

LAB: 2

Practical Pathogenic bacteria

Genus: Staphylococcus

Staph. aureus

Staph. epidermidis

Staph. saprophyticus

***Staphylococcus* is a genus of Gram-positive bacteria in the family Staphylococcaceae from the order Bacillales. Under the microscope, they appear spherical (cocci), and form in grape-like clusters. *Staphylococcus* species are facultative anaerobic organisms (capable of growth both aerobically and anaerobically), none motile, none spore forming, some members have capsule, catalase positive, oxidase negative, coagulase a variable, DNase a variable, mannitol fermentation a variable.**

Staphylococcus includes at least 43 species. Of these, nine have two subspecies, one has three subspecies, and one has four subspecies. Many species cannot cause disease and reside normally on the skin and mucous membranes of humans and other animals. *Staphylococcus* species have been found to be nectar-inhabiting microbes. They are also a small component of the soil microbiome.

General characteristics

- 1- Gram-positive
- 2- Shape: cocci
- 3- Arrangement: grape like clusters
- 4- Facultative anaerobes (capable of growth both aerobically and anaerobically).
- 5- All species grow in the presence of bile salts.
- 6-Catalase positive
- 7- Oxidase negative
- 8- Growth in a 7.5% NaCl solution.
- 9- On Baird-Parker medium, *Staphylococcus* species grow fermentatively, except for *S. saprophyticus*, which grows oxidatively.
- 10-Coagulase: positive \ negative
- 11-Mannitol fermenting positive \ negative
- 12-Motility: negative
- 13-Capsule: positive \ negative
- 14- DNase: positive \ negative
- 15-Blood hemolysis: Beta hemolysis *S.aureus*
- 16- None spore forming

Specimens come to laboratory

Urine, Blood, Stool, swabs from ear, nose .eye, acne, burns and wounds, seminal fluid.

Laboratory Diagnostics

- 1- Gram stain: purple grape like clusters
- 2- Catalase test
- 3- Oxidase test
- 4- Coagulase test
- 5- Motility test
- 6- DNase test
- 7- Grow on milk agar for pigments
- 8- Grow on blood agar for blood hemolysis
- 9- Grow on Mannitol salt agar for mannitol fermenting

Gram stain

What is a Gram stain?

A Gram stain is a laboratory test that checks for bacteria at the site of a suspected infection or in certain bodily fluids. A medical laboratory scientist processes the Gram stain, which gives relatively quick results, so healthcare providers can know if bacteria are present.

Bacteria are a large group of one-celled organisms. They can live in different places in your body and on your skin. While some types of bacteria are harmless or even beneficial, others can cause infections and disease. A Gram stain helps diagnose harmful bacteria.

Under a Gram stain, different kinds of bacteria change one of two sets of colors (pink to red or purple to blue) under a special series of stains and are categorized as “gram-negative” or “gram-positive,” accordingly. Gram staining

works by differentiating bacteria by the chemical and physical properties of their cell walls.

However, not all forms of bacteria can be tested using the Gram stain method, and Gram stains don't usually provide a diagnosis alone. Instead, they help to broadly determine the type of bacteria.

Gram staining is an essential staining technique in microbiology that scientists have used for hundreds of years. It's named after Danish bacteriologist Hans Christian Gram, who first introduced it in 1882, mainly to identify organisms causing pneumonia.

Gram-positive organisms

Gram-positive bacteria have cell walls that contain thick layers of peptidoglycan, a substance that forms the cell walls of many bacteria. The peptidoglycan forms about 90% of the cell wall in gram-positive bacteria. This causes them to appear blue to purple under a Gram stain.

Gram-positive organisms include:

- *Staphylococcus* species.
- *Streptococcus* species.
- *Corynebacterium* species.
- *Clostridium* species.
- *Listeria* species.

