Gram Stain Webinar for the Laboratory Generalist ASCLS 2023

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GRAM STAIN OBJECTIVES:

Upon completion, the participant will be able to:

1. Explain the principle of the Gram stain procedure, including elements which can affect staining results.
2. Interpret Grams stains from direct specimens and pure cultures.
3. Correlate common pathogens in positive Gram stains from blood cultures and direct smears from sterile body fluid or tissue.

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Purpose of Gram Stain

- Assess quality of specimen
- Classify bacteria based on Gram reaction, form, cellular morphology, size
- Identify specific infectious agents based on Gram reaction and morphology
- Correlate with culture growth
- Correlate with culture-independent methodology
- Guide presumptive antibiotic therapy

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Principle of Gram Stain

- Differentiates bacteria based on the composition of their cell wall
  - Gram-positive: thick peptidoglycan layer & teichoic acid
  - Gram-negative: high in lipid content
- Basic premise
  - Crystal violet: all cells take up primary stain
  - Gram's iodine: mordant to form complex
  - Decolorizer: mixture of acetone and alcohol
    - Dehydrates lipids in gram-negative cell walls, washes out complex
    - Gram-positive cells retain stain complex
  - Safranin: counterstain
    - Gram-negative cells take up counterstain

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Preparation of Samples

Specimen Type	Preparation
CSF/sterile body fluids	Cyto/Centrifuge
Blood culture broth	Drop to slide
Tissue	Touch prep
Tissue homogenate	Drop to slide
Swabbed material	Roll to slide
Sputum, exudates	Roll to slide

- Appropriately label slide in pencil or use printed label
- Perform under BSC or face shield

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CSF & Sterile Body Fluids

- Centrifuge >1mL: per laboratory procedure
- <1mL: inoculate directly to slide
- Place 1 drop on slide but do not spread
- Cytocentrifugation: protein absorbed by filter pad, deposits cellular material within small drop-like area
- Mark area on slide!

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Positive blood culture bottles:

- Work under BSC or with face shield
- Aspirate positive blood bottle with syringe or blood culture transfer device*
- Bottles can be pressurized, avoid spray
- Transfer 1-2 drops on slide
- Spread to thin monolayer

* Not performed on direct blood culture before incubation, concentrations too low to detect

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Tissue & Homogenates:

- Touch prep: preserves cellular characteristics (WBC's)
  - Cut tissue with sterile scissor or scalpel
  - Use sterile forceps to hold tissue, touch specimen to sterile glass slide
- Homogenates: tissue grinder
  - Transfer 1-2 drops on slide
  - Spread to thin monolayer

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Smears from Swabs

- Should not be prepared from swab after inoculation of plate media
- Two swabs should be submitted
  - If only one swab, roll onto sterile slide, then inoculate plates, then place in broth
- Gently roll swab back and forth over contiguous area of glass slide to form monolayer
- Do not rub swab back and forth as cellular material can be broken, or bacterial arrangements disrupted

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Sputum, Exudates, Stool

- Immerse swab into specimen, specifically in more representative areas:
  - pus like
  - bloody
- Gently roll swab on glass slide
- Dilute extremely thick specimens in drop of saline and spread to form monolayer

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Colonies from Culture Media

- Place drop of sterile saline onto slide
- Touch top of a single colony with sterile applicator stick, wire needle or loop
- Transfer to saline and gently emulsify
- Spread out to make thin smear

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Smear Fixation

- Allow slides to air dry under BSC/ shield
- Heat fix on 60ºC slide warmer for one minute
  - Overheating can cause over-decolorization
  - Under BSC/shield if possible
- Methanol fix
  - Flood the dried smear with methanol 60 seconds
  - Rinse and dry
  - Produces a cleaner background

