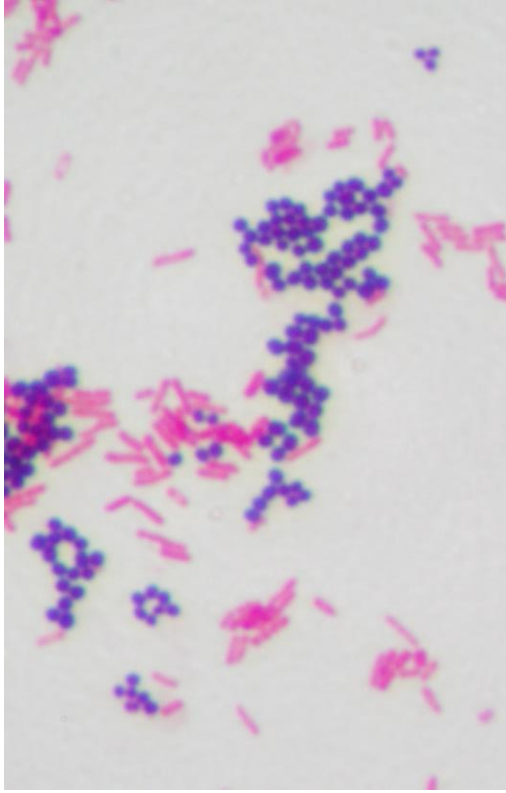
Gram Stain:
From Start to Finish



Mir Noorbash, PhD, D(ABMM)

March, 2019

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Objectives

- Introduction
- Process of Gram Stain
- Reporting format – WBC
- Bacterial Vaginosis
- Wet-Prep

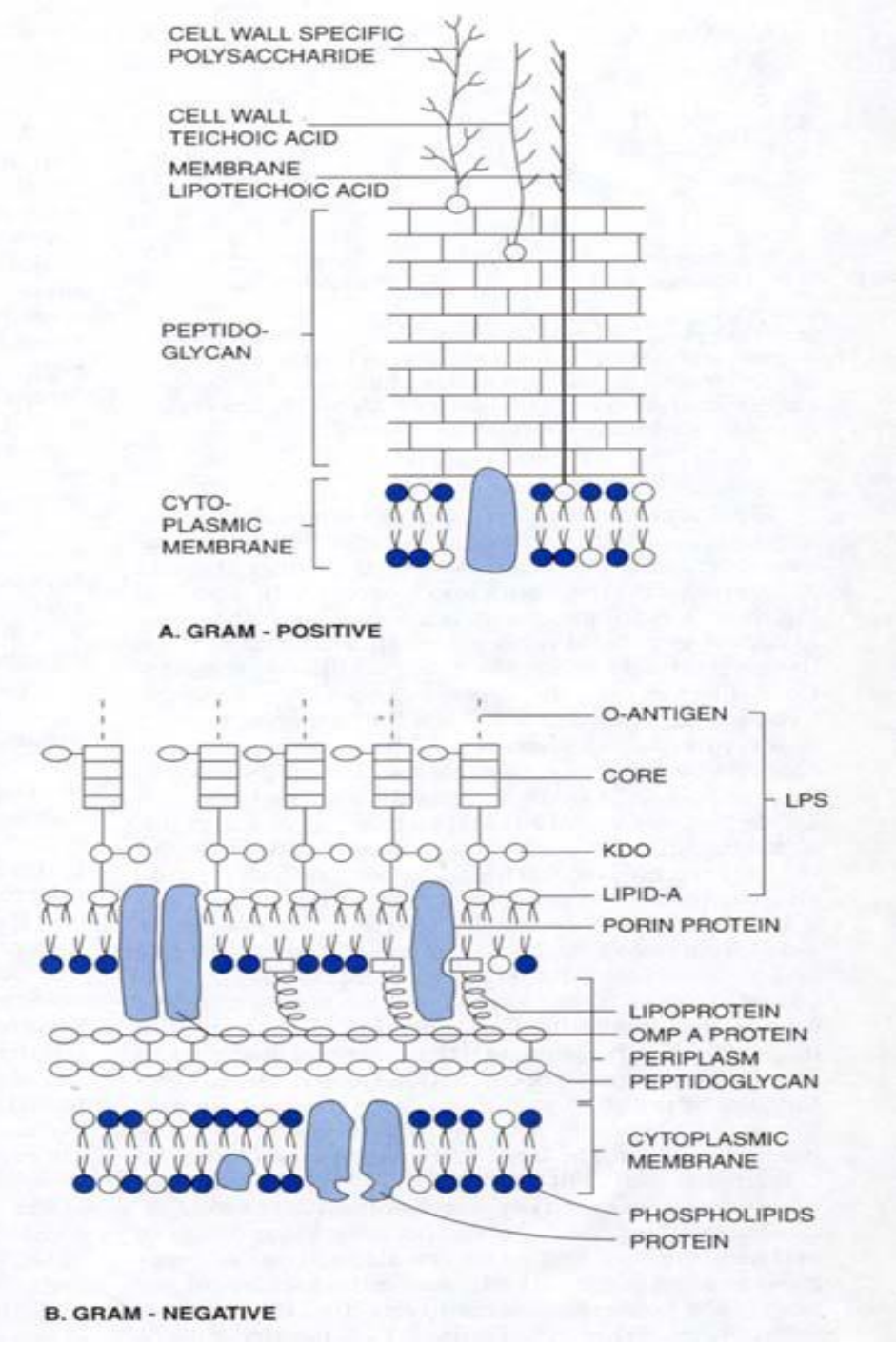
Hans Christian Joachim Gram

- Born in Copenhagen, Denmark
- In **1884** developed **Gram** stain
- Still the most important microbiology test!!

https://en.wikipedia.org/wiki/Hans_Christian_Gram

Gram positive
thick peptidoglycan and teichoic acid in cell wall

Gram negative
asymmetric Lipopolysaccharide phospholipid bilayer interspersed with protein



GRAM STAIN PROCEDURAL HIGHLIGHTS

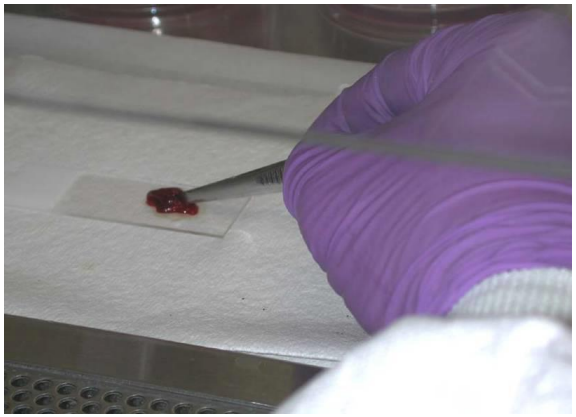
- Selecting a portion of the specimen
- Preparing the smear
- Staining
- Low power (10X) examination
- High power (100X) examination
- Quantitation of cells and microorganisms
- Interpretation of morphotypes
- Minimum competency
- Slides for review

Variables with Gram Stain

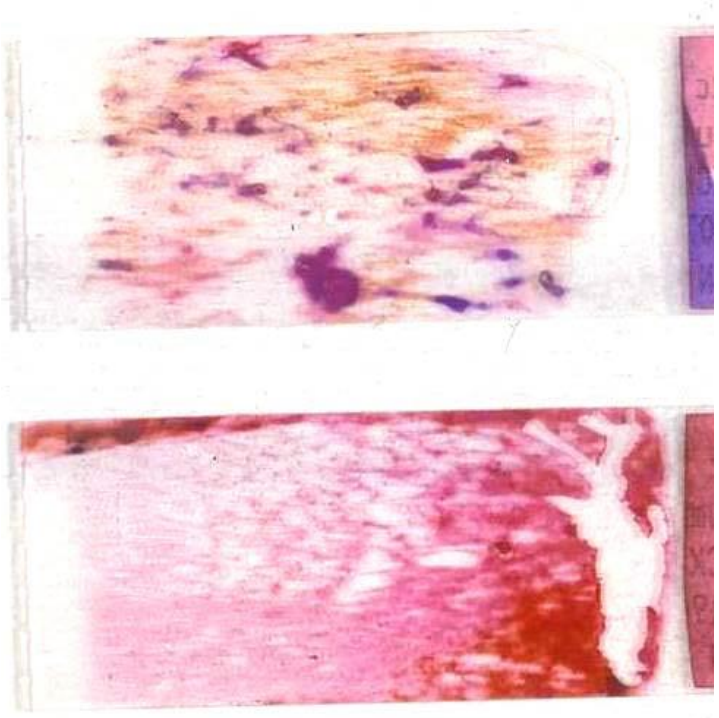
- Inoculum/smear preparation
- Slide preparation: method of cleaning
- Fixation
- Reagents
- Dry with paper or air
- Timing of various steps
- Organism: species, age, antibiotic therapy…

Smear Preparation

- Inoculum: thick/thin smears
- Swab roll
- Liquid drop
- Cytospin
- Touch preparation
- 2-slide thin smear
- Minced biopsy



Sputum or tissue smear – 2 slide method



PREPARING THE SMEAR
Make a monolayer of cells

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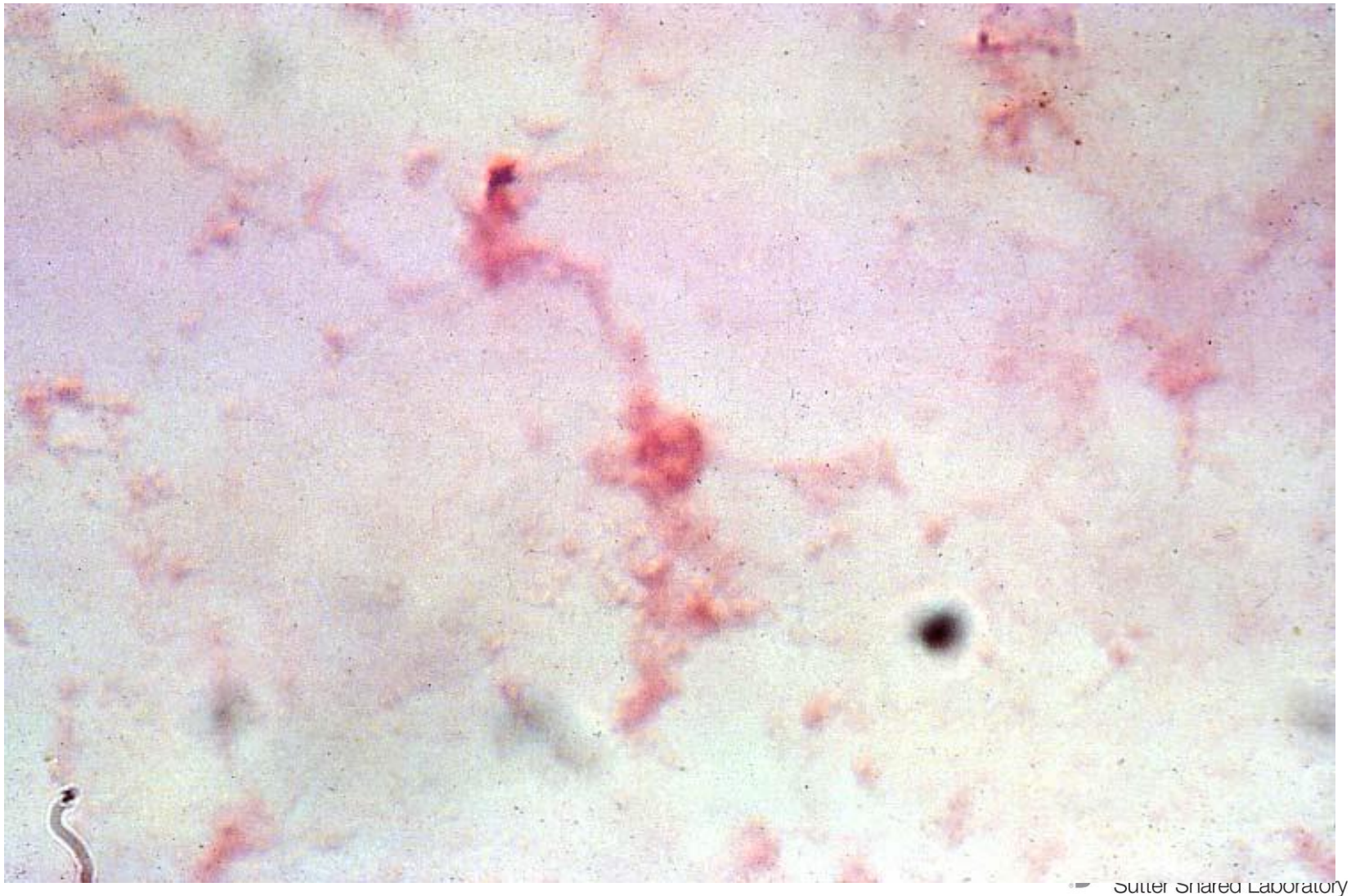
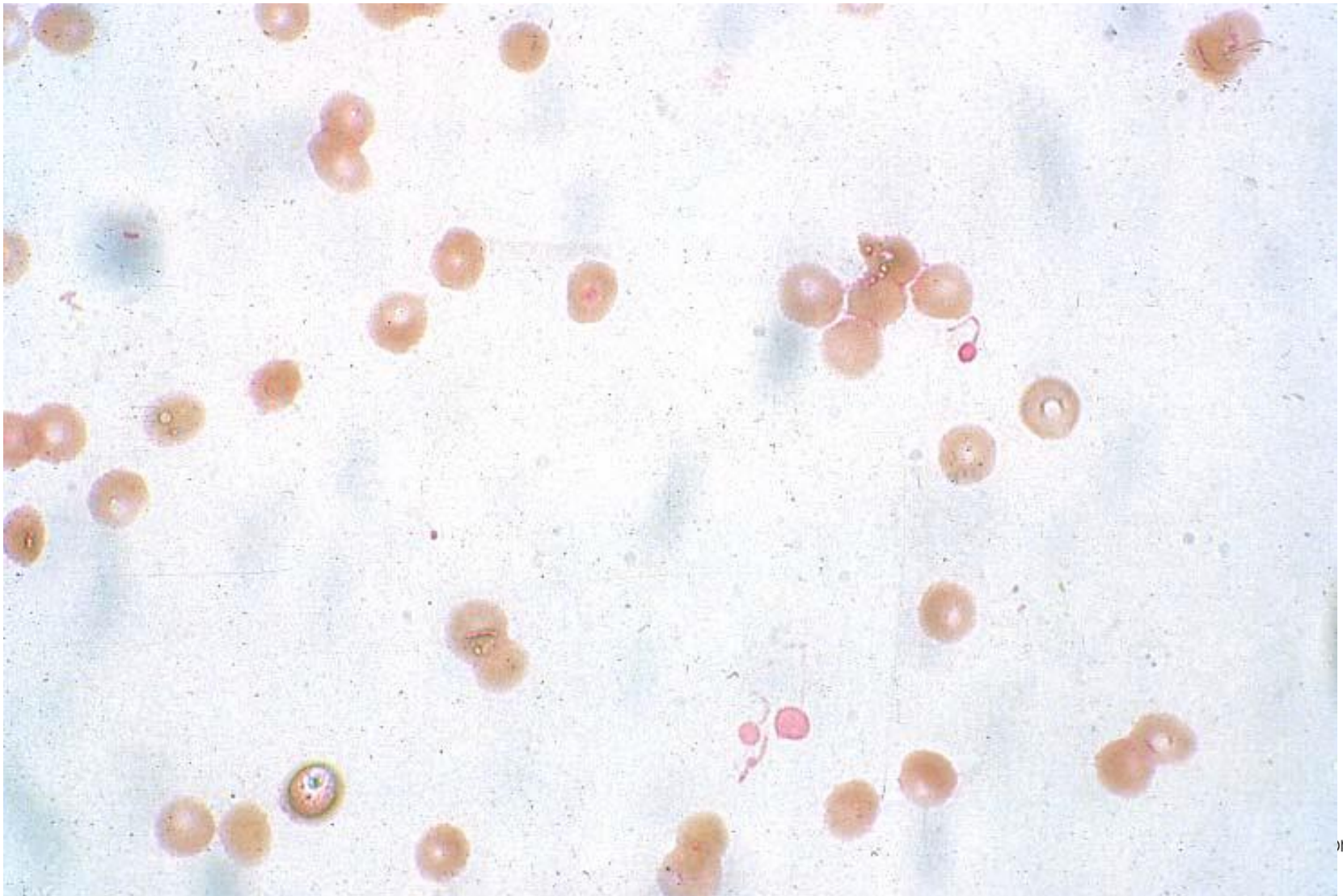
Not methanol fixed

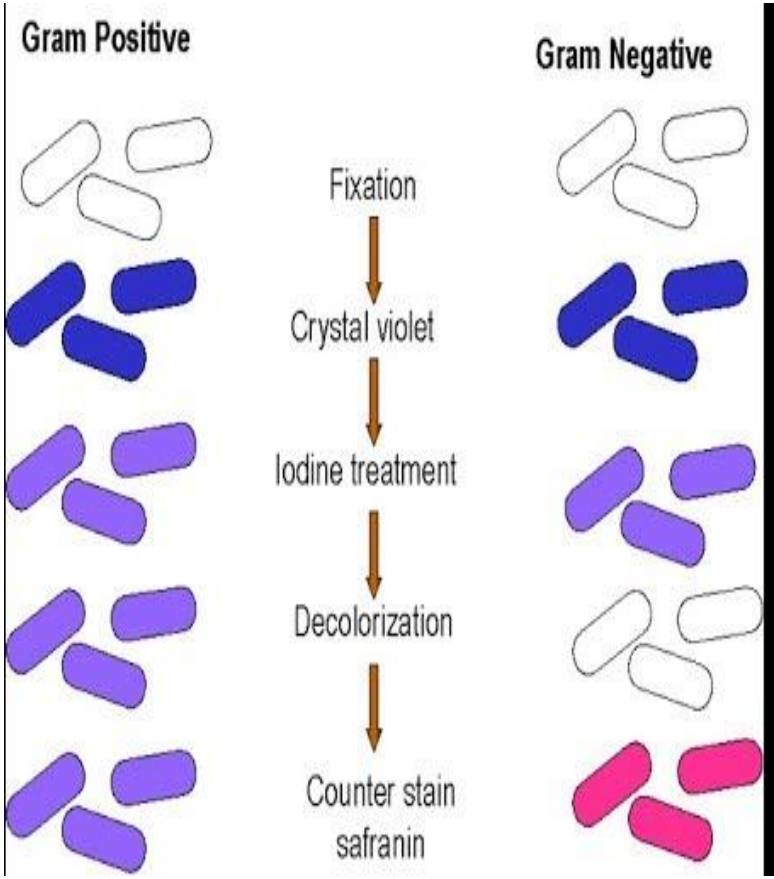


Methanol fixed

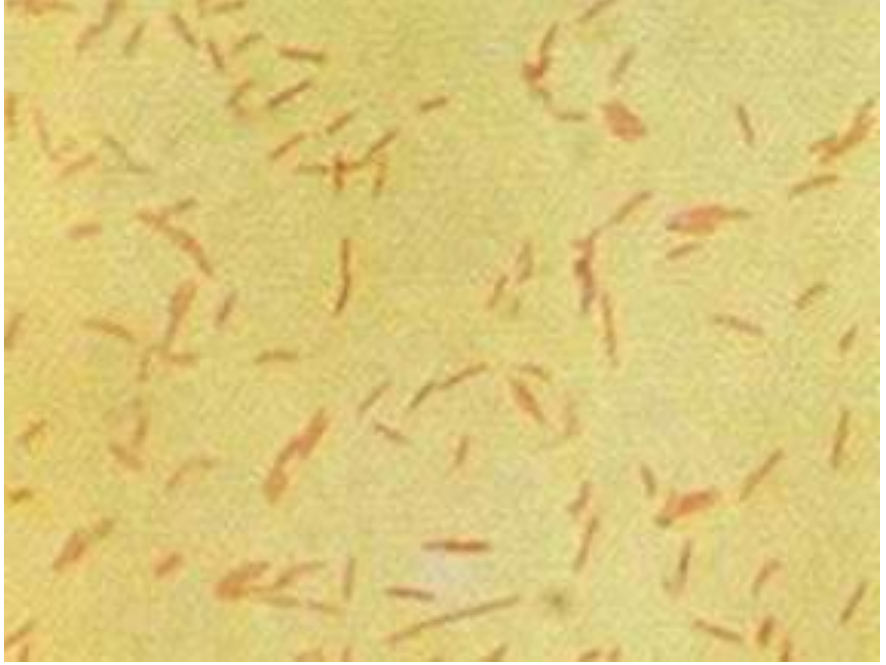


Gram Stain steps

- Make thin film of suspension or sample on slide.
- Allow to dry, then fix with methanol
- Flood slide with crystal violet
- Add iodine (mordant) – 2x time of crystal violet
- Decolorize
- Flood with counterstain (safranin or fuchsin)
- Rinse excess stain off; dry before adding oil

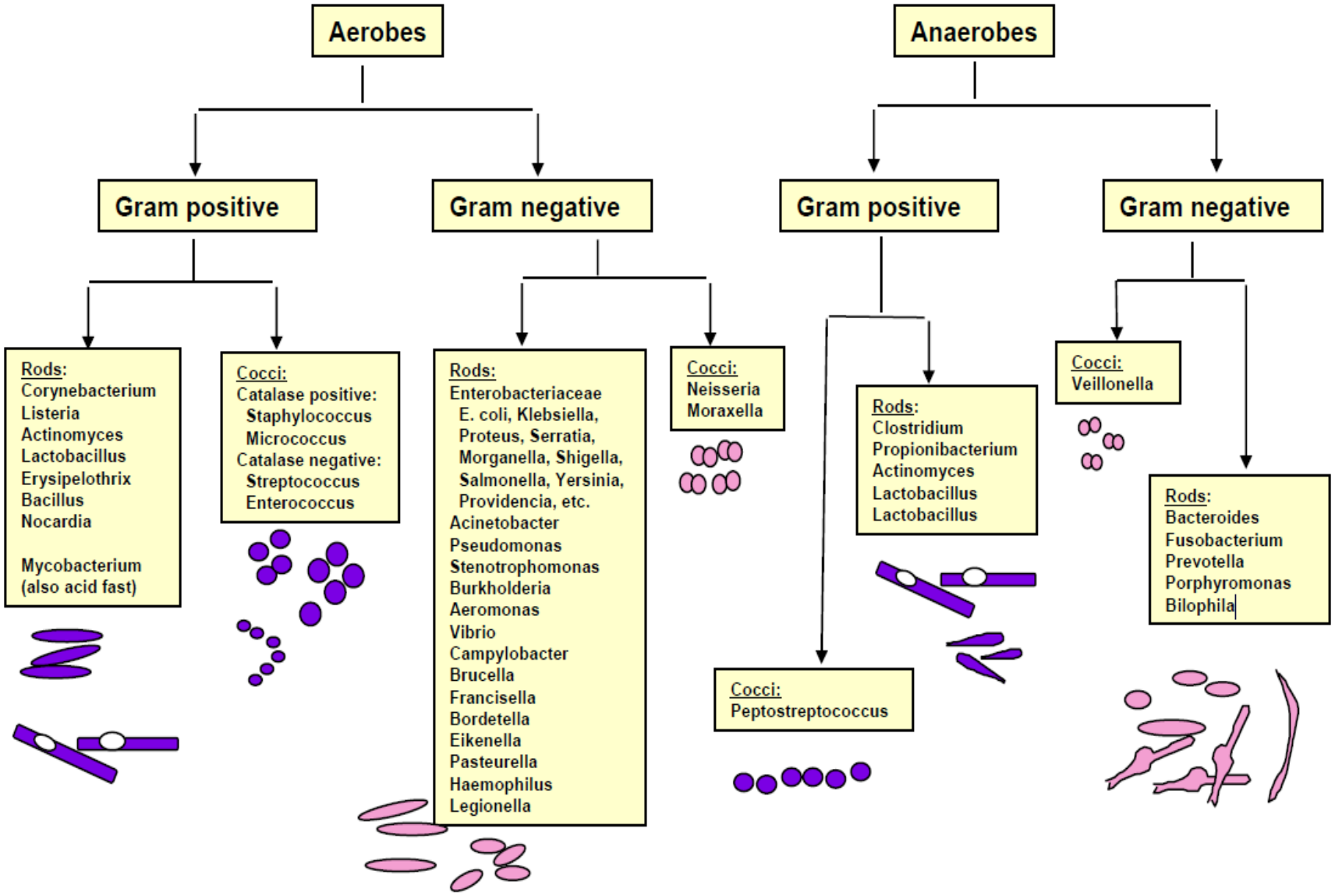


Reagents



- **Decolorization**
  - **Alcohol**
  - **acetone-alcohol**
  - **acetone**
- **Substitutes for crystal violet or iodine reagents**
- **Counterstain reagents:**
  - **0.1% aqueous basic fuchsin stains Gram Negatives, such as *Helicobacter, Campylobacter, Fusobacterium, Bacteroides, Legionella, Brucella* and anaerobes better than safranin**

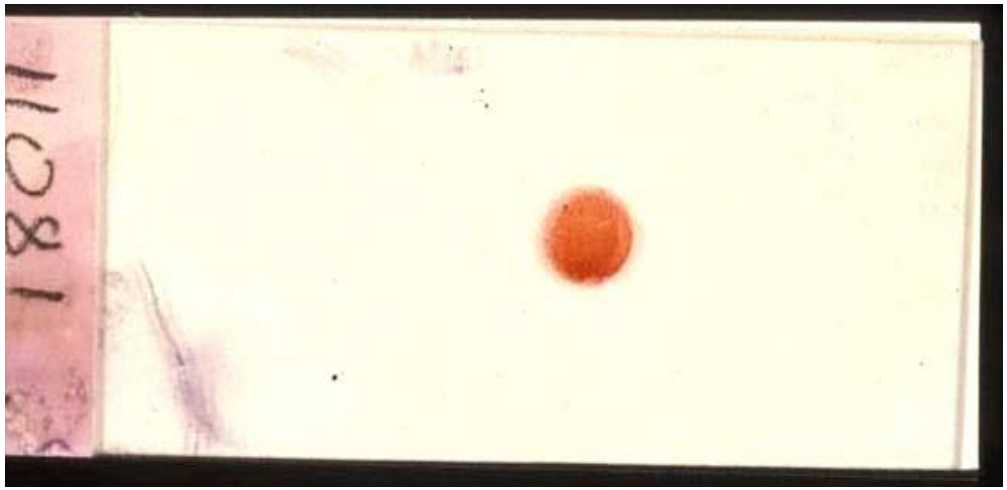
Basic bacterial identification based on cell morphology



FLUIDS THAT ARE NOT THICK PUS

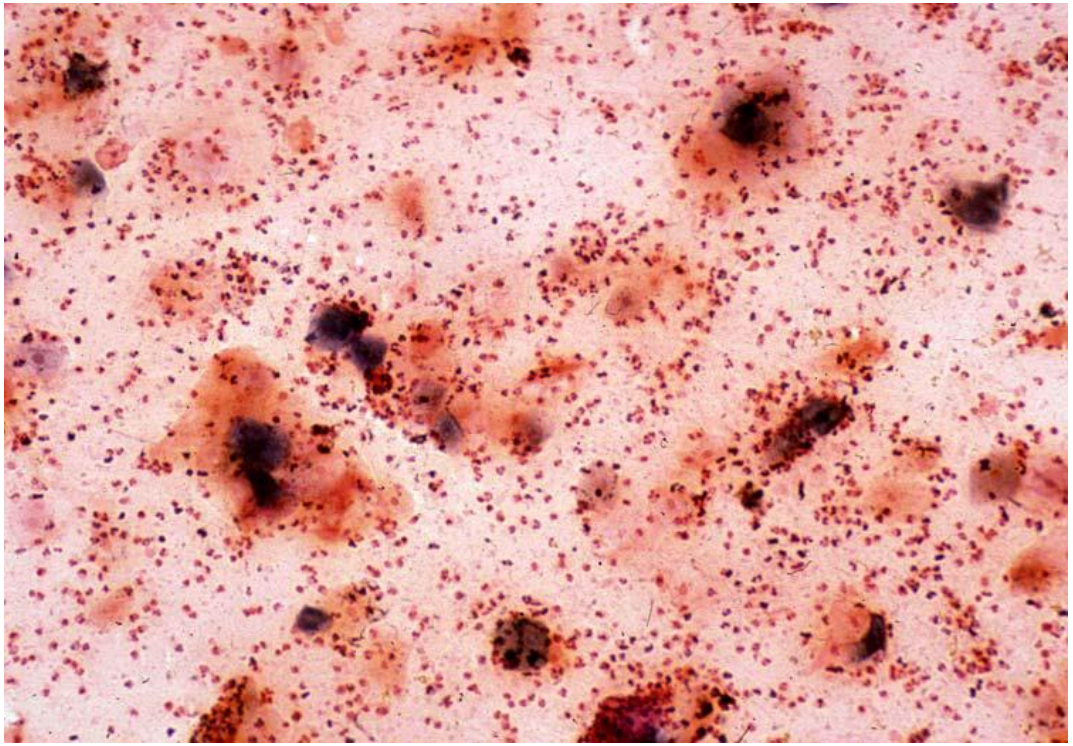
PREPARING THE SMEAR

- **Concentrate fluids with cytospin**
- **If too thick, dilute and make another slide**
- **Cytospin adds one category to quantity, but don’t report differently**



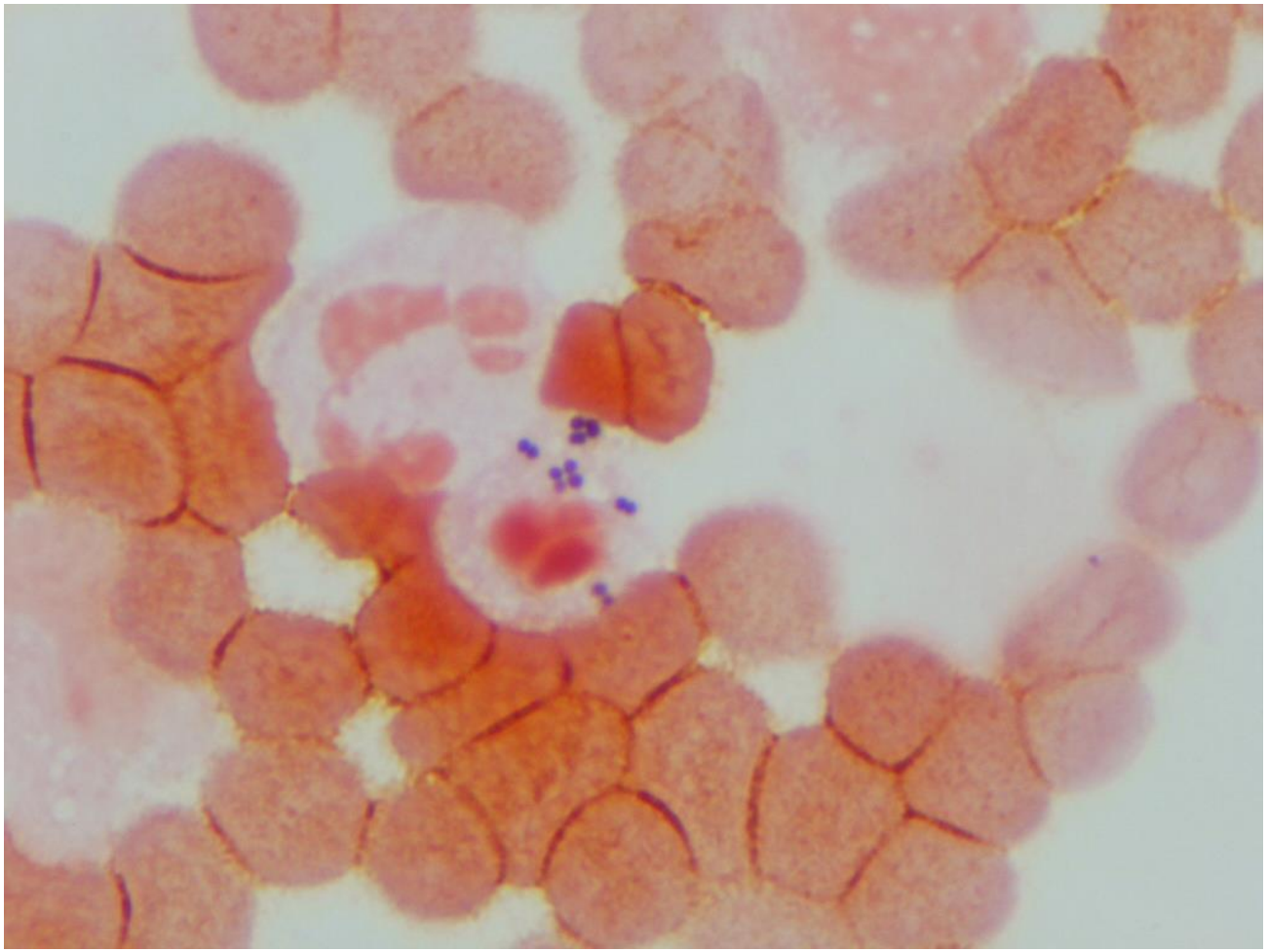
Cytospin

Examine under low power (10x)



