Medical Bacteriology Lab
460 MIC

Practical 1

General Safety Rules for the Microbial Pathogenesis Laboratory

The microorganisms used for instruction in this course are **pathogenic** for humans or animals. The safety of every student depends upon the conscientious observation of rules that must be followed by all who work in the laboratory. Certain precautions must be followed to avoid endangering well being, that of neighbors and those who clean the laboratory. Any student who is in doubt about how to handle infectious material should consult an instructor. Laboratory attendance is mandatory. There will be no way to make up missed work.

The following rules **must be observed at all times**.

1. Always wear a laboratory coat when working in the laboratory classroom.
2. Put nothing in mouth which may have come in contact with infectious material.
3. Eating and drinking in the laboratory are not permitted at any time.
4. Mouth pipetting is not permitted under any circumstances. Use the safety pipetting devices which are provided. Dispose of used pipettes in the appropriate receptacle. Any infectious material which may accidentally fall from pipettes to the laboratory bench or floor should be covered with a disinfectant and reported to any instructor immediately.
5. Any spilled or broken containers of culture material should be thoroughly wet down with a disinfectant and then brought to the attention of an instructor. **There are no penalties for accidents** provided they are reported promptly.
6. Report at once an accident which may lead to a laboratory infection.
7. The microscope issued to you is both an expensive and delicate instrument--treat it accordingly. Always, at the end of each laboratory period, microscope lamp turned

off ,carefully clean oil from the objective and condenser lenses, align the low power dry objective with the condenser and rack condenser up and body tube down. You will be held personally responsible for any defect found on microscope when it is recalled at the semester's end.

8. When finished for the day, dispose of all used glassware and cultures in the appropriate receptacle, clear workbench and wash the top with a disinfectant. Wash hands thoroughly with soap and water before leaving the laboratory.

9. Do not throw refuse of any kind into the sink. Use the containers provided.

10. Be sure all burners are turned off at the end of the laboratory period. Double check to be sure that handles on **all gas** outlets are in the off position.

11. The inoculating needle should be heated until red hot before and after use. **Always flame needle before you lay it down**.

12. Always place culture tubes of broth or slants in an upright position in a rack. **Do not** lay them down on the table or lean them on other objects. They may roll onto the floor and break.

13. All culture containers which are to be incubated should bear the following notations: 1) initials (or last name of the student), 2) specimen (name of organism or number of unknown) and 3) date. When using Petri plates, these notations should be entered on the bottom half, not the lid. Unless otherwise directed, all plates are to be inverted, all plugged tubes should have the plugs firmly set into the tubes, and all screw cap tubes should have the caps loosened one-half turn to permit gas exchange.
14. *Gas tap* is turned off.
15. *Water tap* is closed properly.
16. Finally wash your hands thoroughly

Discard cultures and other infectious materials:

- **Petri dishes→ Plastic bag → Autoclave.**
- **Test tube cultures → wire basket → Autoclave.**

- **Used pipettes → Plastic bag → Autoclave.**
- **Used slides, covers, and pipettes → Jar containing a disinfectant.**
- **Broken glass → swept in a dustpan → container for broken glass.**

NEVER place contaminated material in waste basket.

Broken or spilled living cultures:

- **Clothing → Autoclave plastic bag → Autoclave.**
- **Flood the area with a disinfectant (or paper towels are placed over the spills).**
- **After 20- 30min→ wipe up & discard the waste in autoclavable dustpan→ Autoclave.**

Definitions:

Sterilization: Killing or removal of all living micro-organisms (from a particular location or material).

***Sterile article**:* completely free of all living micro-organisms

Disinfection: Destruction of vegetative conspiring micro-organisms.

Disinfectants: Chemicals which cause disinfection Bacterial spore mycobacteria, some viruses → considerable resistance

Antiseptics: Disinfectants which can be safely applied to skin & mucous membranes.

Contamination: Introduction of undesirable m.o.

Asepsis: Processes designed to prevent m.o. from reaching a protected environment.

***Aseptic technique*:** Practices used by microbiologists to exclude all organisms from contaminating media or contacting living tissues.

