CASE 4

The Microbiology Laboratory

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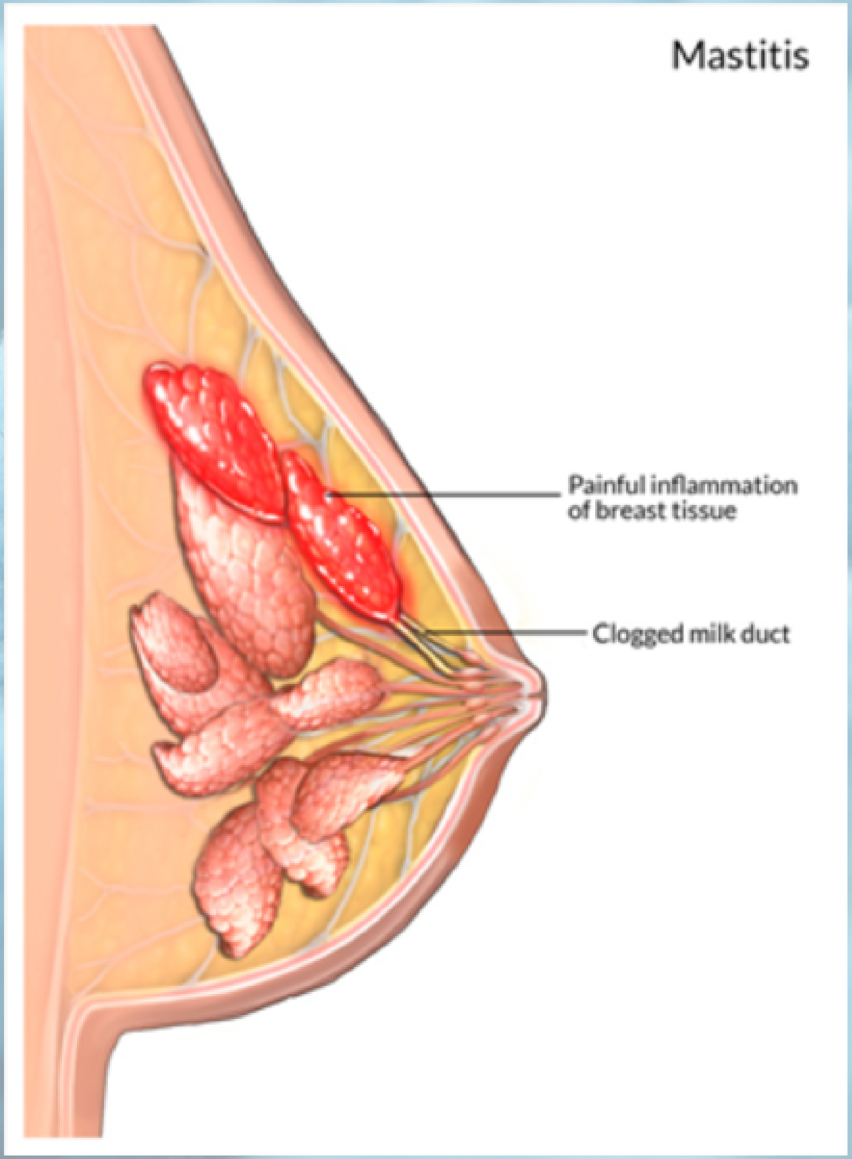
CASE: A NEW BABY

Elizabeth's pregnancy and the birth of Amanda had gone well however, Elizabeth and Amanda were now **struggling with breastfeeding**. Elizabeth was aware from her prenatal classes of the various reasons why breastfeeding might be difficult. She arranged to meet with a lactation consultant and continued trying to 'latch' and feed Amanda in the days leading up to the visit but stopped when she began to experience **breast pain** and noticed that her **right breast was red all around the nipple**. She was feeling stressed and **tired** + a **feeling of general malaise**- she attributed to the stress of trying to breastfeed her newborn baby. Based on Elizabeth's symptoms, the lactation consultant made a **preliminary diagnosis of mastitis** and suggested that Elizabeth see her doctor for a full diagnosis and **possible antibiotic treatment**.

