بسم الله الرحمن الرحيم

كليـــــة نبتــــــه

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Medical Laboratory Sciences Program

Department of Microbiology

Student Practical log book

Semester 4

2018-2019

Name: ……………………………………………

Student ID: …………………...............................

Batch: ………...

Prepared by : Baydaa f. Hammad

Instructions

- **Wear gloves when in contact with body fluids, such as serum, plasma, urine or whole blood**
- **Wash your hands when gloves are removed or changed**
- **Perform procedures carefully to minimize aerosol formation**
- **Wear protective clothing such as laboratory coats or aprons when working with specimens**
- **Keep your hands away from your face**
- **Cover all superficial cuts before starting any work**
- **Dispose of specimens and other contaminated materials according to you laboratory's biohazard control procedure**
- **Keep your work area disinfected, disinfect tools and other items that have been in any contaminated area.**
- **Do not eat or drink or apply cosmetics while in the laboratory**

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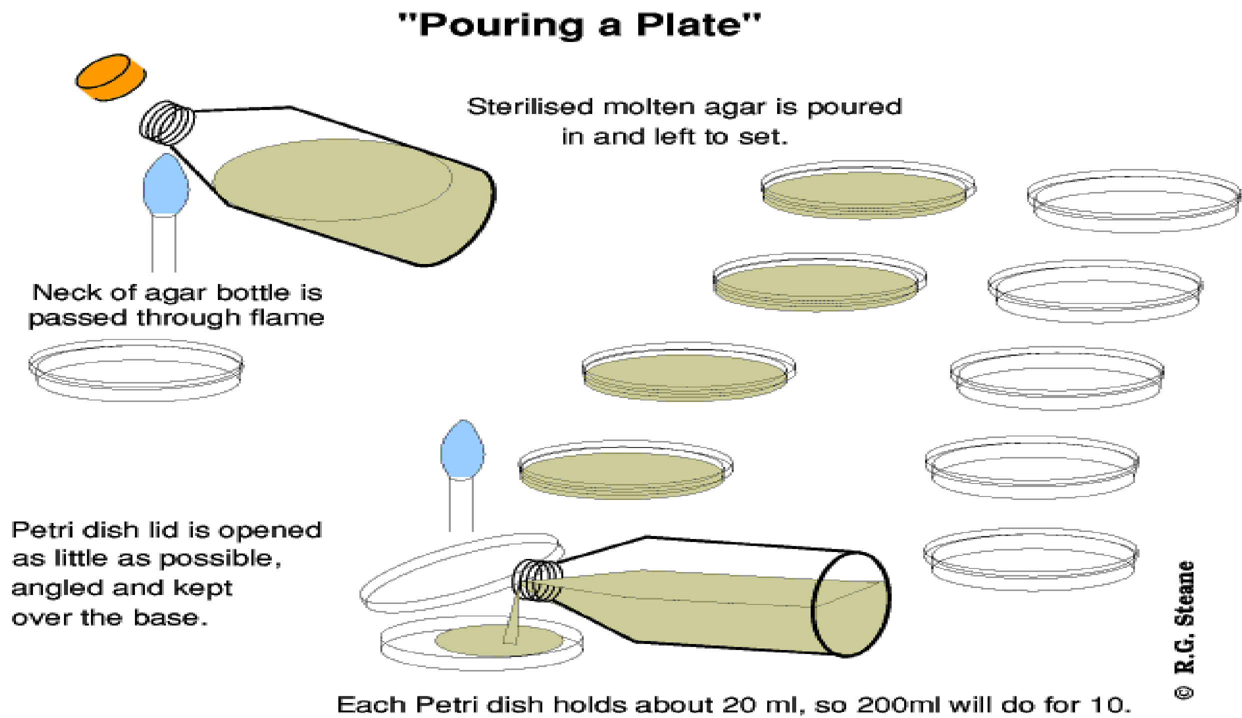
Lab (1) <u>Preparation of culture media</u>

Medium (media, plural): a nutrient blend used to support microbial growth.

Preparation steps:

1. Re-hydrate powder according to manufacturer's instructions.
2. Sterilization of Media by Autoclaving ,Filtration,…etc
3. Pouring (dispensing)



4. Solidification of Media (cooling)
5. Drying of Media (Hot Air Oven)
6. Storage of Media (Refrigerator)

Student activity (1)

A. Preparation of Nutrient Agar Plate
   1) Re-hydration calculations

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B. Preparation of Nutrient Agar slope

Review Questions

1. Types of Media Based on the physical state
   1)……………………………………………
   2)……………………………………………
   3)……………………………………………
   2. Give 2 examples on:
   1) Enriched Media……………………………
   2) Selective Media……………………………
   3) Transport Media……………………………

Student activity (2)

1. Preparation of Blood agar
   Calculations
   ………………………………………………
   ………………………………………………
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   ………………………………………………
   ………………………………………………
   ………………………………………………
2. Preparation of Chocolate Blood agar

Calculations

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Review Questions

1) Component of Blood Agar Media

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2) Difference of Blood Agar & Chocolate Blood Agar

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Lab (2) Aseptic Technique and Inoculation

Aseptic Techniques are practices that prevent the contamination of growth media Inoculation: Producing a pure culture

Inoculation

is the purposeful introduction of bacteria into a sterile growth medium.

A material is sterile when it has no living organisms present.

contamination is the presence of unwantedmicroorganisms.

Isolation: Colony on media, one kind of microbe, pure culture

Incubation: growing microbes under proper conditions

Aseptic techniques include the following practices:

1. Minimize the time that cultures and growth media are open to the environment.
2. Disinfect the work area before and after use.
3. Do not touch or breathe into the sterile culture media or the stock cultures.
4. Loops, needles, pipets, etc. should be sterilized before they are used
5. When working with tubes, the tube caps should not be placed on the table top; they should be held in your hand while inoculating.
6. When removing the caps from test tubes,

flame the lip of the test tube after the cap is removed. This heats the air inside the tube, so the air moves out of the tube, preventing contaminants from entering the tube

Inoculation and Transfer Techniques:

1. Sterilize an inoculating loop or needle in the flame of a Bunsen burner .Remove the loop from the flame after it is properly heated. Let it cool.
2. Pick a small amount of bacteria (only touch one colony).