Gram-negative organisms

Gram-negative bacteria have cell walls with thin layers of peptidoglycan (10% of the cell wall) and high lipid (fatty acid) content. This causes them to appear red to pink under a Gram stain.

Gram-negative organisms include:

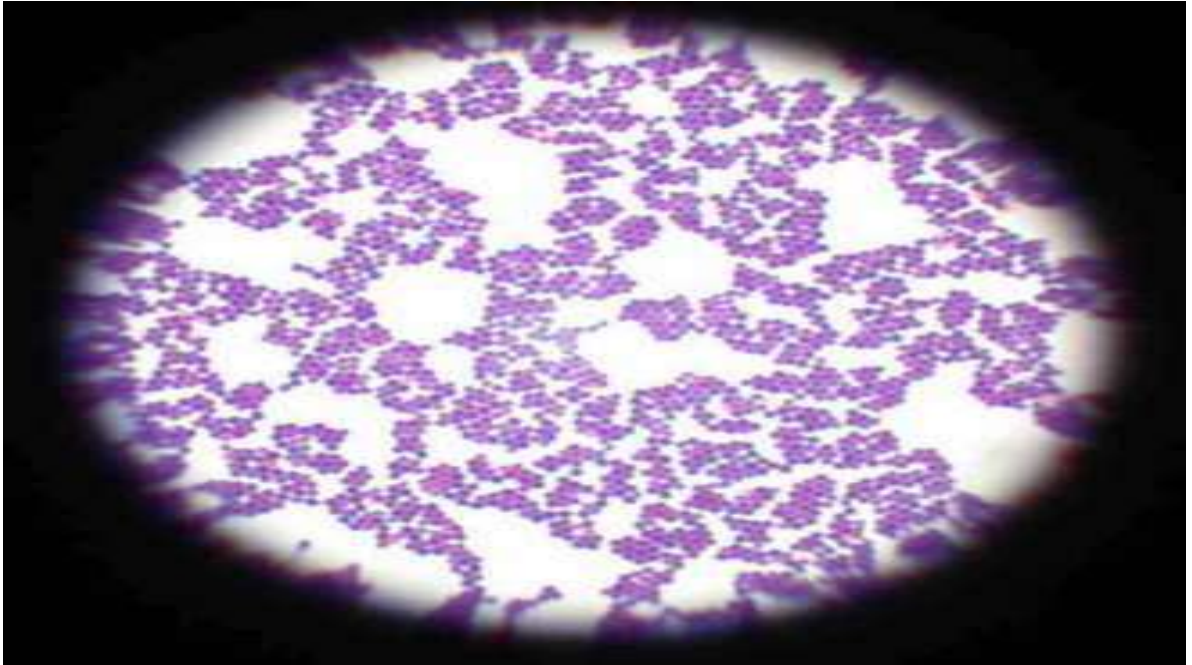
- *Neisseria gonorrhoeae* and *Neisseria meningitides*.
- *Moraxella* species.
- *Escherichia coli* (E. coli).
- *Pseudomonas* species.
- *Proteus* species.
- *Klebsiella* species.

