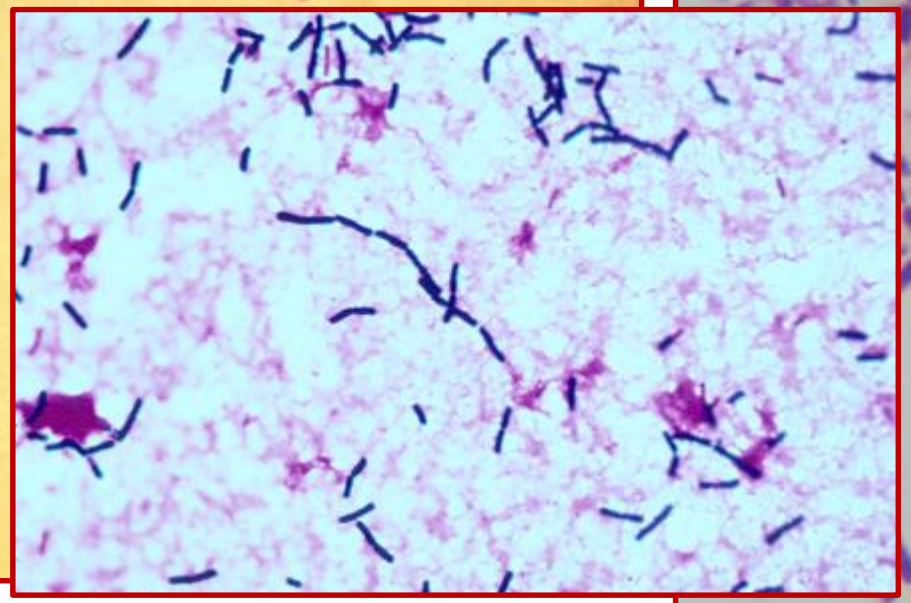
Gram Positive Bacilli/Rods

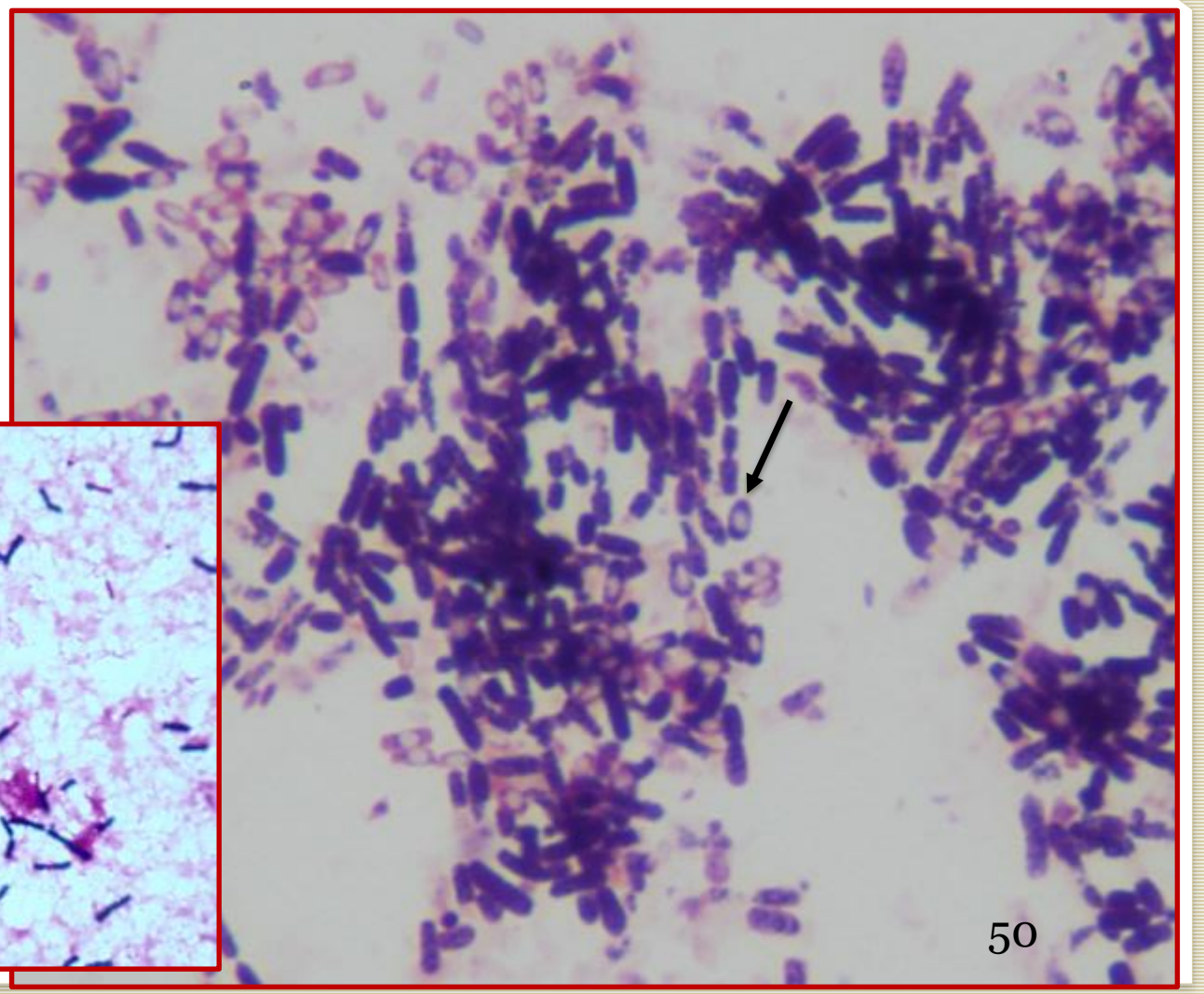