3.Accordingto Media used:

1. Streak-plate technique :
    1) With the loop, spread the inoculum back and forth across the upper 1/4 of the plate, keeping the lines of inoculation very close together (area 1 in figure below).
    2) Isolated colonies are not expected in this area. Do not use strong pressure, which will break the surface of the agar. Use the end of the loop, not its side when streaking. Dispose of the loop in the biohazard bucket on the bench.
    3) Turn plate approximately 90oC. Streak the plate as indicated in the figure (area 2) across about 1/4 of the plate. Dispose of the loop.
    4) Repeat step 2 one or two times more.
    5) In area 3 and/or 4 single colonies should appear.
    6) Label plates on the bottom and incubate inverted at 37oC.

A

Flame loop

Turn plate through 90°

B

Flame loop

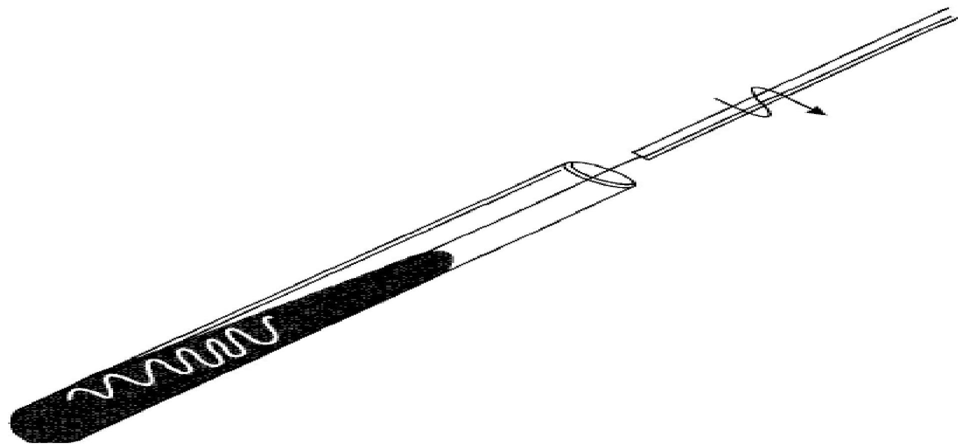
Turn plate through 90°

C

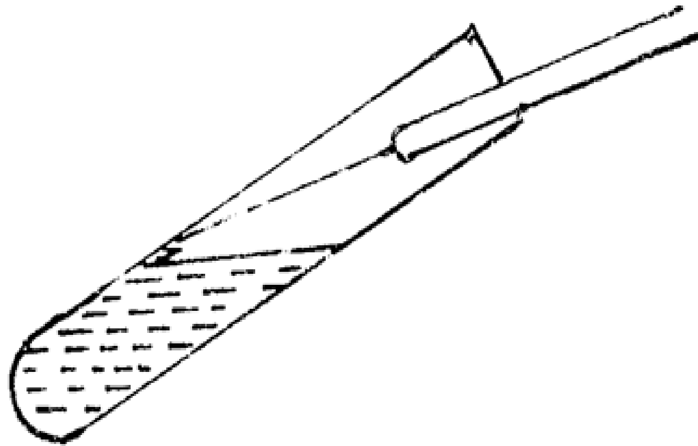
Turn plate through 90°

D

2 .Slant inoculation



7) Liquid medium inoculation

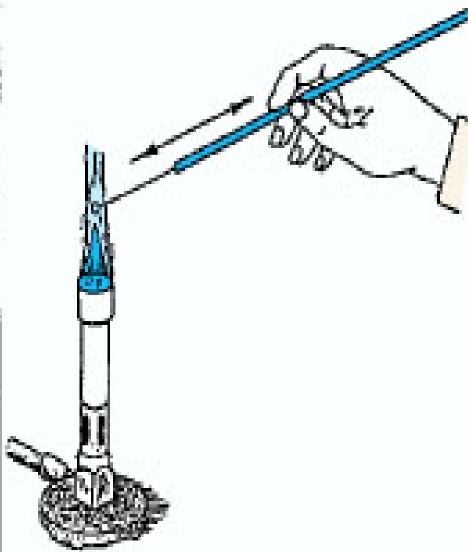


Inoculating from a tube of broth or an agar slant:

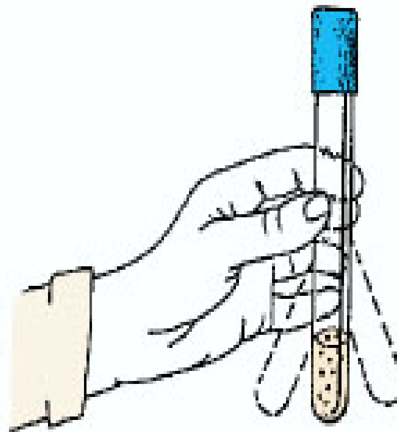
1. Flame the loop.

Without setting the loop down, open the first culture tube and flame the mouth. Do not set the cap on the bench. The cap should be held in the same hand as the loop.

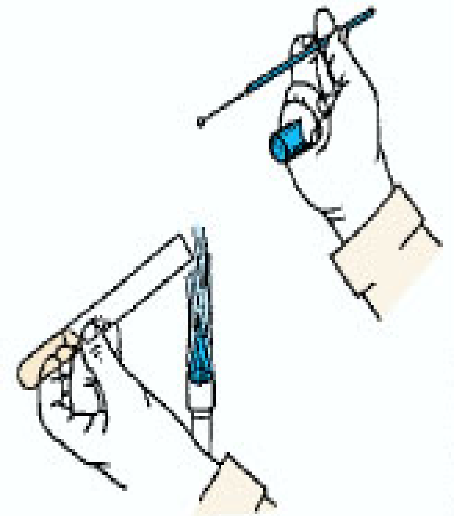
2. Insert the loop into the culture medium, then withdraw it.
3. Flame the mouth of the first culture tube again, and replace the cap.
4. Open the second culture tube and flame the mouth. Do not set the cap on the bench. The cap should be held in the same hand as the loop.
5. Insert the loop into the second culture tube and spread the culture suspension (on the loop) inoculum into/onto the second culture medium
6. Flame the mouth of the second culture tube, then replace the cap.
7. Flame the loop and set on the bench.
8. When in doubt about the sterility of an instrument or container, sterilize it.