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Gram stain procedure

1. Place fixed slide on rack over sink
2. Flood with crystal violet – approximately one minute
3. Rinse gently with flowing distilled or tap water
4. Flood slide with Gram's iodine – approximately one minute
5. Rinse gently with flowing distilled or tap water
6. Run gentle stream of decolorizer over smear, 1-5 sec, until no more color is washed off
7. Quickly rinse with flowing distilled or tap water
8. Flood slide with safranin – approximately one minute
9. Rinse gently with flowing distilled or tap water
10. Drain slide, air dry or gently blot with paper towel or blotting paper
11. Dry completely before examining

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Examination of Cellular Material

- Scan Low Power 10X (lpf)
  - Background, WBC = pink
  - Specimen quality
    - Search for areas with WBC or RBC
    - Avoid thick areas, crystal violet precipitate or squamous cells if sputum
  - Quantitate average number of cells (Epi & WBC)
    - Rare (1+) = <1 cell/lpf
    - Few (2+) = 1-10 cells/lpf
    - Moderate (3+) = 11-25 cells/lpf
    - Many (4+) = >25 cells/lpf

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Examination of Bacteria

- Scan High Power 100X / Oil Immersion (hpf)
  - Background, WBC and RBC = pink
  - Systematically scan good representative areas
    - Carefully observe 10-25 fields
    - *Haemophilus* & *Nocardia* can blend in
    - More than one organism can be found
  - Quantitate average number of bacteria & yeast
    - Rare (1+) = <1 cell/hpf
    - Few (2+) = 1-10 cells/hpf
    - Moderate (3+) = 10-25 cells/hpf
    - Many (4+) = >25 cells/hpf

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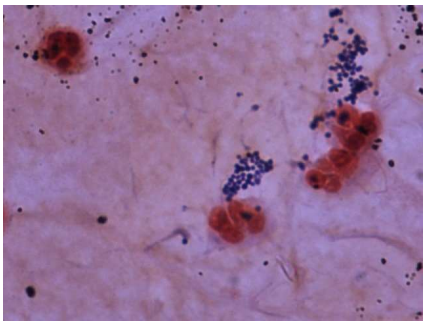
Specimen quality

- Precipitate vs Gram positive cocci



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Interpretation

Classify bacteria on:

1. Gram Reaction
2. Gram Morphology
3. Arrangement

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Gram Reaction

- Gram-positive: purple/ blue
- Gram-negative: red/ pink
- Gram-variable: irregularly stained, appears both blue & pink
  - Gram-positives too old or treated with antibiotics will decolorize
  - Excessive thick areas are difficult to decolorize, portions remain blue

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Gram Morphology

Shape/Size varies greatly

Bacillus = rod like 

Cocci = round or slightly oval 

Yeast = Can be budding, larger than GPC 

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Arrangements

Cocci

- Single 
- Pairs
- Chains – in a line
- Clusters – grape like 
- Tetrads – packets of four 
- Diplococci (gram-negative) –Kidney bean 

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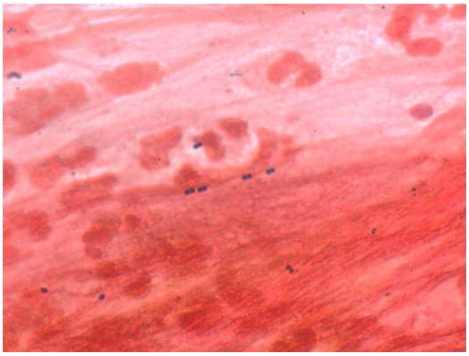
Arrangements: Pairs



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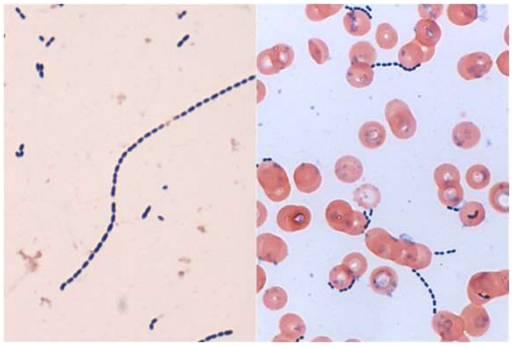
Arrangements: Chains