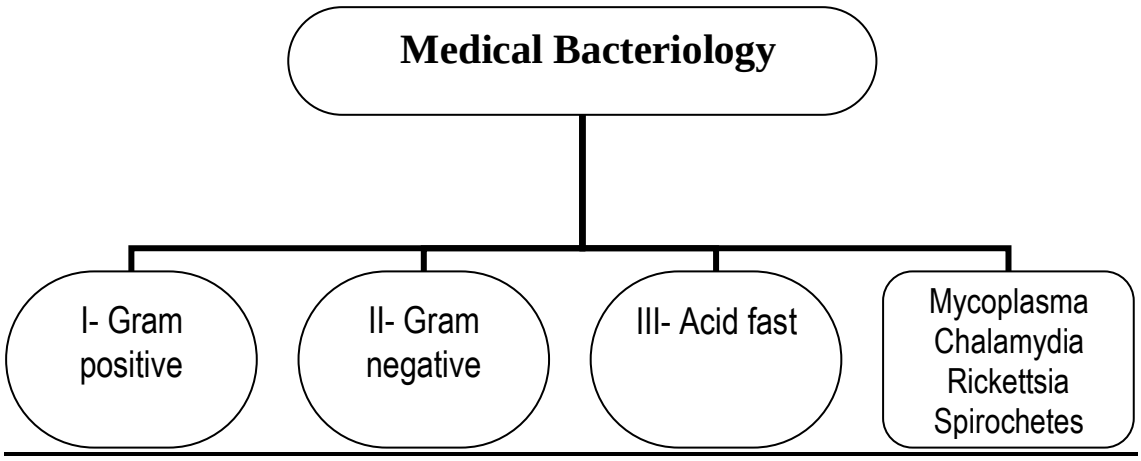
pathogen: is a microorganism that is able to cause disease in a plant, animal or insect.

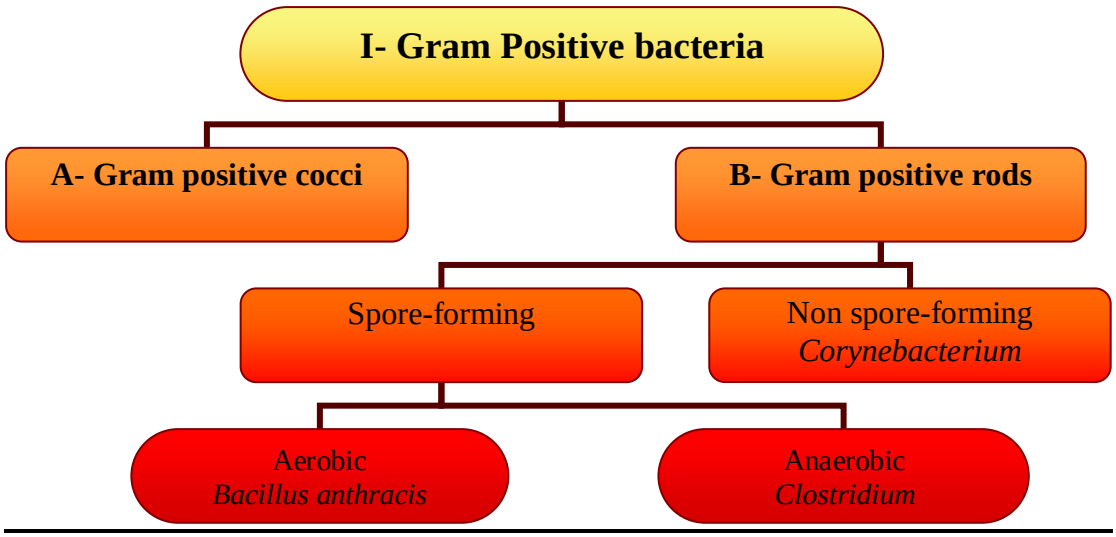
Pathogenicity: is the ability to produce disease in a host organism.