- Count at least 10-20 fields, quantitate cells
- Reject respiratory samples if >10 squam. epi's
- Select area for high power examination

Examine under high power (100x)



20-40 fields; quantitate microorganisms

Interpret Gram stain based on specimen source

- Sterile specimen source (presumed)
  - Report WBC's
  - Report microorganisms
  - > 3 morphologically typical shapes before reporting
- Non-Sterile specimen source
  - Report PMN's
  - Name microorganisms only if potential pathogen
  - Quantitate and report normal flora

PATTERN RECOGNITION

- Respiratory tract
  - Pneumonia/bronchitis
  - Aspiration pneumonia
  - Chronic lung disease /COPD
- Urinary tract
  - UTI
  - Vesicocolonic fistula
- Meningitis
  - Acute bacterial

- Abscess
  - Staphylococcal
  - Mixed aerobic/anaerobic
  - *Streptococcus anginosus*/milleri
  - Toxemia
  - Streptococcal necrotizing fasciitis
  - *Clostridium* gas gangrene
- Miscellaneous
  - BV (bacterial vaginosis)
  - Lemierre's disease (jugular vein thrombosis)
  - Gonococcal urethritis
  - Crystalline joint disease

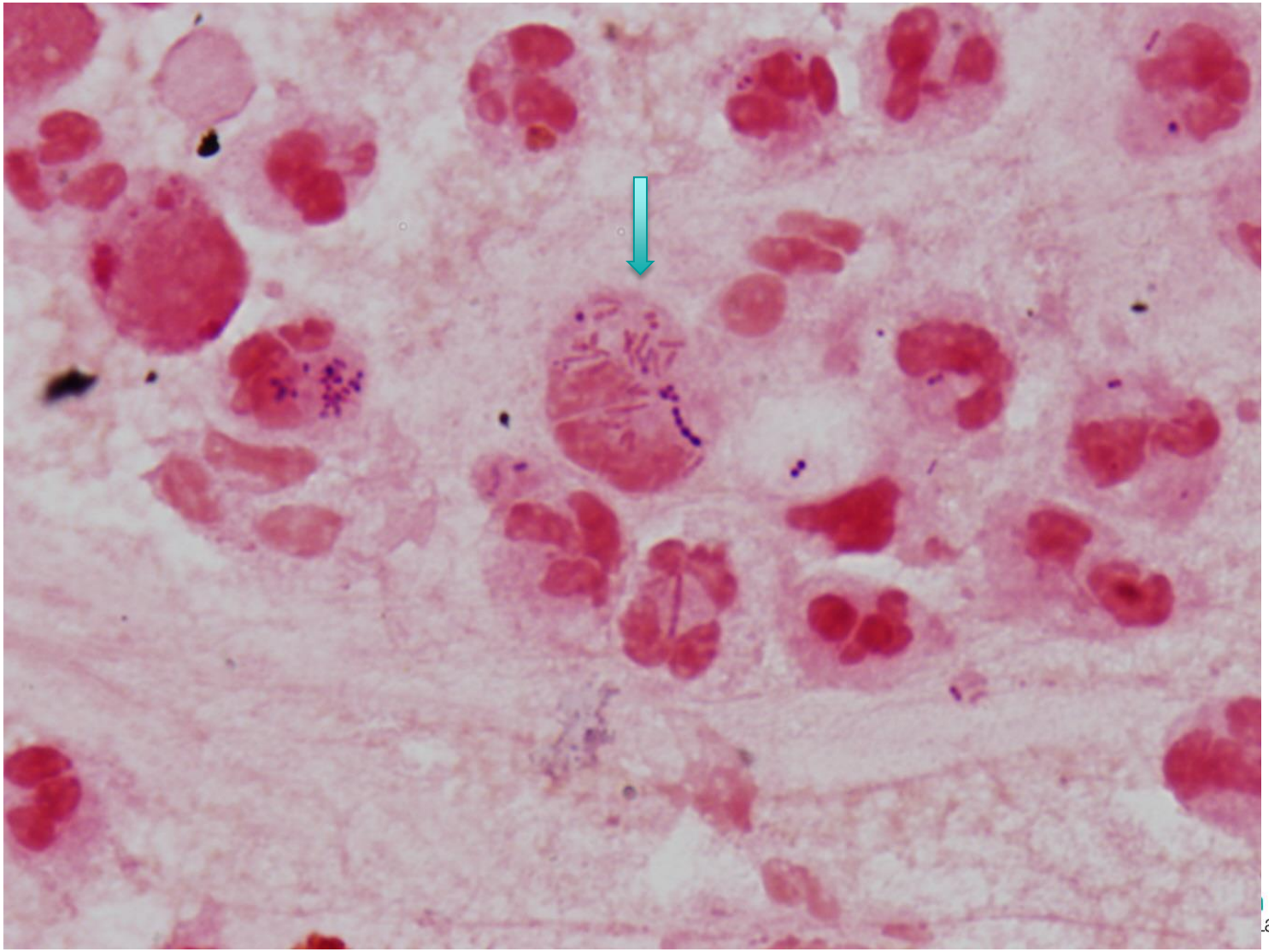
GRAM STAIN QUANTITIES

- Rare (1+)
  - Less than 10 in all fields examined
- Few (2+)
  - 1/field
- Moderate (3+)
  - More than 1/field but < 25/field
- Many (4+)
  - More than 25 in one field

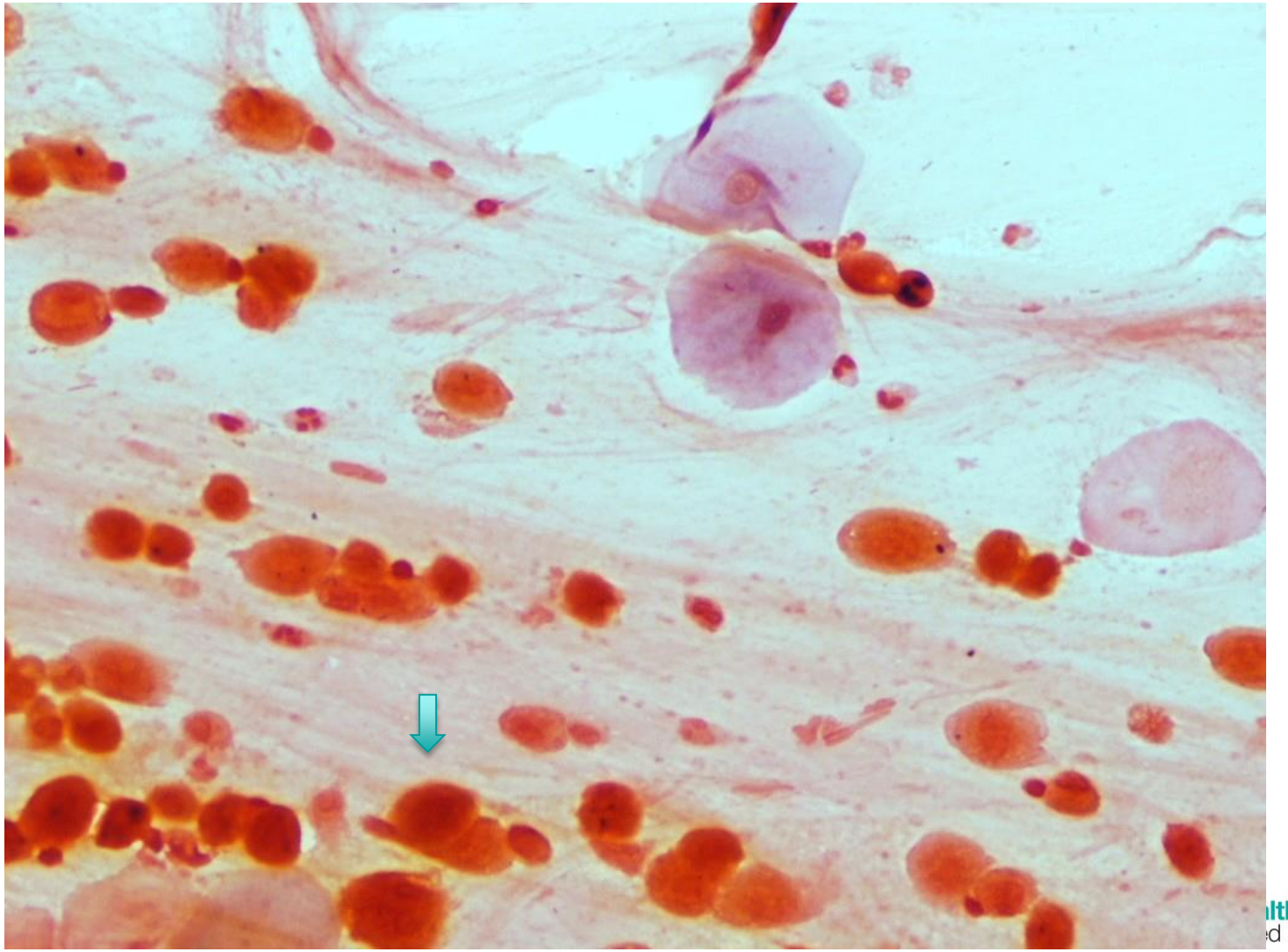
INDICATORS OF PATHOLOGY

- White blood cells (polymorphonuclear leukocytes)
- Alveolar macrophages
- Squamous/columnar epithelial cells
- Elastin/collagen fibers
- Curschmann's spirals
- Corpora amylacea
- Cell necrosis
- Intracellular bacteria/yeasts
- "Antibiotic" treated bacteria
- Crystals
- Charcot-Leyden Crystals
- Respiratory therapy
- Other oddities

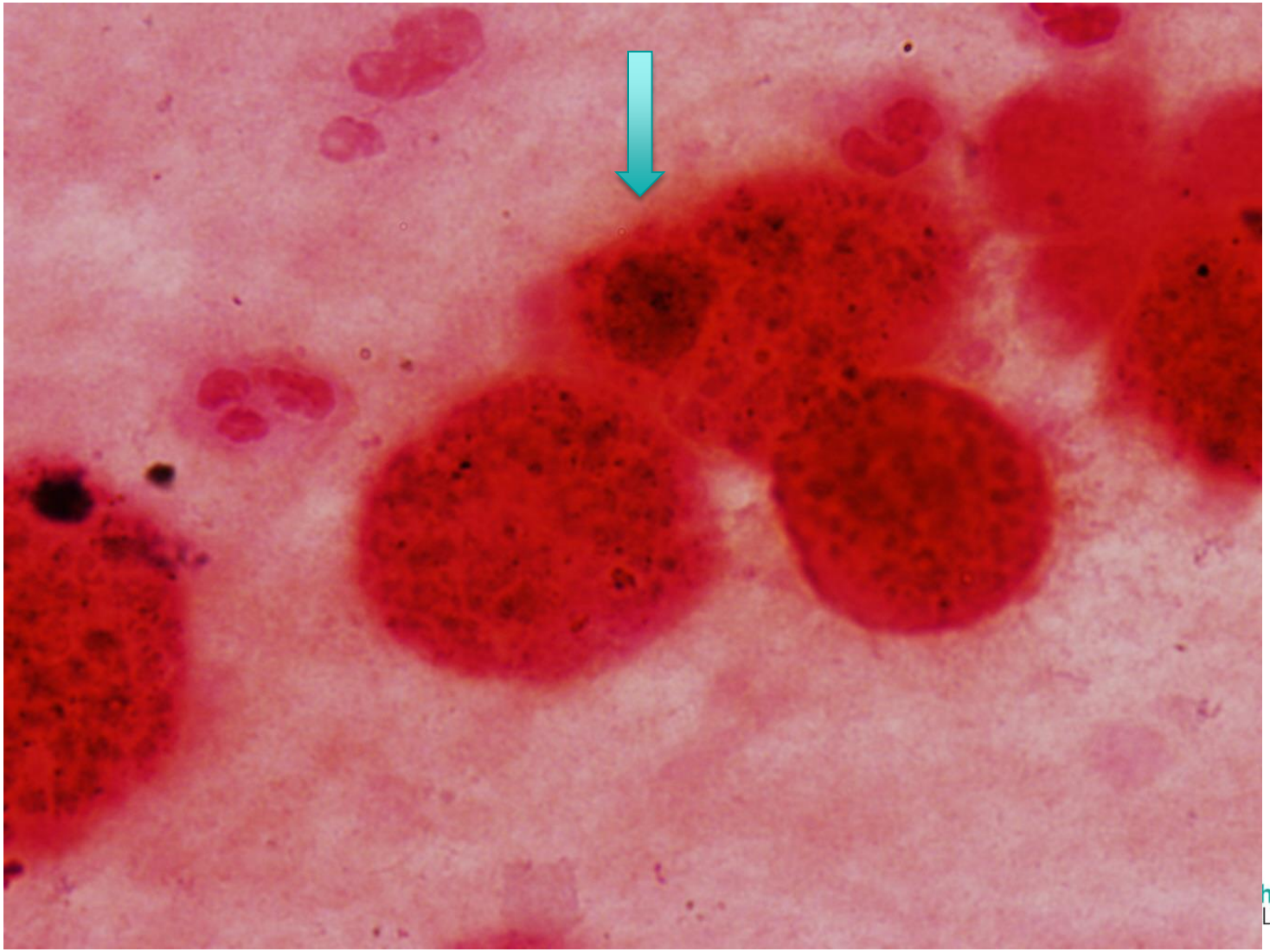
Intracellular bacteria



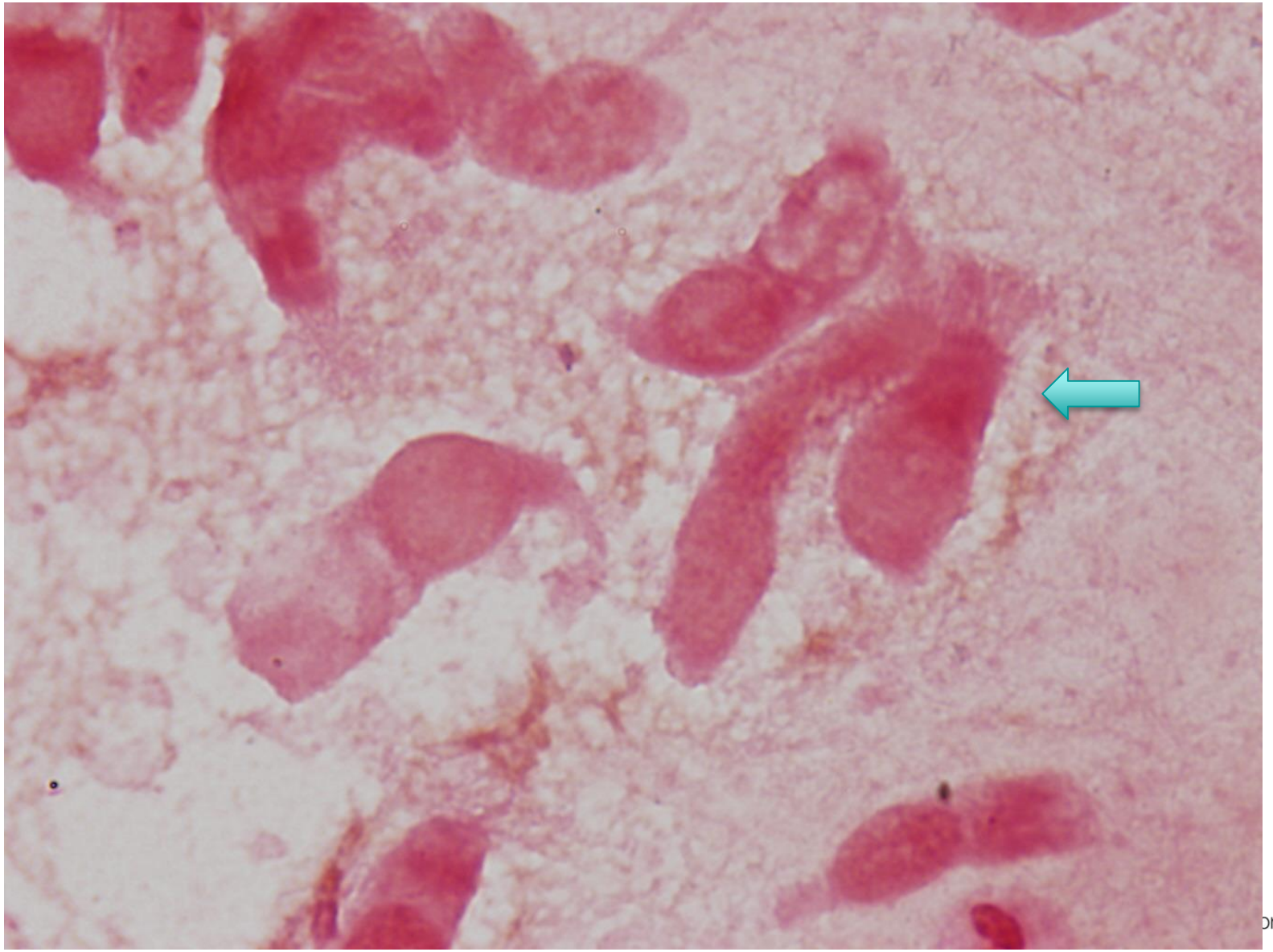
Alveolar macrophages



Alveolar macrophage

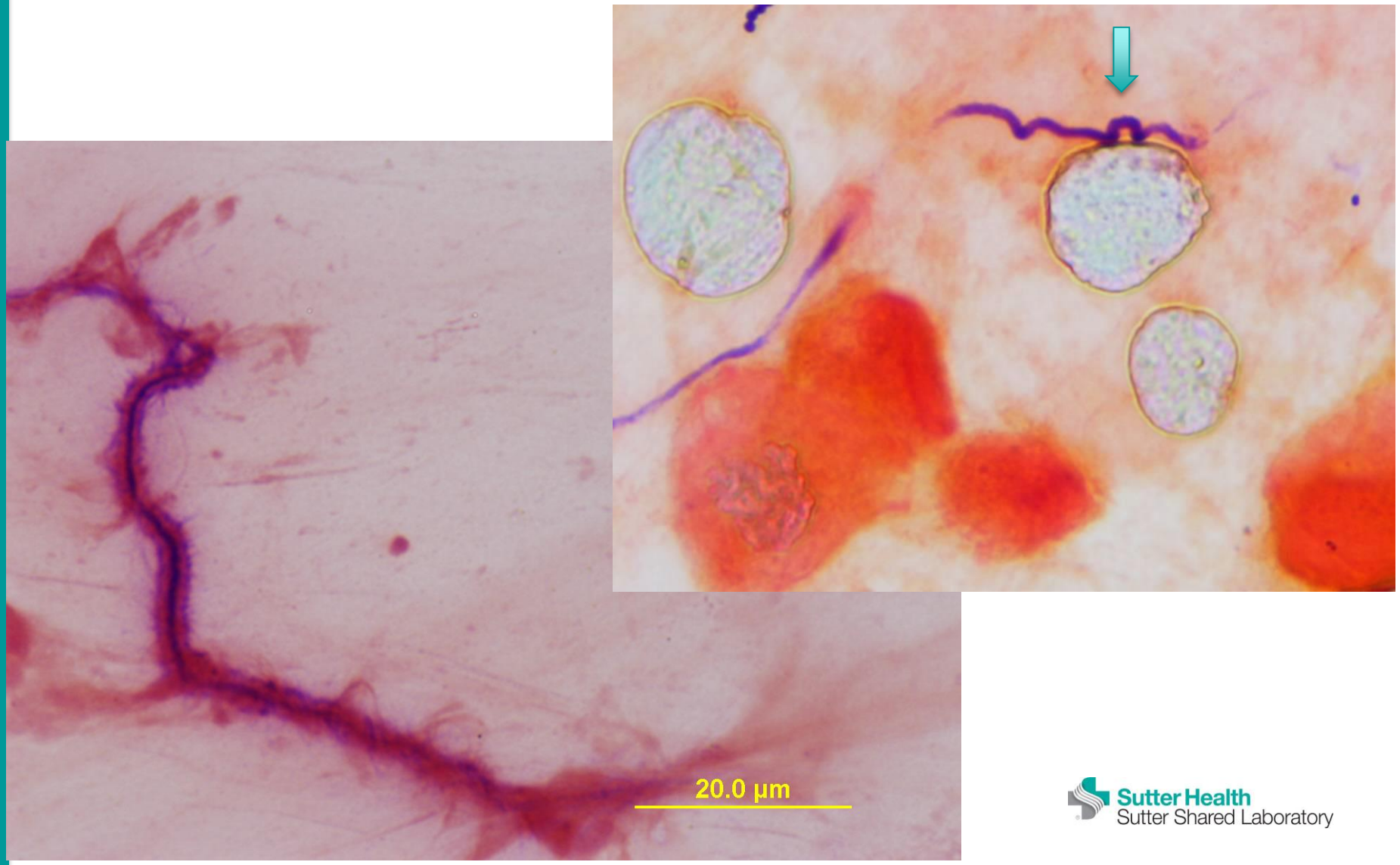


Ciliated columnar Epithelials



Elastin fibers

Curschmann's spiral (distal bronchiole cast)



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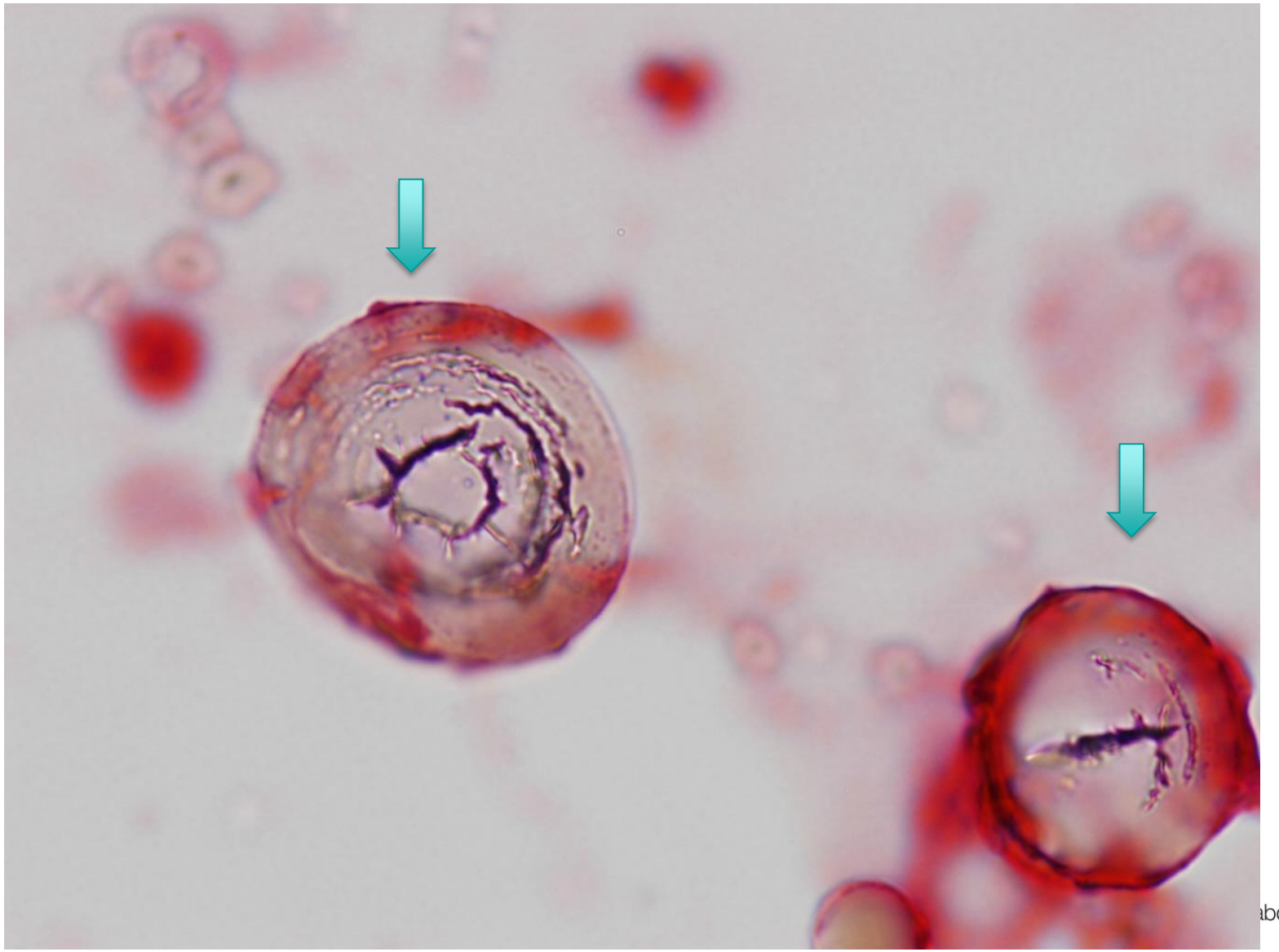
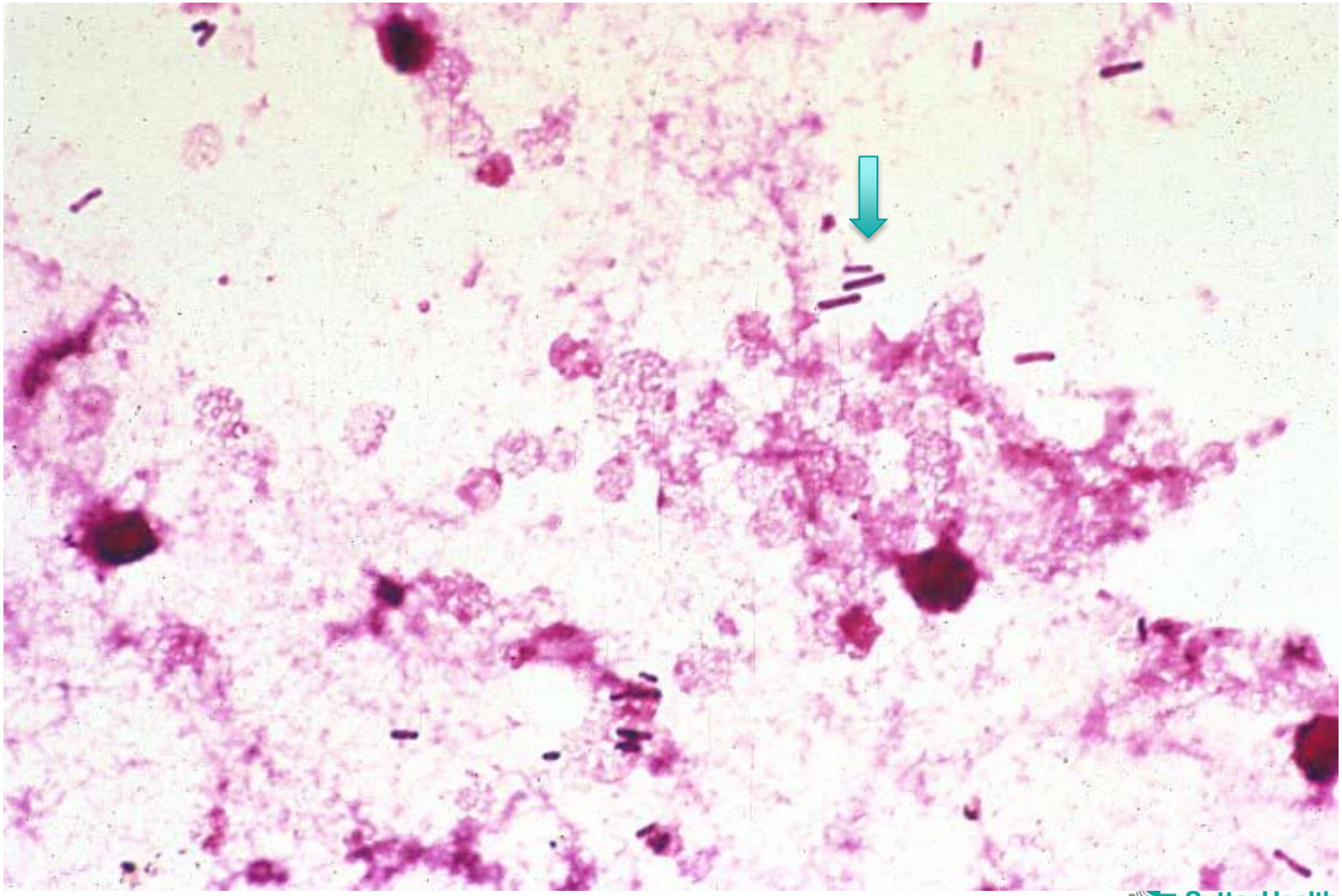
Corpora amylacea (breakdown product epithelial cells)



Gas gangrene
Clostridium perfringens necrotizing myonecrosis



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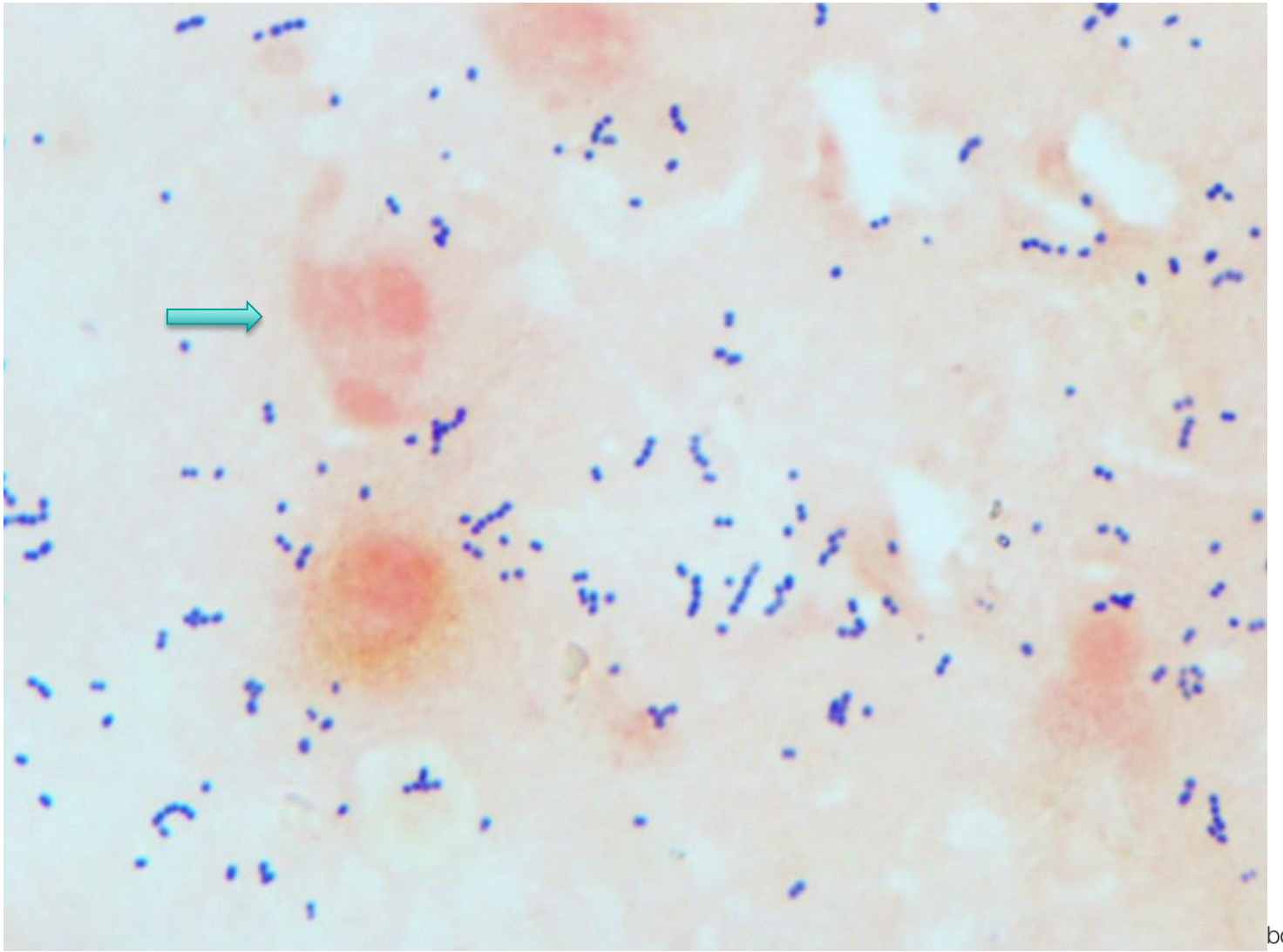
Necrotic PMNs