Gram staining procedure

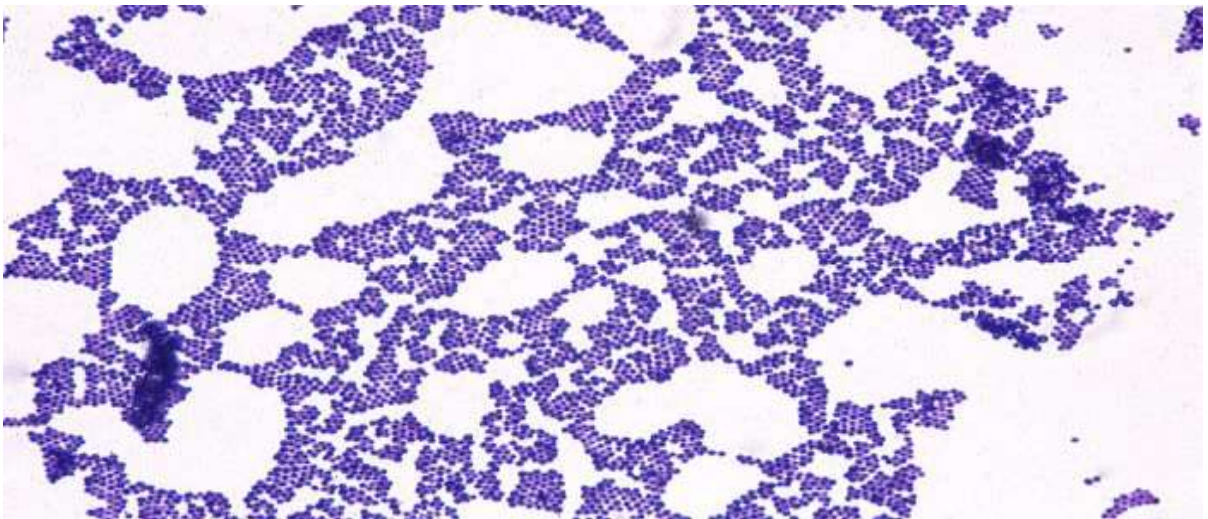
At the lab, a medical laboratory scientist smears or spreads the sample on glass microscope slides. These slides are known as smears. They then apply a series of stains to the smear to perform a Gram stain.

The Gram staining process includes four basic steps, including:

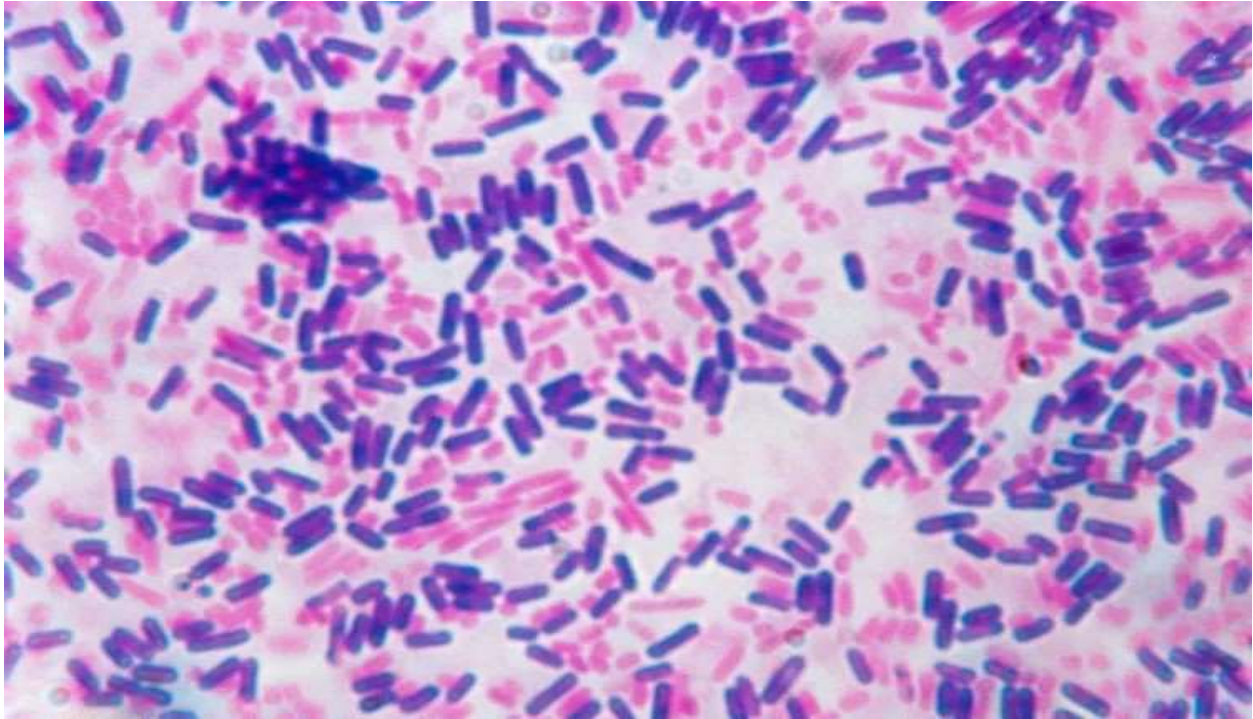
1. Applying a primary stain (crystal violet).1min
2. Adding a mordant (Gram's iodine).1min
3. Rapid decolorization with ethanol, acetone or a mixture of both.20 sec
4. Counterstaining with Safranin.0.5 min