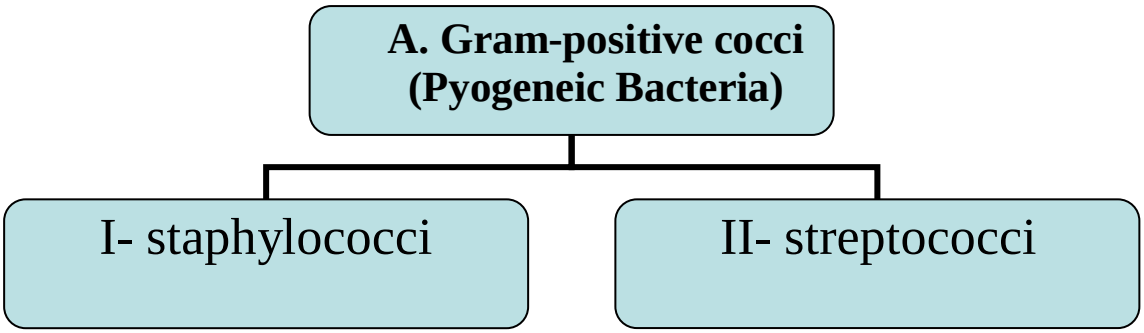
Virulence: a term which refers to the degree of pathogenicity of the microbe.

A microbiological culture medium: is the food we use for culturing bacteria and fungi.

The culture medium: is an artificial preparation that contains the essential elements and nutrients in a proper concentration needed by the microorganism (most bacteria &fungi) to grow







Staphylococci

- **Objective:**

1- Detect the presence of catalase in staphylococci and to differentiate between staphylococci (catalase positive) and streptococci (catalase negative)
2- Learn procedure of Gram stain and to know Gram reaction, shape and arrangement of staphylococci
3- Use of mannitol salt agar as selective and differential medium for staphylococci and to differentiate between mannitol fermenter and mannitol non fermenter staphylococci
4- Learn the use of plasma to differentiate coagulase positive and coagulase negative staphylococci
5- Detect DNase in staphylococci and to compare activity in *S. aureus* and *S. epidermidis*

- **Material requirements**

1- Overnight cultures of *S. aureus*, *S. epidermidis*, & *S. saprophyticus*
2- Grams stain dyes (Crystal violet and Saffranin) and reagents (Iodine and alcohol), filter paper, slides, immersion oil.
3- Nutrient agar plates
4- Mannitol Salt Agar (MSA) plates
5- Deoxyribonuclease (DNase) agar plastes
6- 1N HCl (100 ml)
7- Hydrogen peroxide 3% (10ml)
8- Rabbit plasma
9- Novobiocin disk

- **Practical protocol:**

1- Gram stain:
2- Catalase test
3- Mannitol fermentation on MSA
4- Coagulase test
5- DNase test
6- Novobiocin susceptibility

General characters of Staphylococcus genus

Gram Positive Cocci, 0.5-1.5 μm in diameter
Grape-like clusters, in irregular
Non Motile
Non Spore Forming
Non Capsulated
Grow in simple media (i.e. non Fastidious)
Facultative Anaerobes
Fermentative
Catalase positive

Three species of staphyloccoci have medical importance:

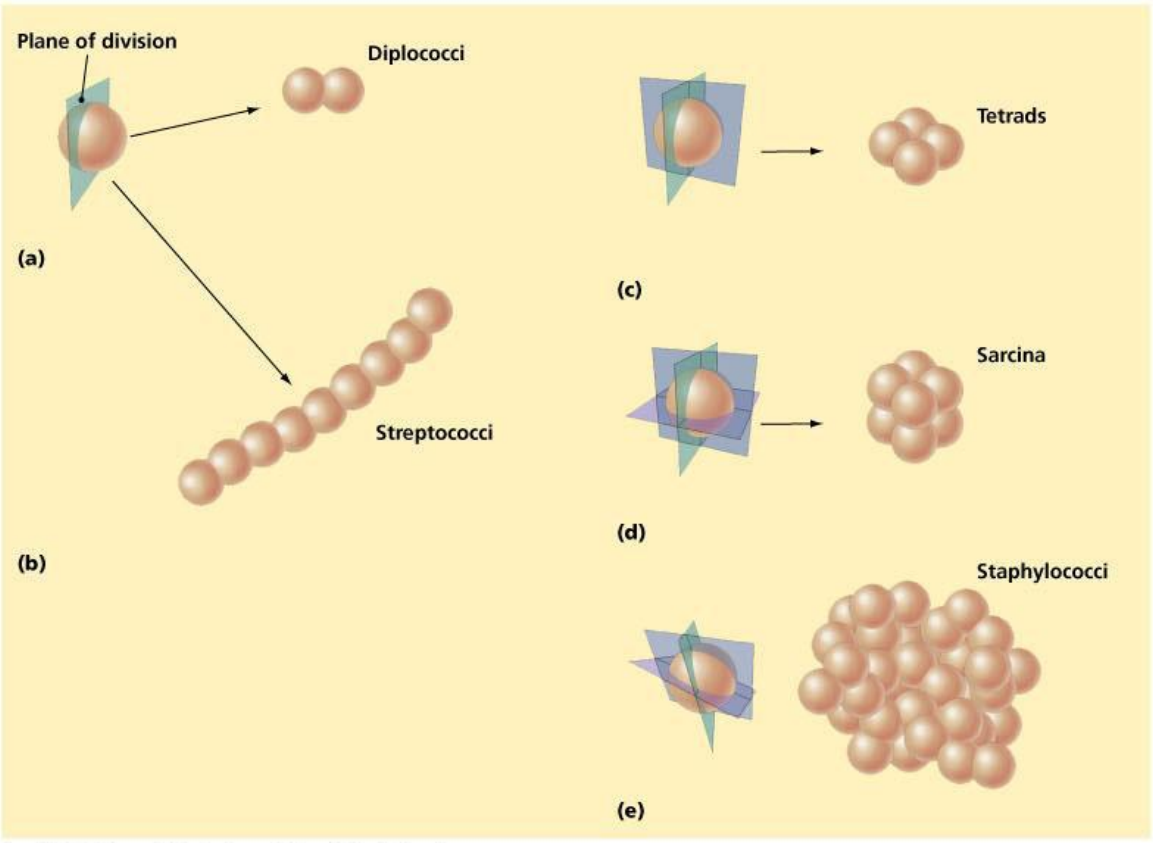
S. aureus: Pathogenic & commensally found in nose (nares)