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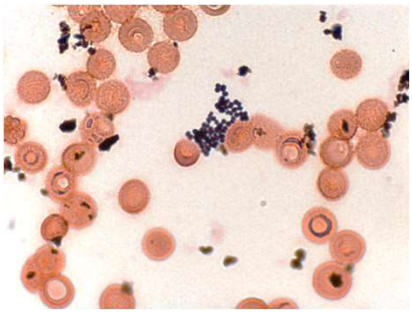
Arrangements: Clusters



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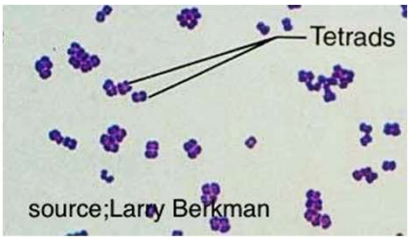
Arrangements: Tetrads



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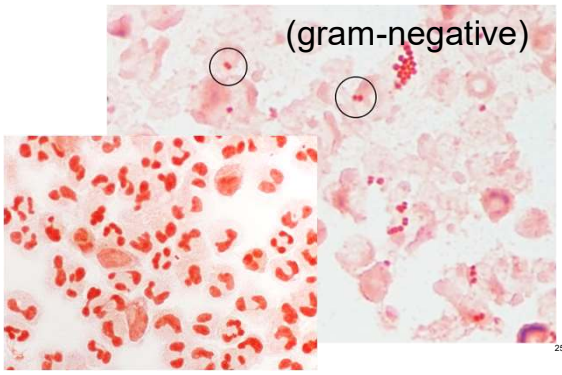
Arrangements: Diplococci



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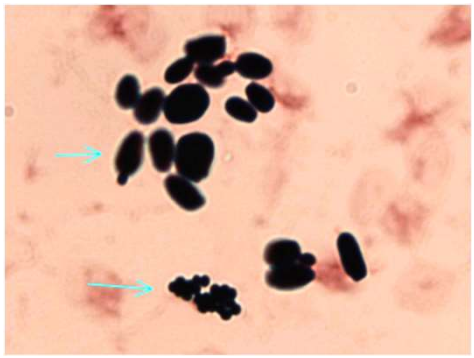
Yeast



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Arrangements

Bacillus/ Rods

- Rod
- Rod with spores
- Coccobacilli
- Palisade
- Stonehenge Like

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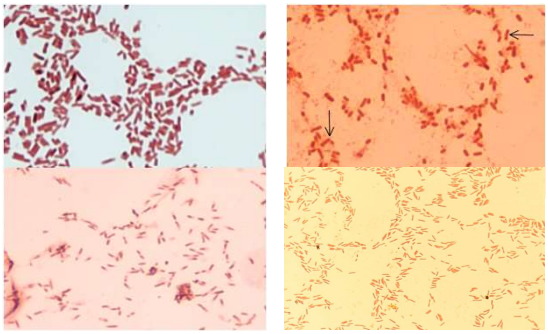
Gram-negative rods



Gram negative rods not described in arrangements

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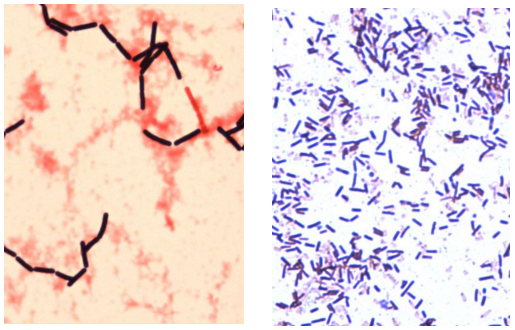
Gram-positive rods