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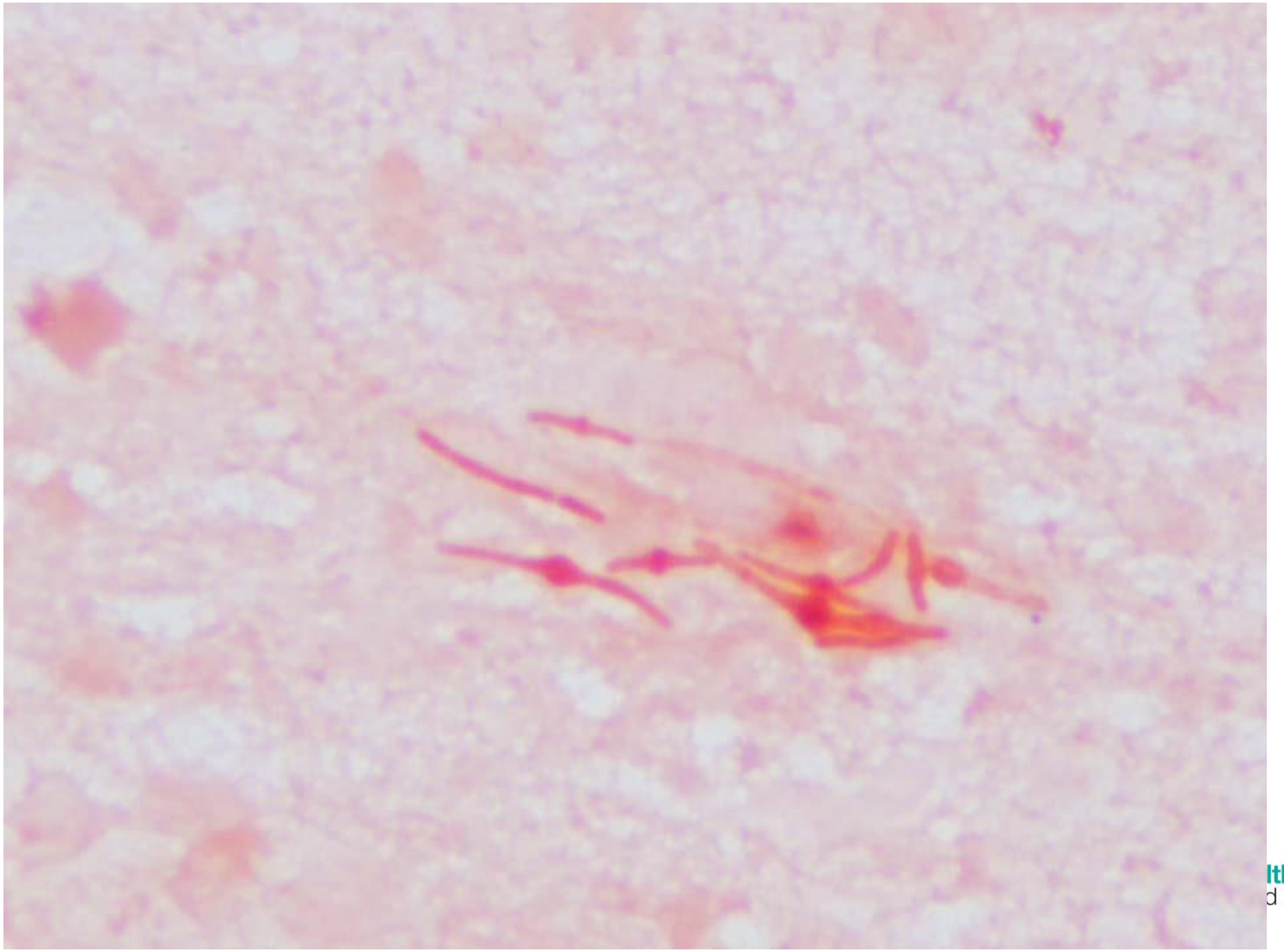
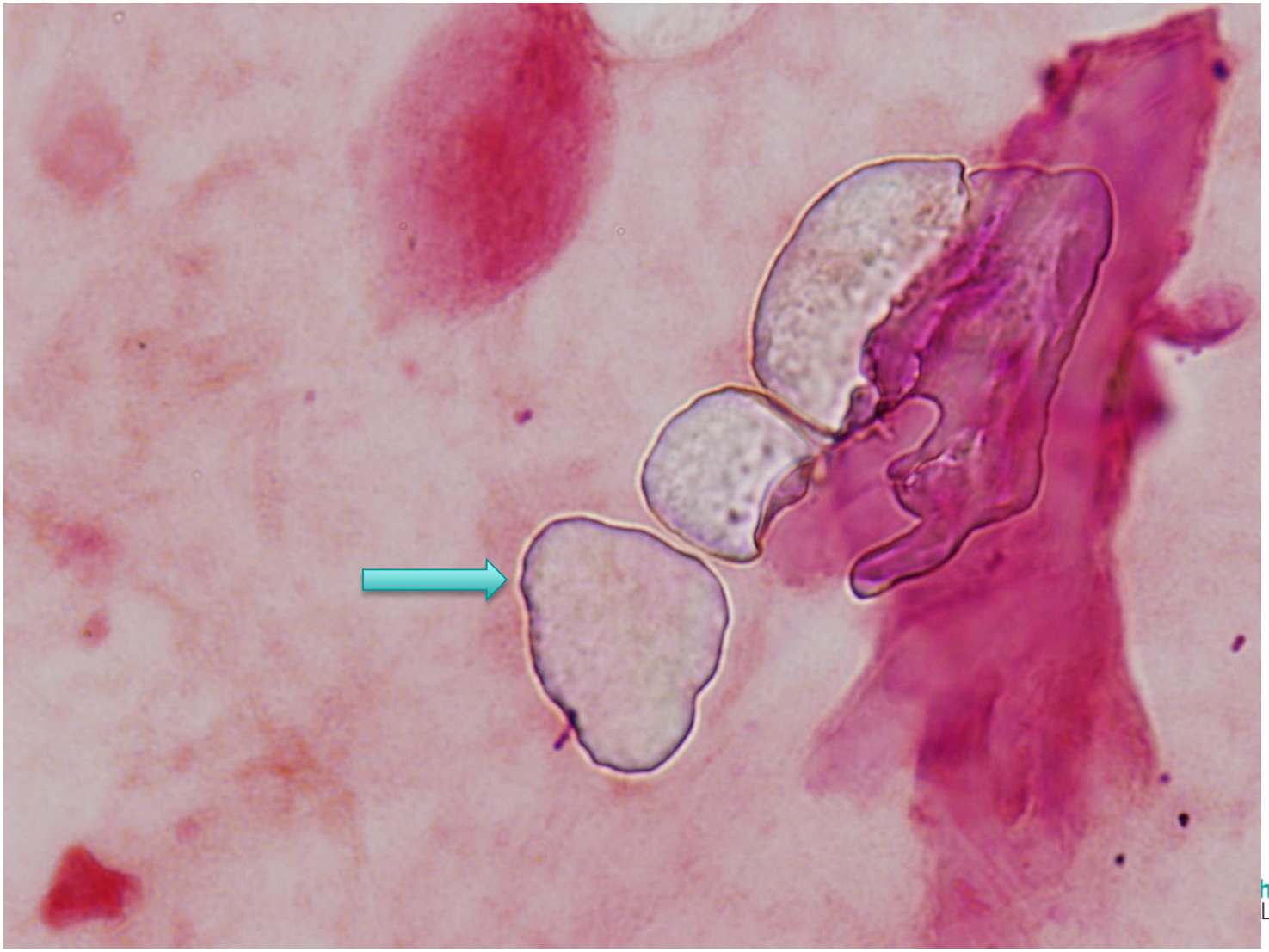
Intracellular yeast cells

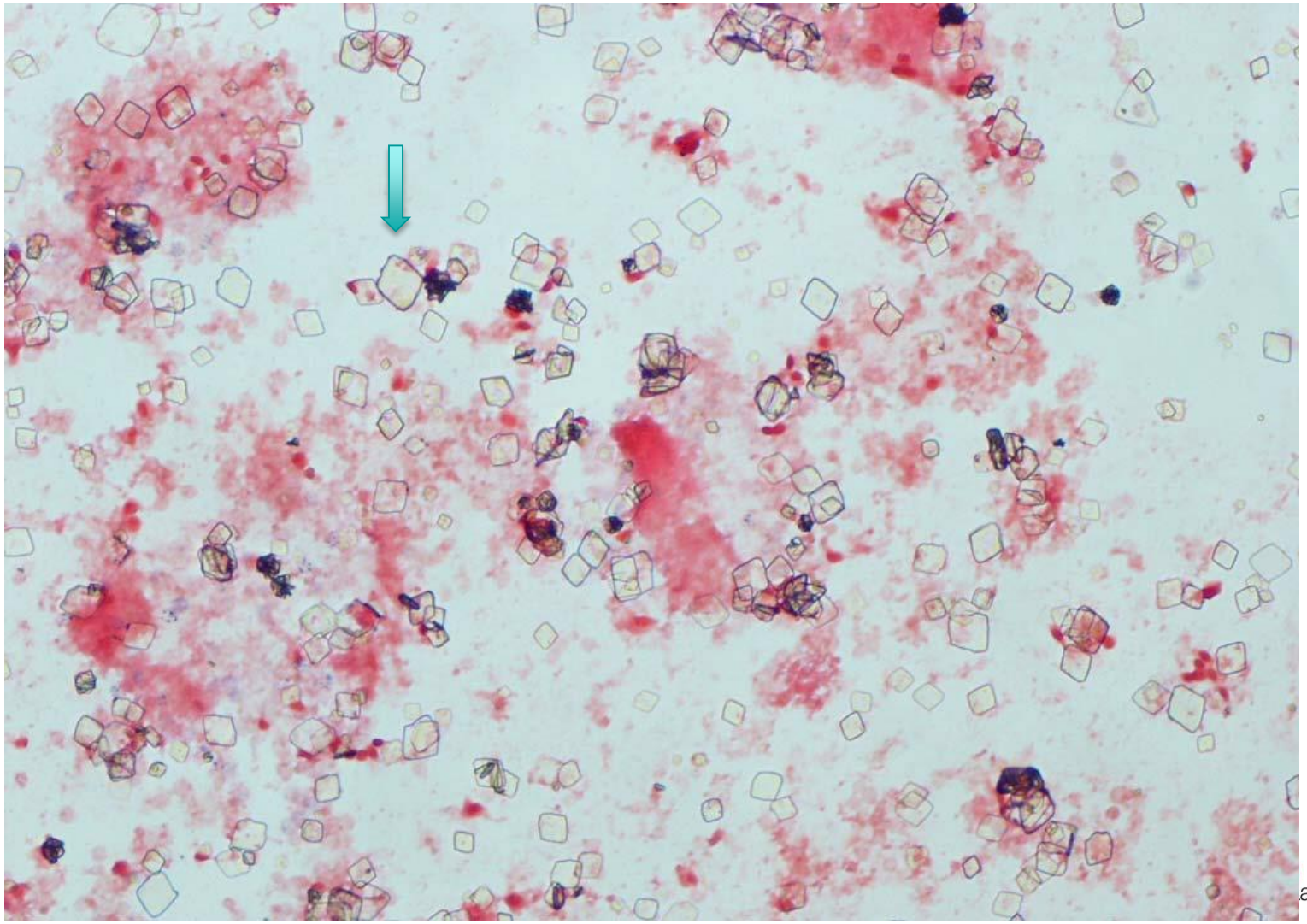
Antibiotic effect



Aerosolized lipid in inhaler



Uric acid crystals



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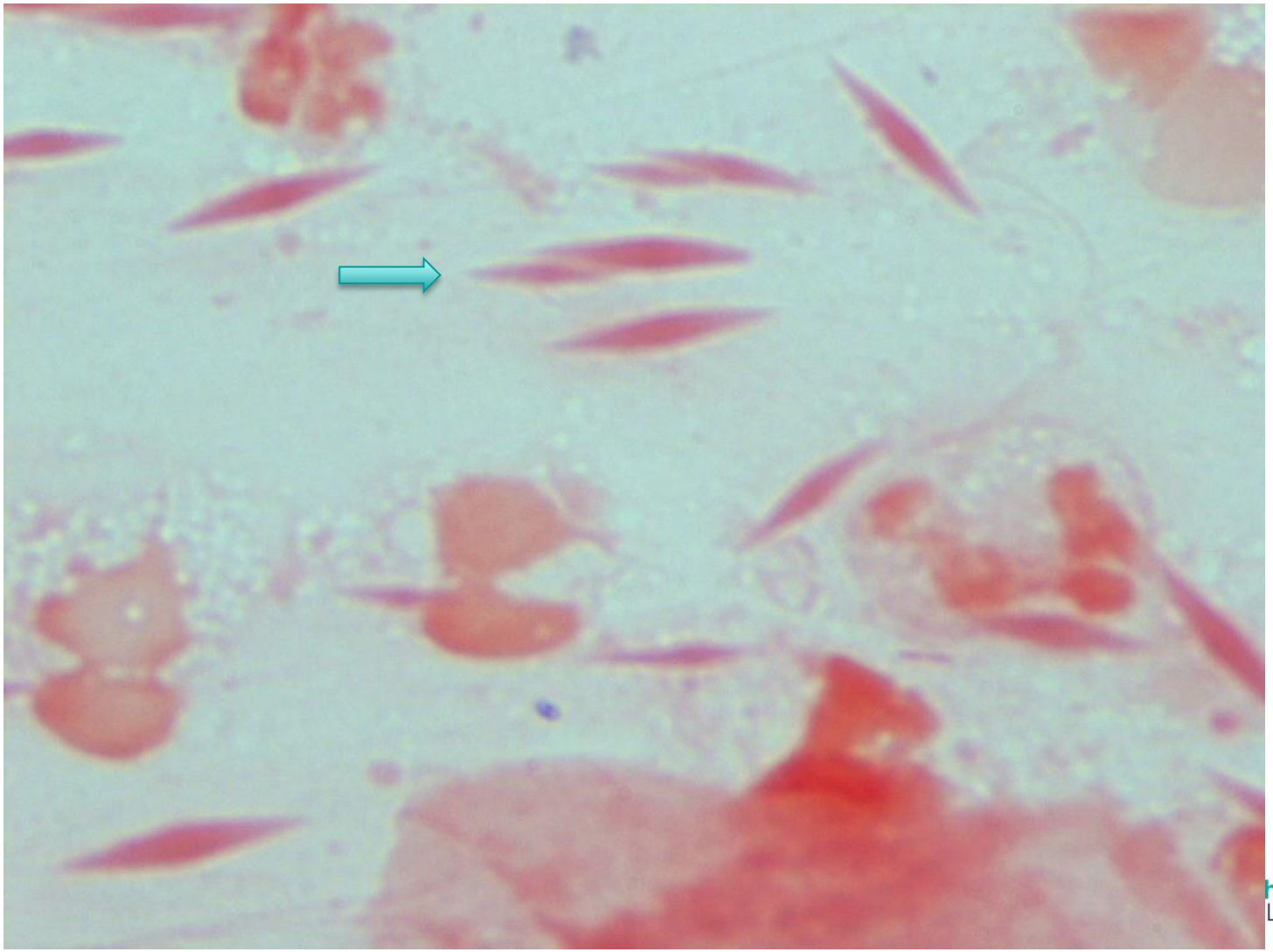
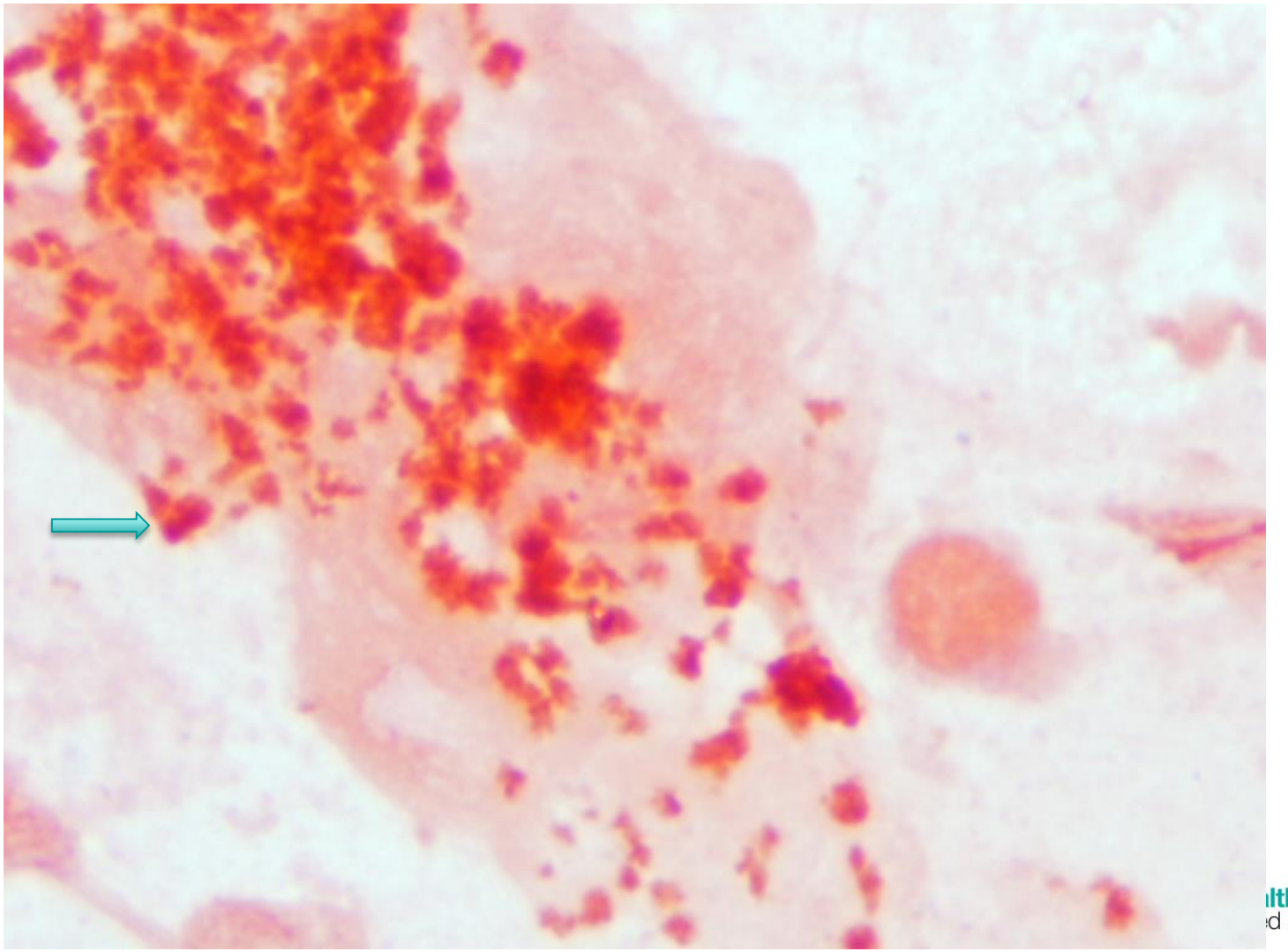
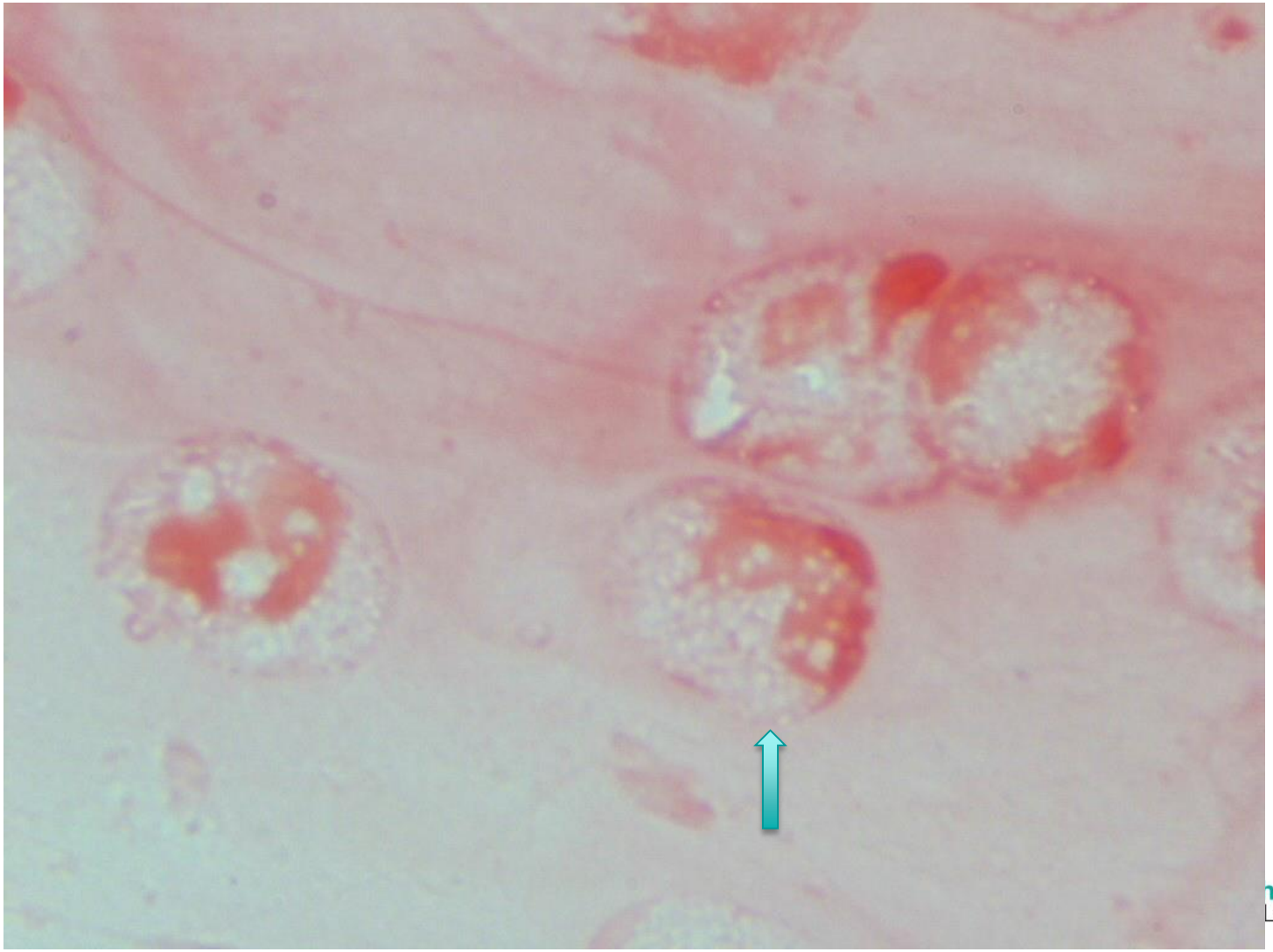
Charcot-leyden crystals
(seen in allergic pulmonary aspergillosis)



Respiratory therapy effect (hypertonic saline aerosol)

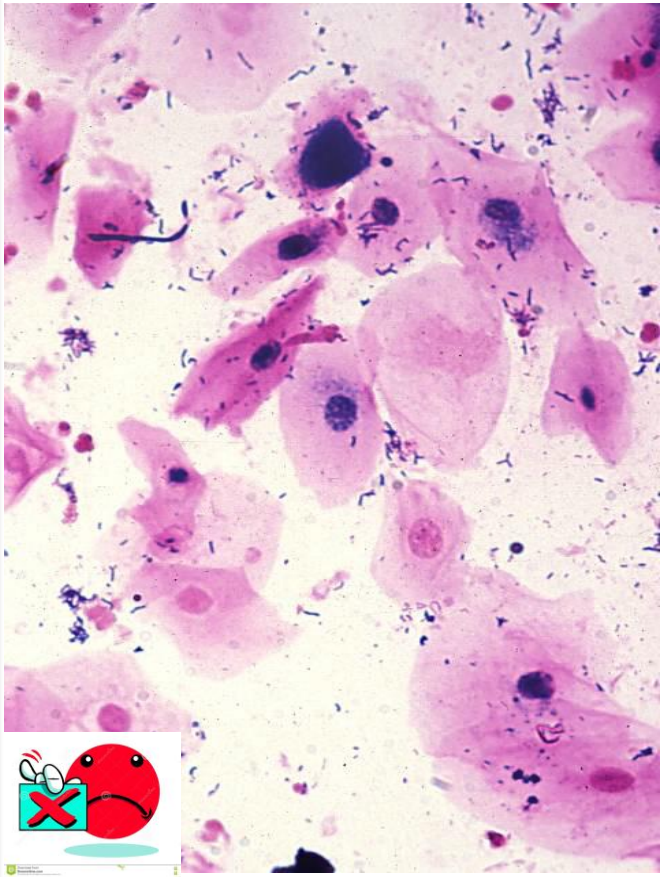


Eosinophils (looks like vacuoles)



Respiratory Specimen Quality

- **Optimal smear preparation and staining essential**
- **Target selection of grossly mucopurulent portions of specimen for Gram stain and culture**



Rejection Criteria

- Use the same criteria for ET aspirates
- Do not reject for BAL, Legionella, AFB or from cystic fibrosis patients
- Add comment on report (Ex: > 10 SEC per LPF indicates the specimen is contaminated by oropharyngeal flora, culture not performed.")
- Notify the caregiver
- Charge for the Gram stain, not the culture
- QA: follow number of sputum ordered and number rejected each month

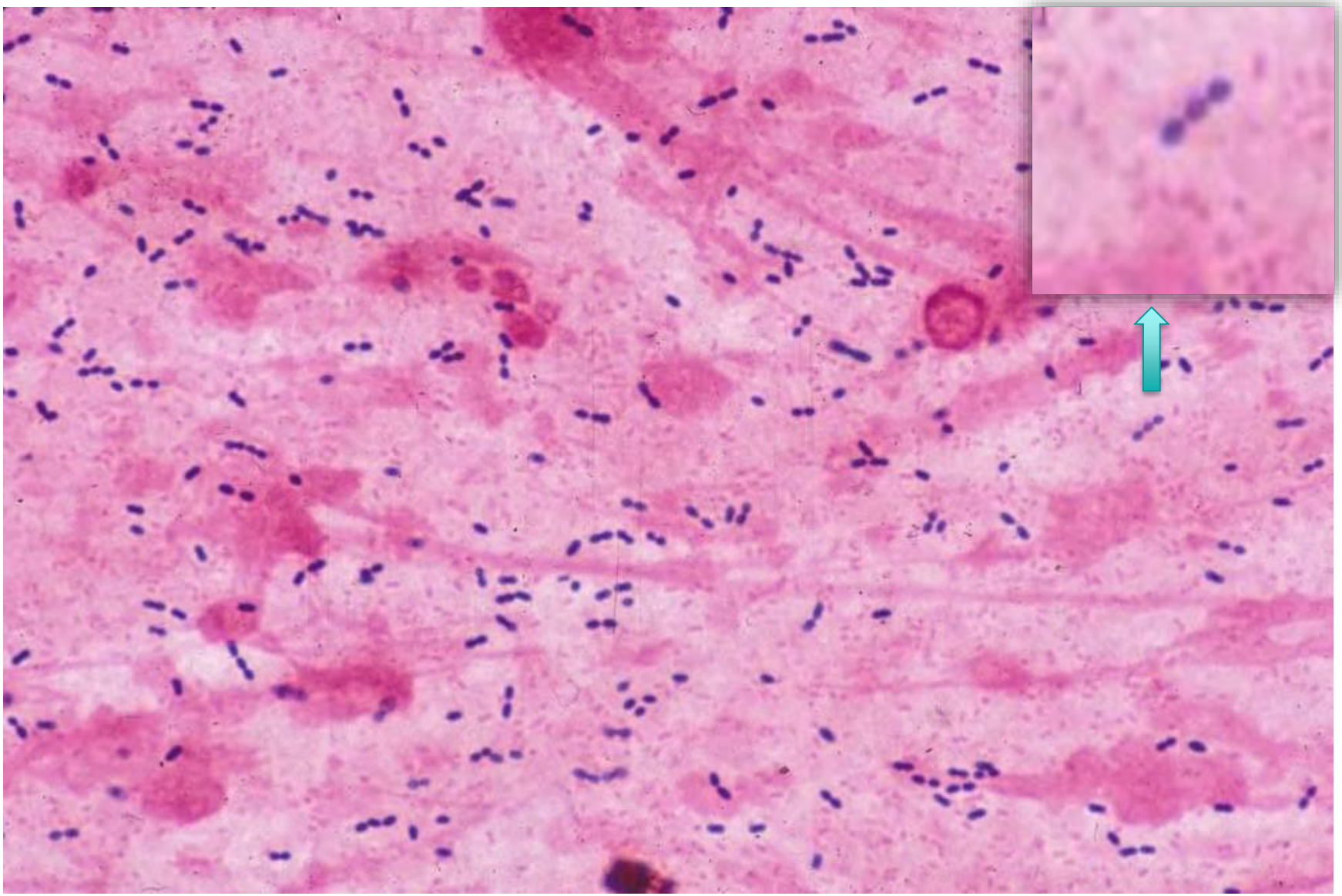
Key patterns in sputum

Numerous PMNs, rare or no squamous Epithelials cells and numerous:

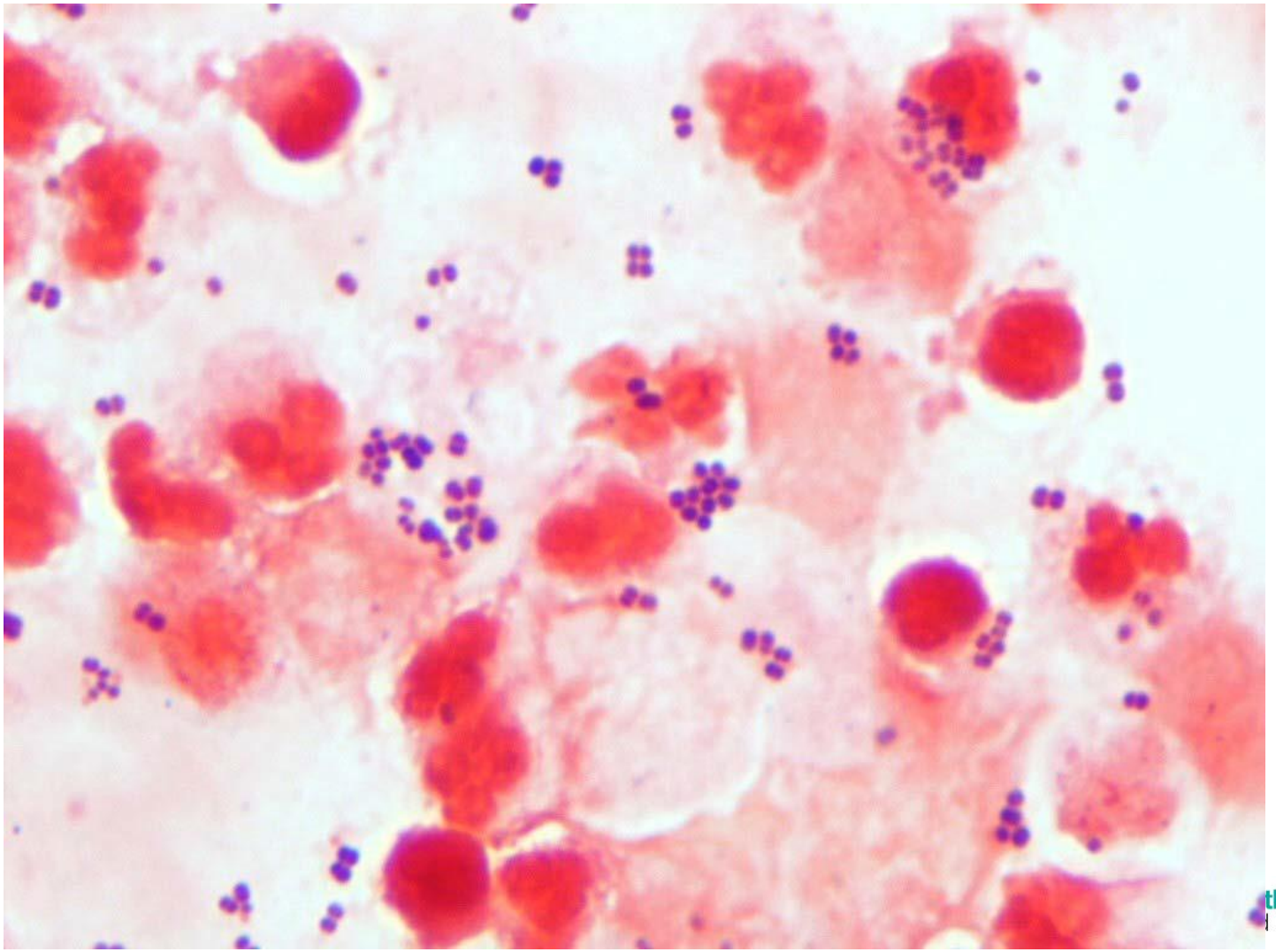
- **Gram-positive cocci in pairs and short chains "Suggestive of *Streptococcus pneumoniae*"**
- **Tiny or pleomorphic Gram-negative rods "Suggestive of *Haemophilus influenzae*"**
- **Gram-Negative Diplococci "Suggestive of *Moraxella catarrhalis*"**
- **Gram-positive cocci in clusters "Suggestive of *Staphylococcus aureus*"**
- **Mixed morphotypes of Gram-positive and Gram negative**

rods, cocci, and coccobacilli "Suggestive of Aspiration Pneumonia"