Staphylococcus spp = Cocci , Grape like clusters and Purple



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Red: Gram negativePurple::Gram positive

Mannitol salt agar

Chapman Agar or Mannitol Salt Agar is a selective medium used for the isolation, enumeration and differentiation of Staphylococcus from clinical, food, antiseptic and cosmetic samples.

This medium is both selective and differential agar. The medium will select organisms that can live in areas with a high concentration of salt (sodium chloride) and the fermentation of mannitol, demonstrated by the yellow turn of the pH indicator (phenol red), makes it possible to guide the diagnosis.

Principle of Mannitol Salt Agar

- ◆ The **selectivity** of this medium is based on the presence of **sodium chloride (7.5%)** which inhibits most Gram negative and Gram positive bacteria.
- ◆ The **differentiation** is based on the ability or not to ferment the **mannitol** (the only sugar in the medium). If there is fermentation, this induces acidification which leads, at pH levels below 6.9, to a yellow coloration of the medium in the presence of **phenol red** (pH indicator).

Note :

- In clinical samples, mannitol positive isolates are suggestive of *Staphylococcus aureus* and should be further tested.
- A non-fermenting bacteria that resists the high salt concentration results in a red to pink area due to the degradation of the peptone.

Staphylococci aureus form lush, pigmented colonies surrounded by a yellow halo due to the fermentation of mannitol. Non-pathogenic staphylococci usually form small red colonies which do not change the color of the medium.

Note: The change in color of the medium demonstrates the fermentation of mannitol, NOT the color of the colony. This is particularly important because many micrococci are pigmented.

- Several species of *Staphylococcus* other than *S. aureus* are positive for mannitol and produce yellow colonies surrounded by yellow areas (eg *S. capitis*, *S. xylosus*, *S. cohnii*, *S. sciuri*, *S. simulans*). Therefore, further biochemical testing is needed to identify *S. aureus* or other species.

1-Sugar : mannitol

2- Gram positive & negative inhibitor: NaCl 7.5%

3-PH indicator: phenol red



Figure : (A).*Staphylococcus aureus*: large yellow halo around growth indicates fermentation of mannitol.