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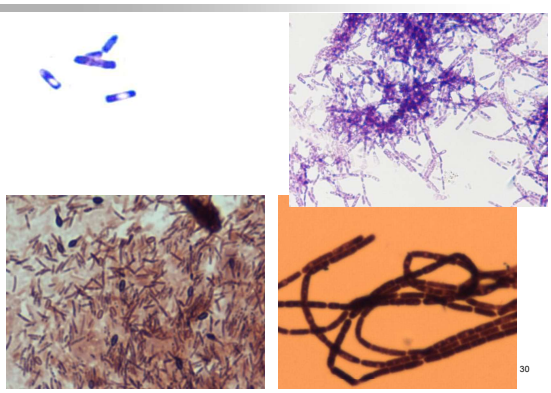
Gram-positive rods with spores



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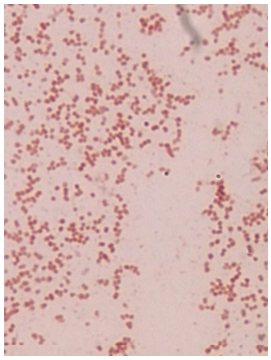
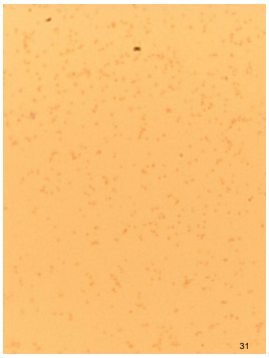
Gram-negative coccobacilli





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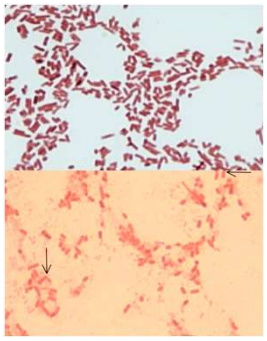
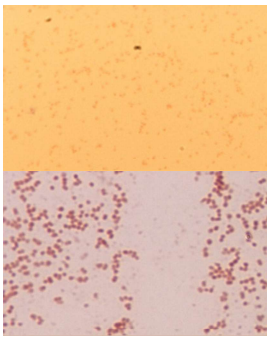
Gram-neg. rods vs, Gram-neg. coccobacilli





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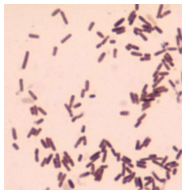
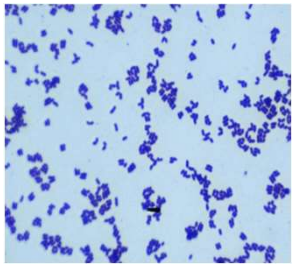
Arrangements - Other

Gram-positive rods

- Palisading
- Stonehenge-like





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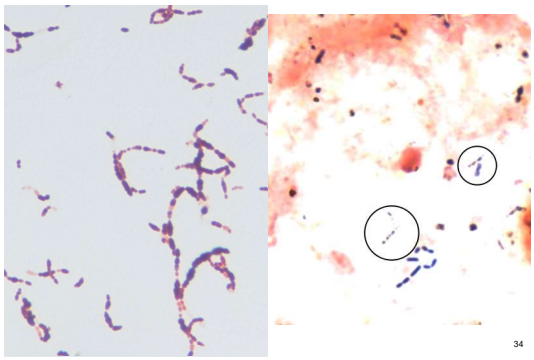
Arrangements – Pleomorphic or Beading



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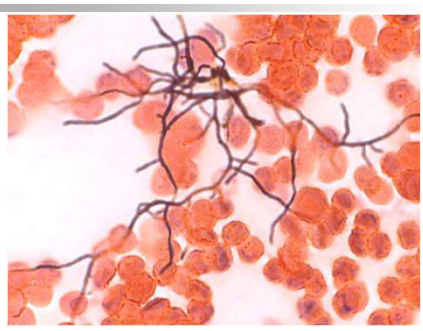
Arrangements – Branching



