II. *LABORATORY SESSIONS*
1ST SEMESTER 2000/2001

ITEM	*TOPIC*
1.	Introduction - Safety rules - simple stains
2.	Gram stain
3.	Cultivation of bacteria
4.	Collection of clinical culture specimens (Throat, Sputum, Blood , CSF, Urine)
5.	Precipitation
6.	Antimicrobial susceptibility testing
7.	Agglutination
8.	Gram - Positive cocci
9.	Use of labels in serology
10.	Gram- positive bacilli and gram - negative cocci
11.	Gram-negative bacilli
12.	Gram-negative bacilli
13.	Seminar of Bacteriology
14.	Seminar of Bacteriology

Bacteriology

Precautions in Microbiology Laboratory

1. A void contaminating your hand, table or any object.
2. Do not permit entrance of foreign organisms from the skin or any object into the culture plate or tube.
3. Always flame the mouth of the test tube after removing the closure and immediately before replacing it. Be sure to flame your wire loop before introducing it into a culture and again after you are through with it.

Exercise 1: Size , Shape , motility , of bacteria

1. A several drops from a broth culture of *Proteus* and *Staphylococcus* is mixed gently with erythrocyte suspension in the test tube.
2. Ring the edge of the cover slip with a very thin layer of Vaseline previously prepared.
3. Place a few loopfuls of the preceding mixture on the cover slip previously prepared.
4. Invert a microscopic slide over the cover slip preparation, and quickly invert the side with the cover slip appearing on the top.
5. For microscopic observation, first focus with the low power objective. Bring to the center of the field and change to the high dry power.
6. Compare size and shape of the bacteria. Relate to the red blood cells in your preparations.
7. The movement of the bacteria is of two types: in the case of *Staph* you find Brownian motion. In the case of *Proteus* you find true active motility characterized by the bacteria actually changing position in relation to each other.

Exercise 2: Bacteriological Staining Techniques

The stains employed in the bacteriology may be categorized as :

A. Simple stain : designed primarily to make the bacterial cells more readily visible microscopically, e.g. methylene blue , crystal violet , basic fuchsin

B. Special stain : designed to stain preferentially same structure of the bacterial cell, e.g. spore stain capsule and flagella stain

C. Differential stain : designed to differentiate types of bacteria to facilitate identification, e.g. gram stain , acid-fast stain.

Simple stain :

This exercise is designed to acquaint you with simple staining operation as well as to give you further practice in the handing of pure culture.

Materials :

Fresh (18-24 hour) agar slant culture of *Staph* .albus, *Esch.Coli, bacillus cereus* , loeffler's methylene blue.

Procedure :

1. In order to stain a satisfactory preparation your slide must be cleaned and free of fat.
2. Transfer the wire loop a small drop of water to a slide .flame the wire loop and remove a small amount of culture from the agar slant or on agar plate. Mix the bacteria in the drop of water by spreading it over an area of 1 cm^2 . flame your loop after this operation before setting it aside for economy place the three types of bacteria on a single slide.
3. Let the smear air-dry them fix the film by passing the slide through the Bunsen burner, flame two or three times. Don't let get too hot .
4. Place the slide on the staining rack in the sink. Flood the surface with methylene blue staining solution. After staining for one minute wash gently with running water to remove the excess methylene blue. Either allow the slide to dry naturally or carefully blot it dry paper toweling.
5. Stain smear of bacteria are examined commonly with oil immersion objective and without a cover-glass. Examine the smear and compare the each organism with respect to size ,shape and intensity of staining.

GRAM STAINING

INTRODUCTION:

The Gram stain is a **DIFFERENTIAL STAIN** which allows most bacteria to be divided into two groups. Gram-positive bacteria and Gram-negative bacteria. The technique is based on the fact that the Gram positive cell wall has a stronger attraction for crystal violet when Gram's iodine is applied than does the Gram negative cell wall. Gram's iodine is known as a **MORDANT**. It is able to form a complex with the crystal violet that is attached more tightly to the Gram-positive cell wall than to the Gram-negative cell wall.

This complex can easily be washed away from the Gram-negative cell wall with ethyl alcohol. Gram-positive bacteria, however, are able to retain the crystal violet and therefore will remain purple after **DECOLORIZING** with alcohol. Since Gram-negative bacteria will be colorless after decolorizing with alcohol, **COUNTERSTAINING** with safranin will make them appear pink.

The Gram stain is probably the most commonly used staining procedure in microbiology. It is extremely useful in identifying bacteria. It is important that you understand the color changes that occur at each step in the Gram stain. It is also important that you understand the function of each reagent used in this procedure. It takes some practice and patience to be able to reliably Gram stain.