Gram Positive Bacilli (Boxcar)

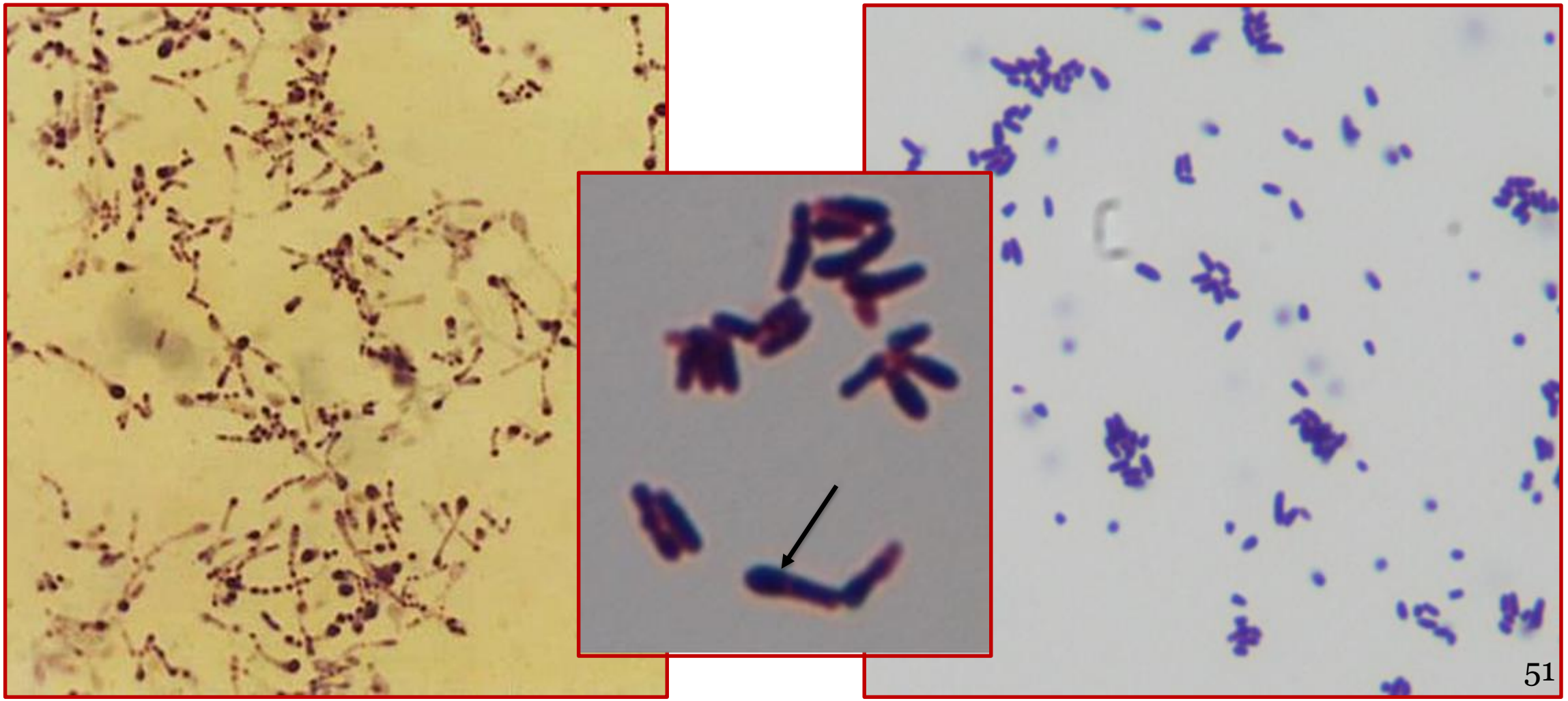
What is the source of the culture?