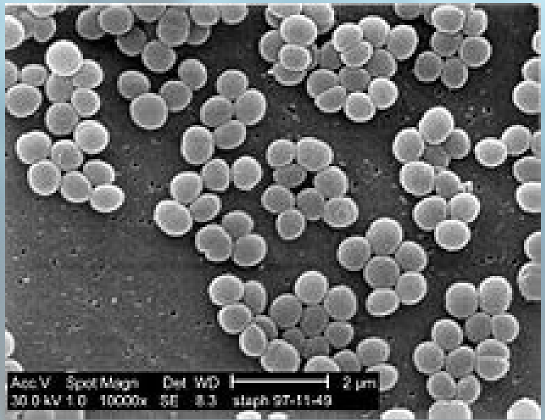
MASTITIS OVERVIEW

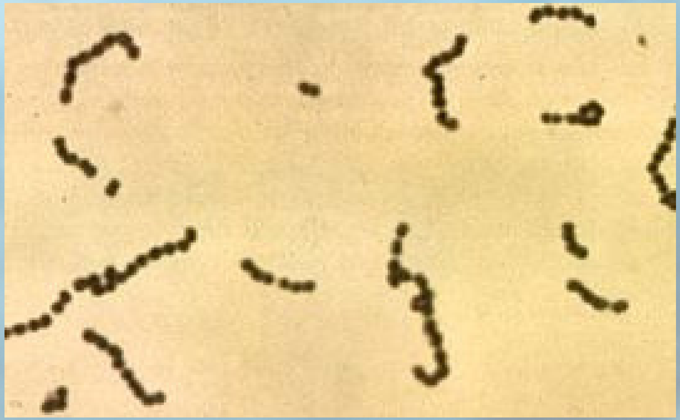
- Characterized by chronic inflammation of the breast which accounts for the redness observed around Elizabeth's right nipple
- Breast pain caused by dilation of the mammary duct leading to milk build-up within the breast
- Infection can be due to a variety of pathogens- including mycoplasmas and algae
- Both aerobic and anaerobic bacteria could lead to infection



MASTITIS: COMMON BACTERIAL PATHOGENS

- There are **three main bacterial species** found in mastic milk samples (Angelopoulou et al., 2018) :
- **1. Staphylococcus (97.57%)**: **gram-positive bacteria** and **facultative anaerobes**, appear round and form in grape-like clusters
- *frequently found on respiratory tract and skin
- **2. Streptococci (70.20%)**: **gram-positive bacteria** and **facultative anaerobes or obligate anaerobes**, grow in chains or pairs
- *primarily part of the salivary microbiome
- **3. Corynebacteria (16.60%)**: **gram-positive** and **aerobic**, rod-shaped
- *usually not pathogenic, unless they opportunistically capitalize on atypical access to tissues



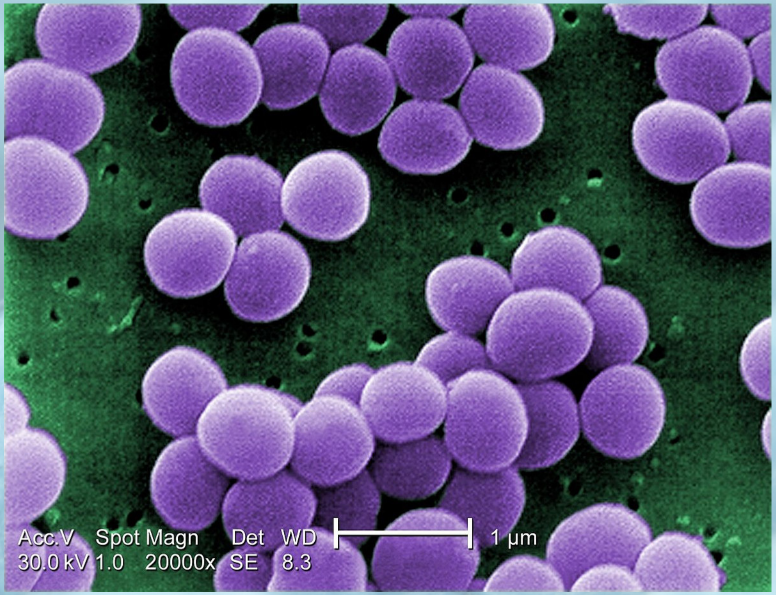


HEALTHY VS. MASTIC MILK MICROBIOME

Healthy Core Milk Microbiome	Mastitis Milk Microbiome
Pseudomonas, Staphylococcus, Streptococcus, Elizabethkingia, Variovorax, Bifidobacterium, Flavobacterium, Stenotrophomonas, Brevundimonas, Chryseobacterium, Lactobacillus and Enterobacter	Predominantly Staphylococcus species Most dominant: S. aureus (acute) and S. epidermidis (subacute) Significantly more Lysinibacillus, Macrococcus, Planococcus, Brevundimonas, Alcaligenes, Acinetobacter