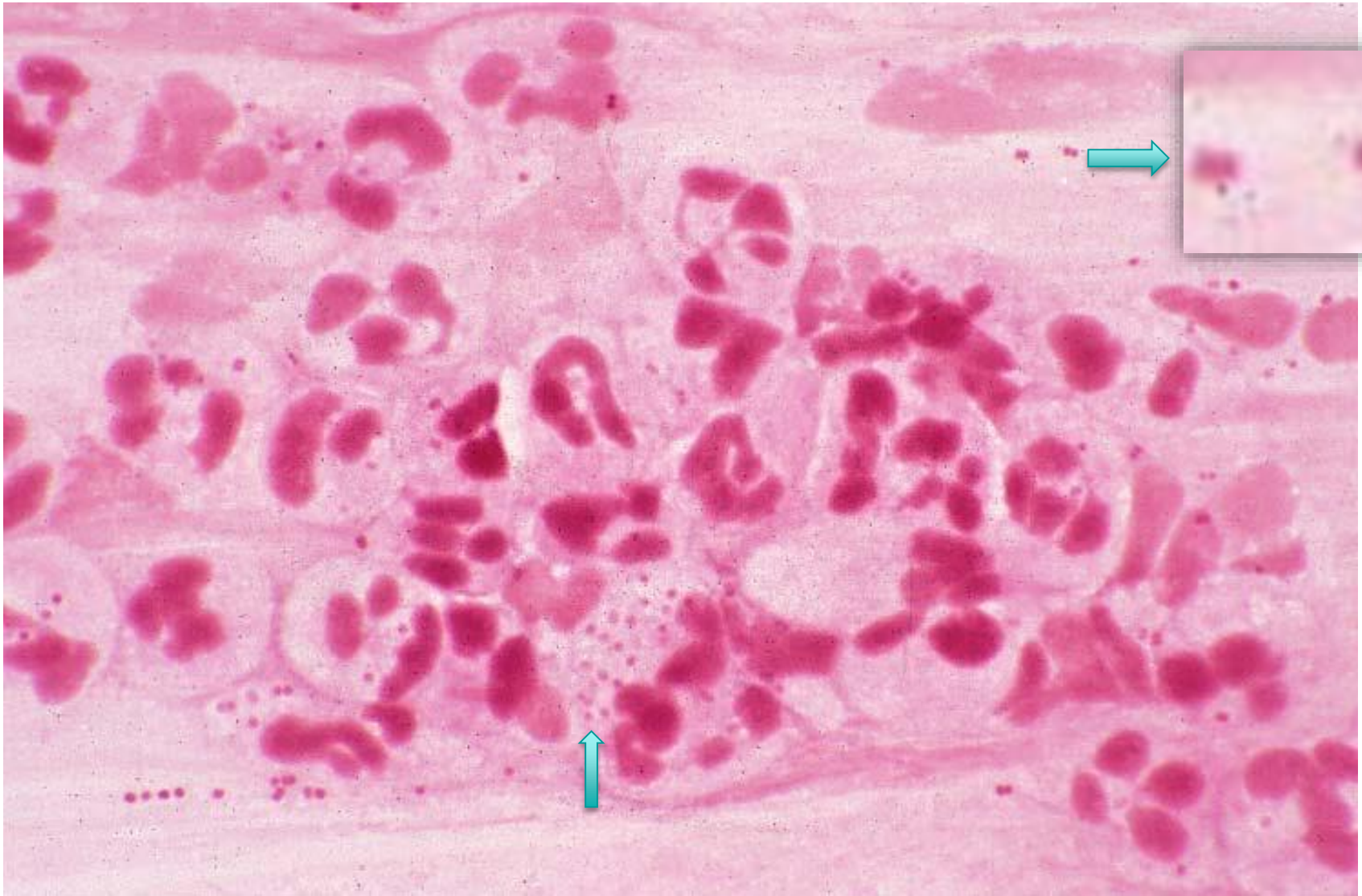
S. pneumoniae and ?? *H. influenzae*



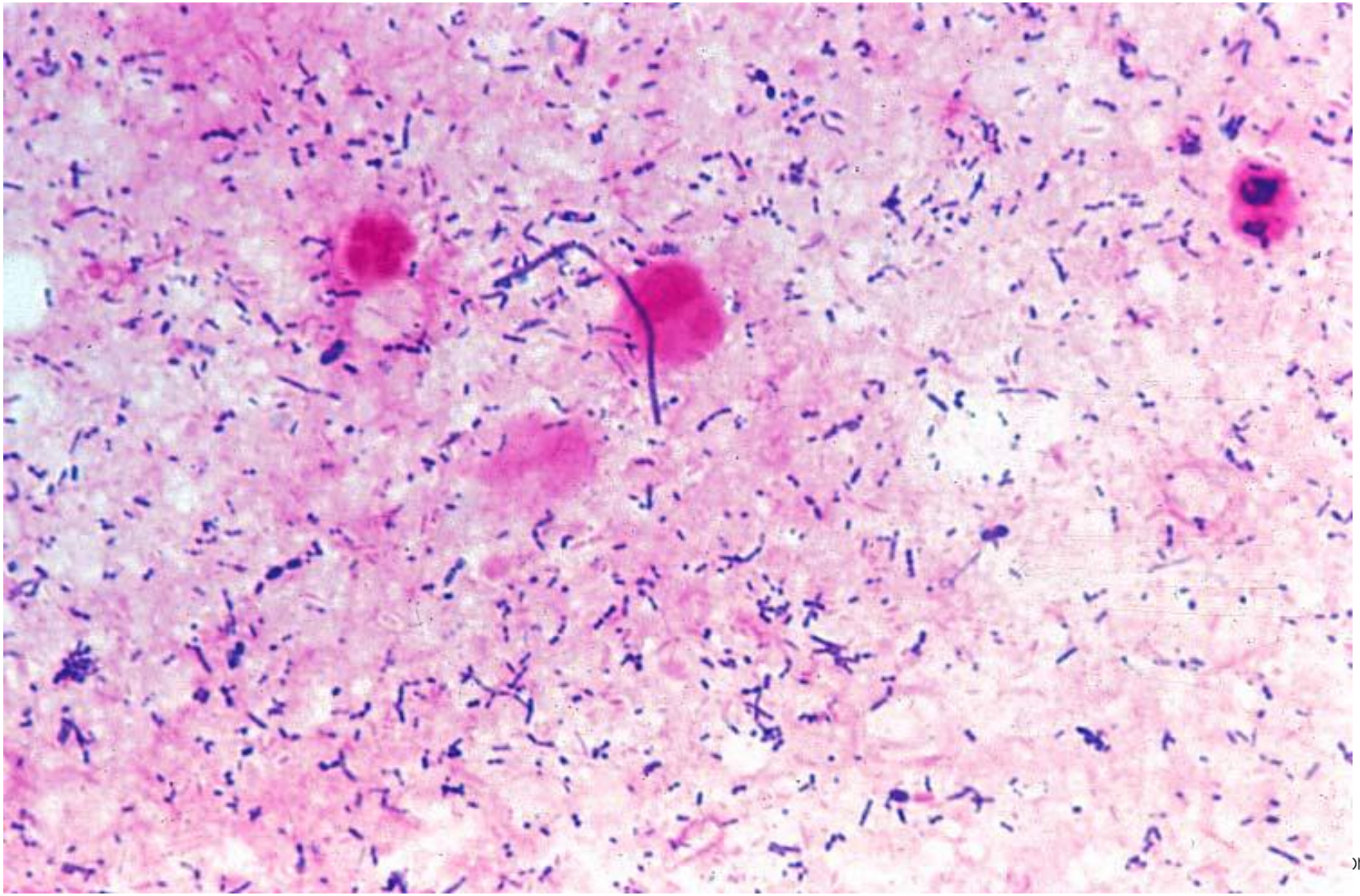
Staphylococcal pneumonia



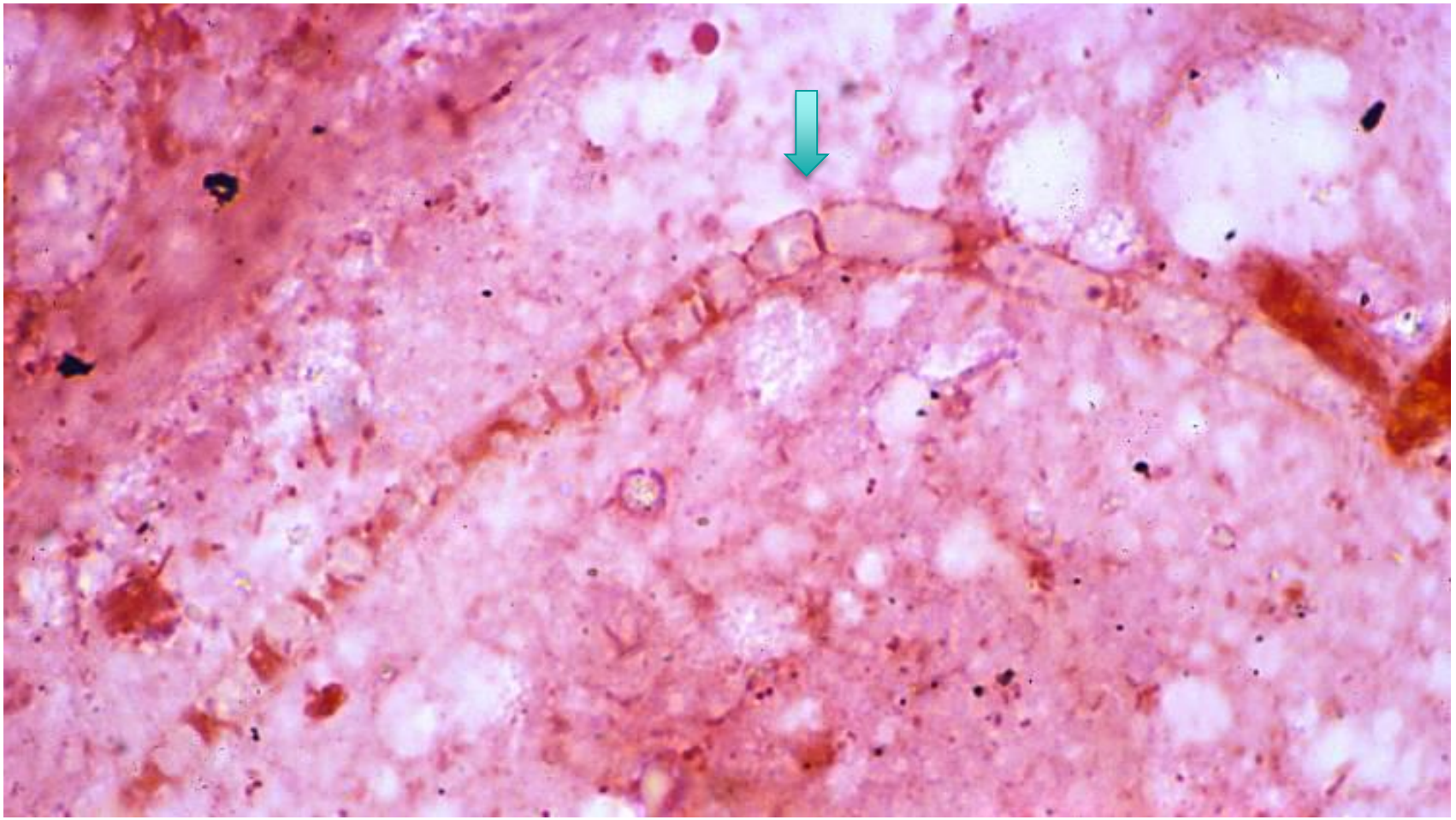
Gram-Negative, Intracellular Diplococci suggestive of *Moraxella catarrhalis* in sputum



Mixed morphotypes, no Epithelial Cells= aspiration pneumonia



Fungal element in sputum



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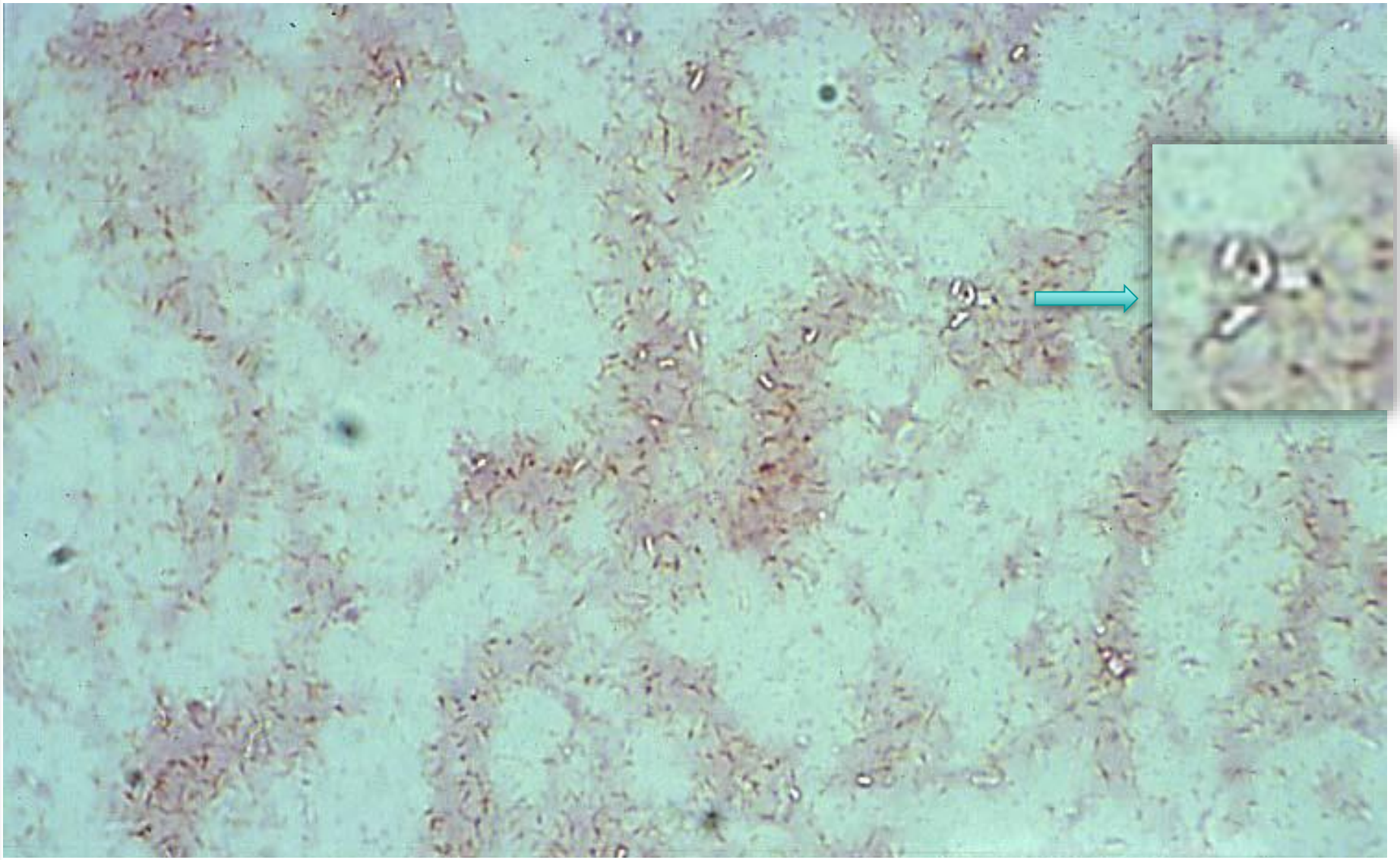
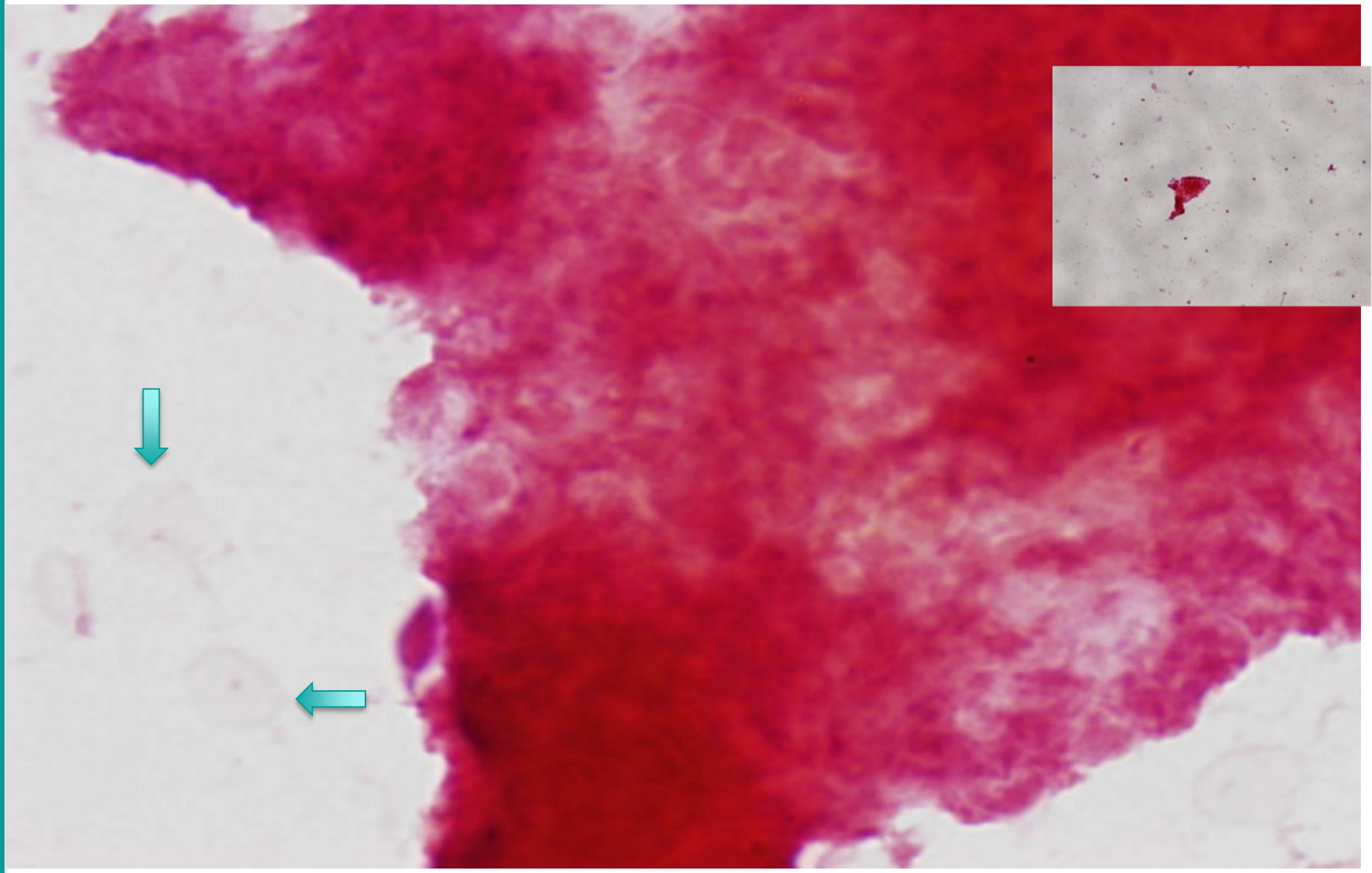
AFB in Gram stain



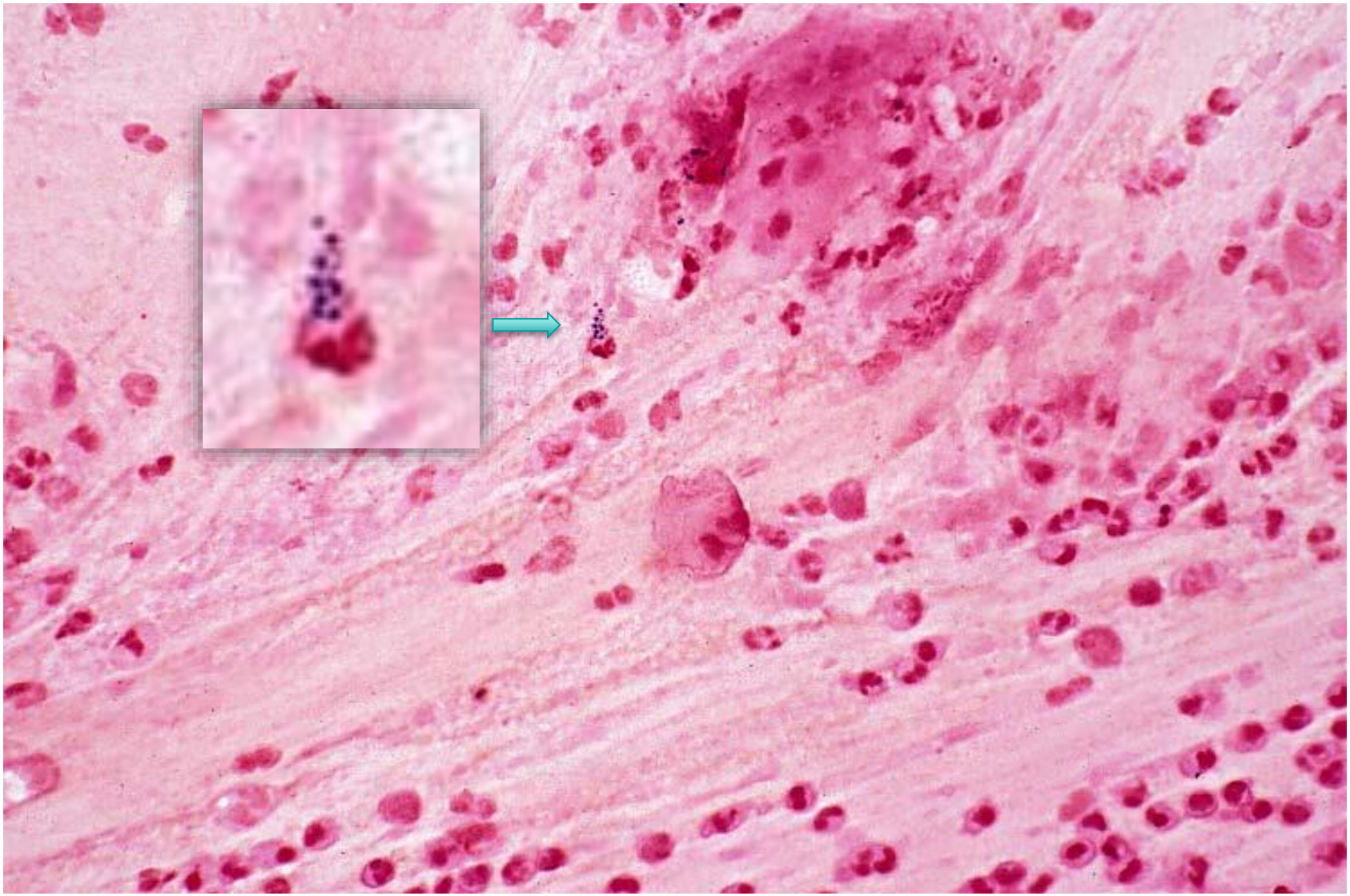
Pneumocystis jiroveci



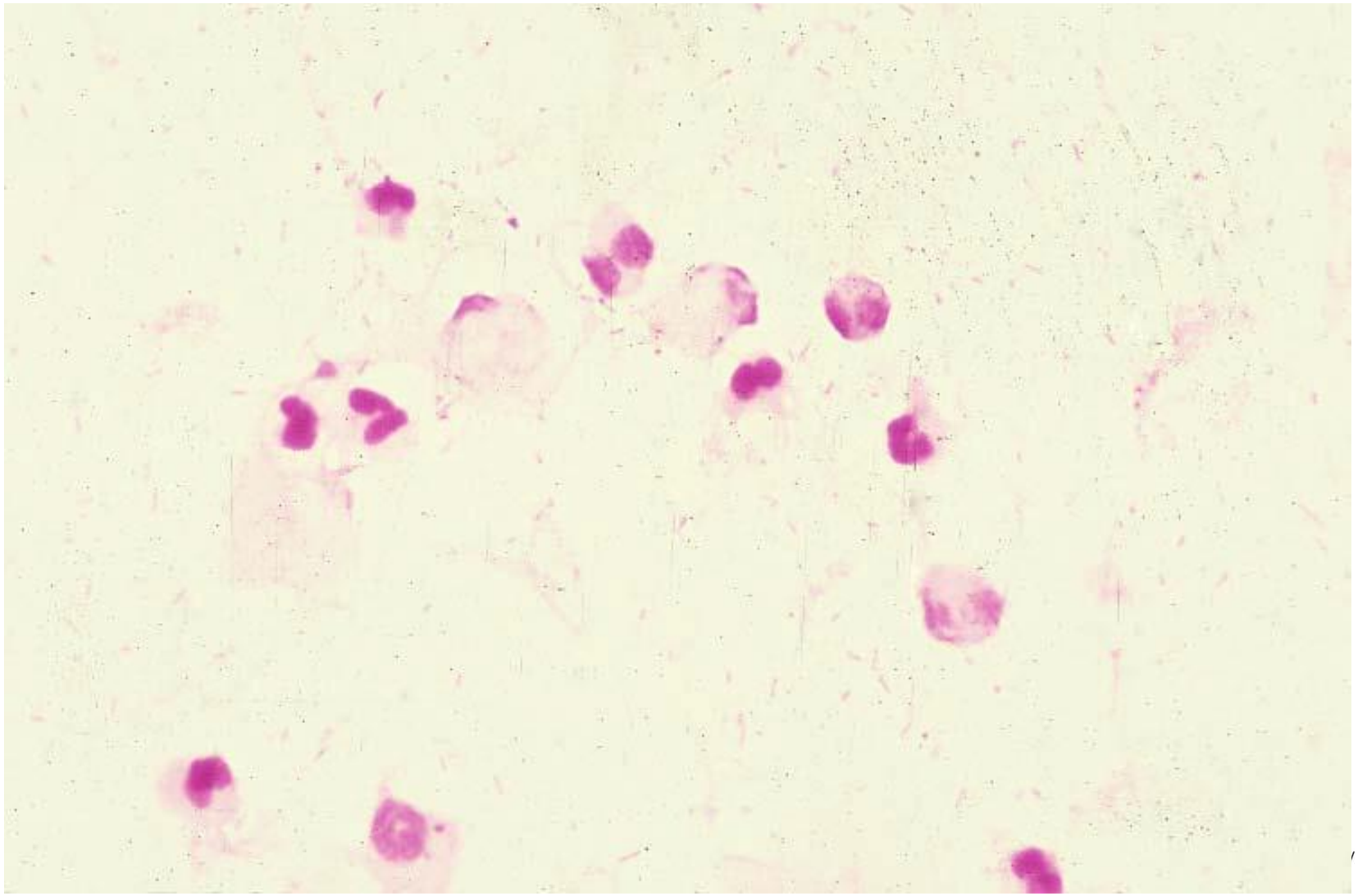
Patterns to look for in evaluating Gram stains from tissue or aspirates

Finding	Comment
Presence of squamous epithelial cells	Suggests poor specimen collection technique; culture may yield skin flora "contaminants"
Numerous PMNs	Look long and hard for a bacterial agent, which may be intracellular
Numerous PMNs and mixed morphologies Gram negative and positive, small organisms	Mixed anaerobic or facultative and anaerobic bacterial infection.
Few or necrotic-looking PMNs and large, boxcar-shaped Gram variable or GPR's	Possible Clostridial tissue necrosis. Surgical emergency. Suggestive of *Clostridium perfringens* gas gangrene.
Rare PMNs but no other somatic cells	Patient may not mount much of an immune response or organism does not elicit PMNs (such as cocci). Bacterial etiology still highly suspected.
Intracellular organisms Active bacterial infection.	Intracellular organisms Active bacterial infection.
Clumps of small, pale and irregular Gram positive cocci in chains	If from an abscess, suggests *Streptococcus anginosus* ("S. milleri") group.
Very tiny Gram negative rods (GNR's)	Particularly if from lymph node or tissue, could be bioterrorism agent (*Brucella, Yersinia, Francisella*) and should be handled in the hood. Plates should be taped and cultures should be handled in a BSC only. *** DO NOT SNIFF PLATES ***.

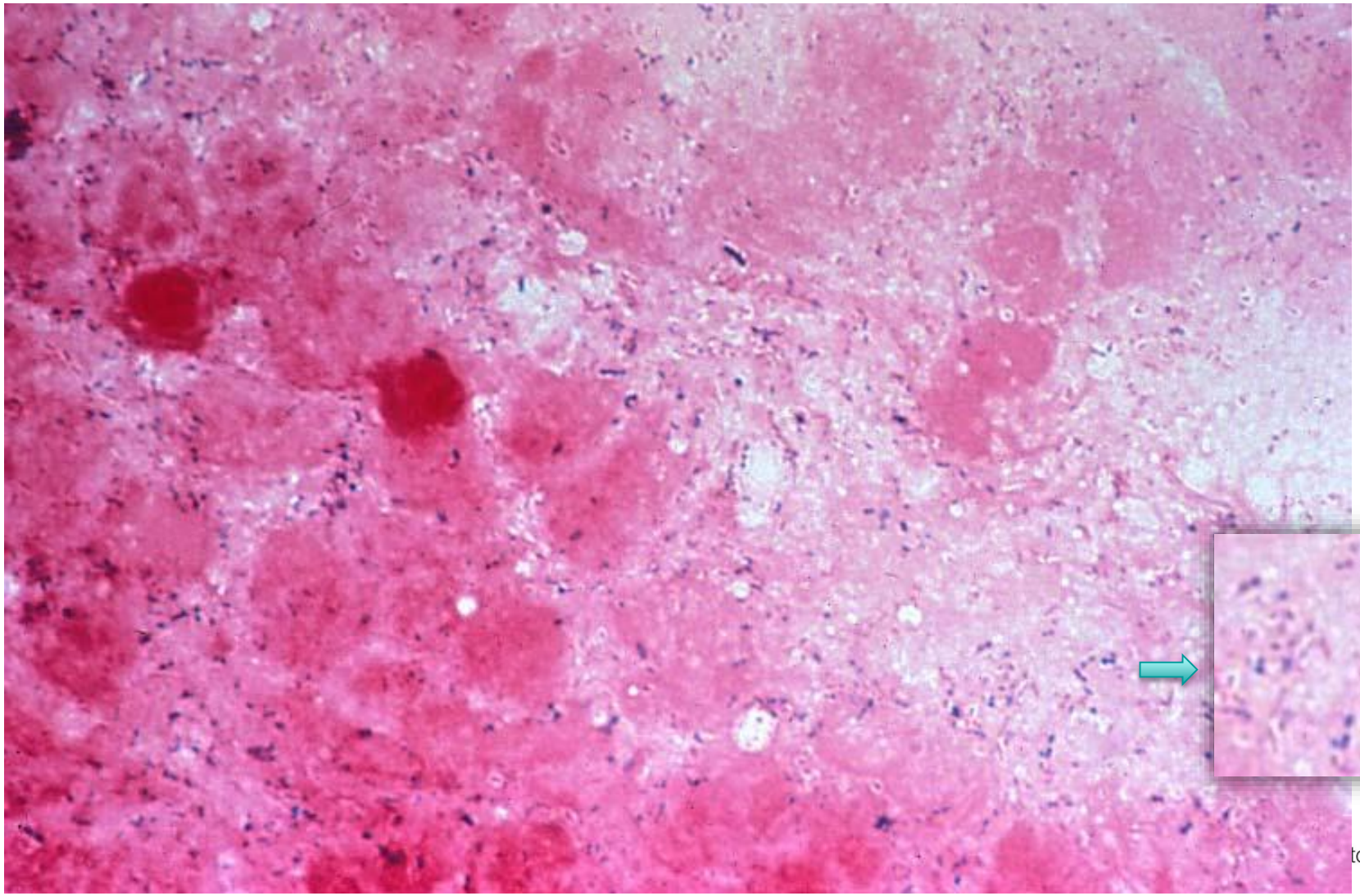
Staphylococcal soft tissue infection



Tiny GNRs in pus – aspirate from lymph node: BE CAREFUL!!