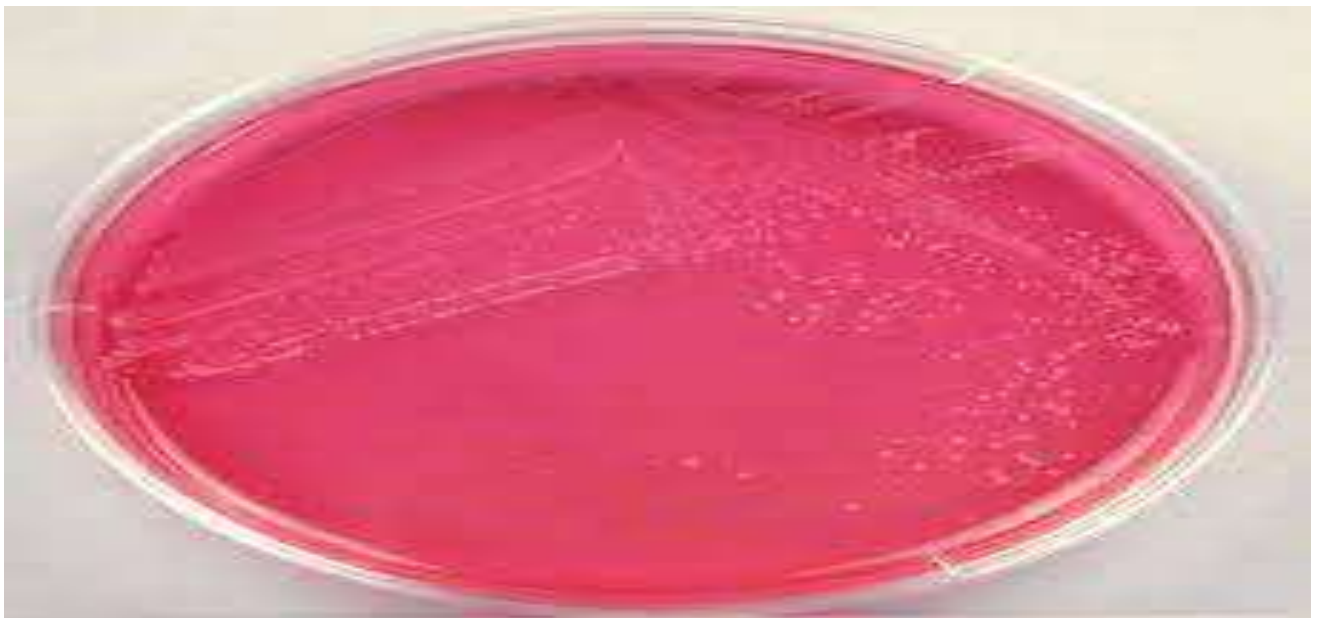
(B). *Staphylococcus epidermidis*: Growth but not color change to the media indicating no fermentation of mannitol.

(C). *Staphylococcus saprophyticus*: small yellow halo around growth indicates fermentation of mannitol. (10% of *S. saprophyticus* ferment mannitol)

(D). *E. coli*: no growth. Inhibited by the 7.5% NaCl



Staphylococcus aureus on mannitol salt agar



Staphylococcus epidermidis on mannitol salt agar

Blood agar

Blood agar is a general purpose, enriched medium often used to grow **fastidious organisms** and to **differentiate bacteria based on their hemolytic properties Beta, Alpha and Gamma hemolysis**. In the U.S., blood agar is usually prepared from tryptic soy agar or Columbia agar base with 5% sheep blood.



Blood agar : Selective differential medium

DNase Test

This test is presumptively used to differentiate *Staphylococcus aureus* which produces the enzyme deoxyribonuclease from other Staphylococci which do not produce deoxyribonuclease (DNase). *Staphylococcus aureus* possesses a heat-stable enzyme, a thermonuclease. To detect this enzyme, first the organisms are destroyed by heat and then the free DNase reacts with the medium. This test is also given positive by *Vibrio*, *Helicobacter*, *Moraxella*, *Serratia*, and *Aeromonas*.

Principle

This test determines the ability of an organism that produce DNase. DNase are extracellular endonucleases that cleave DNA and release free nucleotides and phosphate. To detect these enzymes, DNase agar using no indicators or various indicators (toluidine blue or methyl green) are used to detect the hydrolysis of DNA.

In DNase agar without indicator, the hydrolysis of DNA is observed by a clearing of the agar after addition of HCL (oligonucleotides dissolves in acid but DNA salts are insoluble). The acid precipitates unhydrolyzed DNA making the medium opaque. Therefore, DNase producing colonies hydrolyze DNA and produce a clear zone around the growth.

In case of DNase agar with methyl green, DNA combines with methyl green (act as cation) to produce mint green color. When the DNA is hydrolyzed, the complex is released and the free methyl green is colorless at pH 7.5. So the clear halo is appeared around the areas where DNase producing organism grow.