Characters of *S. aureus*

Production of coagulase
Production of phosphatase
Production of DNase
Ferment Mannitol
Gelatin liquefied
B-hemolysis on blood agar
Acidification & clotting of litmus milk

S. epidermidis: non pathogenic & common commensals in nares & skin

S. saprophyticus: Cause UTI in female & occasionally commensally found skin



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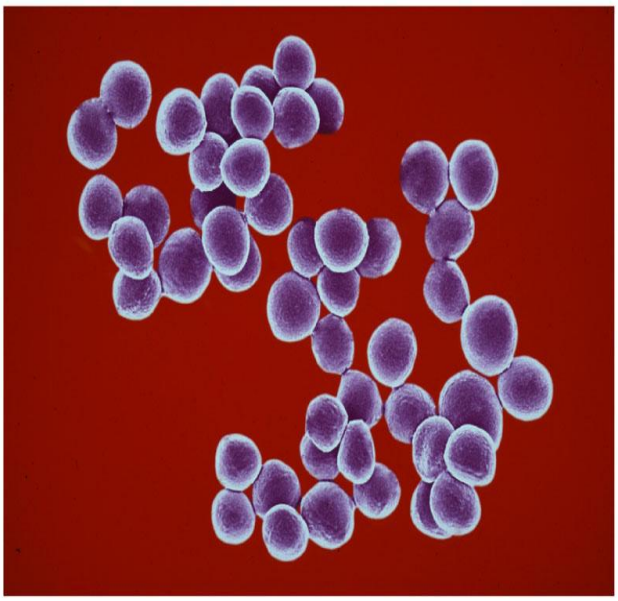
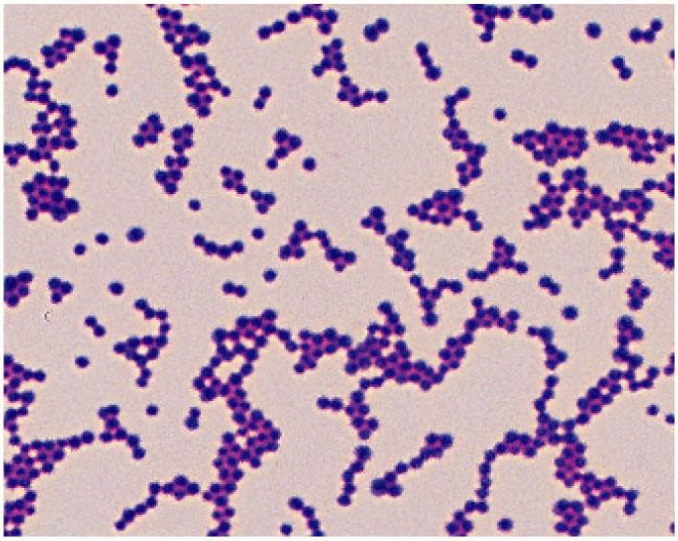
Gram stain of Staphylococcus