Brain aspirate – mixed anaerobic infection



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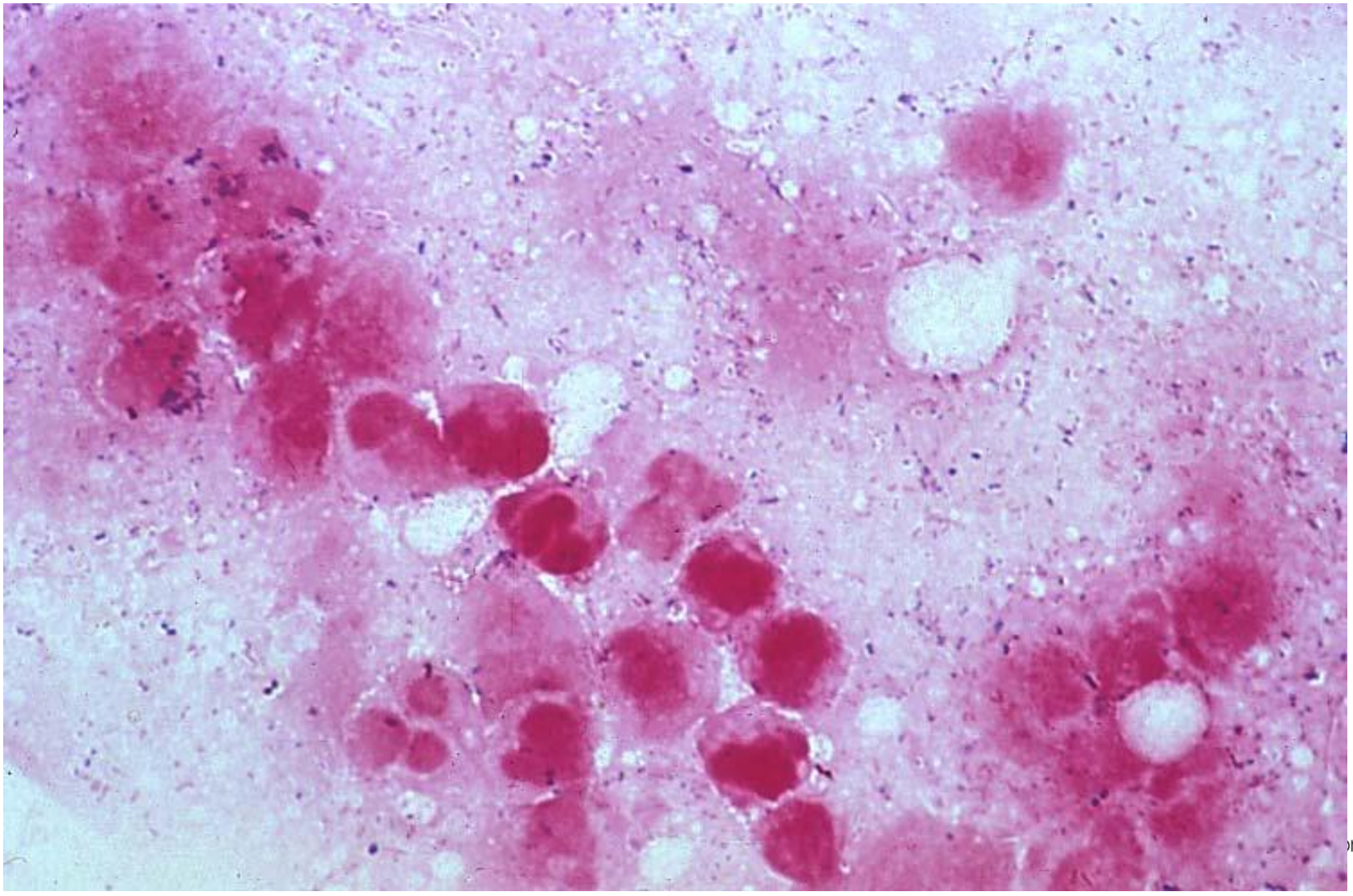
Thigh abscess – mixed anaerobic infection



Gram-positive, beaded branching rod suggestive of *Nocardia* spp.

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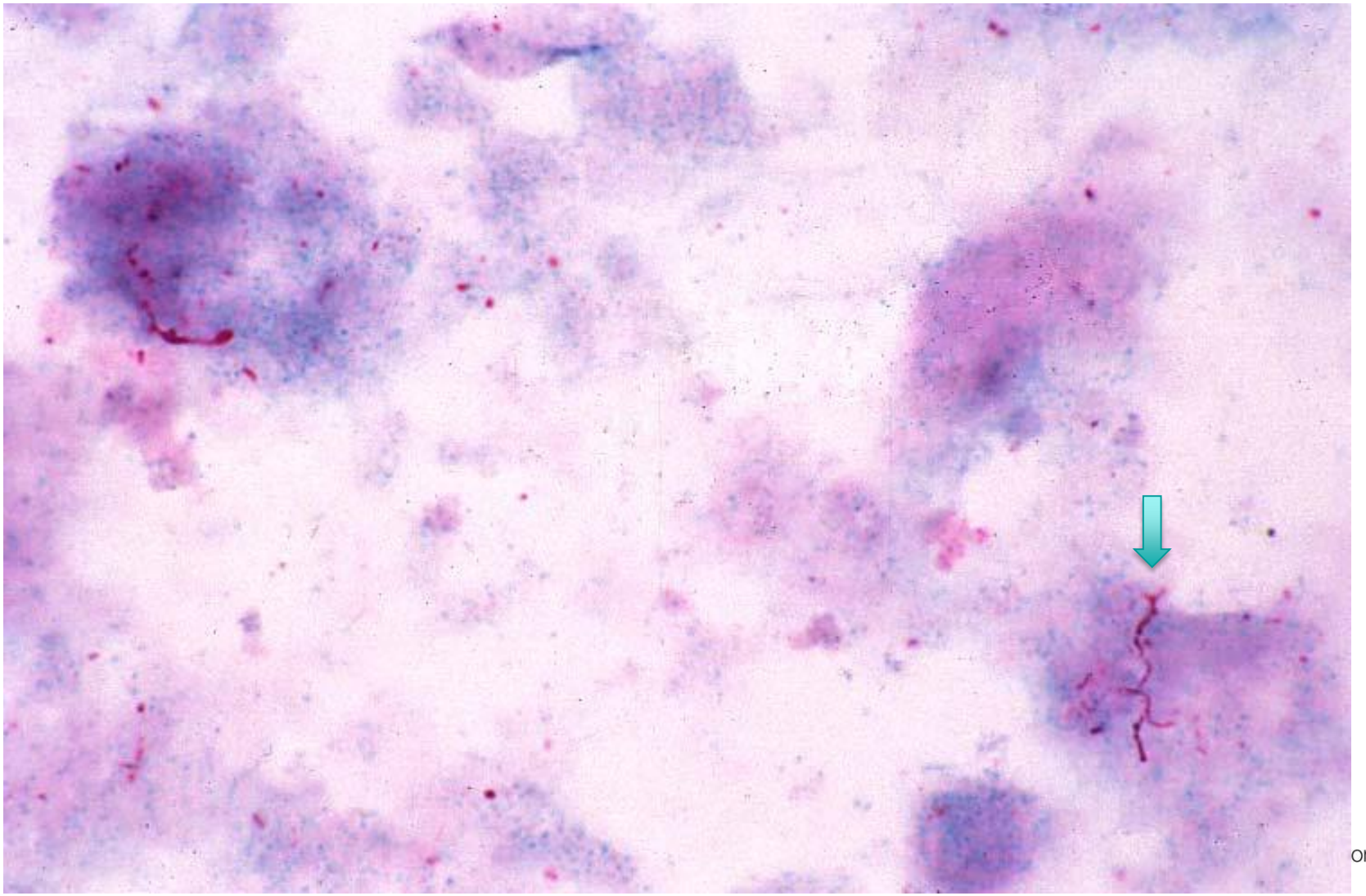
Modified acid fast stain of Branching Gram Positive Rods = *Nocardia* (positive)



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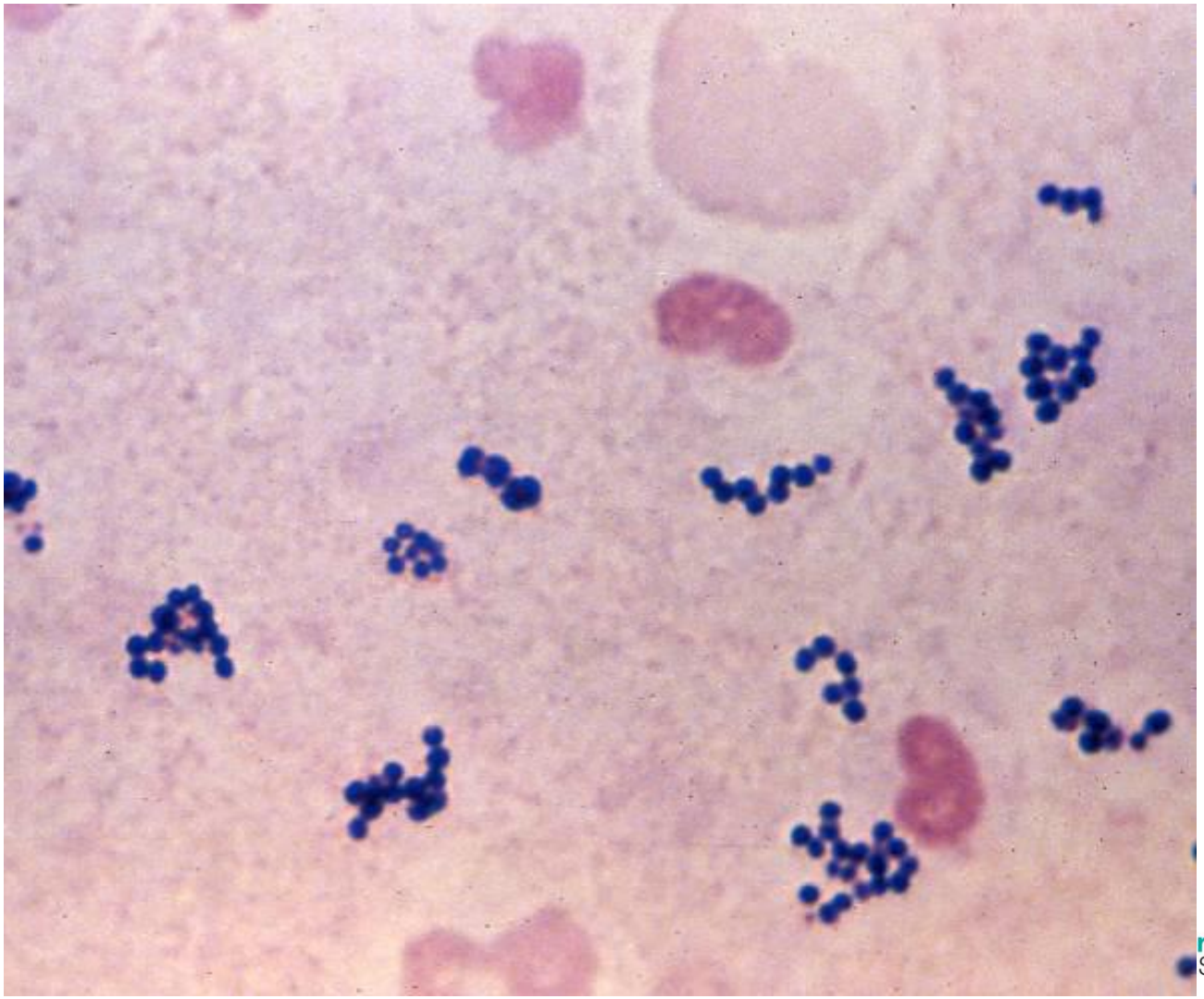
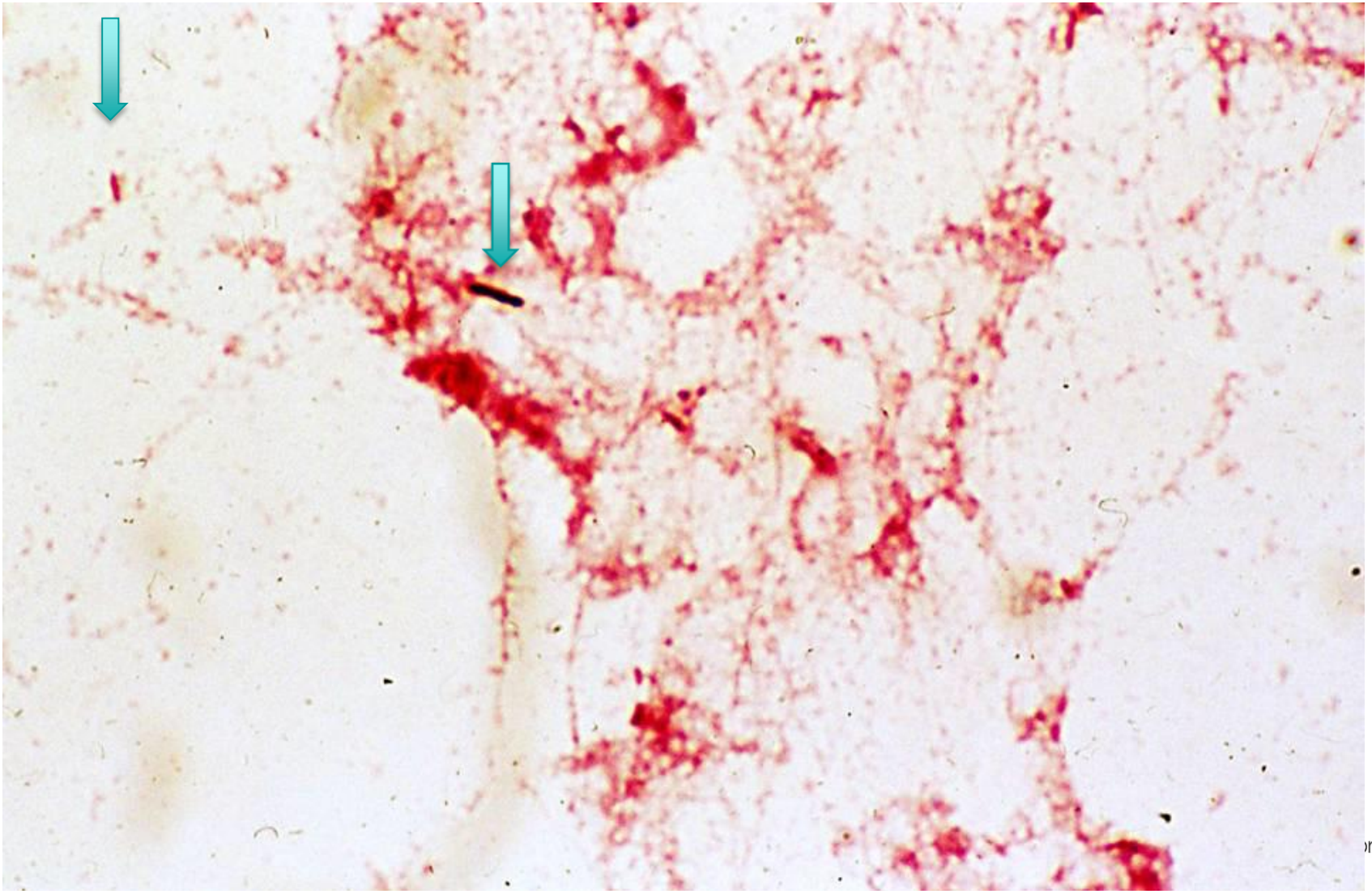
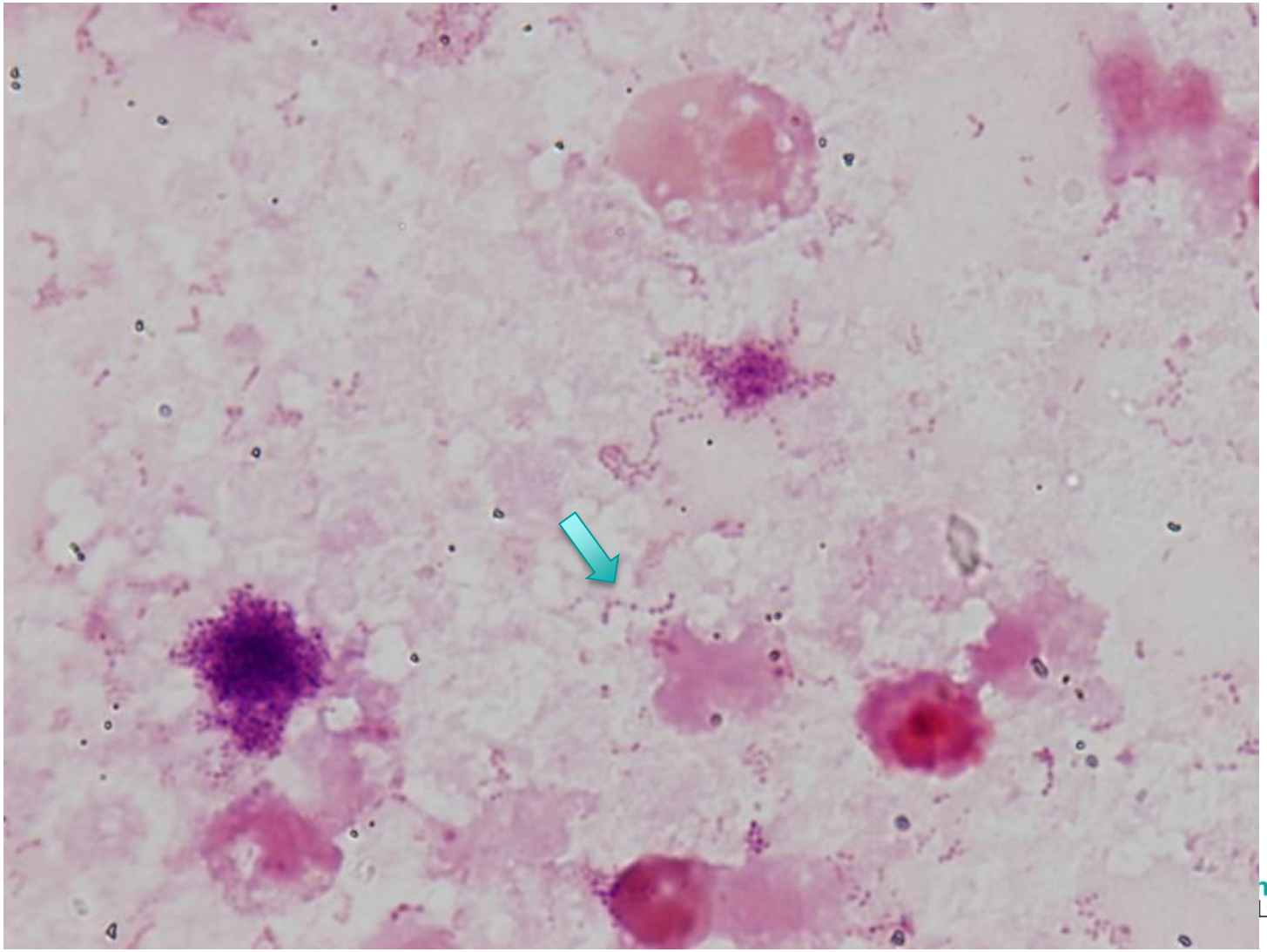
Staphylococcal infection



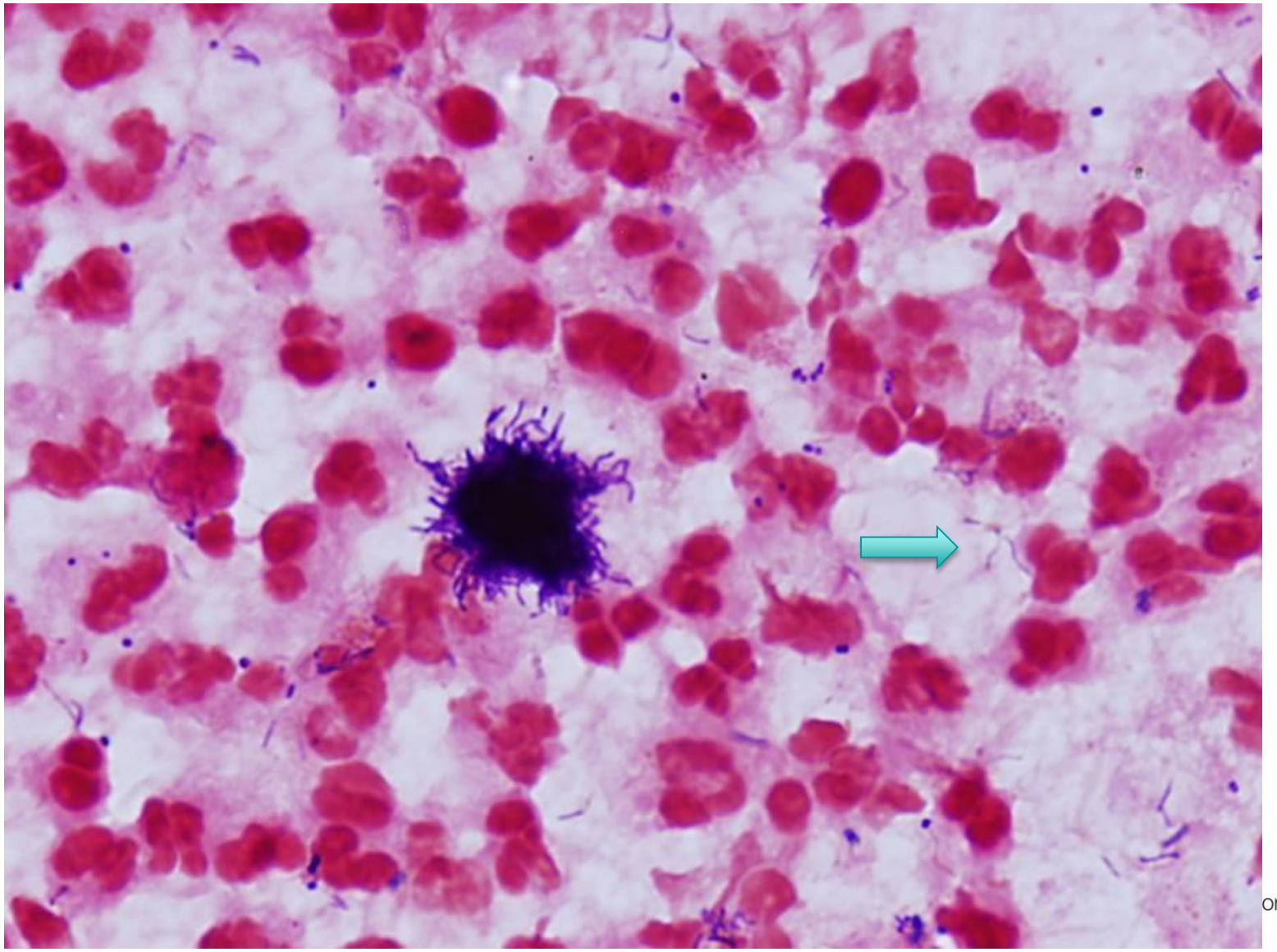
The rods look more Gram positive here but still some GNRs



Intraabdominal abscess aspirate – Long chains and clumps of pale-uneven staining small cocci – suggests *Streptococcus anginosus* (“S. milleri”) group



Tissue from infected IUD Branching GPR's, in clump (sulfur granule), suggests *Actinomyces*



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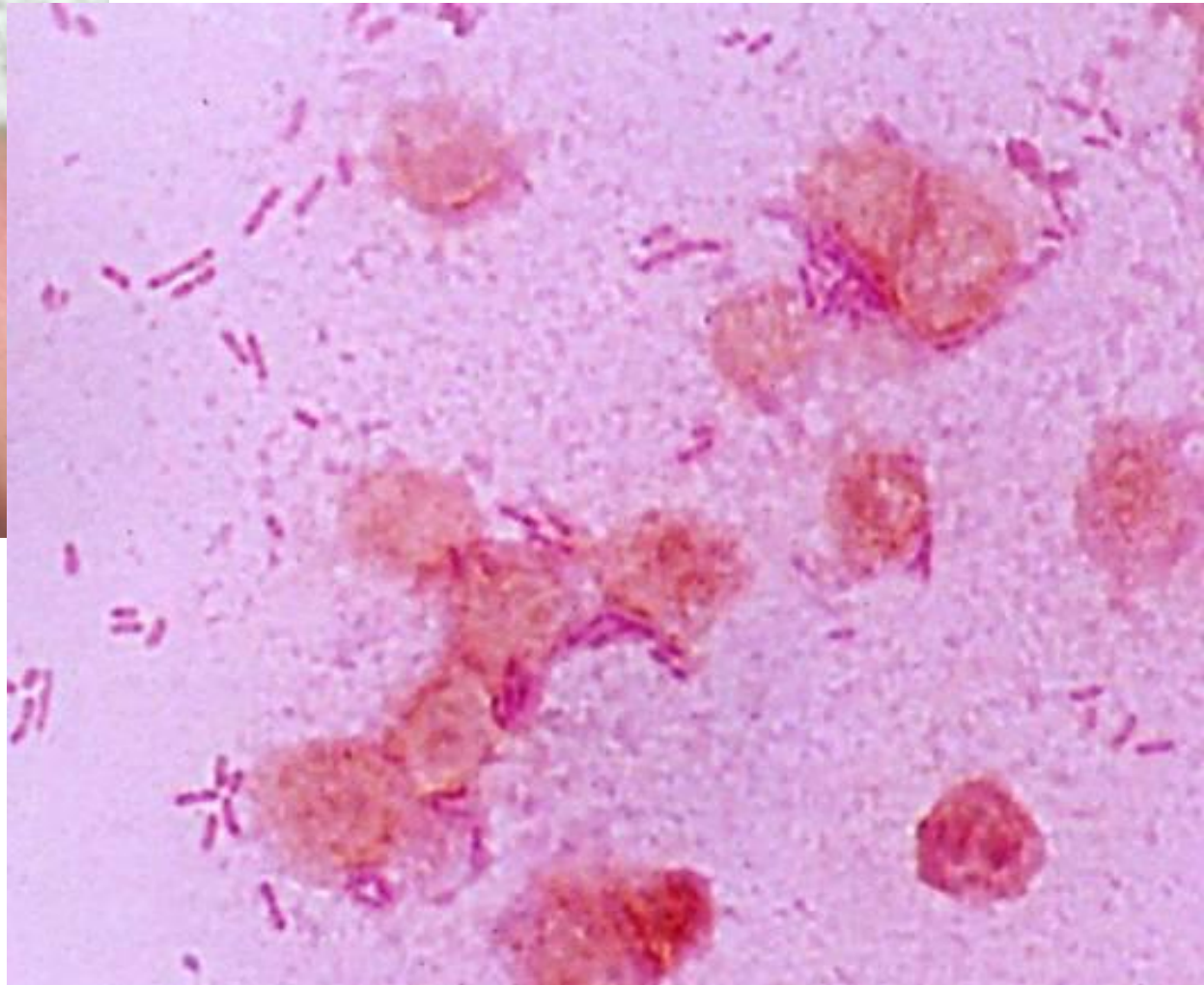
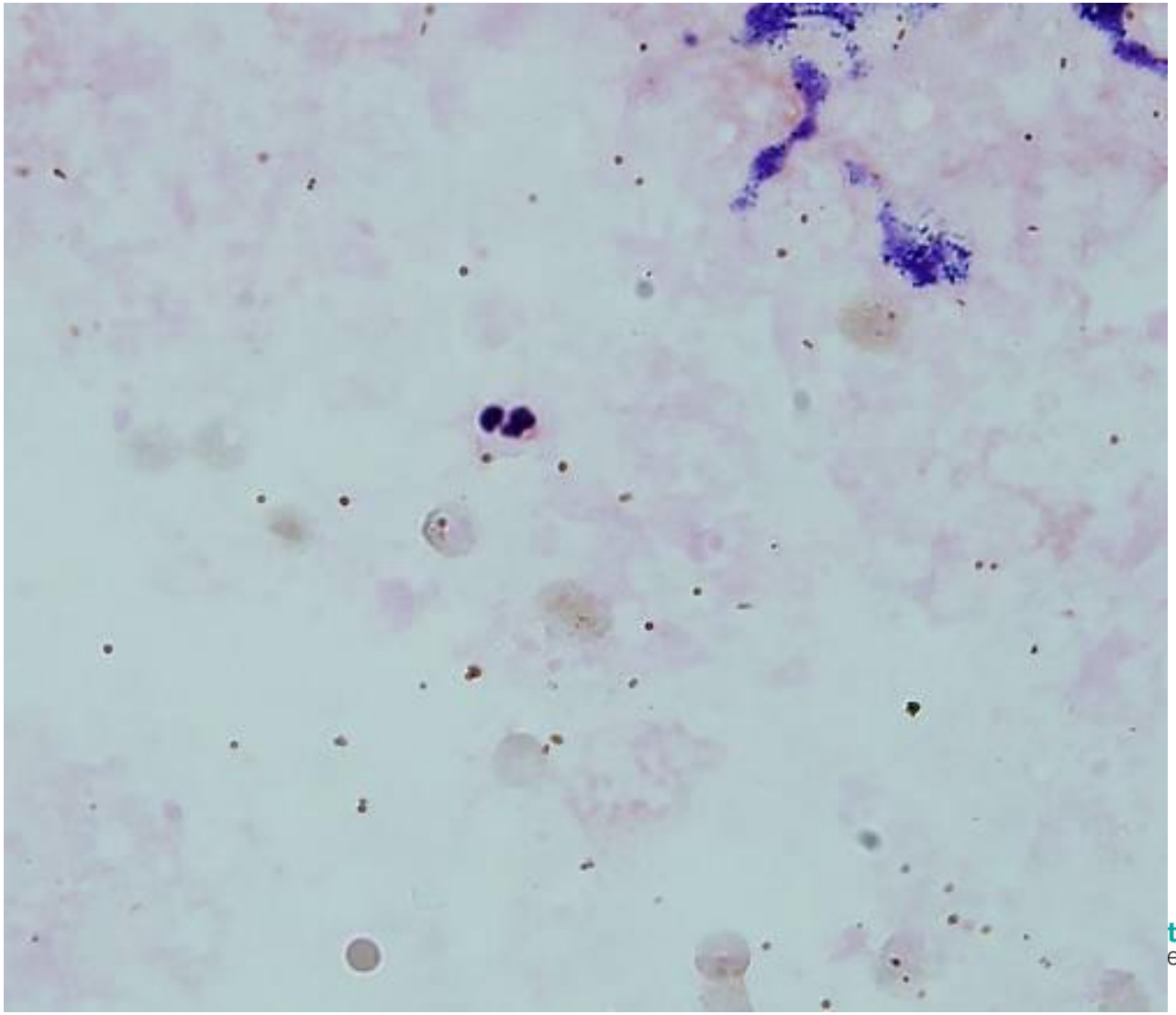
Bipolar staining GNRs