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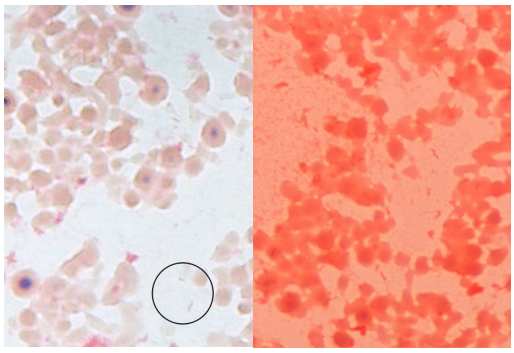
Morphology – GNR “gull winged”



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Grading Materials

- Quantitative and qualitative analysis helpful in culture management
  - Interpretation of poor specimens can lead to delayed or inappropriate treatment
  - Basis for specimen rejection
- Multiple grading systems:
  - Bartlett's Score – compare count WBC, EPI*
  - Heineman's Method – ratio between WBC, EPI
  - Follow facility policy

* Squamous epithelial cells

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Reporting of Direct Specimen Smears

- Turnaround Time (TAT) is important
  - Results can be critical
- Report should be simple and clear
  - Significant elements only
  - Interpretation useful when certain bacteria recognized
    - i.e.) Gram positive cocci in clusters consistent with *Staphylococcus spp*
- Critical results (sterile site) must be called

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Common Pathogens in CSF

- Positive CSF: "critical value"
- Must be communicated directly to physician or follow lab protocol
- Derives presumptive diagnosis on:
  - Patient age
  - Physical exam
  - Local epidemiology
  - Lab analysis

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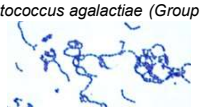
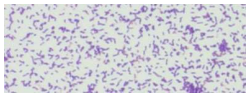
Bacterial meningitis related to age:

- Neonates (<1month)
  - Gram negative rods – *E coli, Klebsiella, Enterobacter*

  - *Streptococcus agalactiae (Group B)*



  - *Listeria monocytogenes*



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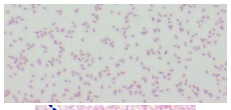
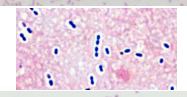
Bacterial meningitis related to age:

- Infant (1-23 months)
  - *Streptococcus agalactiae*
  - *E. coli*
  - *Haemophilus influenzae*



  - *Streptococcus pneumoniae*



  - *Neisseria meningitidis*

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Bacterial meningitis related to age:

- Bacterial meningitis related to age:
  - Children (>2 Years) and Adults
    - *Streptococcus pneumoniae*
    - *Neisseria meningitidis*
  - Older Adults (>65 Years)
    - *Streptococcus pneumoniae*
    - *Neisseria meningitidis*

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Pathogens in Blood

- Most common agents of nosocomial bacteremia:
  - Coagulase negative *Staphylococci*
  - *S. aureus*
  - *Enterococcus ssp*
  - *Candida albicans*
  - *Escherichia coli*
  - *Klebsiella ssp*
  - *Pseudomonas aeruginosa*
- Most common blood contaminates:
  - Coagulase negative *Staphylococci*
  - Diphtheroids
  - *Bacillus spp*
  - *Alpha hemolytic Streptococci*
  - *P. acnes*
- GNDC, GNCB and large GPR: all work done in BSC to avoid open bench exposure

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Quality Control & Management

- Storage
  - Store stains at 15-30°C
  - Decolorizer in flammable closet
  - Protect Gram iodine from light
- Quality Control
  - Background material of clinical smears: pink/red
  - New lot and at least weekly
    - Prepare smear of known organism, fix and stain
      - *E. coli* ATCC 25922 –stains pink/red
      - *S. aureus* ATCC 25923 – stains blue/purple
  - Correlate with culture growth, both in type and amount of bacteria

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Quality Control & Management