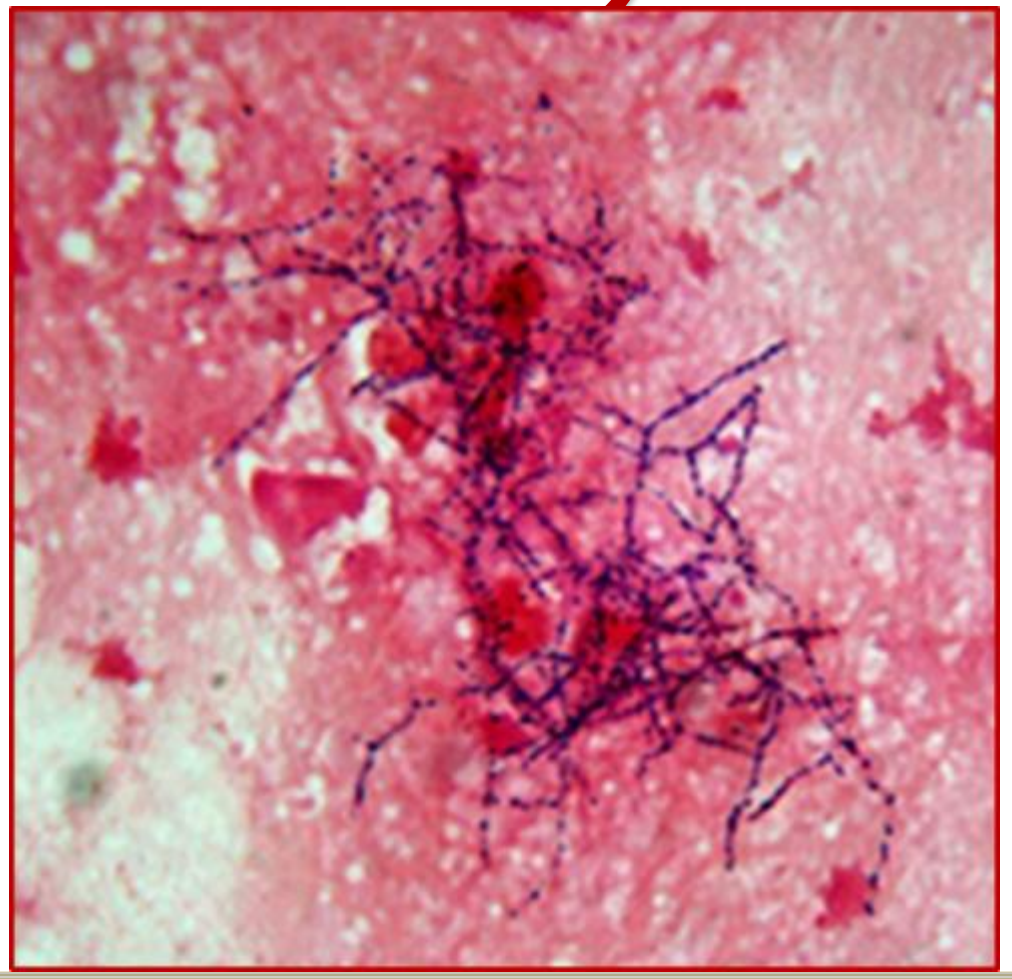
Gram Positive Bacilli (Diphtheroids)



Gram Positive Bacilli (Branching)

Gram Positive Bacilli (Beaded and Filamentous)