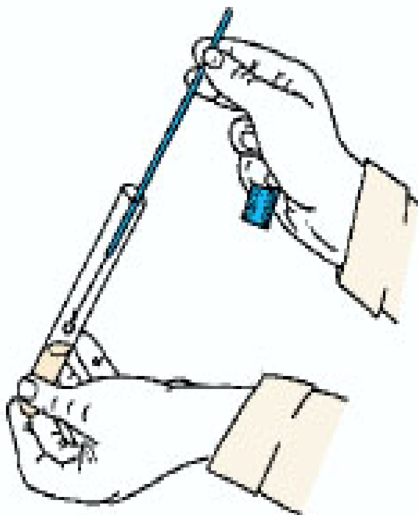
1 Inoculating loop is heated until it is red-hot.



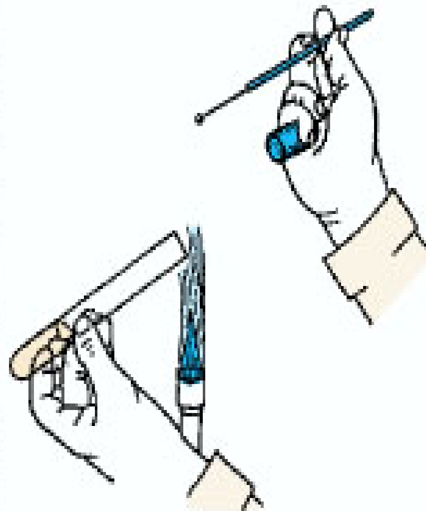
2 Organisms in culture are dispersed by shaking tube.



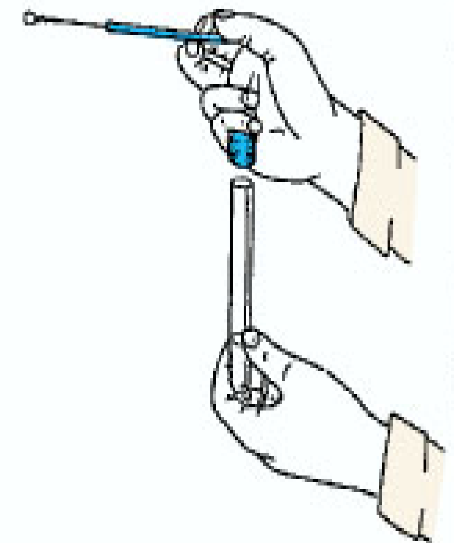
3 Tube enclosure is removed and mouth of tube is flamed.



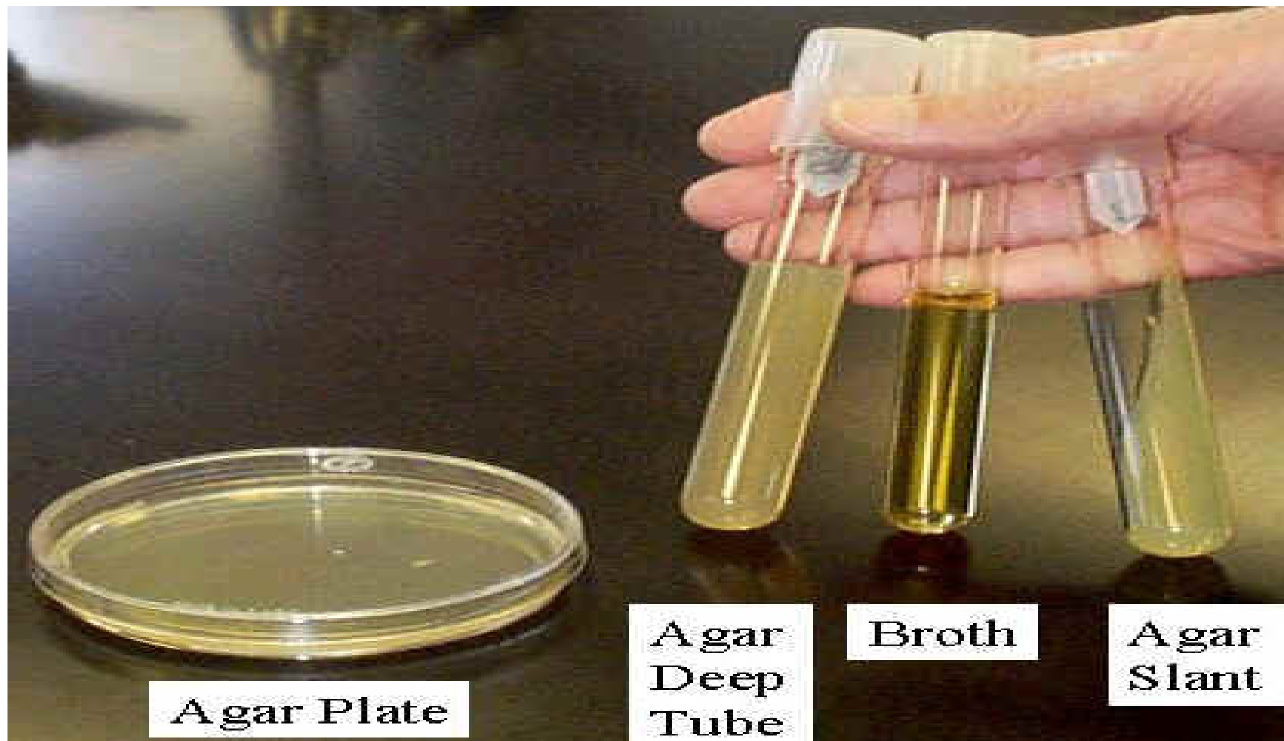
4 A loopful of organisms is removed from tube.