- Quality Improvement: possible corrective action
  - Poor specimen quality: maintain grading/ specimen rejection policy
  - Poor stain quality: repeat from original specimen
    - Too thick or thin
    - Age of bacteria (older bacteria decolorize easily)
    - Precipitate in reagents (will be seen in multiple stains)
    - Patient is being treated with antibiotics
  - Does not correlate with culture growth, in either type or amount of bacteria
    - Establish system to review Gram stain reports
      - Correlate by bench personnel when reading out cultures
      - Supervisory review of final culture reports
      - Supervisory arbitrary review of CSF/ Sterile sites
      - Investigate discrepancies – issue corrective report
        - Aid in correlating relevant clinical information
        - Determine training needs
      - Maintain set of reference slides for competency training
  - Multiple poor specimens consistently being submitted from same location: Director should be contacted to determine plan of action

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Morphology & Associated Organisms

- Gram-positive stain implies possible:
  - Cocci in clusters – *Staphylococcus spp*
  - Cocci in pairs – *Streptococcus pneumoniae*
  - Cocci in chains – *Streptococci or Peptostrep.*
  - Rods, large – *Clostridium (anaerobe)* or *Bacillus***

** Trigger point: Continue work under BSC. Correlate growth and biochemical test results to rule out and refer algorithm.

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Morphology & Associated Organisms

- Gram-negative stain implies possible:
  - Rods, medium – *E. coli*
  - Rods, thin/ long – *Pseudomonas spp*
  - Coccobacilli – *Haemohilus spp* or *Francisella***
  - Diplococci – *Neisseria spp***

** Trigger point: Continue work under BSC. Correlate growth and biochemical test results to rule out and refer algorithm.

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Triggers Points - BSC Required

- GNCB, GNDC or large boxcar shaped GPR's seen in direct Gram stain
- Slow growth @ 24-48hrs
- Rapid growth, non-hemolytic @12-16hrs
- Begin "rule out/ refer" workup
- All presumed fungal or mycobacterium specimens
- Procedures that emit aerosols
- Inoculation of all direct specimens to culture plates/ slides, especially blood or sputum

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Who You Gonna Call?

- Follow Laboratory Policy – notify supervisor immediately
- Nebraska Public Health Laboratory
- Attending Physician
- Infection Control
- Infectious Disease Physicians
- County Health Department
- State Epidemiologist

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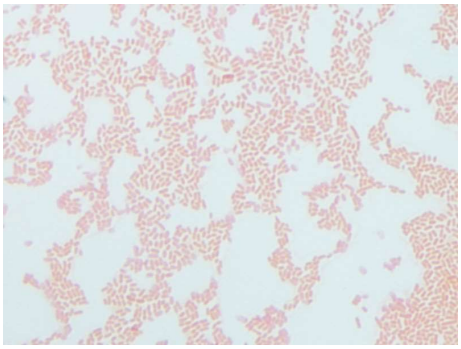
How would you interpret?



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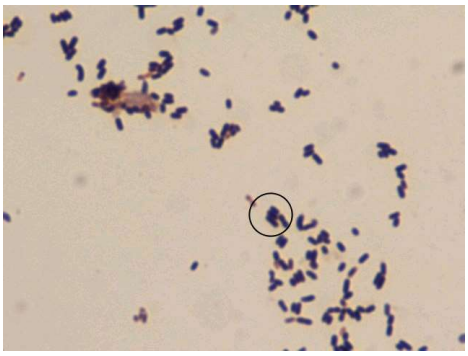

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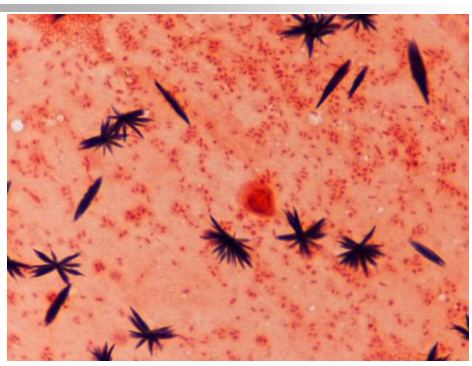