(Morphology)





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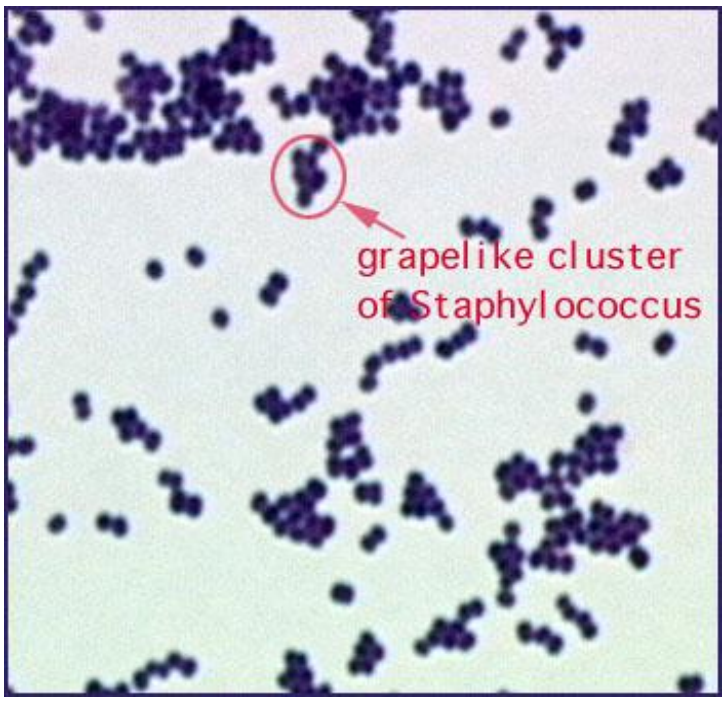


Virulence factors of *S. aureus*

Coagulase:
Converting fibrinogen into fibrin

Exofoliative toxin:
Desquamation of skin in case of exofoliative dermatitis in SSSS

TSST:
Fever, hypotension, & skin rash followed by desquamation of skin

Leucocytes
Kills white blood cells (WBCs)

Polysaccharide A and Protein A
Antiphagocytic and Adhesion

Enterotoxins (A,B,C,D, & E)
Food poisoning (Diarrhea, and Vomiting)

Hyaluronidase
Destroy hyaluronic acid (constituent of connective tissues)

α,β,γ and δ Toxins
Destroy variety of cells (Polymorph)

Disease caused by *S. aureus*

Localized suppurartive (Pyogenic) inflammation:
Folliculitis Infection of hair follicles
Carbuncle Larger abscess
Deep Lesions (Osteomyelitis, Endocarditis & Meningitis)

Toxigenic infection
Scalded Skin Syndrome (SSS)
Toxic Shock Syndrome

Food poisoning
Nausea, Vomiting, Diarrhea without Fever within 8 h after ingestion of toxins in the contaminated food

Laboratory diagnosis of *Staphylococcus*

Diagnosis

Specimen
Smear
Culture
Film
Biochemical Reactions
Antibiogram

Specimen:
Pus, wound, Urine, Stool, Blood, CSF

Gram Stain:

Gram Positive Cocci, arranged in cluster

Smear preparation:

The preparation of a smear is required for many laboratory procedures, including the Gram-stain. The purpose of making a smear is to fix the bacteria onto the slide and to prevent the sample from being lost during a staining procedure. A smear can be prepared from a solid or broth medium. Below are some guidelines for preparing a smear for a Gram-stain.

1. Place one needle of solid bacterial growth or two loops of liquid bacterial growth in the center of a clean slide
2. If working from a solid medium, add one drop of water to your specimen with a water bottle. If using a broth medium, do not add the water.
3. Now, with your inoculating loop, mix the specimen with the water completely and spread the mixture out to cover about half of the total slide area.
4. Allow the smear to dry at room temperature. Gently heat-fix by passing the dried smear through the flame of Bunsen burner. Caution must be exercised not to overheat the slide. Remember to gently heat-fix and **not to cook the bacteria on the slide.**

Gram-staining Procedure:

Gram-staining is a four part procedure which uses certain dyes to make a bacterial cell stand out against its background. The specimen should be mounted and fixed on a slide before you proceed to stain it. The reagents you will need to successfully perform this operation are:

- Crystal Violet (Primary Stain)
- Iodine Solution (Mordant)
- Decolorize (Ethanol is a good choice)
- Safranin (Counter stain)
- Water (preferably in a squirt bottle)

Before starting, make sure that all reagents, as well as the squirt-bottle of water, are easily accessible because you won't have time to go get them during the staining

procedure. Also, make sure you are doing this near a sink because it can get really messy.