5 Loop is removed from culture and tube mouth is flamed.



6 Tube enclosure is returned to tube.

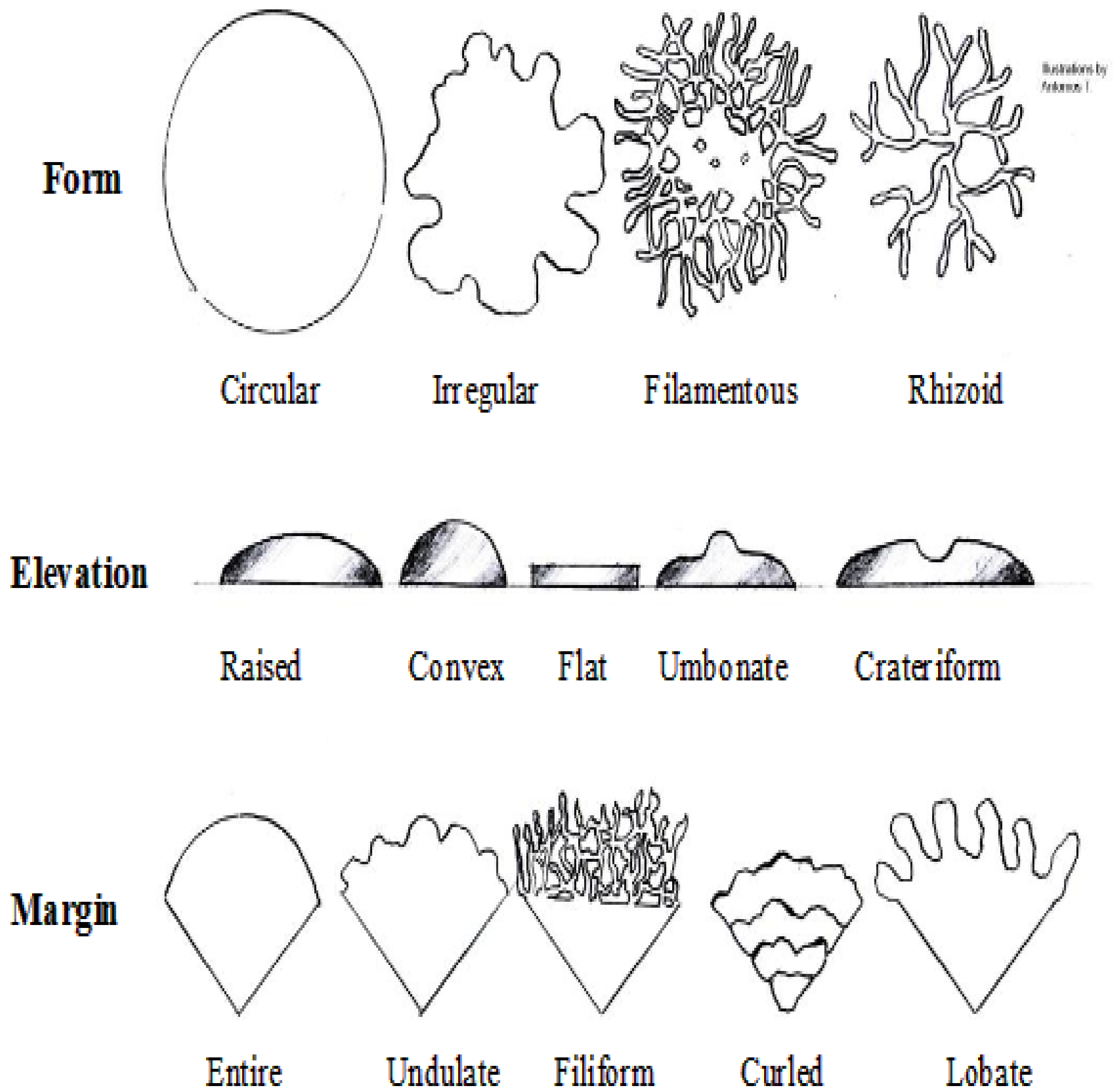


Notes about Labeling and Incubating Plates

1. Always label your plates/tubes BEFORE you do your inoculations. You can use Sharpies on the plates, but
wax markers ONLY on tubes. When labeling tubes, label the tube itself—don't label the cap!
2. Make sure you label the bottom of the plates (the part of the plate that holds the agar).
3. Place plates inverted (upside down) for incubation.

Growth Characteristics:

Growth Characteristics on Plates



Student Activity

Day One

1. Inoculating of Agar Plate
   ……………………………………………………
   ……………………………………………………
   ……………………………………………………
2. Inoculating of Slant

…………………………………………………….

…………………………………………………….

…………………………………………………….

3. Inoculating of Broth
   ……………………………………………………..
   ……………………………………………………..
   ……………………………………………………..
4. Colonial Morphology:

Media	Color	Size	Reaction	Elevation	Pigment	Margin

Day Two

Detection of Bacterial Growth

1. Agar Plate
   …………………………………………………
   …………………………………………………
   …………………………………………………
2. Slant

…………………………………………………

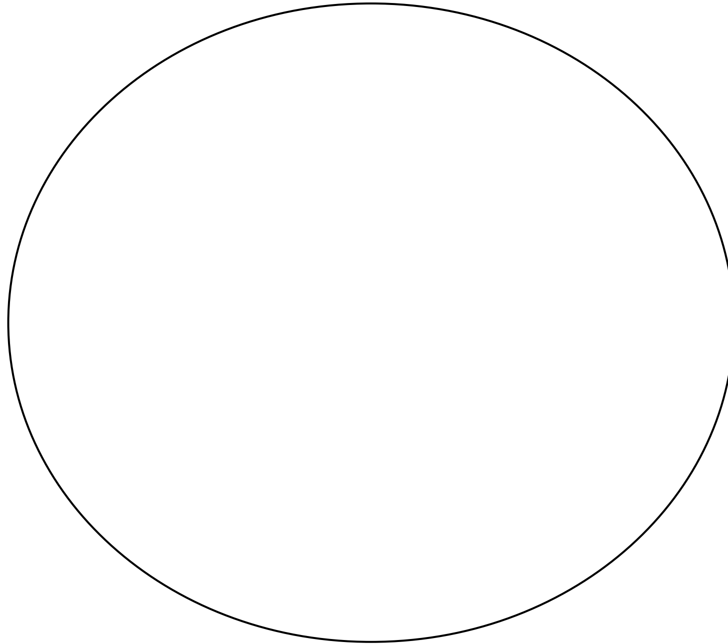
…………………………………………………

…………………………………………………

3. Broth
   …………………………………………………
   …………………………………………………
   …………………………………………………

Review Questions

1. Draw a streaking pattern of plate Agar



Lab (3) Biochemical Tests For Gram Positive Bacteria

1. **Bile solubility test**

Principle

A heavy inoculum of the test organism is emulsified in physiological saline and the bile salt sodium deoxycholate is added. This dissolves *S. pneumoniae*as shown by a clearing of the turbidity within 10–15 minutes. Viridans and other streptococci are not dissolved and therefore there is no clearing of the turbidity.