- Table includes information regarding genera detected in healthy human milk and mastitic human milk via culture-dependent and culture-independent analyses.
- One study demonstrated the presence of 17 genera and 30 distinct species in mastitic milk (Patel et al., 2016)
- Many healthy breastfeeding women have potentially pathogenic bacteria in their breast milk. Increasing bacterial counts did not affect the clinical manifestation of mastitis

MAIN CAUSITIVE AGENT: STAPHYLOCOCCUS AUREUS

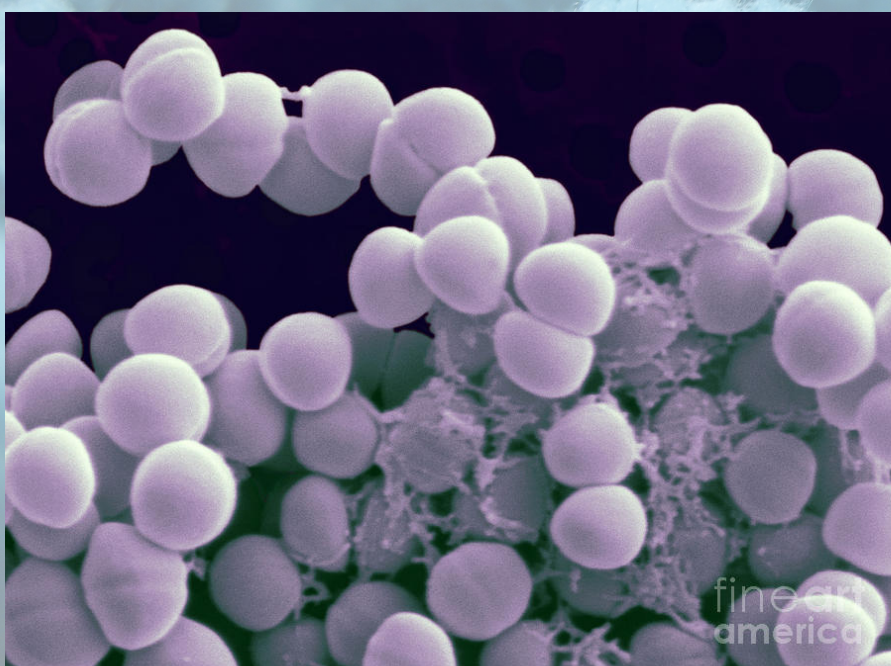


- Women with **acute mastitis** were found to have **higher** levels of *S. aureus* (approximately 106 cfu/mL) in their milk compared to those with subacute mastitis
- Configuration of the cocci helps to distinguish between staphylococci and streptococci

- S.aureus establishes itself after coming into contact with infected objects and can be spread by contact between non-infected and infected glands
- May display resistance to several relevant antibiotics
- **Virulence factors**: biofilms and surface proteins that promote adherence and growth of bacteria

STAPHYLOCOCCUS EPIDERMIDIS

- *S. epidermis* was found to be the most abundant staphylococcal species in women with **subacute mastitis**
- Bacteria found in colonies, 0.8-1 μm in diameter
- Commonly appears in tetrads, phenotypically gray or grayish-white in colour, and translucent
- Large prevalence of *S. epidermis* increases the risk of potential infection within the mammary ducts