Cat bite wound



Tissue from nodule on a wrist – spherule of *Coccidioides immitis*

Aspiration from vitreous fluid (eye) – Pigment granules, not bacteria



BUGS IN BLOOD CULTURES

https://www.google.com/search?q=bactec+bottles&safe=active&rlz=1C1GGRV

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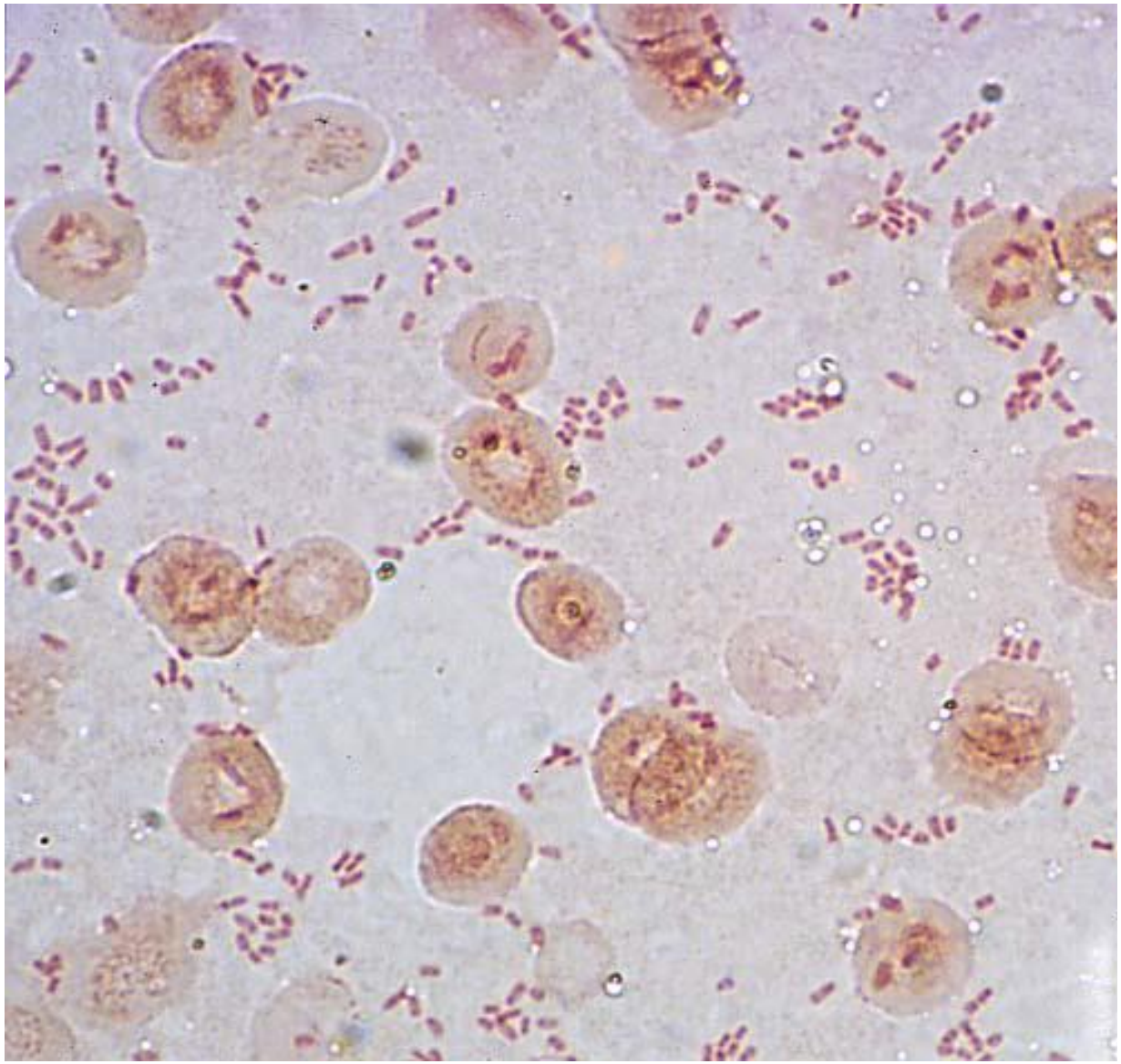
Enteric Gram-negative rod in blood



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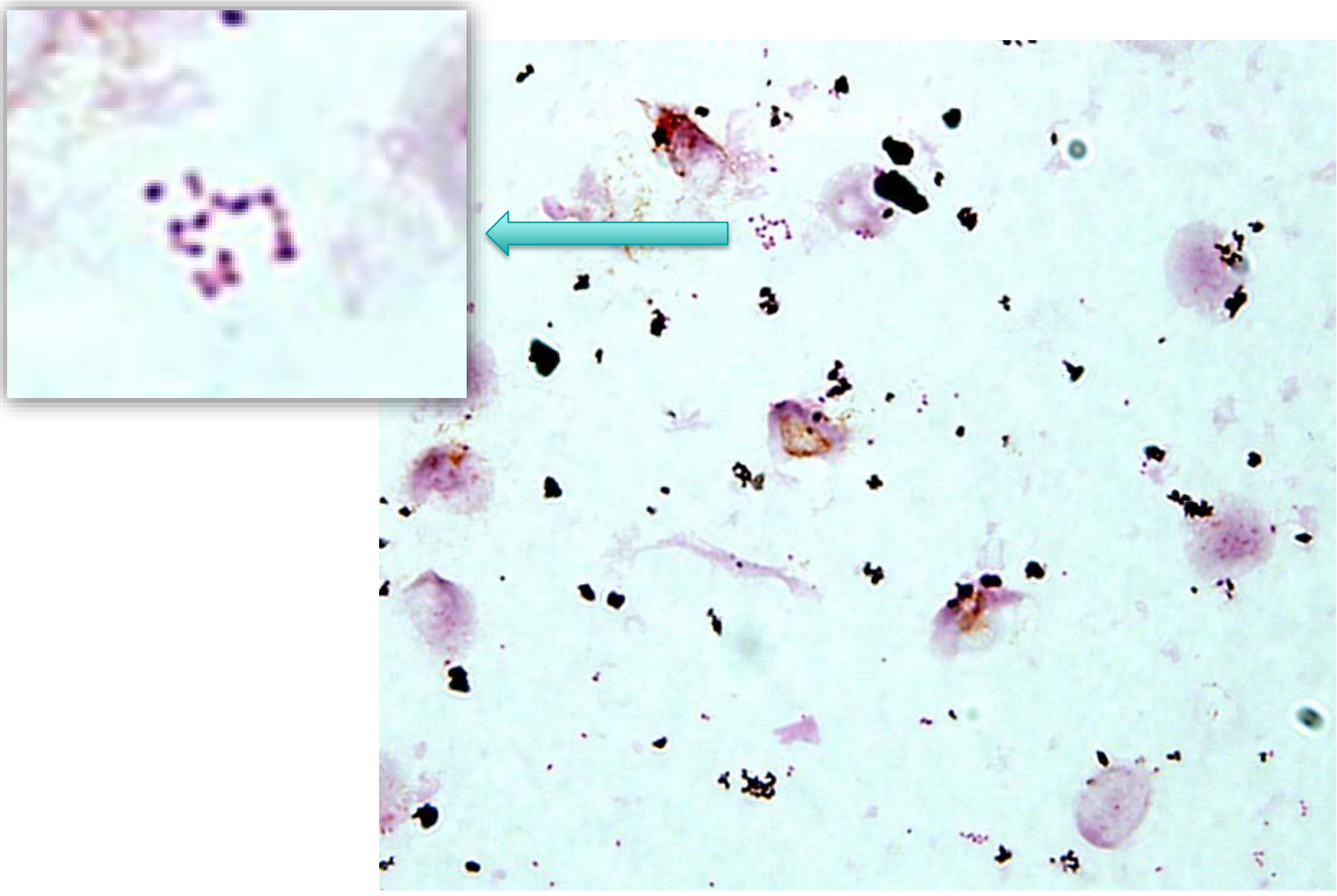
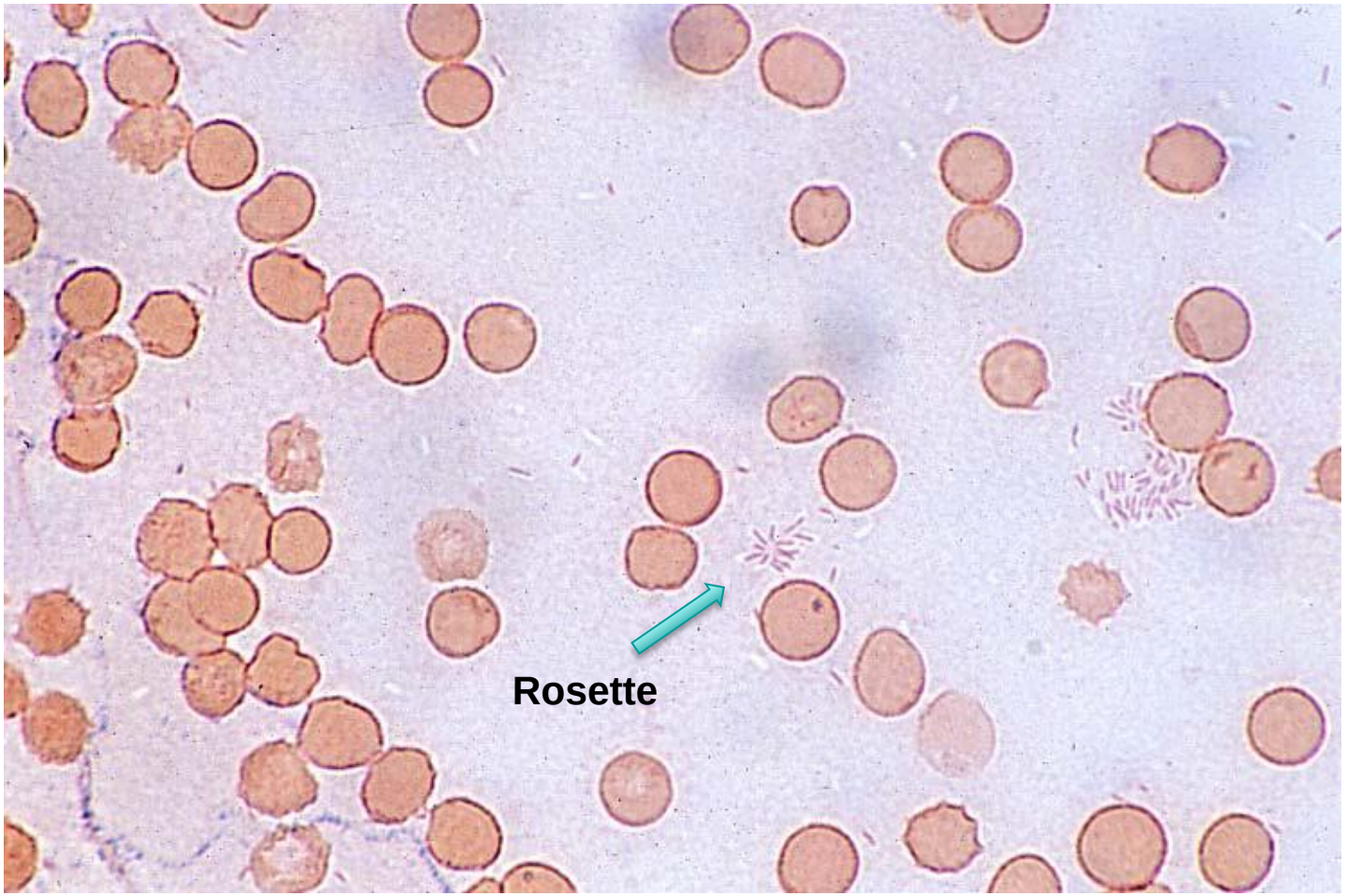
Brucella in blood



Cardiobacterium hominis in blood



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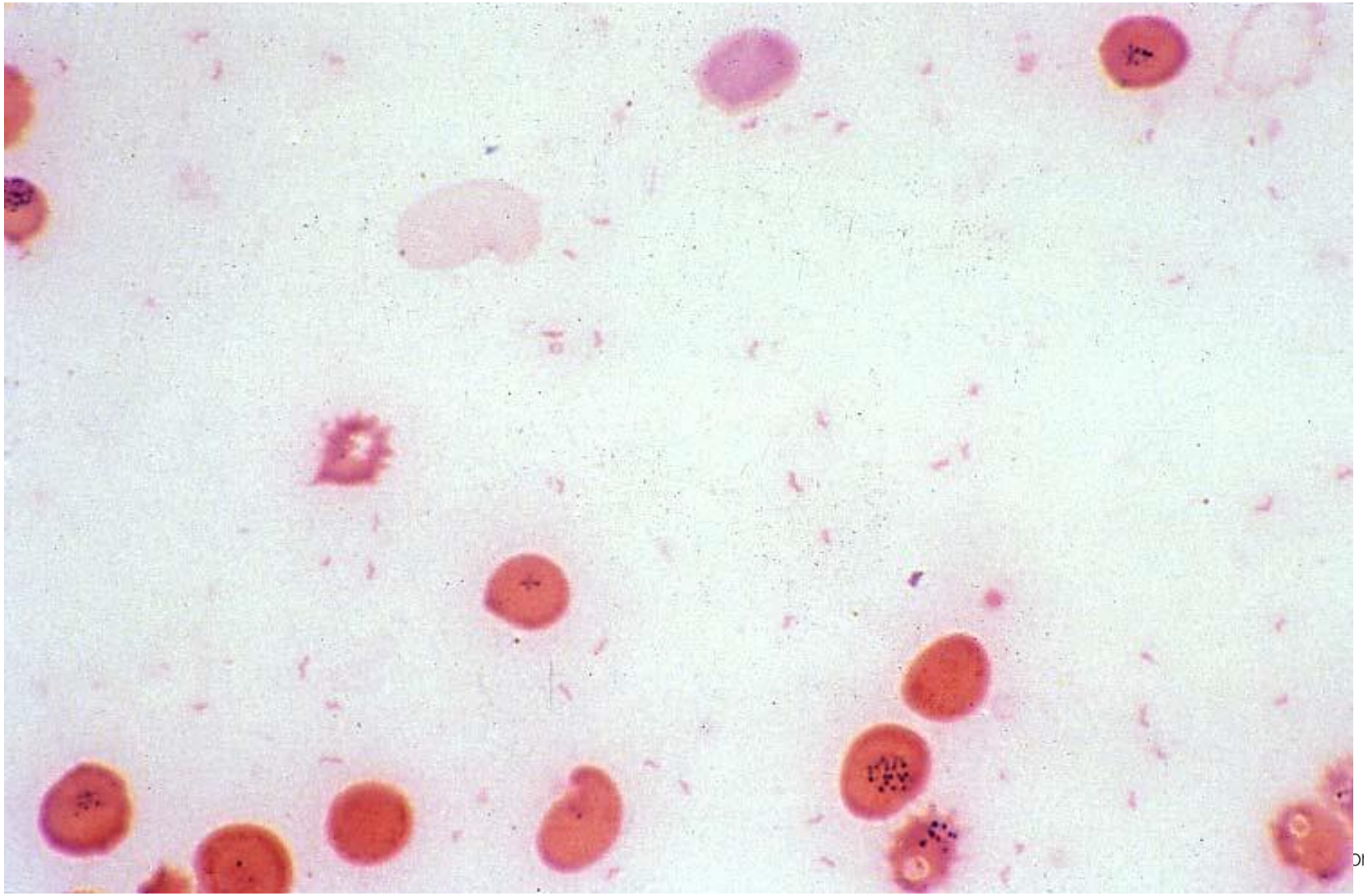
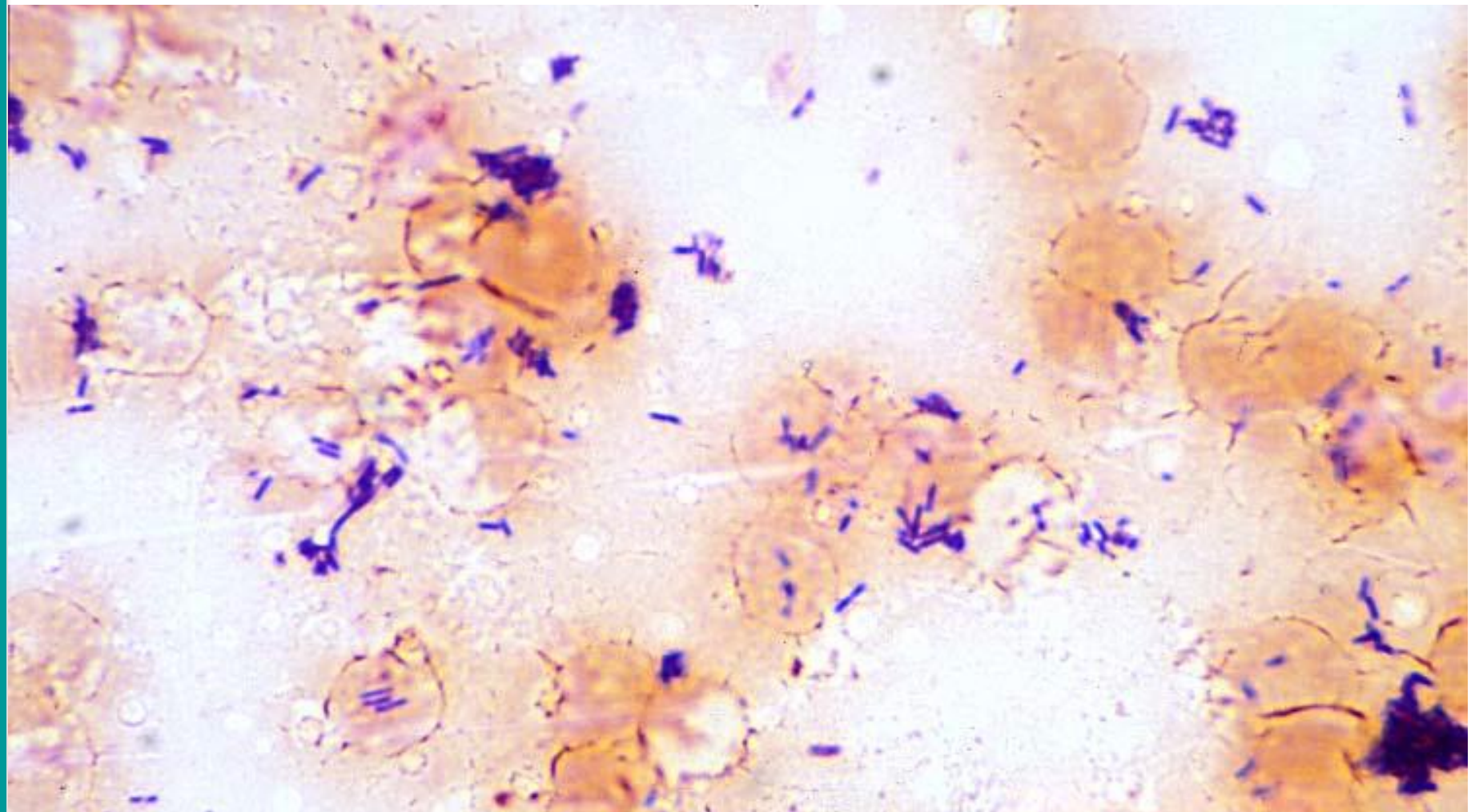
Campylobacter in blood



Listeria in blood culture



Streptococcus
(partially treated) blood culture

Genital Specimens

- **Discharge:**
  - **Use a swab to collect from vaginal fornix or use self collected swab**
  - **Roll the swab on the slide**
  - **Touch slide directly to discharge from penis if possible, then roll with slide if too thick**
  - **Slides made at the bedside are best**
    - **Provide the physician with a sterile slide and a slide box**
- **Lesions:**
  - **Blot with gauze wet with sterile saline**
  - **Firmly press slide onto lesion**

Normal	Bacterial Vaginosis (BV)	Comments
Many squamous epithelial cells Many squamous epithelial cells	Many squamous epithelial cells Many squamous epithelial cells	If rare squamous epithelial cells seen on slide, then probably specimen was not well collected. Could report “sparse epithelial cells; sample inadequate for assessment of BV.”
Clean, clear background between squamous epithelial cells	“Dirty”, crowded background with many bacterial cells between squamous epithelial cells	Numbers of bacteria in vaginal secretions may increase 3-4 logs during BV
Edges of squamous epithelial cells sharp and clearly demarcated	At least 75% of the edges of some epithelial cells totally obscured by adherent Gram variable coccobacillary bacteria (looks shaggy). At least 20% of cells should show adherent bacteria.	*Gardnerella vaginalis* and anaerobic bacteria adhere to edges of epithelial cells. This type of cell is called a “clue cell” in a wet preparation.
Presence of few or many relatively long, parallel-sided rods, Gram positive or Gram variable, suggestive of Lactobacilli.	Virtual absence of the regular shaped rods suggestive of lactobacilli.	Absence of most types of Lactobacilli is a hallmark of BV. Unclear whether this happens first or is a consequence of BV.
Besides Lactobacilli, other bacterial morphotypes are rare, especially curved rods.	Numerous morphotypes present including Gram variable short coccobacilli, small Gram positive cocci, and occasionally curved rods of two types: short, comma shaped and long, fusiform.	The mixed morphologies represent anaerobic bacteria and Gardnerella vaginalis. The curved rods represent *Mobiluncus* species, which are rarely seen in women without BV.
Other structures, PMNs, yeast, sperm, etc. may be present but are not contributory to diagnosis of BV. They should be reported.	Other structures, PMNs, yeast, sperm, etc. may be present but are not contributory to diagnosis of BV. Their presence should be reported.	*Trichomonas* cannot be recognized in a Gram stain.

Gram Stain-WBC

- look for and report WBC
  - Do NOT use PMN
- RBC report helps the clinicians
- Organisms
  - First exam the slide by low-power for “good” areas
  - Never report one single bug!
  - Never report yeast if not-budding
  - Carefully exam the pink background for GNR’s

GS: Bacterial Vaginosis

- A quick and accurate alternative to culture
- Uses Nugent scoring system
- Make sure to report other elements
  - Yeast
  - WBC
  - Clue Cells

GS: Bacterial Vaginosis

Organism Morphotype	Number/oil immersion field	Score
Lactobacillus - like (parallel sided, gram positive rods)	>30	0
	5-30	1
	1-4	2
	<1	3
	0	4
Mobiluncus - like (curved, gram negative rods)	>5	2
	<1-4	1
	0	0
Gardnerella/bacteroides - like (tiny, gram variable coccobacilli and pleomorphic rods with vacuoles)	>30	4
	5-30	3
	1-4	2
	<1	1
	0	0

[Table/Fig-2]: Nugent scoring of Gram stained smear for bacterial vaginosis.
Total score:- 0-3 Normal; 4-6 Intermediate, repeat test later; 7-10 Bacterial vaginosis.

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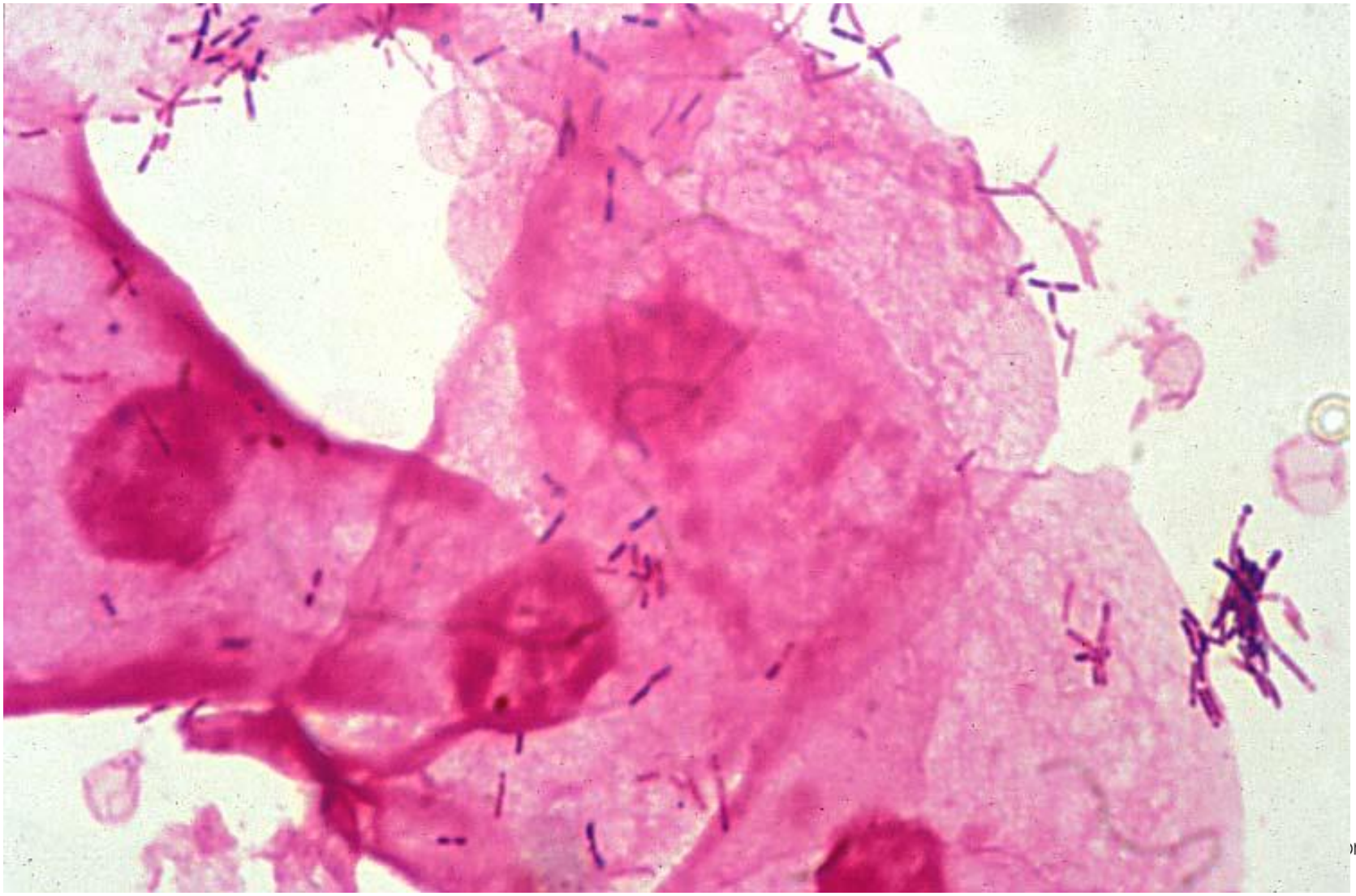
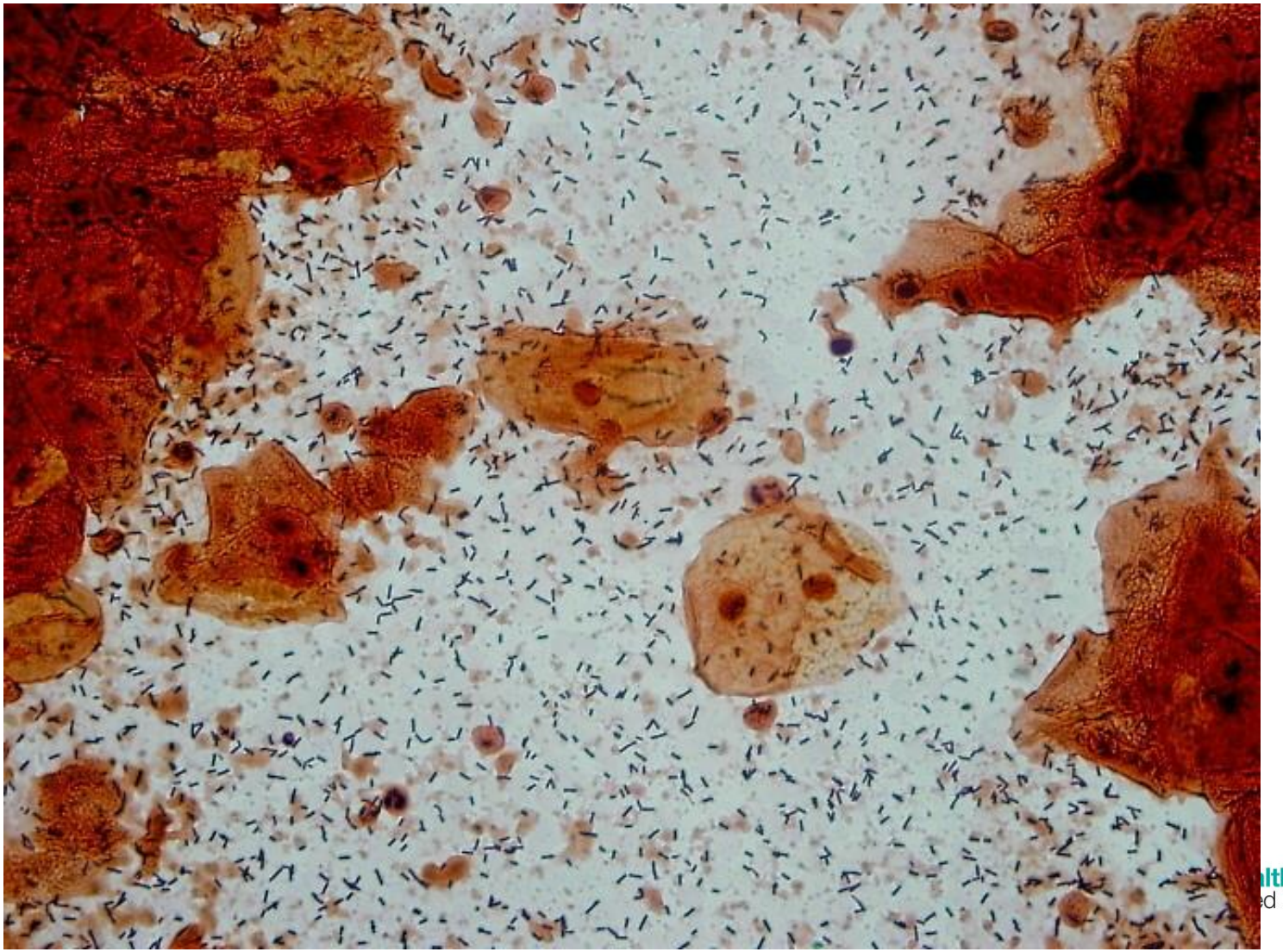
http://www.njlm.net/articles/PDF/2095/10-%2017330_F(GH)_PF1(Vsu_Om)_PFA(Om)_PF2(PVSU).pdf