Required

— 10% Sodium deoxycholate

— Physiological saline (sodium chloride, 8.5 g/l)

1. Emulsify several colonies of the test organism ina tube containing 2 ml sterile physiologicalsaline, to give a turbid suspension.

2. Divide the organism suspension between twotubes.

3. To one tube, add 2 drops of the sodium deoxycholatereagent and mix.

4. To the other tube (negative control), add 2 dropsof sterile distilled water and mix.

5. Leave both tubes for 10–15 minutes at 35–37 C^0.

6. Look for a clearing of turbidity in the tube containingsodium deoxycholate,

Results

Clearing of turbidity positive result

No clearing of turbidity Negative result

2. Catalase test

This test is used to differentiate those bacteria thatproduce the enzyme catalase, such as staphylococci, from non-catalase producing bacteria such as streptococci.

Principle

Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer. The culture should not be more than 24hours old.

Required

Hydrogen peroxide, 3% H_2O_2 (10 volume solution)

Method

1. Pour 2–3 ml of the hydrogen peroxide solution into a test tube.

2. Using a sterile wooden stick , remove several colonies of the test organism and immerse in the hydrogenperoxide solution.

3. Look for immediate bubbling

Results

Active bubbling . . . . Positive catalase test

No bubbles . . . . Negative catalase test

Note: false positive result when using blood culture (catalase is present in red cells)

3. Coagulase test

This test is used to identify *S. aureus* which produces the enzyme coagulase.

Principle

Coagulase causes plasma to clot by converting fibrinogen tofibrin. Two types of coagulase are produced by most strains of*S. aureus*:

1. Free coagulase which converts fibrinogen to fibrin by activatinga coagulase-reacting factor present in plasma. Free coagulase is detected by clotting in the tube test.
2. Bound coagulase (clumping factor) which convertsfibrinogen directly to fibrin without requiring a coagulasereactingfactor. It can be detected by the clumping ofbacterial cells in the rapid slide test.

Required

EDTA anticoagulated human plasma

<u>Slide test method (detects bound coagulase)</u>

1. Place a drop of distilled water on each end of a slide or on two separate slides.

2. Emulsify a colony of the test organism (previouslychecked by Gram staining) in each of thedrops to make two thick suspensions.

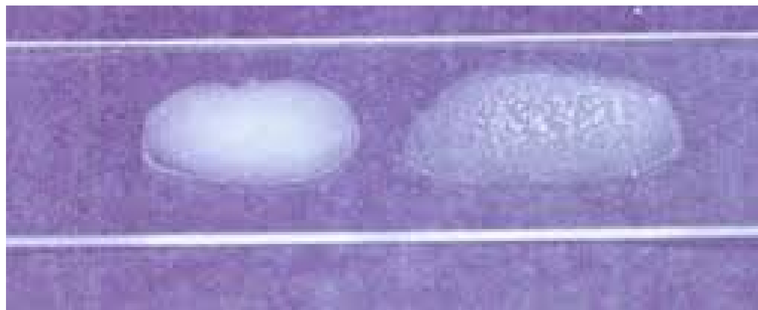
3. Add a loopful of plasma to one of thesuspensions, and mix gently. Look for clumpingof the organisms within 10 seconds.

No plasma is added to the second suspension.

Results

Clumping within 10 secs . . . . .Positive*S. aureus*

No clumping within 10 secs . . . . Negative No boundcoagulase



Tube test method (detects free coagulase)

1. Take three small test tubes and label:

   (T) Test organism (broth culture)

   (P) Positive control (*S. aureus*brothculture)

   (N) Negative control (sterile broth)

2. Pipette 0.2 ml of plasma into each tube.

3. Add 0.8 ml of the test broth culture to tube T.

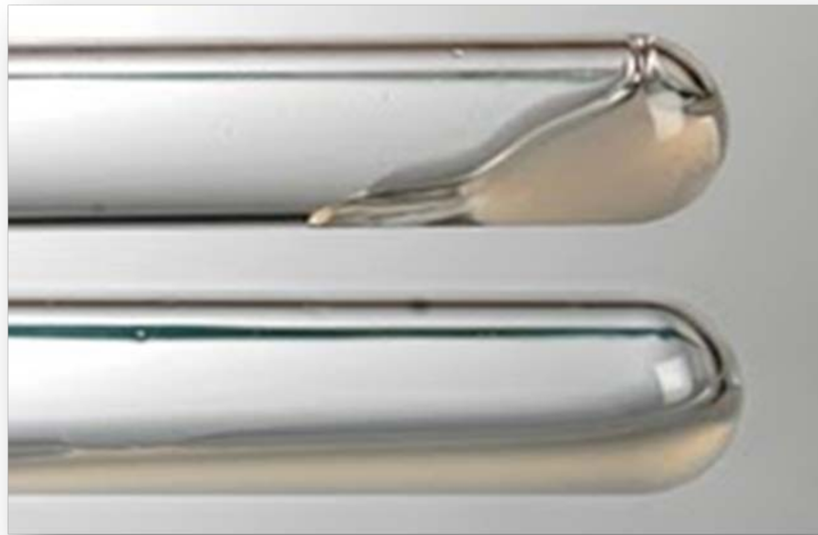
4. Add 0.8 ml of the *S. aureus*culture to the tubelabelled(P).

5. Add 0.8 ml of sterile broth to the tube labeled (N).

6. After mixing gently, incubate the three tubes at35–37 C^0. Examine for clotting after 1 hour If no clotting has occurred, examine after3 hours. If the test is still negative, leave the tube at room temperature overnight and examine again.

Results

Clotting of tube contents   positive result



4.DNA-ase test

Principle

Deoxyribonuclease hydrolyzes deoxyribonucleic acid (DNA). The test organism is cultured on a medium which contains DNA. After overnight incubation, the colonies are tested for DNA-ase production by flooding the plate with a weak hydrochloric acid solution. The acid precipitates unhydrolyzed DNA. DNA-ase-producing colonies are therefore surrounded by clear areas due to DNA hydrolysis.