When toluidine blue O (TBO) is added to the DNase agar, a complex is formed with the DNA, which changes structure when DNA is hydrolyzed, resulting in a bright pink color.

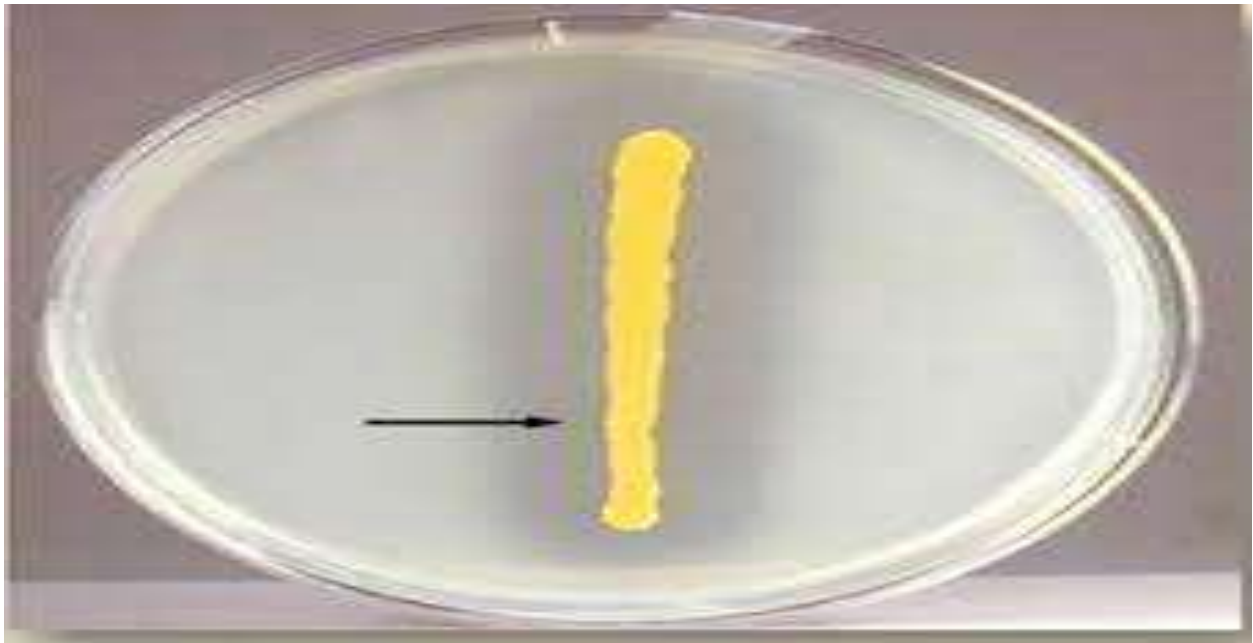
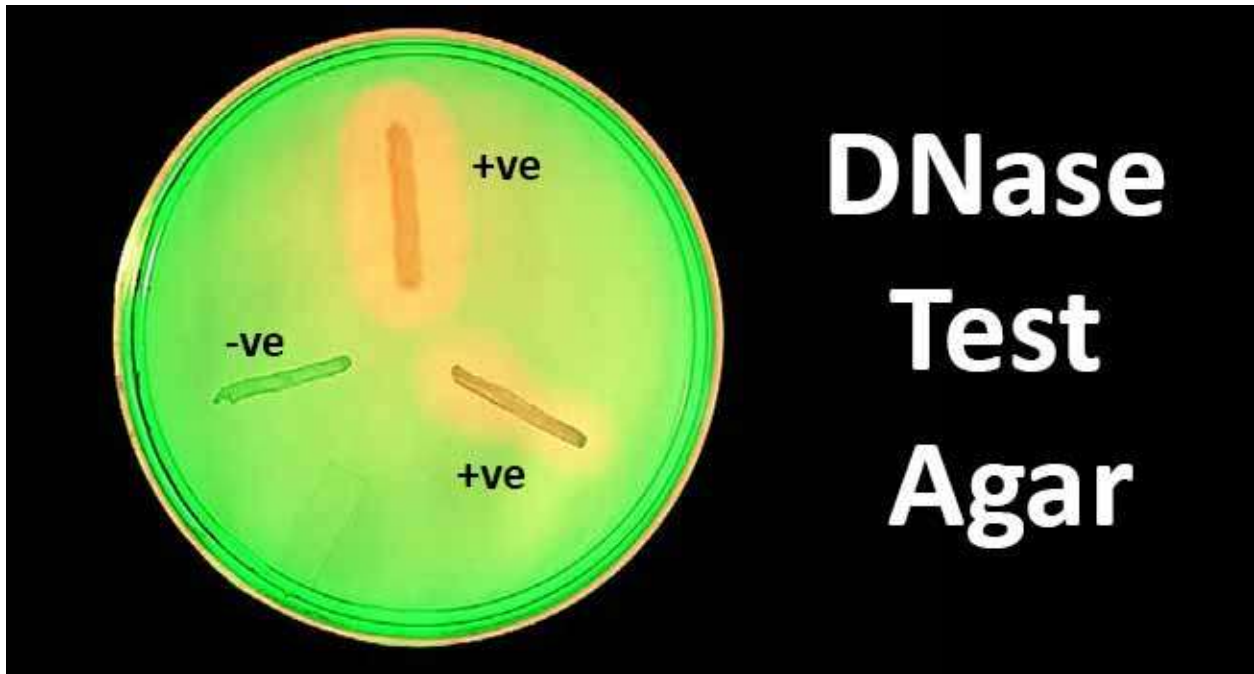
Uses