MATERIALS:

1. 2 microscope slides Gram stain reagents (crystal violet, Gram's iodine, 95%.
   ethyl alcohol, and safranin)
2. Fresh cultures of ***S. epidermidis*** **and** ***E.coli*** **mixture**
3. Fresh cultures of ***Bacillus subtilis and Diphteroid bacilli***

PROCEDURE:

SMEAR PREPARATIONS: **Remember to label the slides.**

1- S. epidermidis **and E.coli mixture:**
2- B. subtilis and **Diphteroid bacilli mixture:**

Prepare smear using aseptic technique.SEE PINK EXERCISE for SMEAR PREPARATION!! **THE FIRST LOOPFUL OF ORGANISM IS NOT SMEARED OUT UNTIL THE SECOND ORGANISM HAS BEEN ADDED.** The two organisms are then smeared out together. After air drying and heat fixing the Gram staining procedure is followed.

Prepare smears using aseptic technique. These organisms are growing on TSA slants. A loopful of distilled water is first placed on each slide. Bacteria are obtained from the slants using a sterile **needle** and proper aseptic technique. SEE PINK EXERCISE for SMEAR PREPARATION!! After air drying and heat fixing the Gram staining procedure is followed.

GRAM STAINING PROCEDURE:

1. Cover smear with **CRYSTAL VIOLET** for 20 seconds.

(PRIMARY STAIN)

2. Gently rinse off the stain with water and shake off the excess.

3. Cover with **GRAM'S IODINE** for one minute (MORDANT)

4. Pour off the Gram's iodine.

5. Run 95% **ETHYL ALCOHOL down the slide** until the solvent runs clear (about 10-20 seconds). THIS STEP IS CRITICAL! THICK SMEARS REQUIRE MORE TIME

THAN THIN ONES (DECOLORIZING AGENT)

6. Rinse with water to stop the action of the alcohol.

7. Cover with SAFRANIN for 20 seconds (COUNTER STAIN).

8. Gently rinse off the stain with water. Blot with filter paper and clean off the bottom of the slide with 95% alcohol.

HELPFUL SUGGESTIONS to perform good smears

a) DO NOT make your smears too thick!

b) Be very careful when you decolorize.

c) Be sure your cultures are young, preferably 18-24 hours old. Older cultures tend to lose the ability to retain stains.

RESULTS:

Observe your smears in the microscope using oil immersion lens as follow:

Staphylococcus Gram-positive cluster of cocci

E.coli Gram-negative bacilli

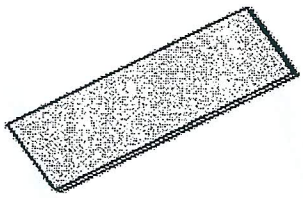
Bacillus Gram-positive, spore-forming bacilli

Diphteroid Gram-positive pleomorphic small cocci to bacilli

GRAM STAINING

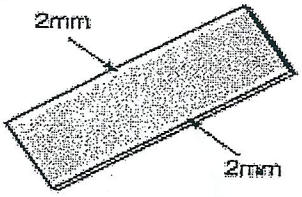
Flow Through Procedure

1



Wipe bottom of biofilm slide clean

2

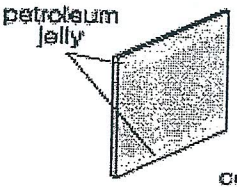


Clean top edges of slide about 2mm

3

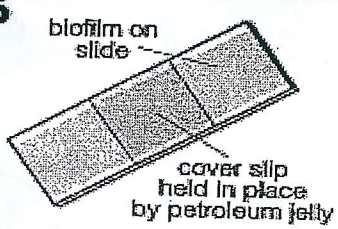
Build up a ridge of petroleum jelly on the top and bottom of a cover slip

4



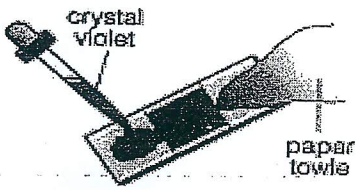
Cover slip with petroleum jelly

5



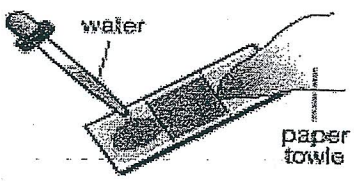
Biofilm on slide with cover slip

6



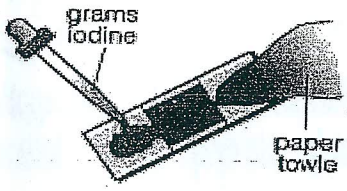
Add crystal violet-wait 30 sec.

7



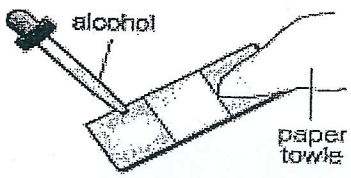
Wash with water

8



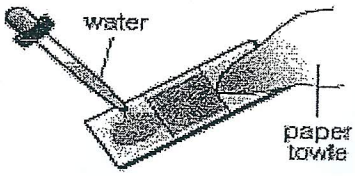
Add Grams Iodine-wait 1.5 min.