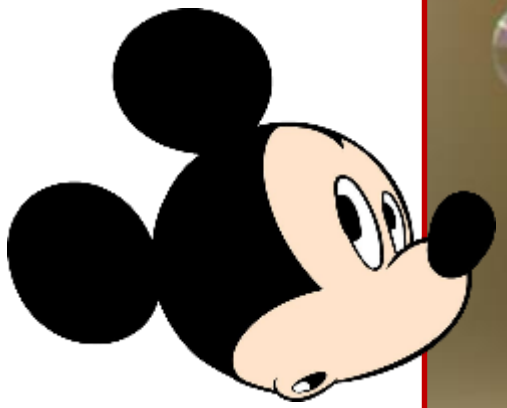
J. Clin. Microbiol. **49:** 1195; 2011



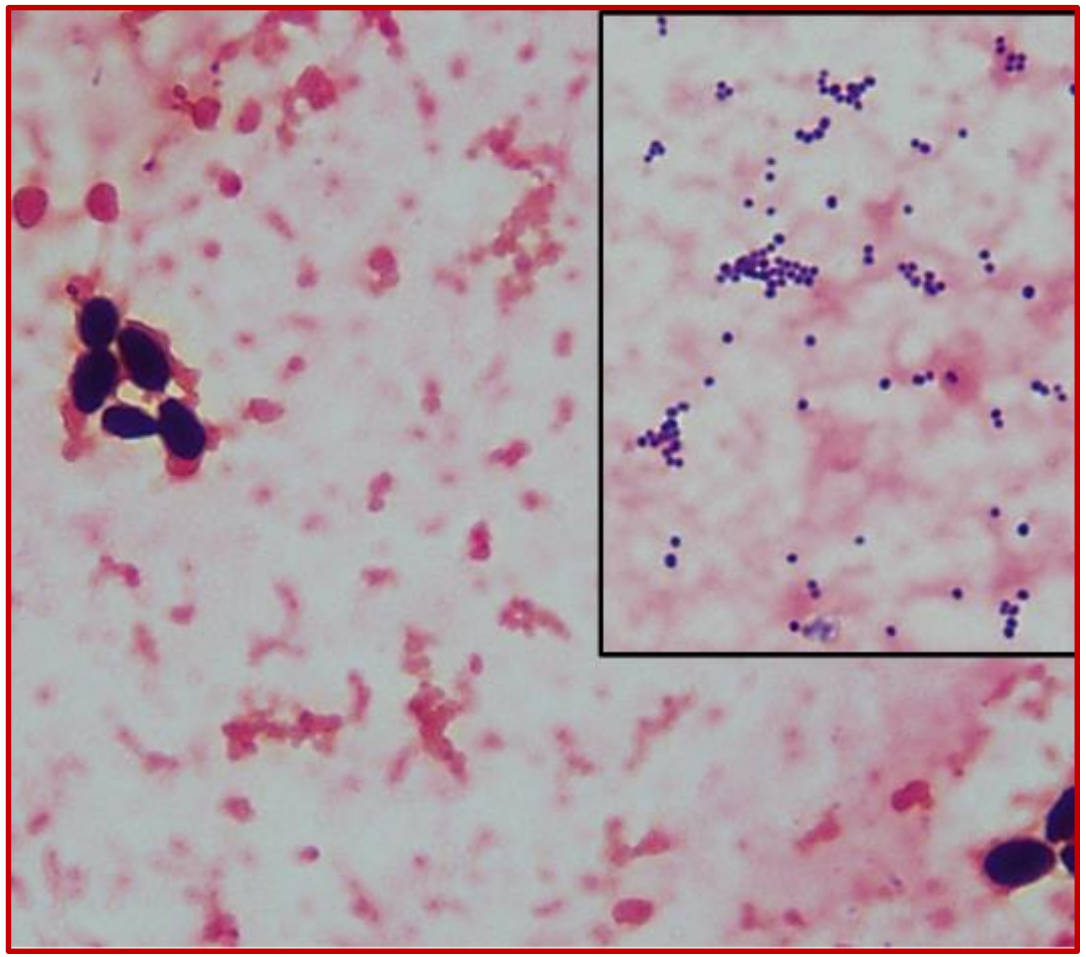
Lets Talk Yeast!