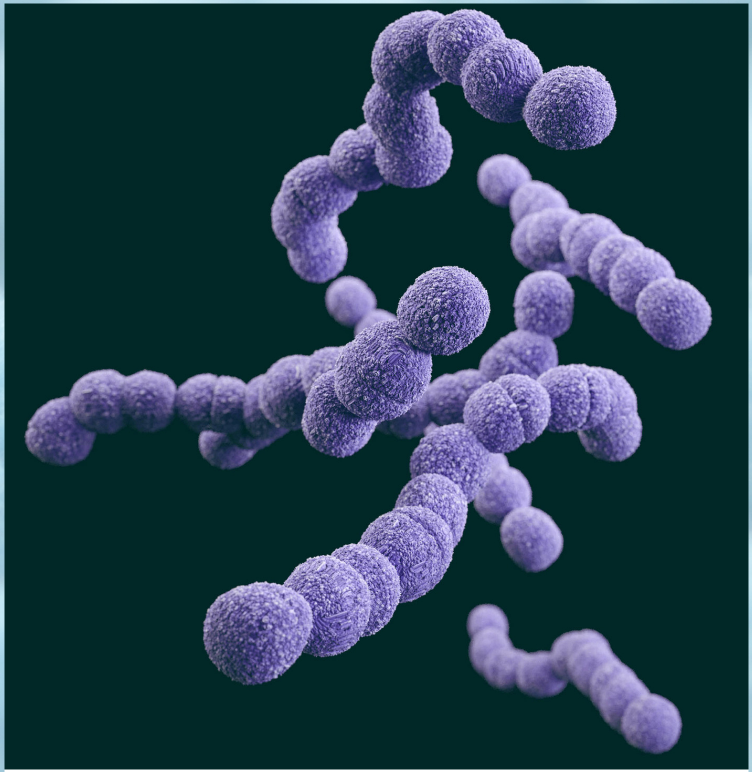


How can you differentiate *S. aureus* from *S. epidermis*?

- *S. epidermis* **lacks** the **enzyme coagulase -** it is considered to be a part of the coagulase-negative staphylococci (CNS) group

GROUP B STREPTOCOCCI (GBS)

- GBS is normally a harmless commensal bacterium part of the human microbiota
- Often colonizes the gastrointestinal and genitourinary tracts of up to 30% of healthy human adults (asymptomatic carriers)
- **Three types of hemolysis** on blood agar: **beta, alpha, and gamma** hemolytic (ranging between complete, incomplete, and hemolysis)
- *S. agalactiae* is a common veterinary pathogen: often causes bovine mastitis
- No defined virulent factors



Most GBS strains are encapsulated and serologically classified based on type-specific capsular polysaccharides

CORYNEBACTERIA

- Corynebacteria prominent in women with **Idiopathic granulomatous mastitis (IGM)**- rare, inflammatory disease characterized by painful, **tender lump** or **mass in the breast**
- Isolation of several strains of corynebacteria from breast mass samples of patients with IGM: ***C. kroppenstedii*, *C. tuberculostearicum*** and ***C. freneyi*** (Dobinson et al., 2015)
- Corynebacteria exist in **lipid-filled vacuoles** inside the granuloma, not present on the tissue itself
- Treatment is difficult- requires agents effective against corynebacteria with the ability to survive in lipid environments

Clinical appearance of the patient's breast including skin ulcerations, abscesses and fistulae.

SAMPLES TAKEN FOR LABORATORY TESTING

1. **Breast Milk Samples: Rare**

   *Culture can help identify specific bacterial source of infection

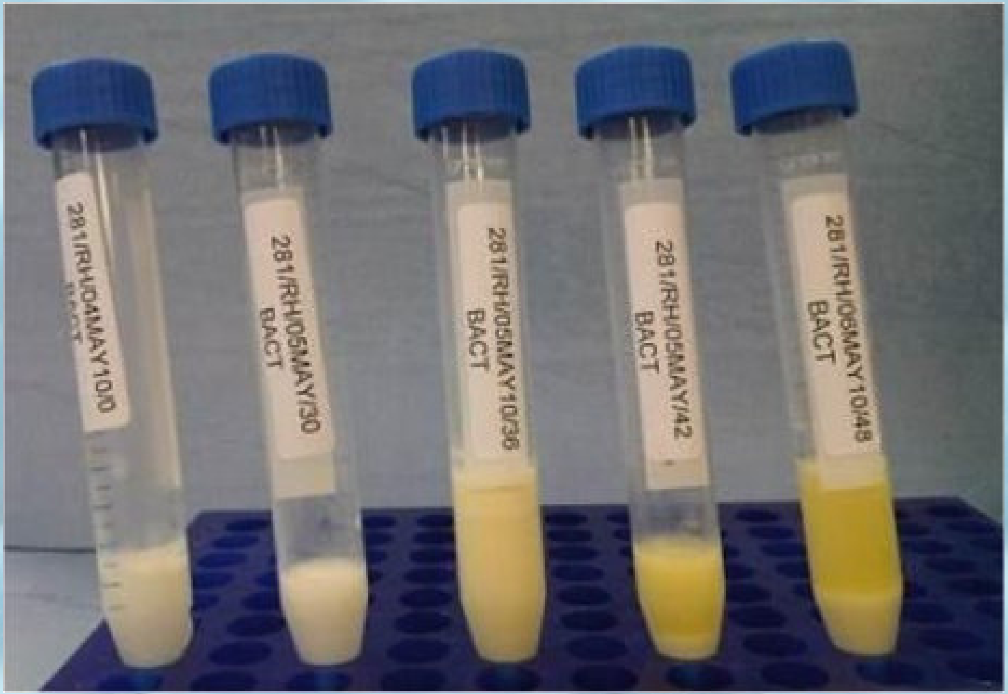
   * Milk samples must be taken from patient when (WHO, 2014):
     1. No response to antibiotics within 2 days
     2. Mastitis recurs or it is a hospital acquired case of infection
     3. Patient is allergic to therapeutic antibiotics
     4. Severe or unusual cases, ex. MRSA