Required

— DNA-ase agar plate

— Hydrochloric acid Reagent (1 N)

Method

1. Using a sterile loop or swab, spot-inoculate thetest

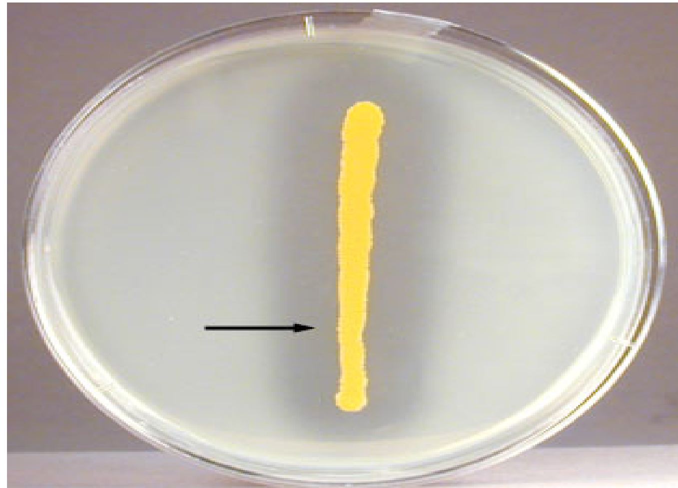
3. Incubate the plate at 35–37 C^0 overnight.

4. Cover the surface of the plate with 1 mol/lhydrochloric acid solution. Tip off the excessacid.

5. Look for clearing around the inoculate within 5minutes of adding the acid.

clearing around the colonies . . . . positive result

No clearing around the colonies . . . . Negative result



5.Litmus milk decolorization test

Principle

A heavy inoculum of the test organism is incubated for up to4 hours in a tube containing litmus milk. Reduction of the litmus milk is indicated by a change in colour of the medium from mauve to white or pale yellow.

Method

1.Using a sterile loop, inoculate 0.5 ml of sterilelitmus milk medium with the test organism.

2 .Incubate at 35–37 C^0

Results

White or pale yellow-pink colour . . . . Positive result

No change or a pink colour . . . . Negative result

6.Aesculin hydrolysis test

Requirement:

Bile esculine agar

Method:

1. inoculate the test organism on bile aesculin agar using wire loop
2. inacbate at 37C^{O} overnight

Result

Blacking of Media	Positive result
No Blacking of Media	Negative result

Biochemical Tests for Gram Negative

1.Indole test

Principle

Bacteria that possess tryptophanase can utilize tryptophan by deamination and produce the indole.

Free indole is detected by p-dimethylamino-benzaldhyed, whose aldhyde group reacts with indole forming Red color complex .

Aim:

Identification of Enterobacteriacae

Requirement:

Broth media contain typtophane.g: peptone water ,neutrient broth .

Kovac reagent or Ehrlichk's reagent

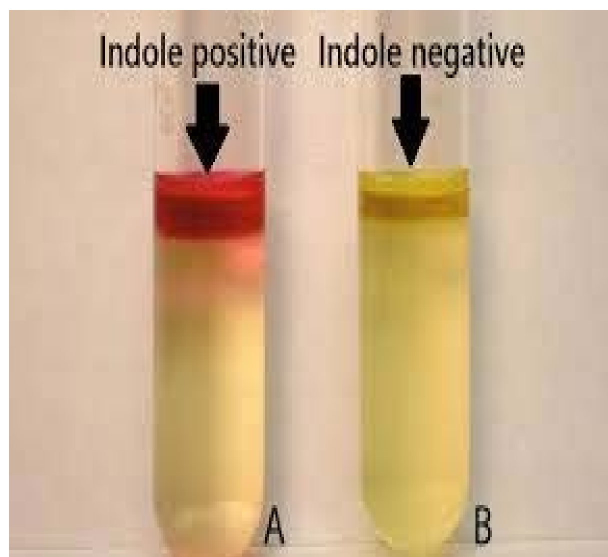
Method:

1. inoculate organism on pepton water
2. Inculation at 37c over night
3. Add covac's reagent

Result :

Red color ………….. Positive test

No red color ….. Negative test



2.Sugar fermentation test

This test is used to assist in the identification of gram negative rods.

Principle:

Certain organism ferments the carbohydrate and produce acid which change the color of indicator used.

Some bacteria produce acid and gas from sugar fermentation, the gas can be detected in inverted durham tube.

Required:

1. Sugar peptone water medium (phenol red as indicator).
2. 4 test tube contain different sugar.
3. Durham tubes.

Method:

1. Inculate a colony of the test organism in the sterile media.
2. Incubate the media at 37c for 24 hrs.

result:

color change from red to yellow:……..positive test.

Color remain red……negative.

Durham tube raised….gas production.

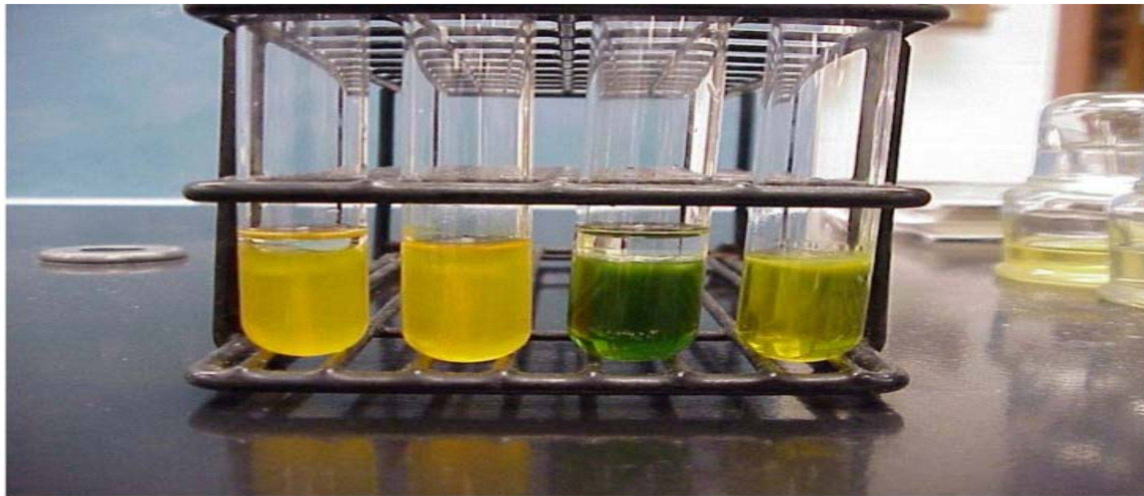
3.Oxidation Fermentation Test (OF test) :

-Oxidation: utilization of carbohydrate in the presence of oxygen.