9



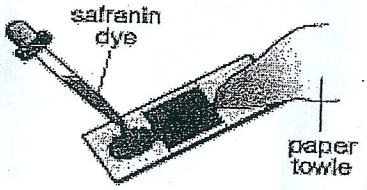
Decolorize with alcohol

10



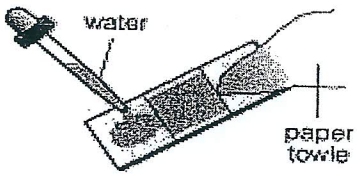
Wash with water

11



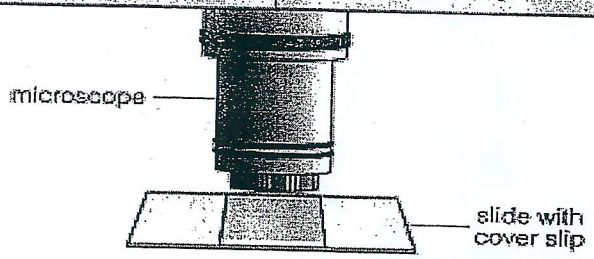
Stain with Safranin dye-wait 30 sec.

12

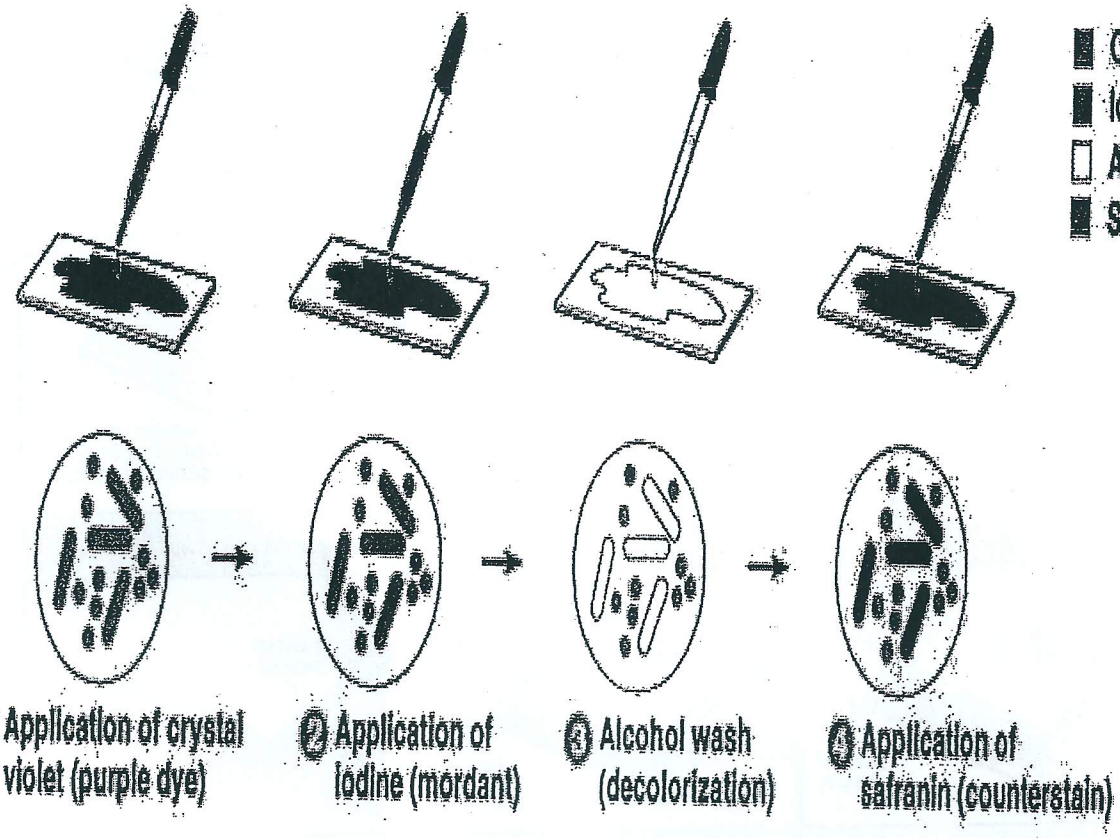


Wash with water

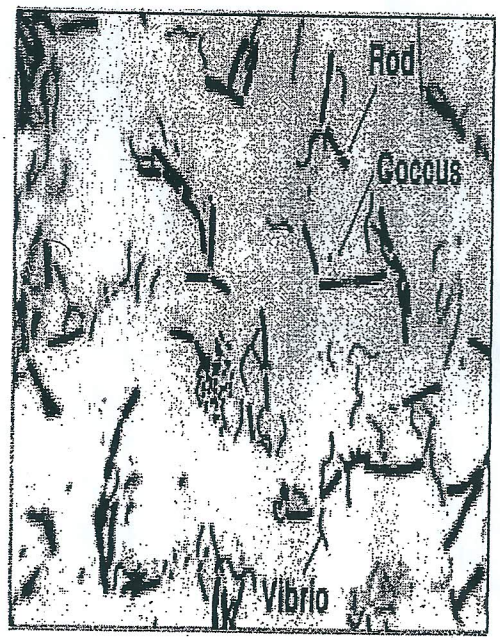
13



Examine under oil immersion through the cover slip



(a)



(b)