   * Confirm infection by identifying specific antibodies found coating the bacteria. Ex. IgA and IgG coated bacteria indicates presence of an immune response mounted against that bacteria

   ***Problems:** positive results may be due to normal bacterial colonization + negative results do not confirm mastitis infection

HOW TO ACQUIRE SAMPLE

1. **Mother must cleanse nipple with alcohol**
2. **Express some of the milk, and discard it**
3. **Collect milk sample in a sterile container- ensure nipple is not touching container to prevent contamination with the normal skin flora**



- If the expression of milk from the mother is painful, painkillers (ex. Advil) may be taken to ease pain.
- Collection should be performed in breast milk banks (BMBs) when available, or at hospitals
- Despite precautions, only 50% of milk acquired is considered sterile

IMPORTANCE OF LAB TESTING

- Physical examinations of the breast area are not always sufficient to conclude the presence of infection
- Signs and symptoms of mastitis such as redness, malaise, unilateral breast tenderness and fatigue may be shared with other illnesses
- Identifying specific bacterial species causing infection can help develop a stronger treatment plan - broad-spectrum antibiotics are often ineffective
- Microbiology lab testing can help prepare for treatment against resistant populations such as strains of *S. aureus*

TESTS PERFORMED ON THE SAMPLES

1. Cultures
2. Gram-Staining
3. Morphology Analysis
4. Biochemical Testing
5. Genotypic/Molecular Testing
6. Antibiotic Susceptibility Testing

CULTURES

Columbia blood agar

- Can identify Streptococci, Staphylococci, and other gram-positive bacteria
- Differentiate three types of hemolysis: alpha (green discharge), beta (clear appearance), gamma (no change in medium)
- Plates must be incubated for 48 hours at 37°C

Sabouraud chloramphenicol (SDC) agar

- Presence of chloramphenicol inhibits gram-positive and gram-negative bacterial growth, allows identification of yeast and fungi
- Yeasts = white or creamy colonies
- Plates are incubated for 5 days at 25°C

CULTURES

Kanamycin Aesculin Azide agar

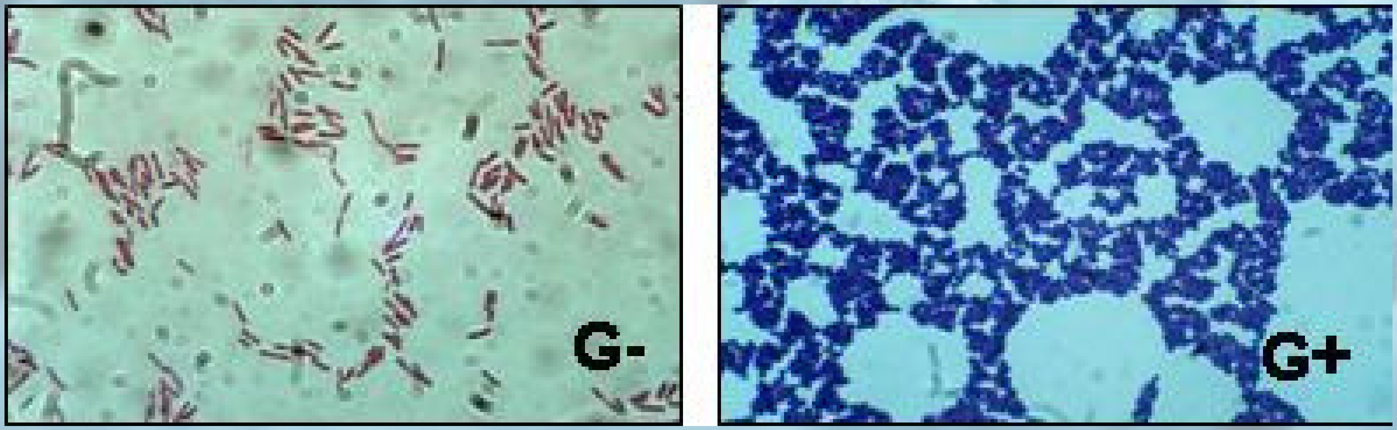
- Identification of enterococci- round, white or grey colonies surrounded by dark zones
- Kanamycin sulphate + sodium azide are **selective inhibitors** - only allow the growth of enterococci
- Plates are incubated for 48 hours at 37°C