-Fermentation: utilization of carbohydrate in the presence or absence of oxygen.

-Carbohydrates utilization produce acid , this lower the pH change indicator color .

-2 tubes huge &lephthon medium & sterile wax are used



O-F glucose media
http://academic.mwsc.edu/jcbaker/bio390sec01/bio390_laboratory_study_images.htm

Dr.T.V.Rao MD 75

4.PPA:

Phenylalanine deaminase tests the ability of an organism to produce this enzyme remove the amine group from the amine acid phenylalanine and releases the amine group as free ammonia, as a result of this reaction, phenylpyruvic acid also produced.

Phenylalanine agar , also known as phenylalanine deaminase medium, contains nutrients and DL-phenylalanine. It is used to differentiate members of the genera proteus ,Morganella (which were originally classified under the genus proteus), and providencia from other enterobacteriaceae.

5.Methyl red test (MR) :

This test is used to assist in the identification of gram negative rods .

Principle :

It depends on the ability of certain organisms to ferment glucose with the production of sufficient acidity to give a red color with the indicator methylered .

Required :

Glucose phosphate peptone water medium

Method:

1- Using sterile straight wire inoculate a colony of a test organism into sterile 2%Glucose phosphate peptone water medium

2- Incubate the medium at 37°C for 24 hrs.

3- Add 5 drops of methyl red indicator .

4- Mix and look for color change of medium

6.Voges-proskauer test :

This test is used to assist in the identification of gram negative rods

Principle :

Determine whether the product of glucose is

Acetyl methyl carbinol(acetoin)

Acetoin reacts with omera reagent to form a pink to rosy red ring in the medium

In negative result the tube remains brown to yellow

Required :

*2%Glucose 6-phosphate peptone water medium .

*Alpha naphthol .

*40% KOH .

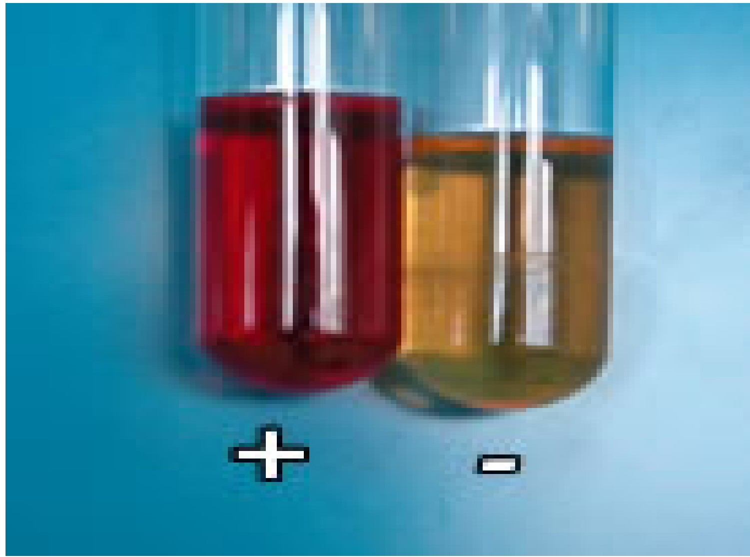
Method :

1- Using sterile straight wire inoculate the test organism into Glucose phosphate peptone water medium .

2- Incubate the medium at 37°C for 24 hrs.

3- Add 0.3 ml of alpha naphthol and 0.1 ml of 40% KOH solution .

5- shake well and look for color change of medium after 2- 5 mins.



7.Urease

Urease hydrolyzes urea releasing amonia

amonia alkalinize the medium by forming amonium and carbon dioxide .

The PH indicator is phenol red becomes red .

Proteus ,morgenella , and providencia are strong urease producer .

Klebsilla and enterobacter a weak urease producer

Yersinia enterocolitica frequently a urease producer .

Required

Christensen urea agar or broth

Method :

Inoculate the media with tested organism

Incubate inoculated media at 37c over night .

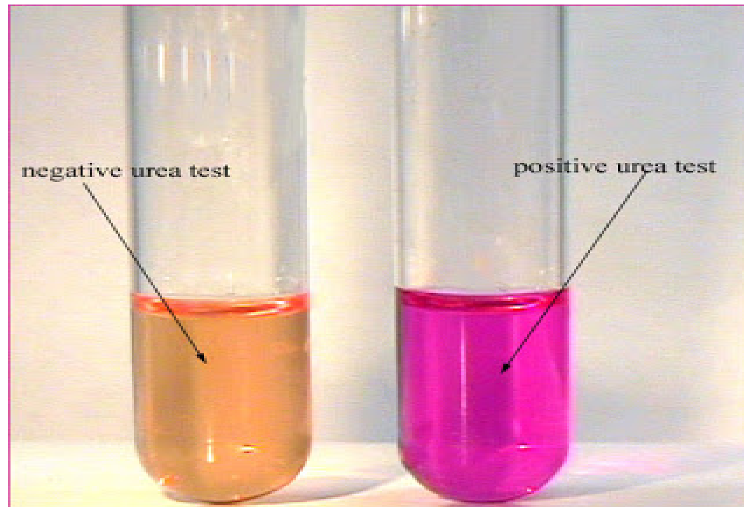
RESULT

Red-pink color ……… positive result

No red –pink color …… negative result

Control positive urease :proteus vulgaris

Control negative urease: Escheria coli



8.Oxidase test

This laboratory test is based on detecting the production of the enzyme cytochrome oxidase by Gram-negative bacteria.

It is a hallmark test for the *Neiserria*

It is also used to discriminate between aerobic Gram-negative organisms like *Pseudomonas aeruginosa* and other *Enterobacteriaciae*

Procedure:

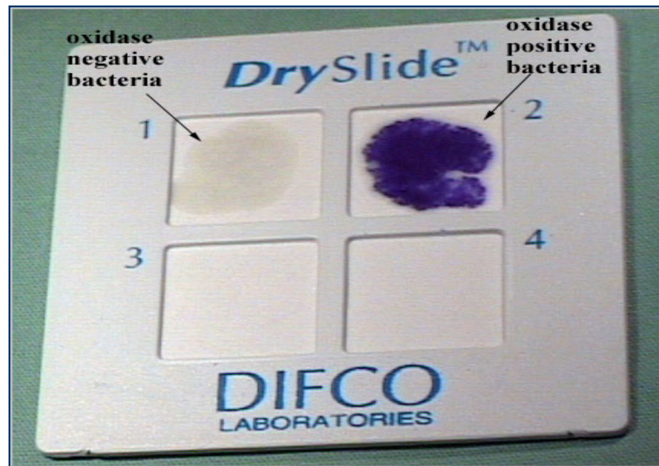
Pick a colony for testing using a sterile wooden toothpick.