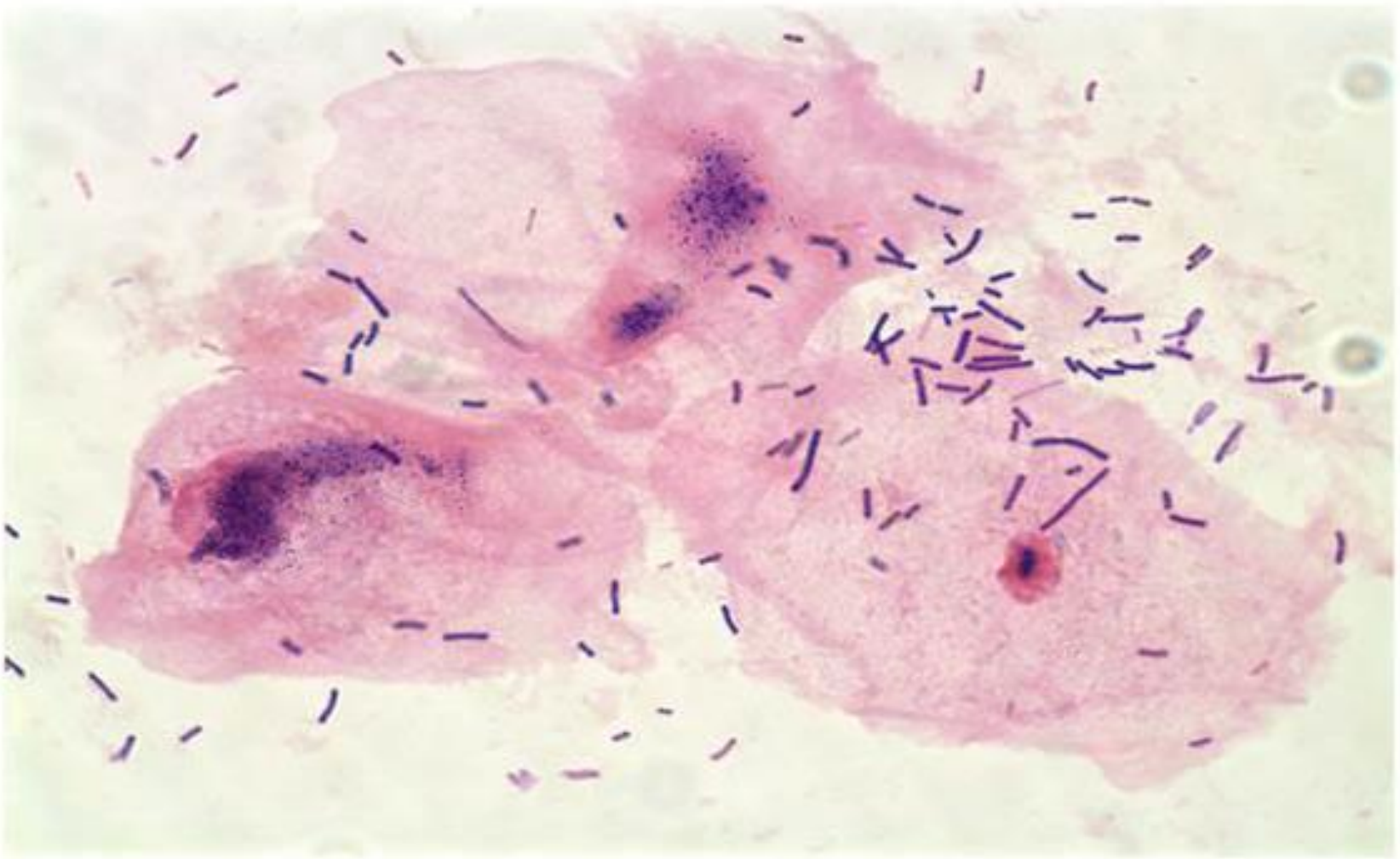
Normal vaginal secretions



Normal vaginal secretions

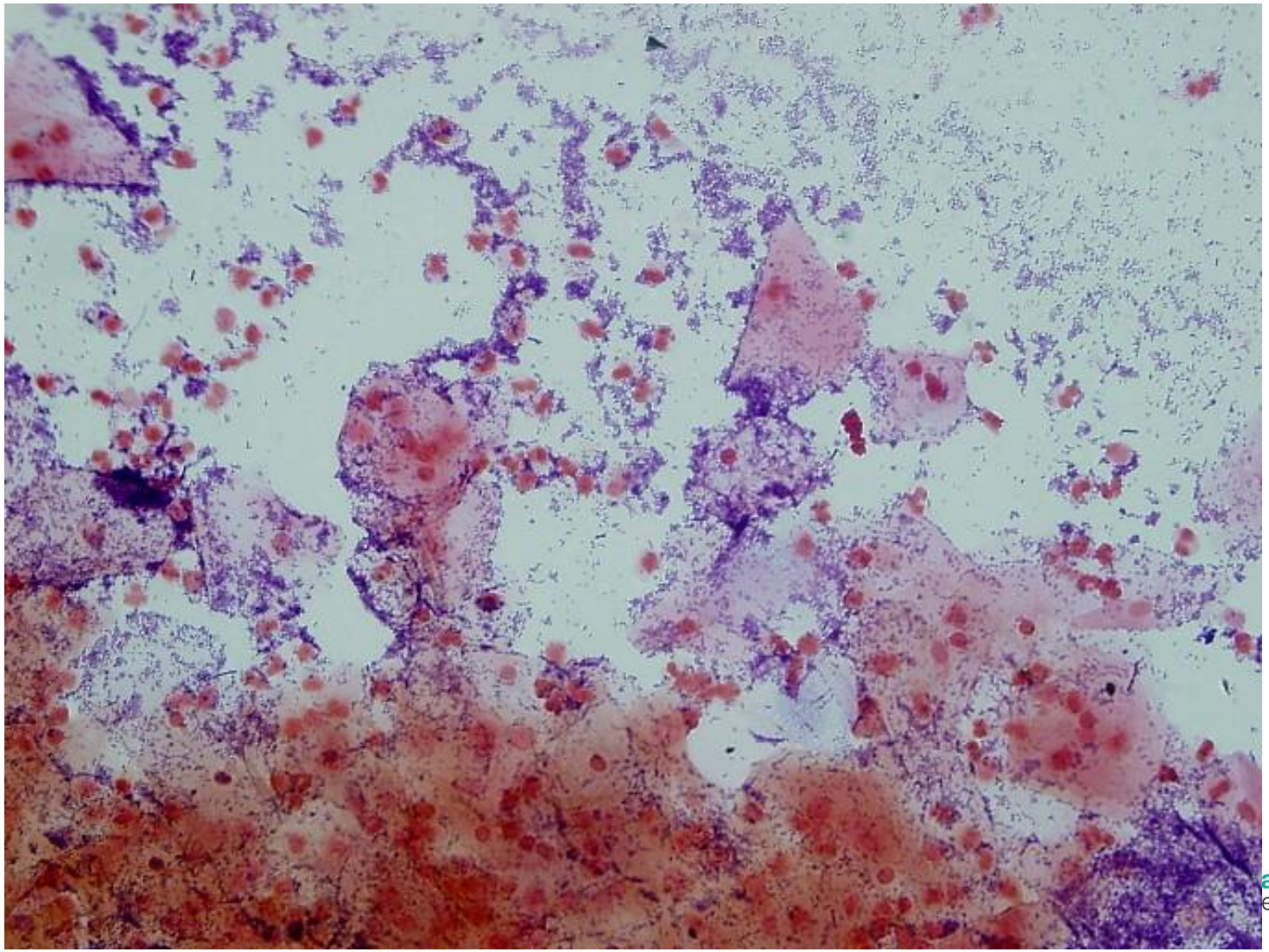


GS: Normal Vaginal Epithelial Cells

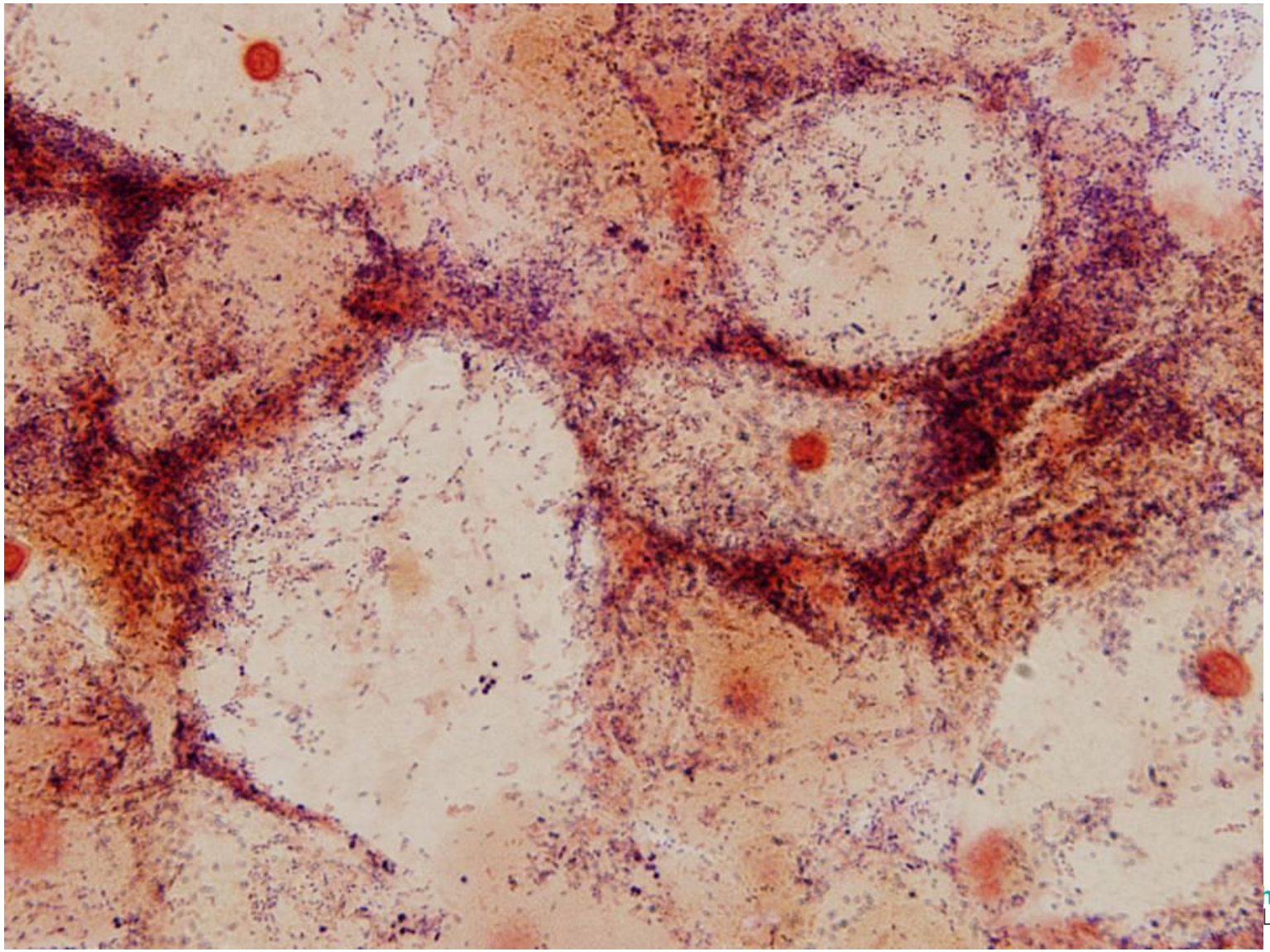


http://www.njlm.net/articles/PDF/2095/10-%2017330_F(GH)_PF1(Vsu_Om)_PFA(Om)_PF2(PVSU).pdf

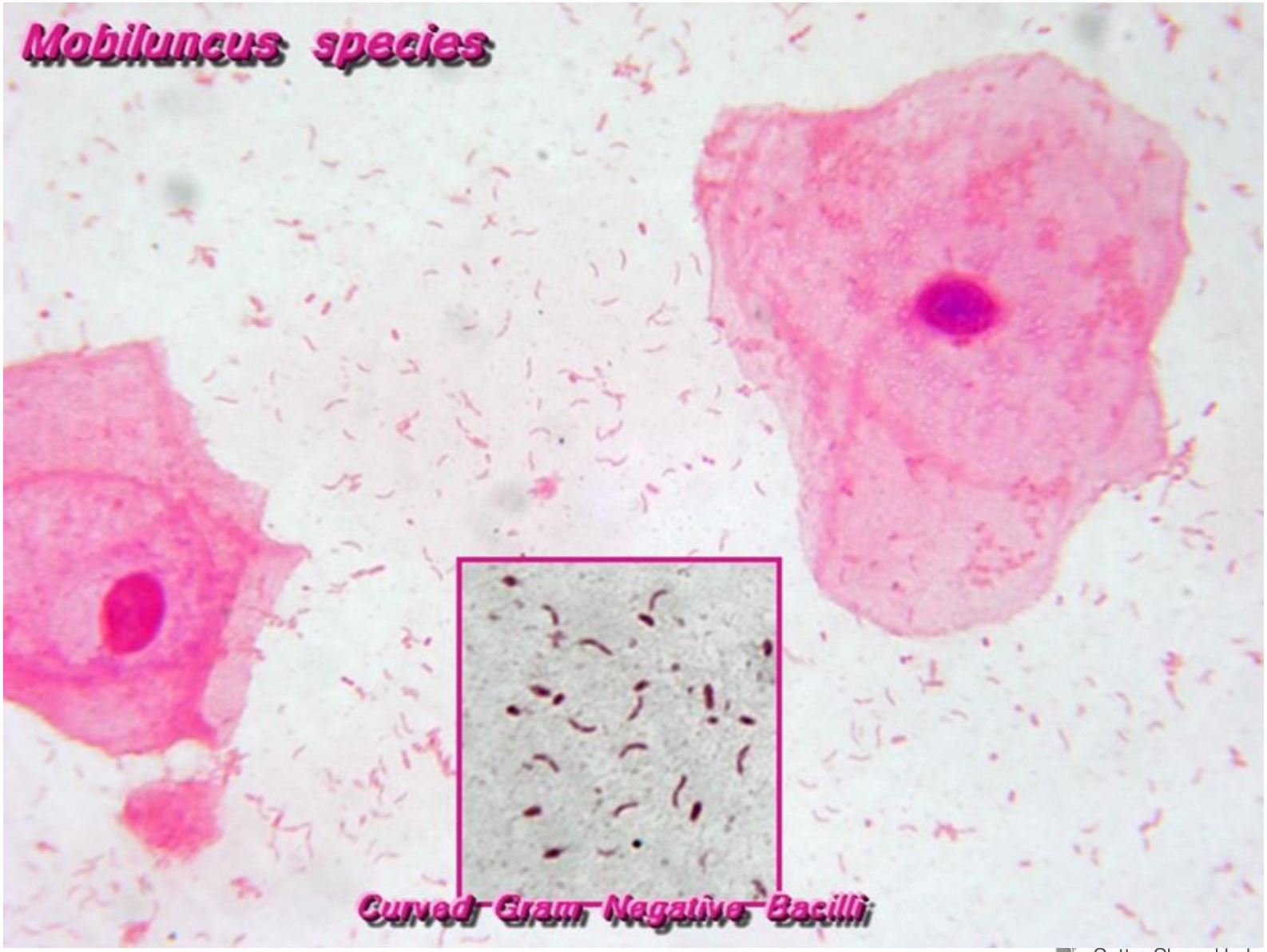
BV: predominant GPC-looking



BV with predominant Gram Variable Coccobacilli

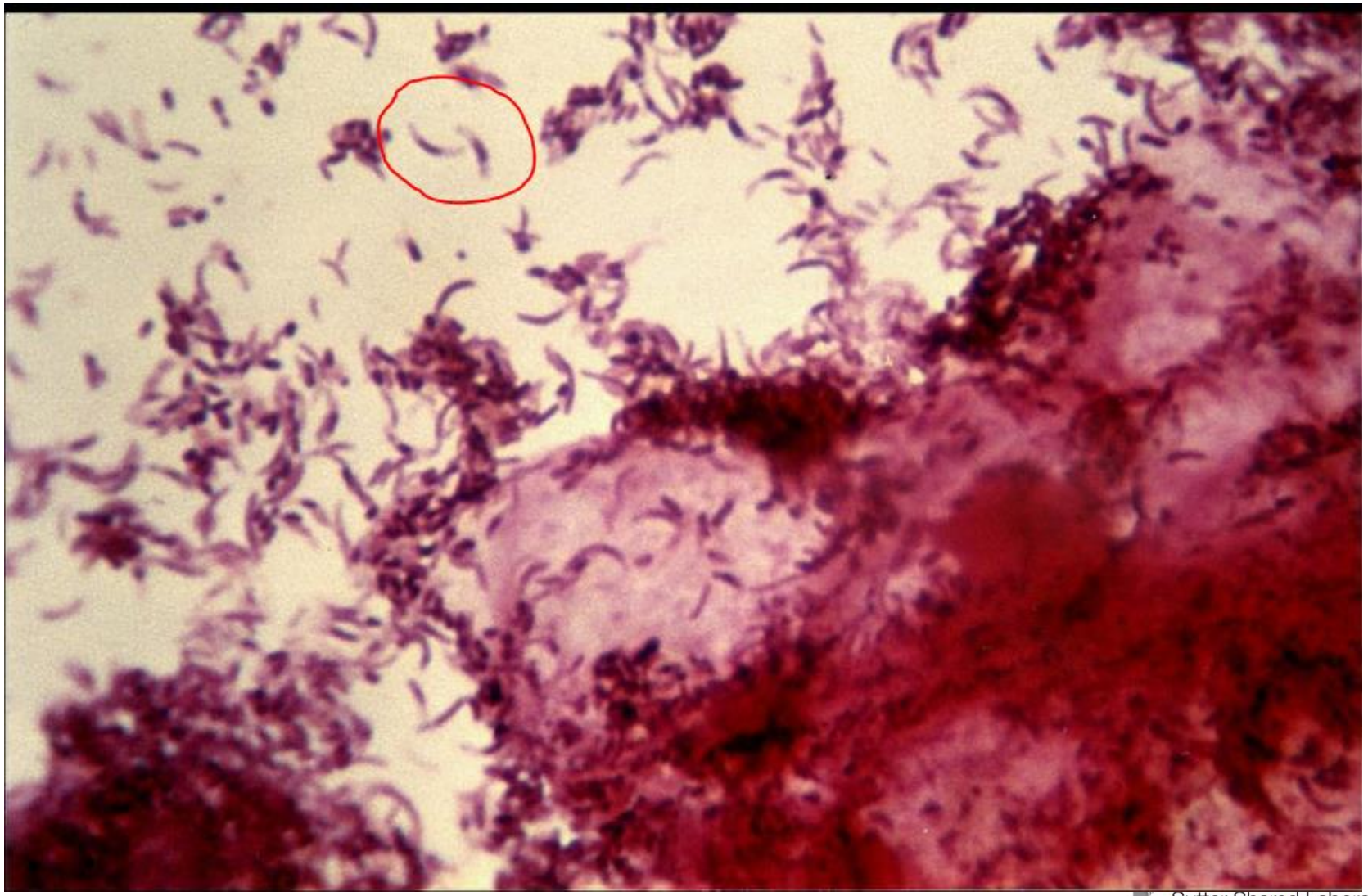


GS: Bacterial Vaginosis- *Mobiluncus*



http://thunderhouse4-yuri.blogspot.com/2010/11/bacterial-vaginosis.html

Bacterial Vaginosis: ***Mobiluncus***



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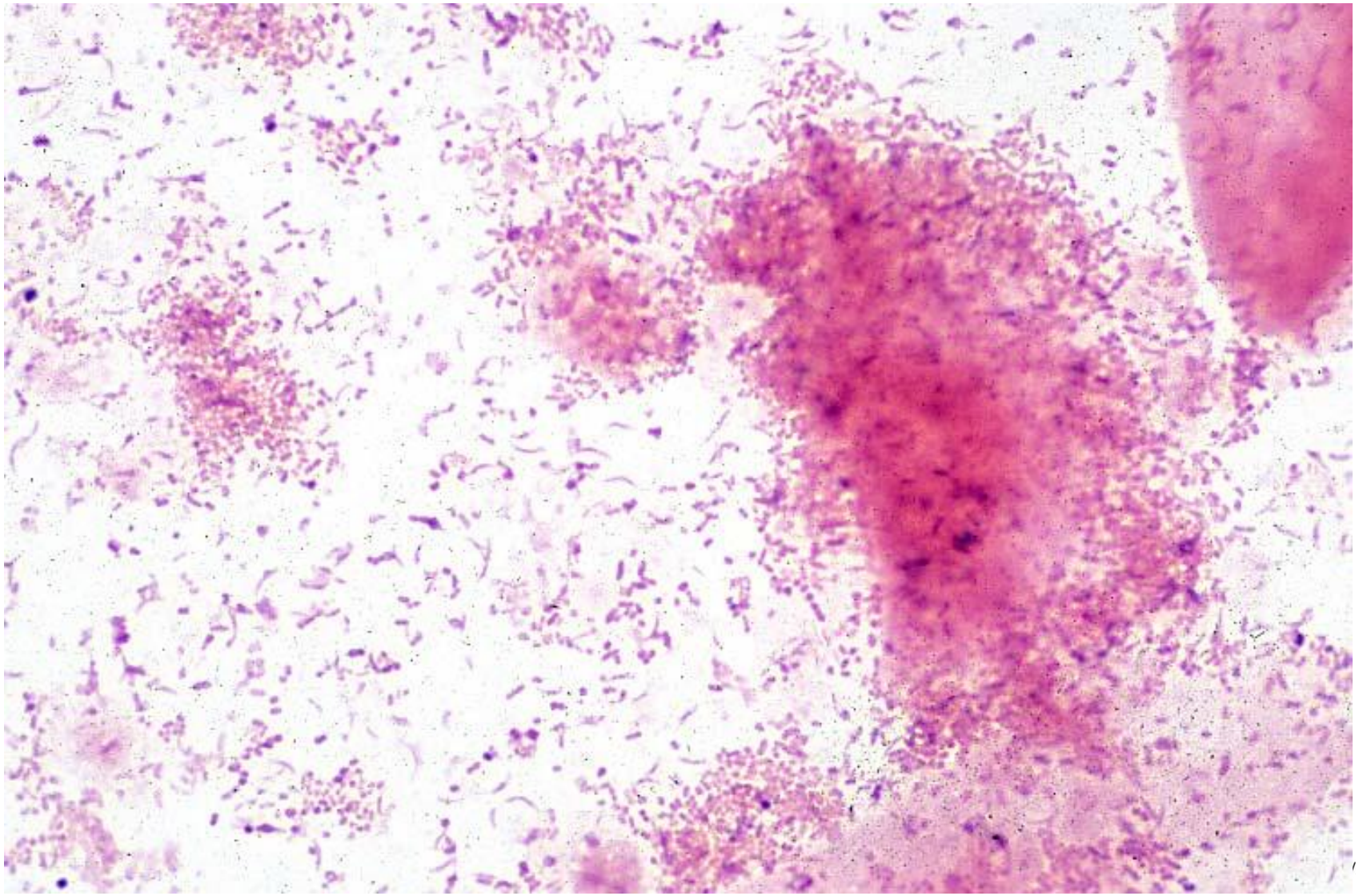
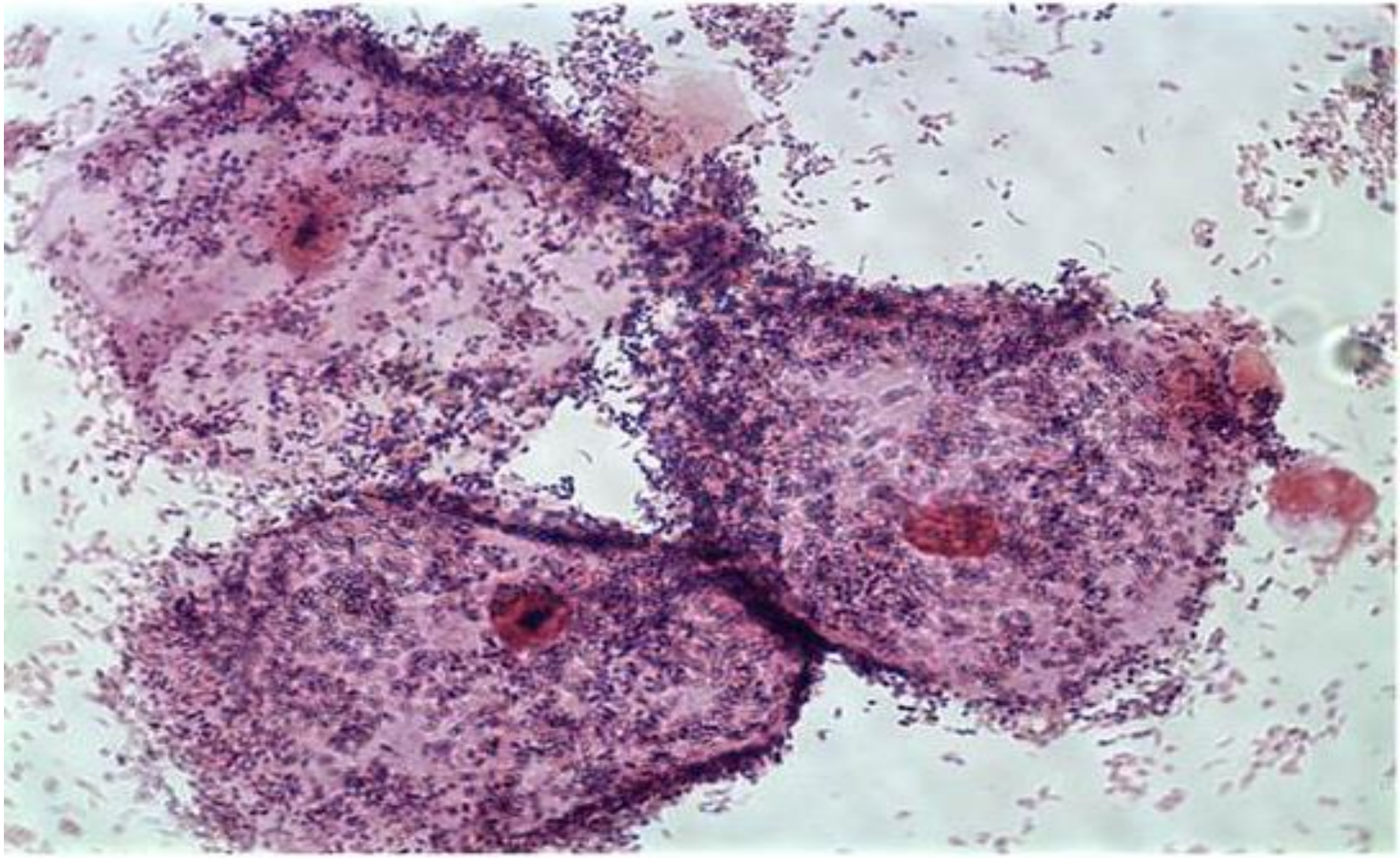
https://www.flickr.com/photos/52859996@N07/4997037695/