Violet red bile glucose plate

- Identification of gram-negative bacteria involved in glucose fermentation
- Bile salts + crystal violet inhibit the growth of gram-positive bacteria
- Presence of glucose fermentation indicated by red and purple colonies
- Plates are incubated for 48 hours at 37°C

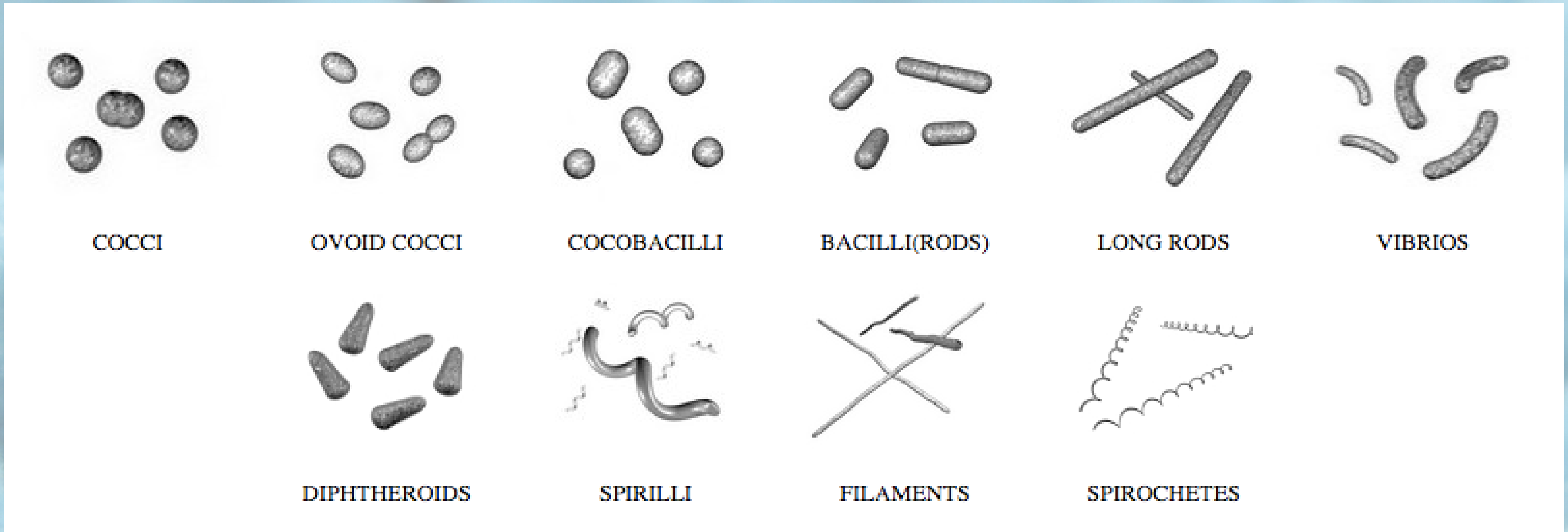
GRAM-STAINING

- Help identify bacteria as gram-positive or gram negative based on cell wall composition
- Performed on isolated bacterial species
- Crystal violet stain applied to a heat-fixed smear of a bacterial culture - trapping agent (Gram's iodine) added, followed by rapid decolorization with alcohol or acetone