1. Used to determine the ability of an organism to hydrolyze deoxyribonucleic acid.
2. Used to differentiate *Staphylococcus aureus* which produces the enzyme deoxyribonuclease from other Staphylococci which do not produce DNase.
3. Particularly useful if plasma is not available to perform coagulase test or when the result of coagulase tests are difficult to interpret.

Procedure

DNase test method

1. Using a sterile loop, several colonies from an 18-24 hours culture is picked.
2. Inoculate the test and control organism in each test area.
3. Incubate the plate at 35-37°C for 24 hours.
4. After incubation observe the color change in DNase with methyl green.
5. In DNase agar without indicators:
 - Flood the surface of agar with 1N HCL solution. Tip off the excess acid.
 - Allow the reagent to absorb into the plate.
 - Observe for clear zone around the colonies within 5 minutes.



DNase test positive : *Staphylococcus aureus*

DNase test negative : *Staphylococcus epidermidis*

S. epidermidis:

- **Nutrient agar:** White colonies
- **Blood agar:** Non haemolytic
- **MSA:** Non fermentative

S. saprophyticus:

- **Nutrient agar:** Yellow colonies
- **Blood agar:** Non haemolytic
- **MSA:** Non fermentative

Habitat:

- ***S. aureus:***
 - Nasal passages, skin, oral cavity and gastrointestinal tract.
- ***S. epidermidis:***
 - skin.
- ***S. saprophyticus:***
 - Rarely found in healthy humans.

Differentiation between
Staphylococcus species

Tests	<i>S.aureus</i>	<i>S.epidermidis</i>	<i>S.saprophyticus</i>
Gram stain	Gram+ve cocci	Gram+ve cocci	Gram+ve cocci
Catalase	Positive	Positive	Positive
Oxidase	Negative	Negative	Negative
Blood agar	Beta	Gamma	Gamma
Coagulase	Positive	Negative	Negative
DNase	Positive	Negative	Negative
Mannitol fermentation	Ferment mannitol	Dose not ferment	Dose not ferment
Novobiocin sensitivity	S	S	R
Colony	Golden	White	Light yellow