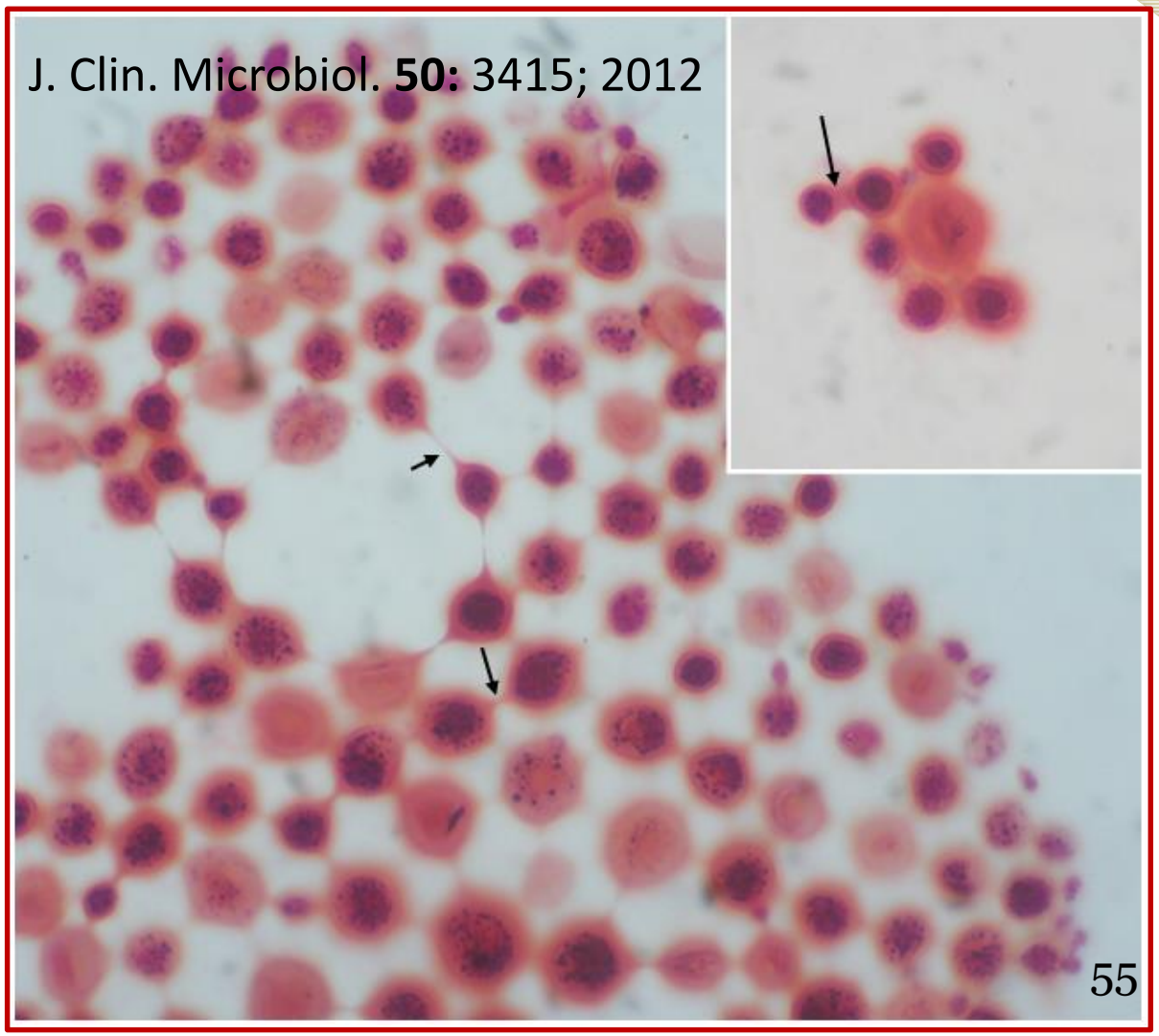
Important Considerations:

- Size
- Budding
- Pseudohyphae

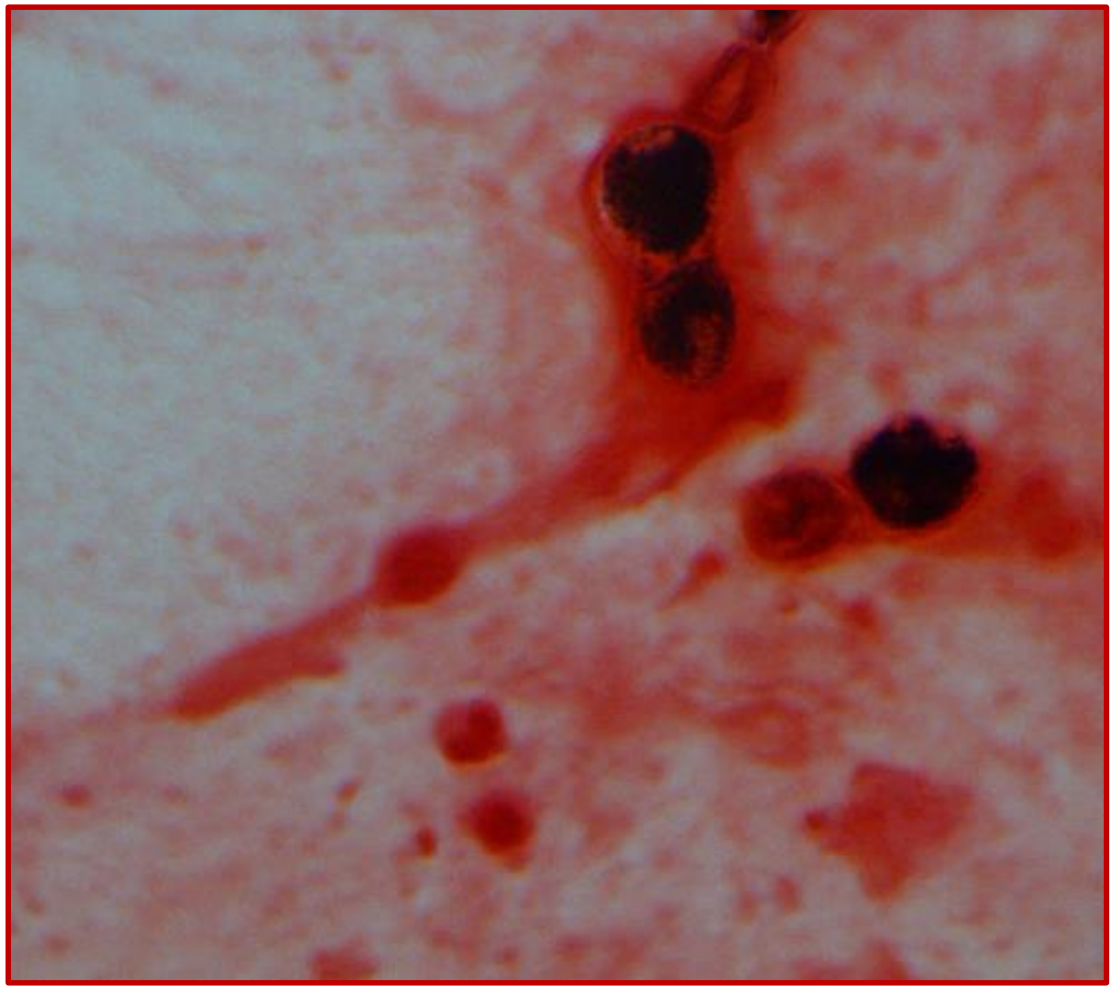


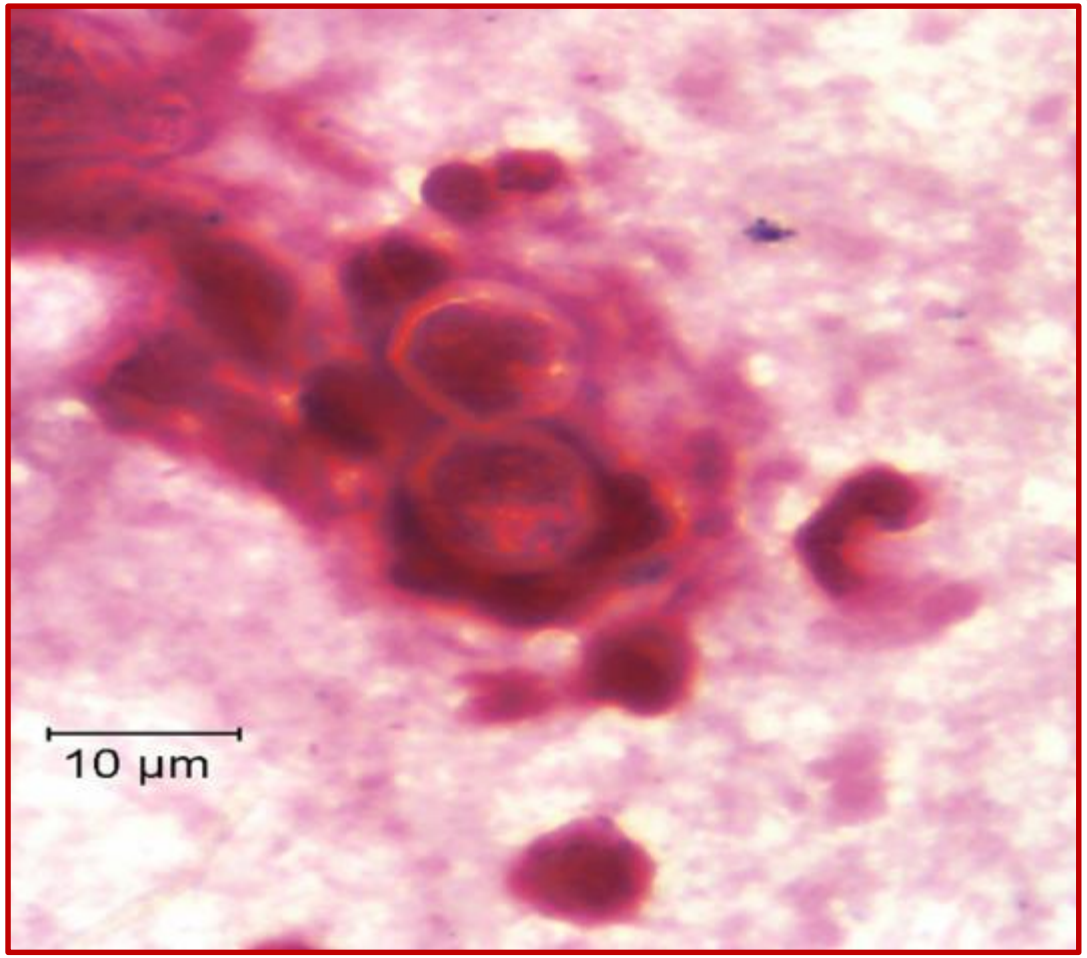
Budding Yeast