Gram-positive bacteria: stains purple/blue due to thick peptidoglycan wall (retains dye)
Gram-negative bacteria: stains pink/red due to thin peptidoglycan cell wall (loses dye)

MORPHOLOGY ANALYSIS

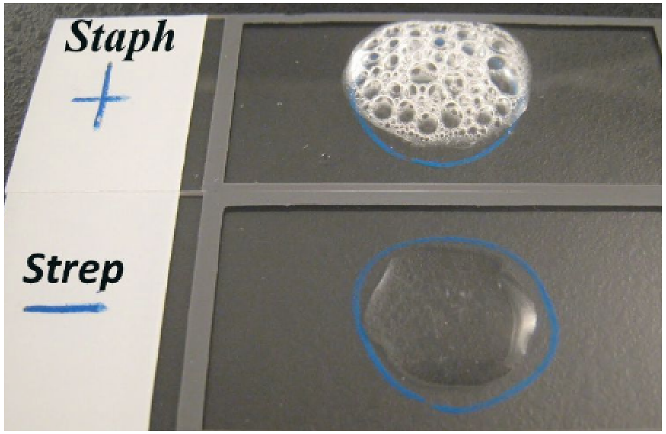


- Bacteria can be identified based on cell shape, size and special structures; ex. capsules, endospores and granules
- Morphology table contains information regarding external characteristics of bacteria
- Ex. Staphylococcus appear round and form in grape-like clusters unlike Streptococci which grow in chains or pairs

BIOCHEMICAL TESTS

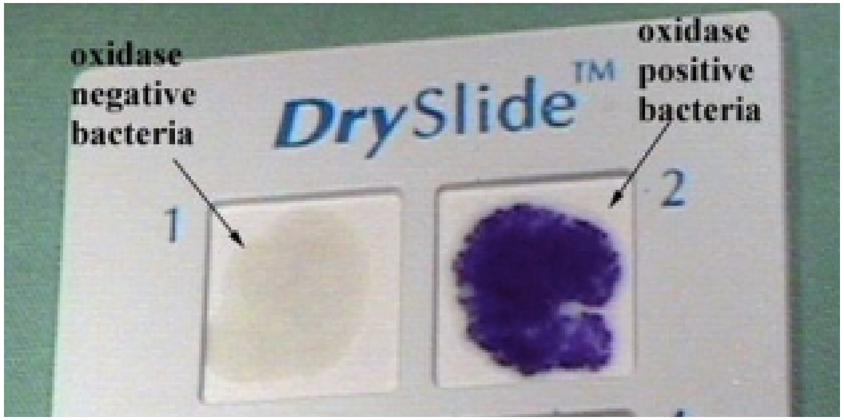
Catalase Test

- Used to detect the presence of **catalase:** can differentiate staphylococci from streptococci
- Demonstrates if the bacteria can use oxygen as a terminal electron acceptor
- Positive test characterized by rapid bubbling- indicates formation of oxygen gas



Oxidative Test

- Used to detect the presence of **cytochrome oxidase** in bacteria
- Cytochrome oxidase is an important component of the electron transport chain- facilitates addition of electrons to oxygen
- Positive test characterized by colour change to dark purple



GENOTYPIC/MOLECULAR TESTING

- Phylogenetic markers help in bacterial characterization-- predominantly the 16S rRNA or 23S rRNA genes
- These regions are favoured for amplification because they are **highly conserved** within the species but differs at broader levels of classification
- Time efficient compared to biochemical tests-long incubation time
- Milk samples are centrifuged to extract the genomic DNA and Polymerase Chain Reaction generates several copies of the template DNA

Mastitis inducer	Genetic markers for diagnosis	
	Diagnosis	typing
Staph. aureus	*nuc* or *16S rRNA*	*coa, aroA, clf, cna* and the *spa* gene and its Xr-region.
CNS	16S *rRNA*, *tuf*, and *rpoB*	RFLP on *gapC* gene, PCR for tRNA intergenic spacer, AFLP, and (GTG) 5-PCR typing.
Streptococci	*cpn60*	*cpn60*

Genetic markers for the identification and typing of mastitis inducing pathogens

ANTIBIOTIC SUSCEPTIBILITY TESTING

- Results help identify bacteria and can help inform antibiotic treatment plans

- **Two predominant methods:**
  1. VITEK 2 system: uses sterile swabs of the bacterial culture placed in a suspension
  2. Diffusion Antibiotic Disc Assay: broth culture of the isolated bacterial species is spread onto an agar plate before antibiotic discs are placed ontop
  Plates analyzed for any zones of inhibitions- areas with no bacterial growth