Step 1: Place your slide on a slide holder or a rack. Flood the entire slide with crystal violet. Let the crystal violet stand for about 60 seconds. When the time has elapsed, wash your slide for 5 seconds with water. The specimen should appear blue-violet when observed with the naked eye.

Step 2: Now, flood your slide with the iodine solution. Let it stand about a minute as well. When time has expired, rinse the slide with water for 5 seconds and immediately proceed to step three. At this point, the specimen should still be blue-violet.

Step 3: This step involves addition of the decolorize, ethanol. Step 3 is somewhat subjective because using too much decolorizer could result in a false Gram (-) result. Likewise, not using enough decolorizer may yield a false Gram (+) results. To be safe, add the ethanol drop wise until the blue-violet color is no longer emitted from your specimen. As in the previous steps, rinse with the water for 5 seconds.

Step 4: The final step involves applying the counter stain, safranin. Flood the slide with the dye as you did in steps 1 and 2. Let this stand for about a minute to allow the bacteria to incorporate the saffranin. Gram positive cells will incorporate little or no counter stain and will remain blue-violet in appearance. Gram negative bacteria, however, take on a pink color and are easily distinguishable from the Gram positives. Again, rinse with water for 5 seconds to remove any excess of dye.

Step 5: After you have completed steps 1 through 4, you should blot the slide gently with bibulous paper or allow it to air dry before viewing it under the microscope. DO NOT RUB THE SMEAR.

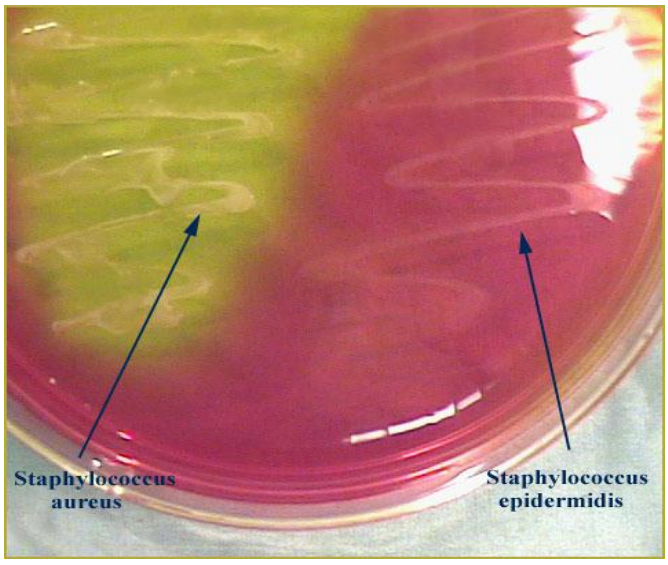
Step 6: Apply a drop of immersion oil directly to the smear and place the slide on the stage of the microscope and examine the film using oil immersion lens (x100).

Culture:

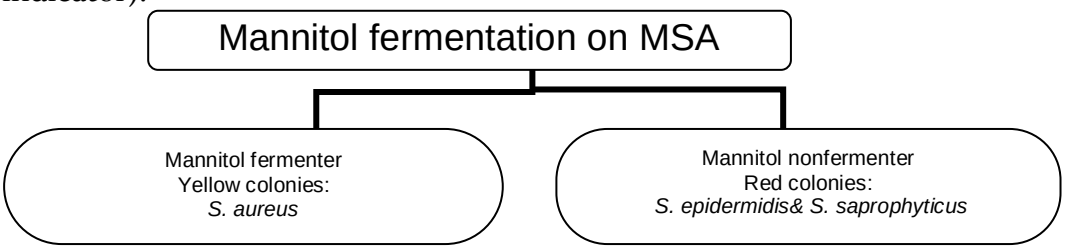
1- Colonial appearance:

Blood agar (Non-Selective Media)
Coagulase Positive Staphylococci are Pigmented & hemolytic
Coagulase Negative Staphylococci are non-pigmented & non-hemolytic

MSA is selective differential medium for staphylococci. It contains: NaCl (7.5%), Mannitol, & Phenol Red. The cause of selectivity due to presence of high salt concentration. The cause of differential because contains mannitol (sugar) and phenol red (pH indicators turns yellow in acidic pH and turns red in alkaline pH). *S. auerus* ferment mannitol and release acid which decreased in the pH of medium. The resultant acidic pH is detected by phenol red and colonies of *S. aureus* appear yellow. On the other hand, other staphylococci do not ferment mannitol and subsequently no changes occur in the pH of medium, therefore colonies of *S. epidermidis* appear red.



S.aureus is the only species of staphylococci that can ferment mannitol→acid production →yellow color around the growth (due to change the color of the pH indicator).



1- **Microscopical examination:**

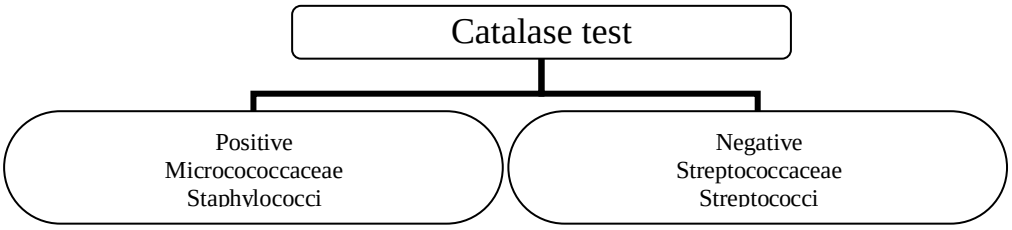
Staphylococci are Gram positive cocci, which occur in irregular "grape-like" clusters

Catalase test

The catalase test is important in distinguishing streptococci (catalase-negative) from staphylococci which are catalase positive. The test is performed by flooding an agar slant or broth culture with several drops of 3% hydrogen peroxide. Catalase-positive cultures bubble at once. The test should not be done on blood agar because blood itself will produce bubbles.

Positive

Negative



Coagulase Test

Principle:

The ability to clot plasma continues to be the most widely used and generally accepted criterion for identification of pathogenic staphylococci. The enzyme act by converting fibrinogen into fibrin. Coagulase test used to classify staphylococci into **1- Coagulase-positive staphylococci** (CPS) (e.g. *S. aureus*) and **2- Coagulase negative staphylococci** (CNS) (*S. epidermidis, S. saprophyticus*). It is thought that coagulase-positive staphylococci may avoid host defense mechanisms by forming this fibrin clot around them.

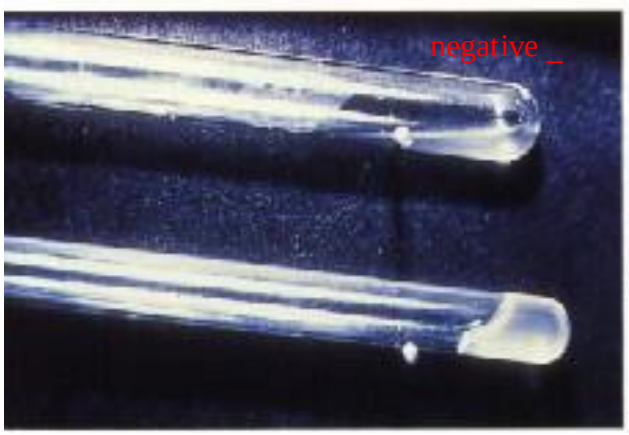
Two different coagaulase tests can be performed:

Test tube or slide test

The tube coagulase test (Free coagulase):

Procedure:

Mix 0.1 ml of culture + 0.5 ml of plasma
Incubate at 37C for 4 h
Observing the tube for clot formation