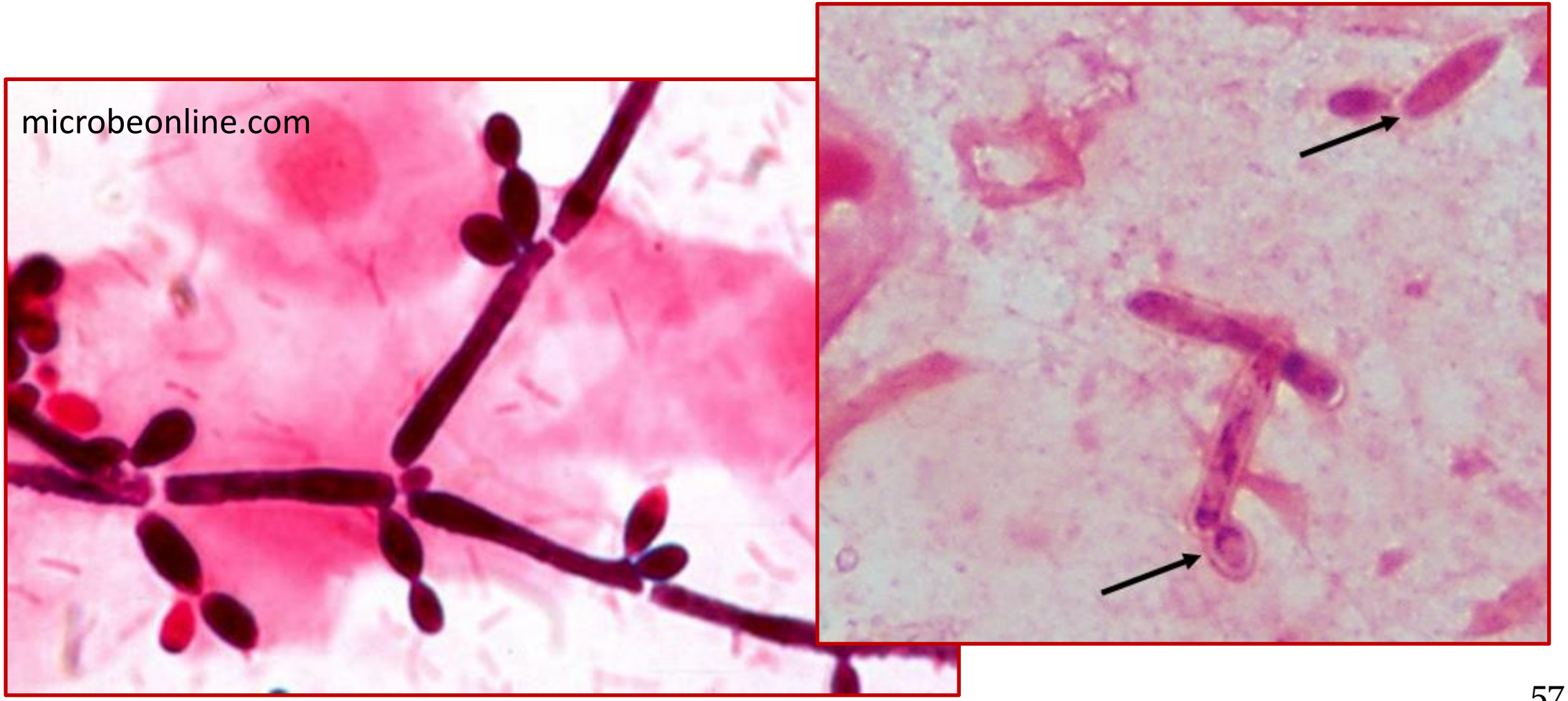


Budding Yeast





Budding Yeast with Pseudohyphae

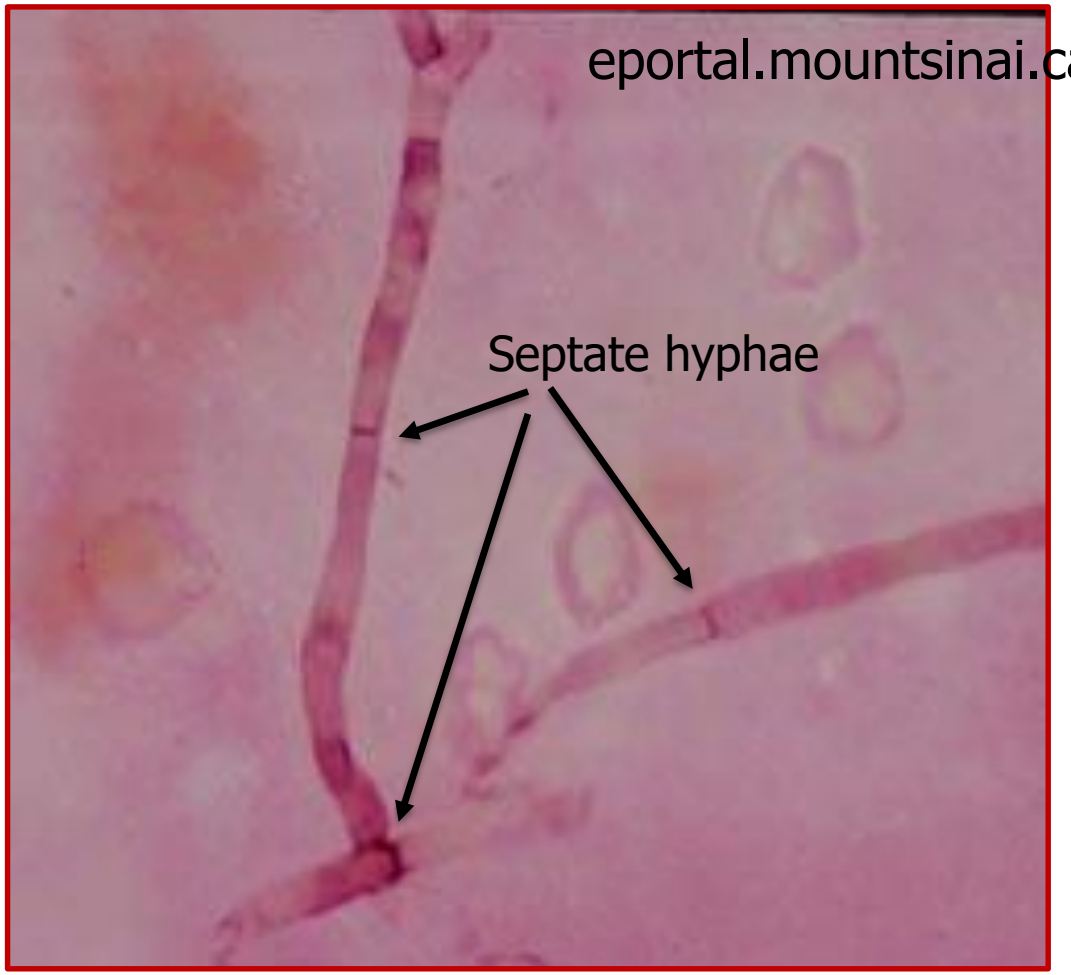


Lets Talk Mold!

Important Considerations:

- Septate hyphae
- Aseptate hyphae

Sepatate vs. Aseptate Hyphae




WHAT ARE YOU LOOKING AT?