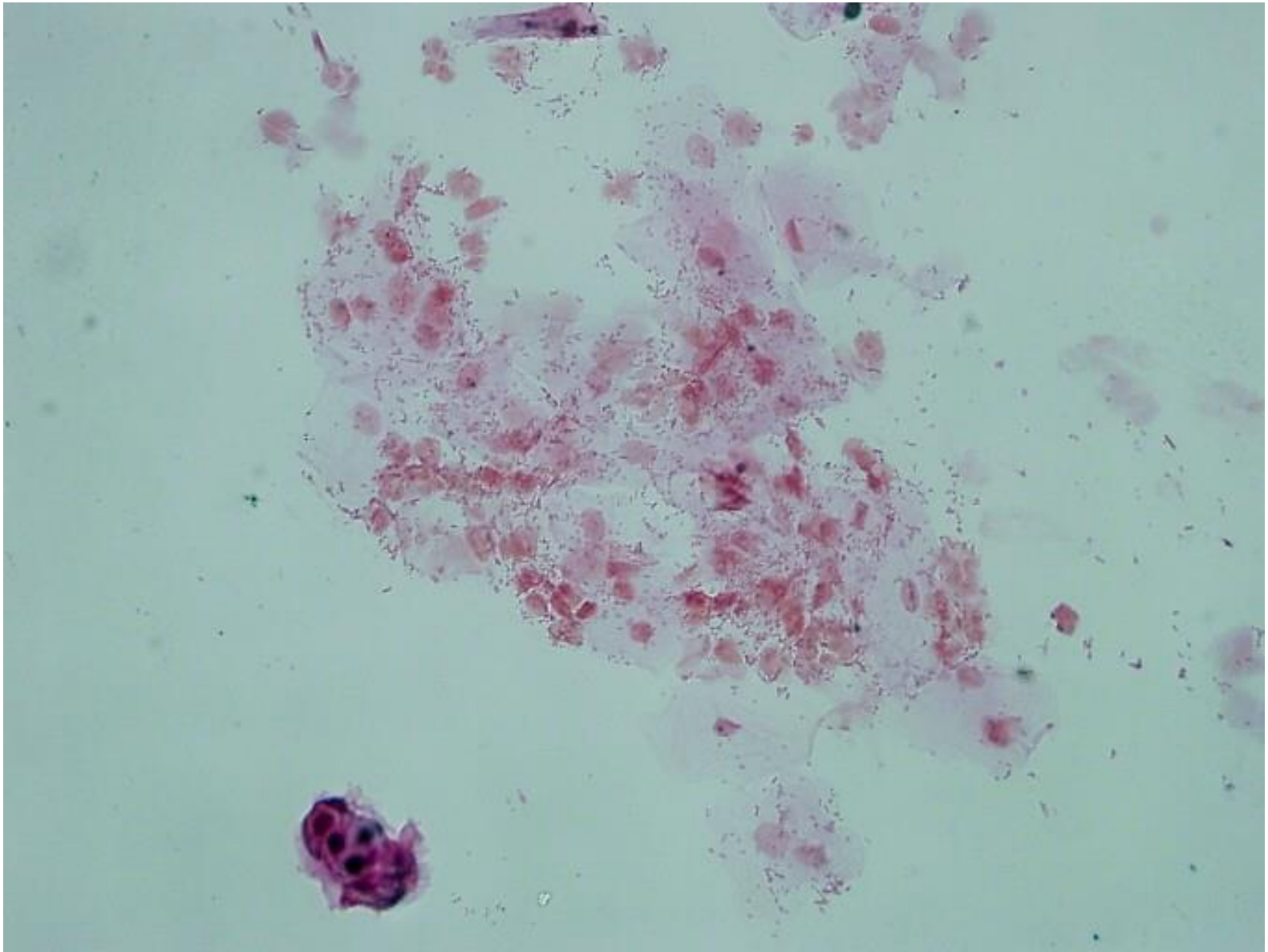
BV with Mobiluncus



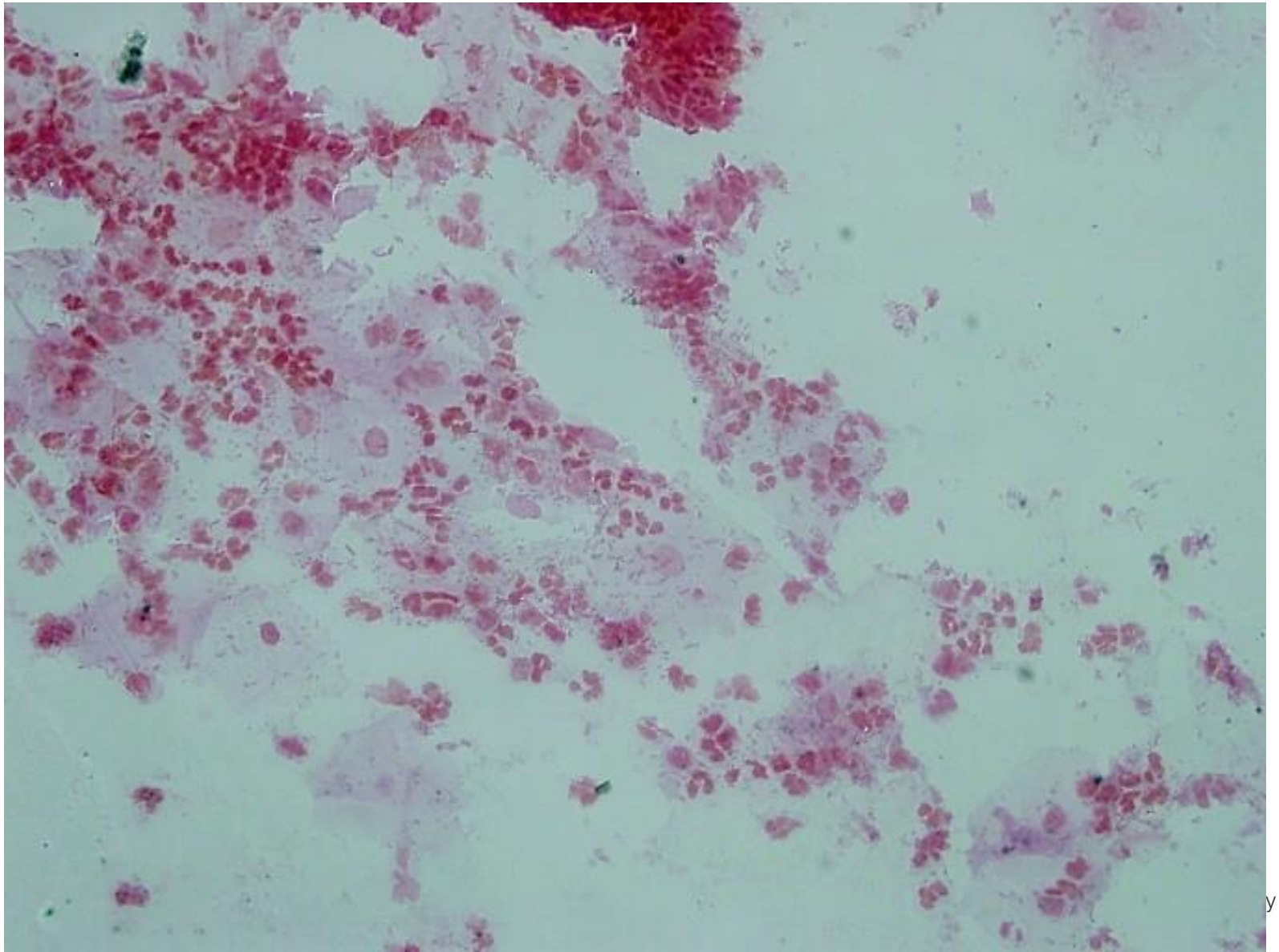
GS: Bacterial Vaginosis



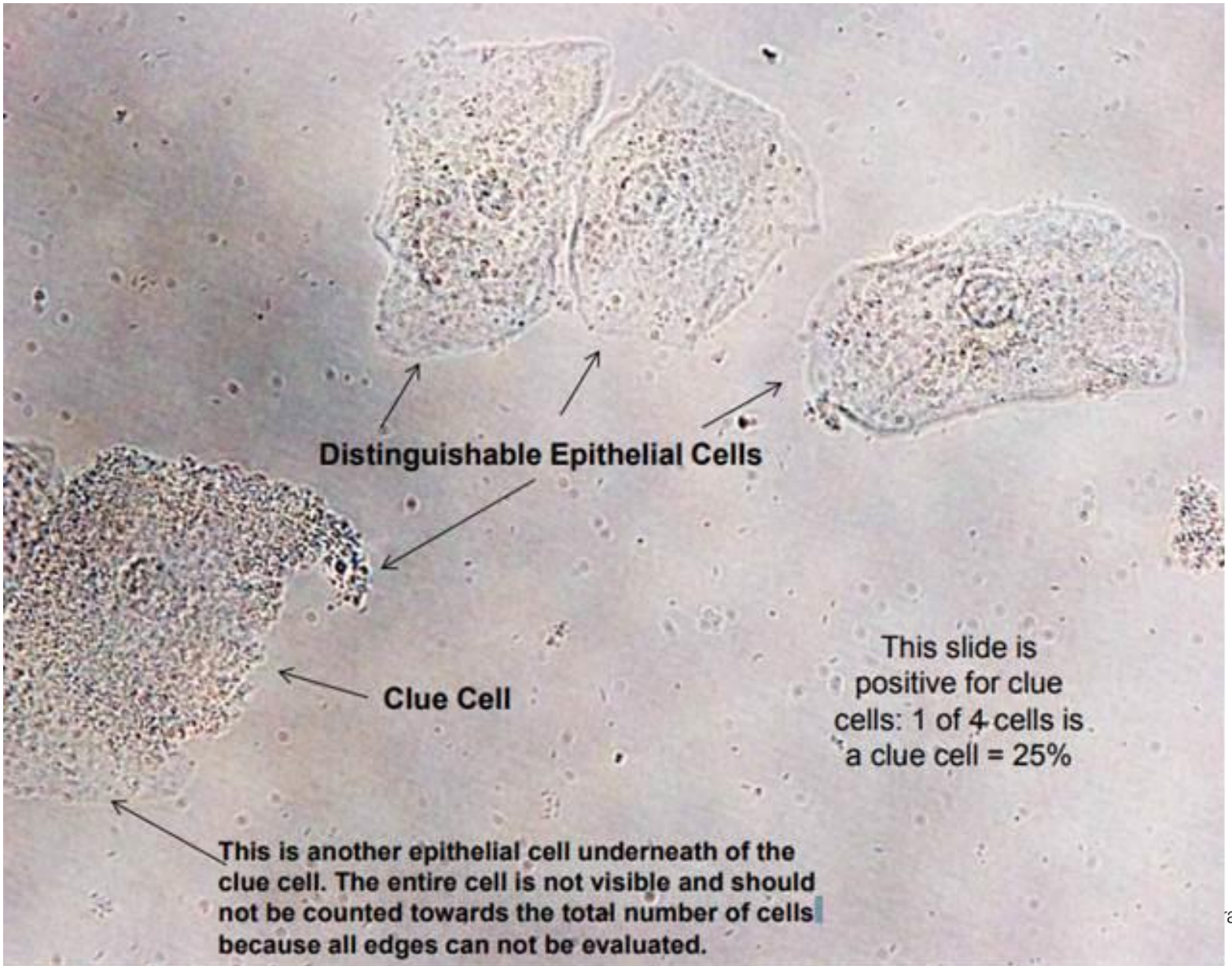
Intermediate score



Vaginal with PMNs



GS: Bacterial Vaginosis



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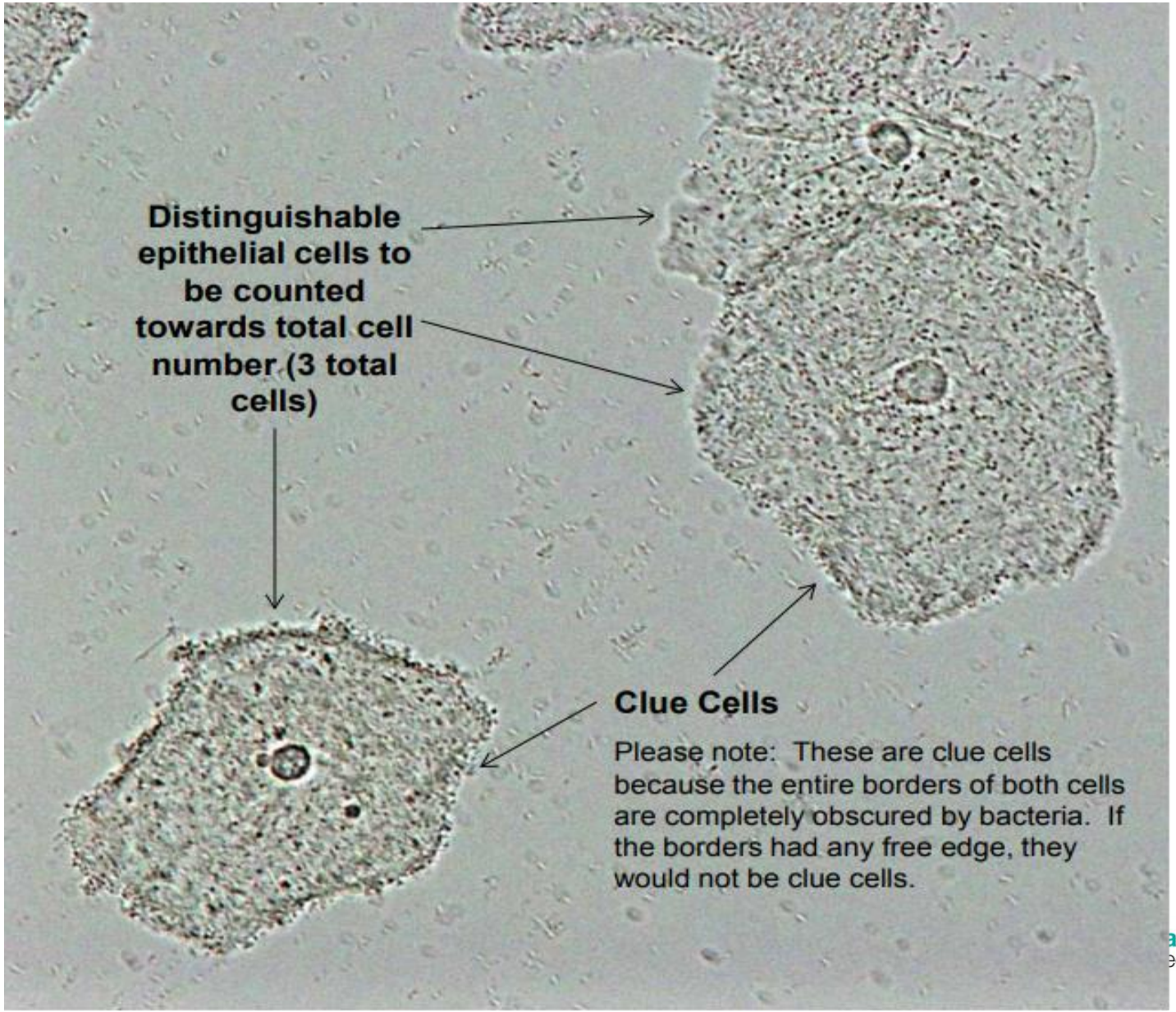
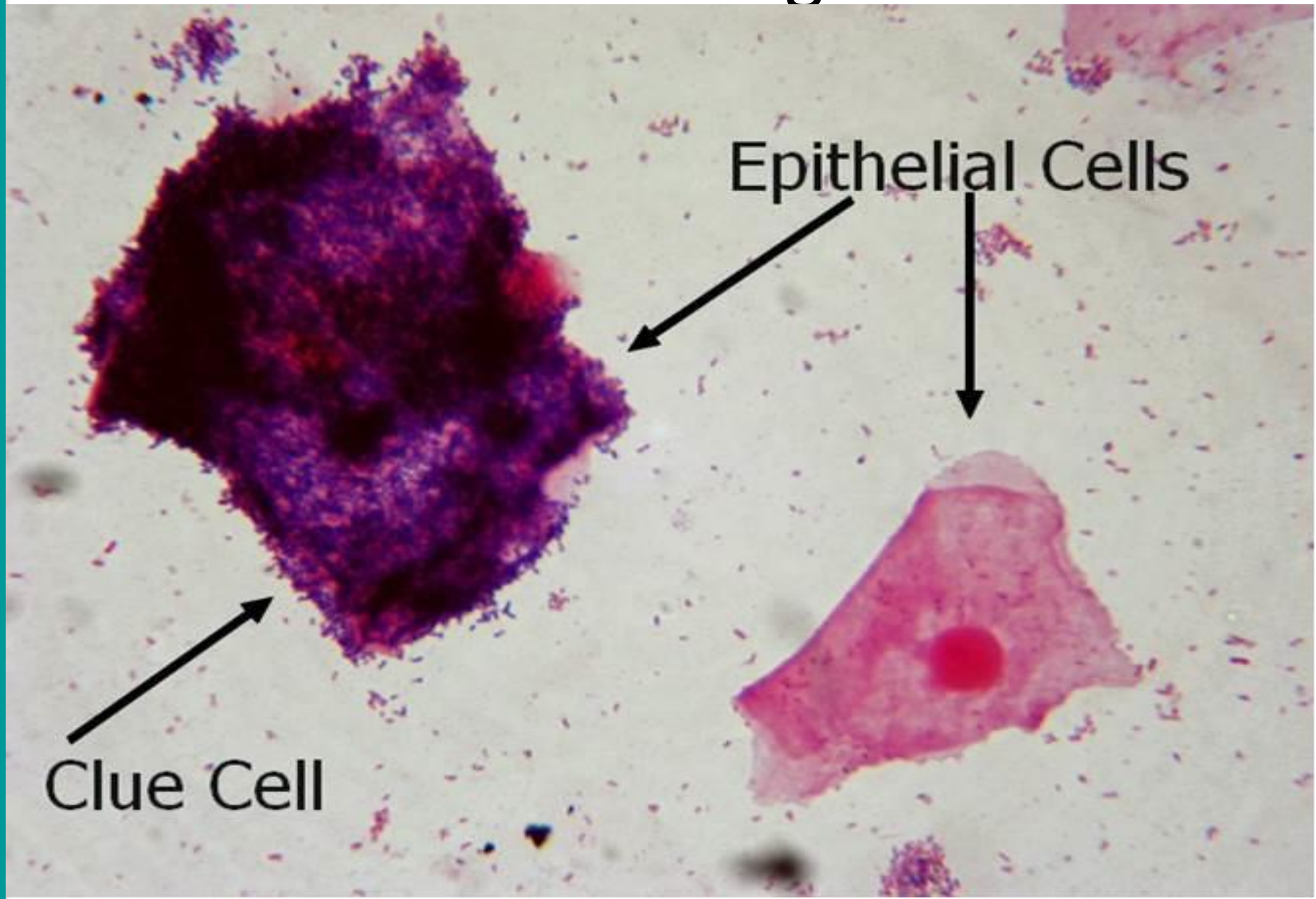
GS: Bacterial Vaginosis



GS: Bacterial Vaginosis

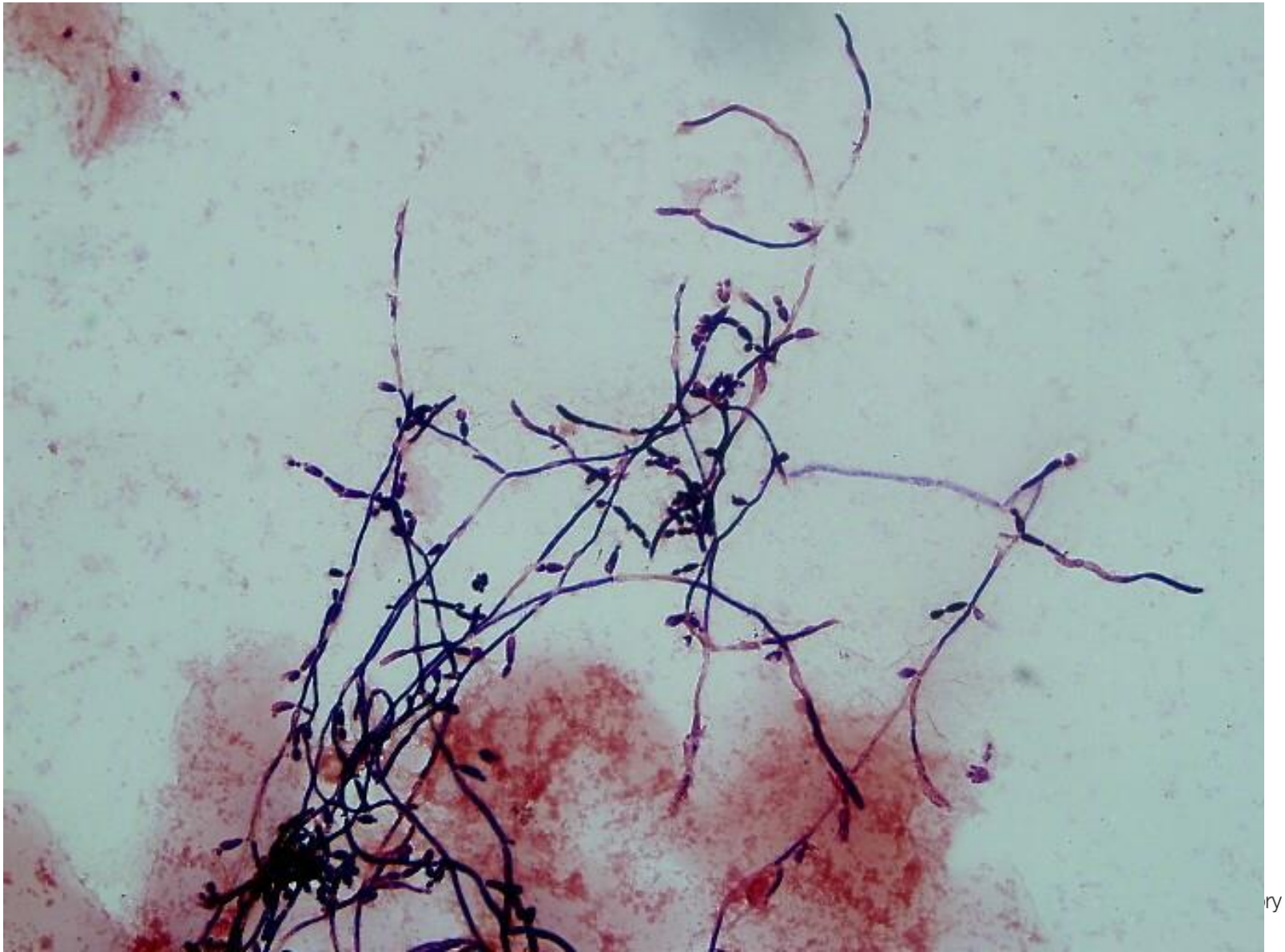


GS: Bacterial Vaginosis Results

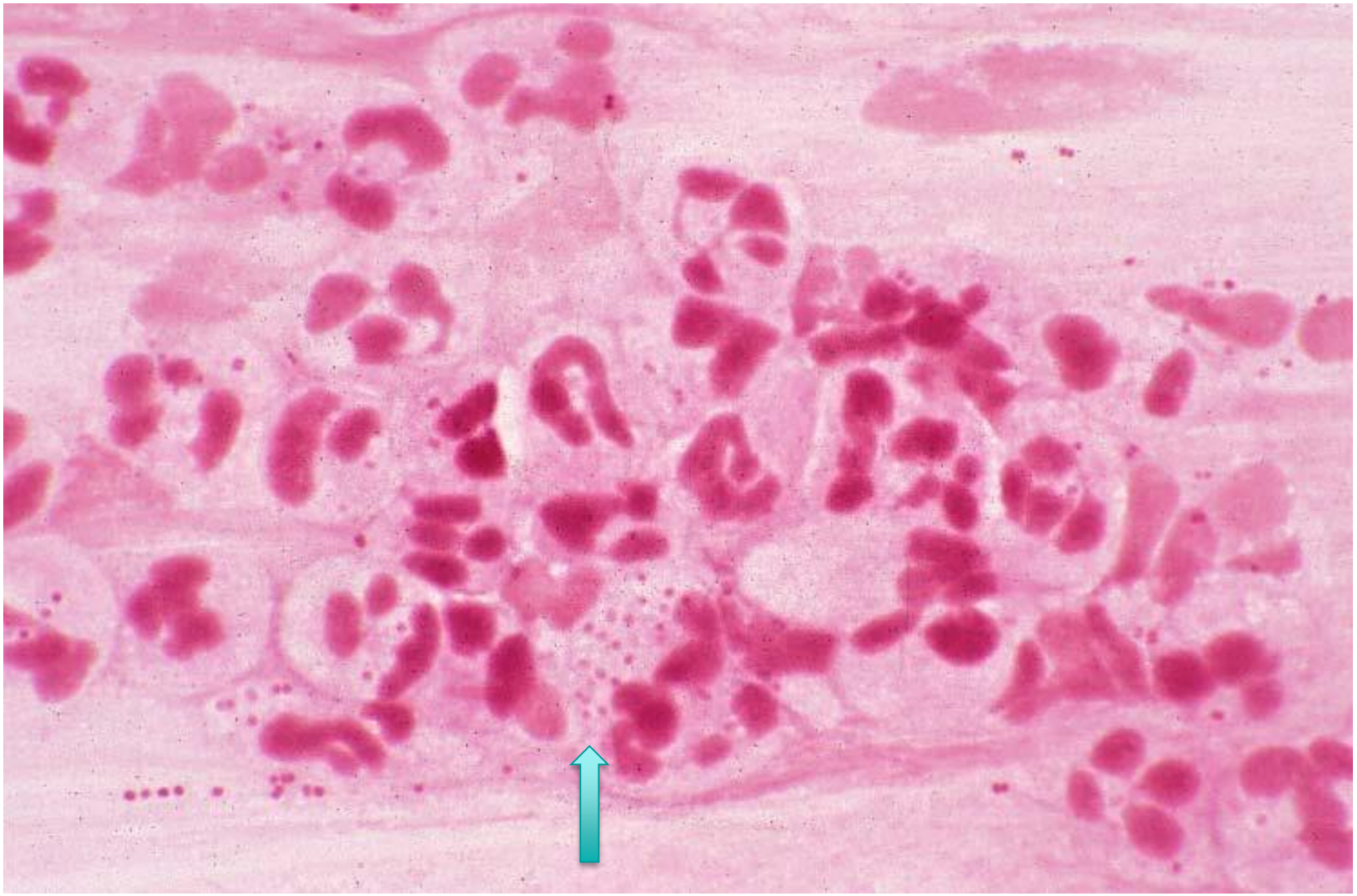
- **Report:**
  - **Cells**
  - **Organisms**
  - **Convert Bacterial Vaginosis score to RESULT:**
    - **(0-3) NBV**: Morphotypes consistent with normal vaginal flora
    - **(4-6) INDBV**: Gram stain results are indeterminate for bacterial vaginosis.
    - **(7-10) BV**: Mixed morphotypes consistent with bacterial vaginosis.

[Do NOT report the scores!]

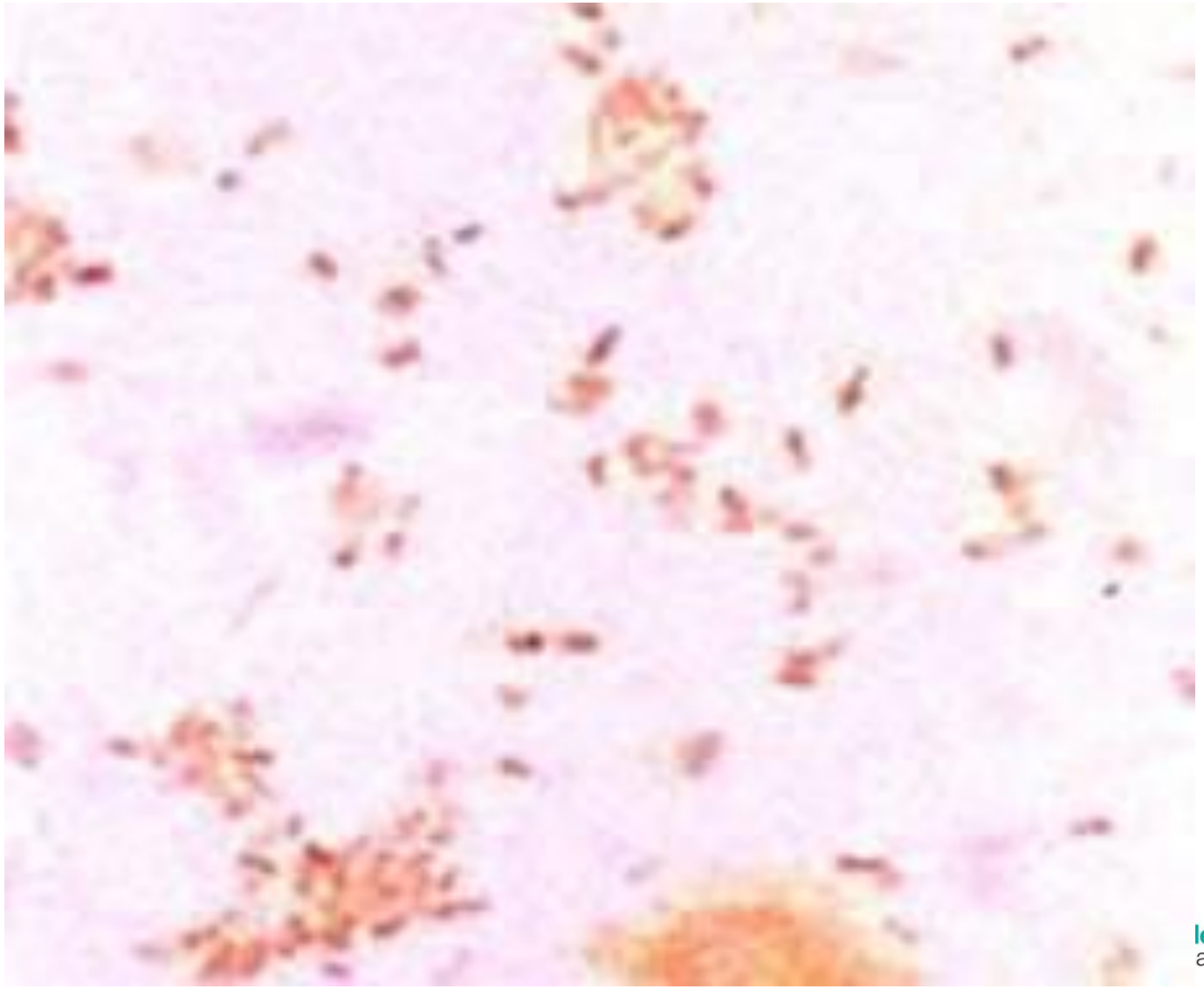
Budding yeast and pseudohyphae



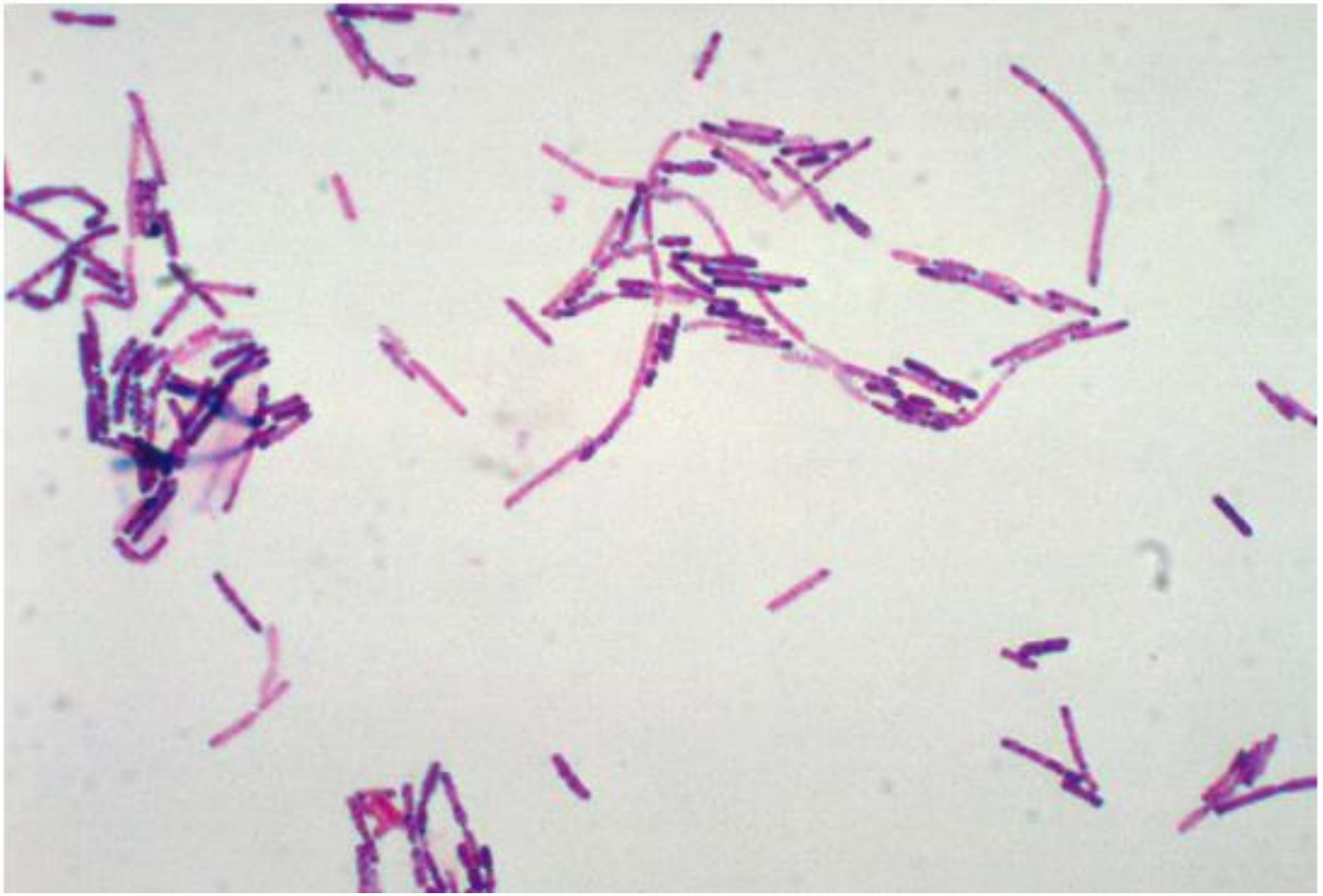
In a male: Gram Negative Intracellular Diplococci



Haemophilus ducreyi

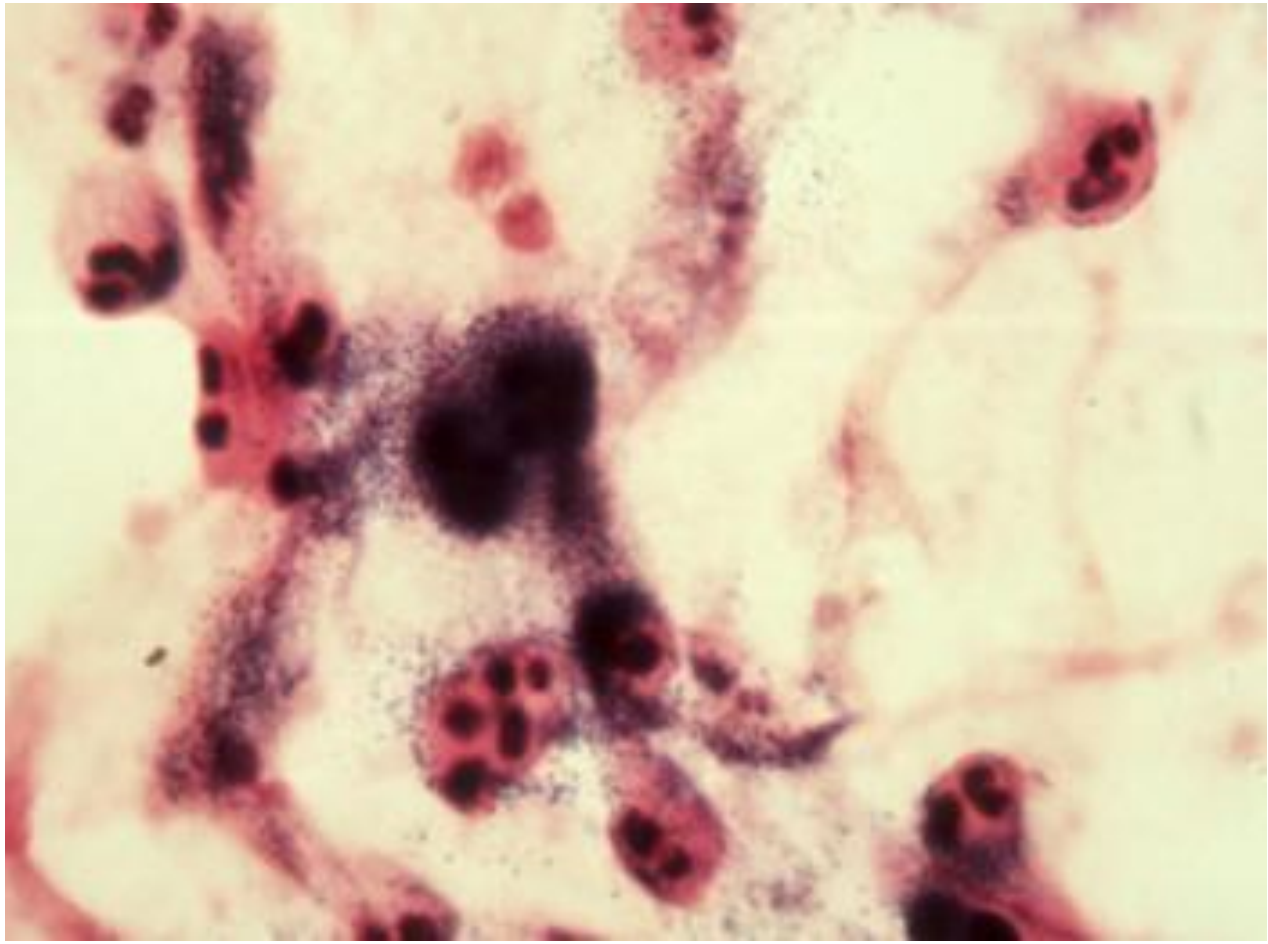


GS: Over decolorized



https://www.google.com/search?safe=active&rlz=1C1GGRV_enUS751US751&biw=1430&

GS: Under-decolorized



https://www.labce.com/spg327552_under_decolorized_or_over_decolorized_smears.aspx

Trichomonas Vaginalis

https://www.youtube.com/watch?v=rim2dXF3Oac

https://www.youtube.com/watch?v=yk0P7IpSiIg

Thank You

- Dr. Ellen Jo Baron for providing special slides
- Pat Rodrigues-Wong, Microbiology Technical Director
- SHSL Microbiology staff for providing pictures and videos

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