- Even within a single species, different strains of bacteria have various responses to antibiotics
- Given the rise in antibiotic resistant bacteria- these results can help create better antibiotic treatment plans

RESULTS: IF S.AUREUS

Test Performed	Expected Result
Columbia blood agar	presence of clear zones to indicating β-hemolysis
SDC agar	expect no growth of *S. aureus*
Kanamycin Aesculin Azide agar	expect no growth of *S. aureus*
Violet red bile glucose plate	expect no growth of *S. aureus*

Above image highlights the presence of clear zones on the **Colombia Blood Agar** plate: indicating the presence of β-hemolysis in *Staphylococcus aureus*

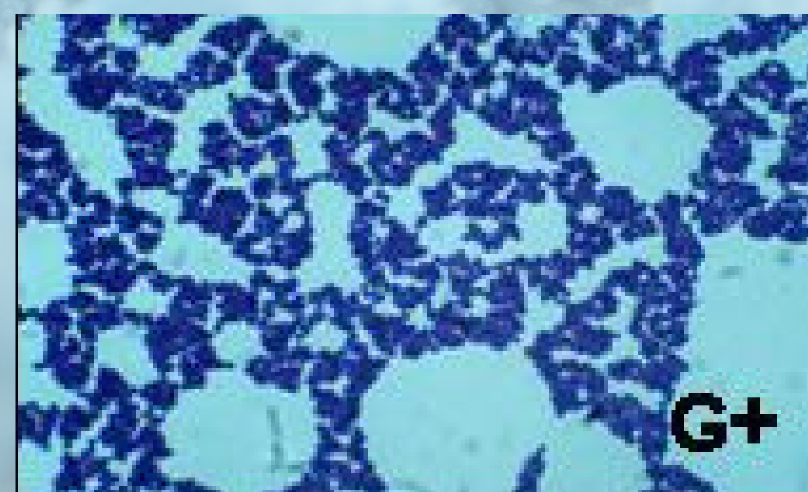
RESULTS: IF S.AUREUS

Catalase Test	*S. aureus* is an aerobic bacterium so a **positive** catalase test is expected. Rapid bubbling indicates the presence of the enzyme catalase.
Oxidase Test	*S. aureus* is known to contain cytochrome oxidase so a **positive** test is expected. Colour change to dark purple due to the interaction with the oxidase agent should occur.

Positive results for the catalase and oxidase tests respectively.

RESULTS: IF S.AUREUS

Test Performed	Expected Result
Gram-Staining	*S. aureus* is a gram-positive bacterium, its thick peptidoglycan wall retains the crystal violet stai n appears purple.
Morphology	*S. aureus* often observed in pairs or clusters. Pre dominant morphological features: circular cocci
Molecular Testing	Expect PCR product to match with the sequence of the 16s rRNA sequence of *S. aureus*.
Antibiotic Disc Assay	*S. aureus* expected to be susceptible to ampicillin, streptomycin and erythromycin



RESULTS: IF S. EPIDERMIDIS

Test Performed	Expected Result
Colombia Blood Agar	Expect to no destruction to hemoglobin indicating γ -hemolysis
SDC Agar	Expect no growth of *S. epidermis*.
Kanamycin Aesculin Azide agar	Expect no growth of *S. epidermis*.
Violet red bile glucose plate	Expect no growth of *S. epidermis*.

The image shows the difference in results following culturing on blood agar plates between *S. aureus* (Left) and *S. Epidermis* (Right)

RESULTS: IF S. EPIDERMIDIS

Test Performed	Expected Result
Catalase Test	*S. epidermidis* is an aerobic bacterium and the presence of catalase is expected (indicated by a positive test result), similar to *S.* aureus.
Oxidase Test	*S. epidermidis* is not known to contain cytochrome oxidase, therefore a negative test result is expected.
Gram-staining	*S. epidermidis* is a gram-positive bacterium is expected to stain purple due to its thick peptidoglycan wall
Morphology	Elevated, round, gray or translucent bacteria found in colonies, visible in pairs or tetrads

The image shows the difference in oxidase test results between *S. aureus* (Right) and *S. Epidermis* (Left)


THANK YOU