Any degree of clotting constitutes a positive test

Advantage

More accurate

Disadvantage

Time consumed

The slide coagulase test positive +

Used to detect bound coagulase or clumping factor

Add one drop heavy bacterial suspension and one drop of plasma on clean slide

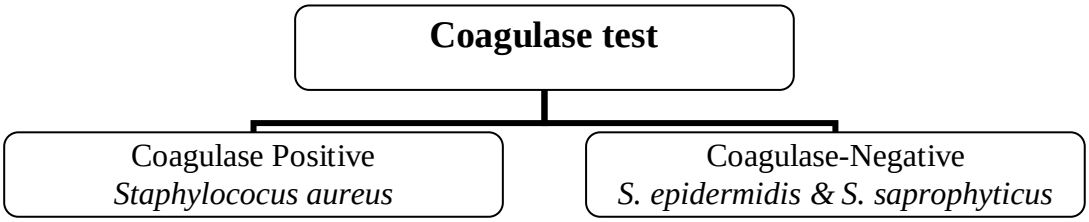
Mixing well and observing for clumping within 10 seconds

Advantage

Rapid diagnosis

Disadvantage

Less accurate



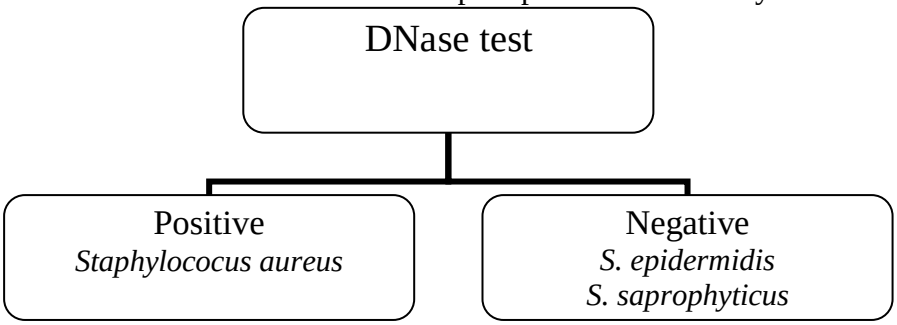
Deoxyribonuclease (DNAase) test:.

Principle:

- DNA is insoluble in acid
- DNA is hydrolyzed into oligonucleotides by the action of DNase
- Nucleotides soluble in acid

Procedure & result:

- Inoculate DNA agar with tested organism in circular motion
- Incubate at 37C for 24-48h
- Observe DNase activity by adding 1N HCl to the agar surface, a zone of clearing indicates a positive test
- The zone represents the absence of DNA
- The medium around colonies not producing DNase remains opaque, which is a reflection of the precipitation of DNA by the added acid.

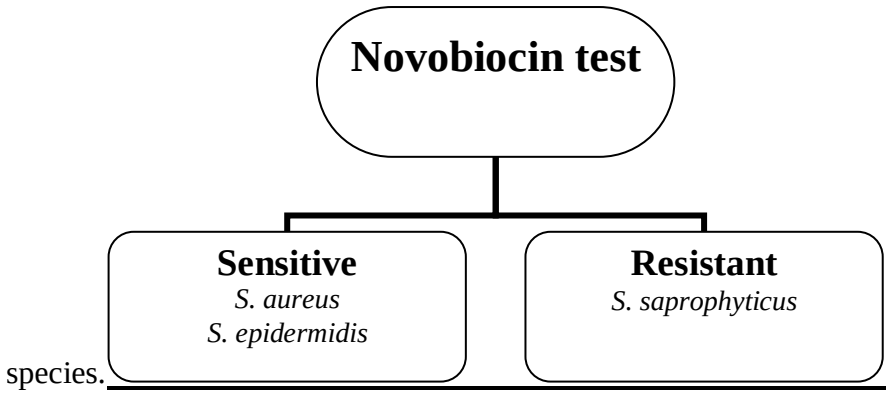


Novobiocin Sensitivity

A simple disk diffusion test for estimating novobiocin susceptibility used to distinguish *S. saprophyticus* from other clinically species

Inoculated overnight culture on Mueller-Hinton agar

Add novobiocin disk on inoculated plate
Incubate at 370C overnight
Novobiocin resistance is intrinsic to *S. saprophyticus* but uncommon in other clinically important



species.

Treatment, and Prevention (cont.)

Hand antisepsis is the most important measure in preventing nosocomial infections

Also important is the proper cleansing of wounds and surgical openings, aseptic use of catheters or indwelling needles, an appropriate use of antiseptics

Gram's +ve Cocci

Irregular Clusters

Tetrads

Chains or Pairs