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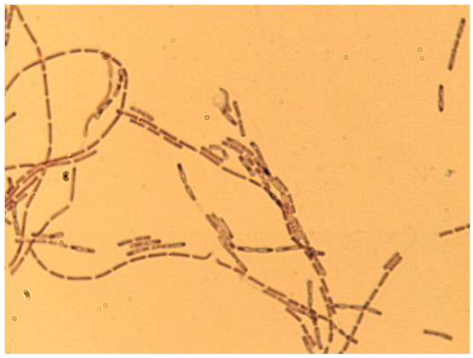

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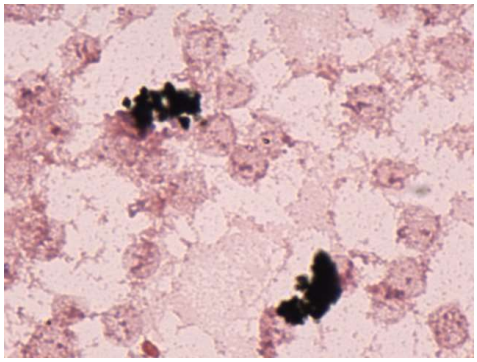

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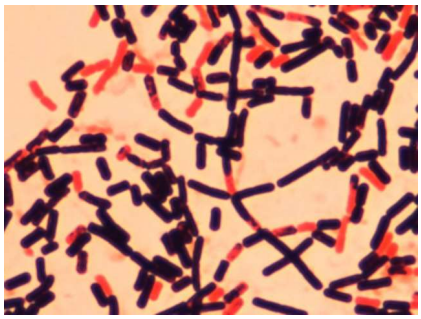

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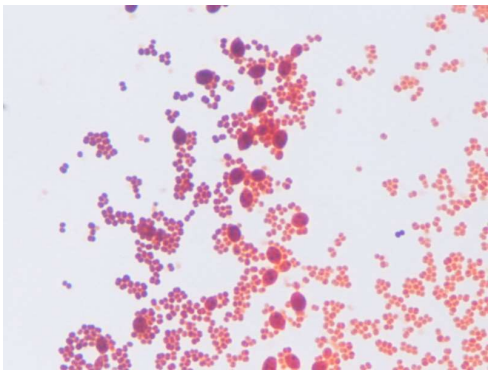

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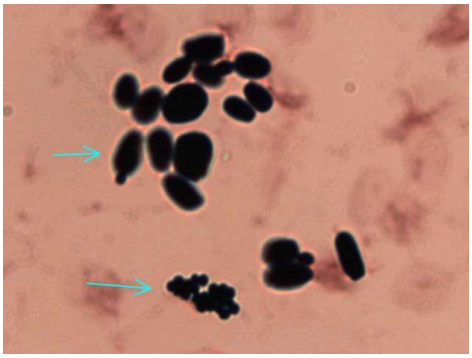
Arrangements - Yeast



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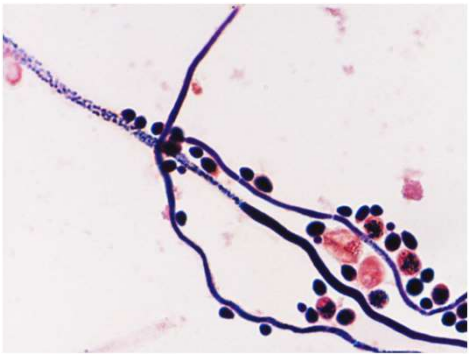

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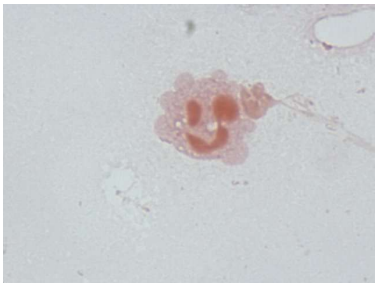
How would you interpret?



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Resources

1. Garcia, L; *Clinical Microbiology Procedures Handbook*,3rd Edition
2. CLS 552 Human Microbiology & Immunology Laboratory, module 3
3. Mahon, Lehman, Manuselis; Textbook of Diagnostic Microbiology
4. Images from NPHL STATPack™ unless otherwise marked
5. http://www.nphl.org

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