Transfer the colony to the surface of one of the four grid areas on the Oxidase Test slide .

The test slide surface is impregnated with the reagent tetramethyl-*p*-phenylenediaminedihydrochloride. This reagent causes a dark purple color to appear in the presence of cytochrome oxidase .

Observe the color change. The reaction color will change from pink to maroon to dark purple .

Read the test results within 10 seconds. Some organisms may show slight positive reactions after this period and such results are NOT considered definitive.



9.Citrate utilization test

This test is use to assist in the identification of gram negative rods.

Principle:

It depend on the ability of the organism to utilize citrate as its only source of carbon and the ammonium phosphate as its only source of nitrogen

Bacteria that can utilize citrate will extract nitrogen from ammonium phosphate releasing ammonia, produce an alkaline pH & the indicator bromo_thymol blue.

Biochemical property of salmonella, klebsiella, citrobacter, enterobacter, serratia.

Required:

Kossers citrate medium or Simmons citrate agar

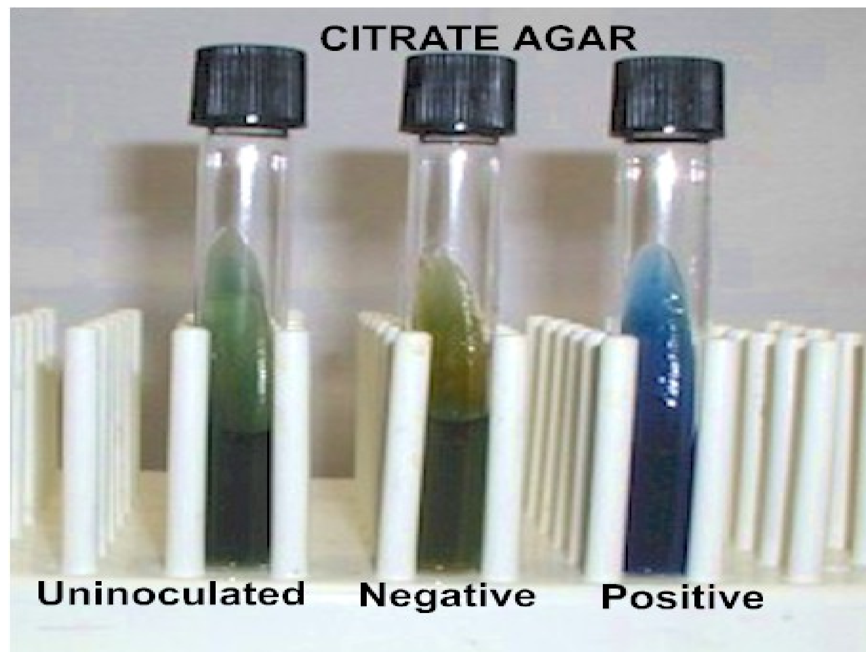
Method:

Inculate the media with test organism.

Incubate at 37c over night.

Turbidity and blue color: positive test.

No growth: negative test.

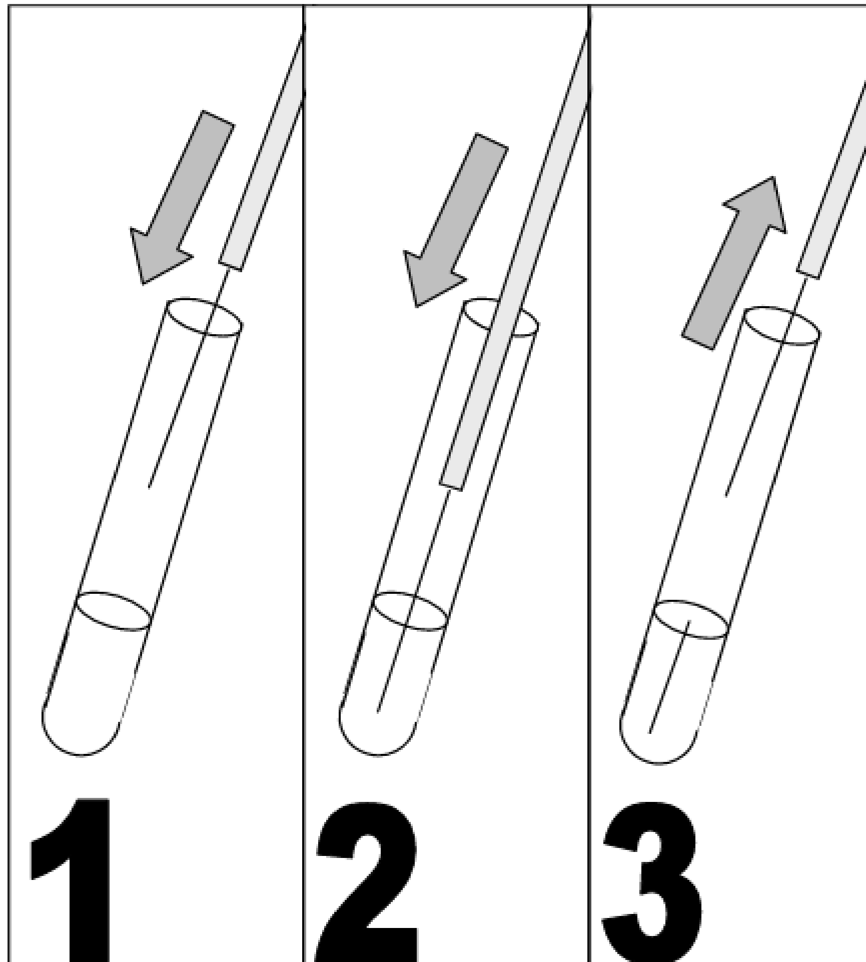


10.Motility test

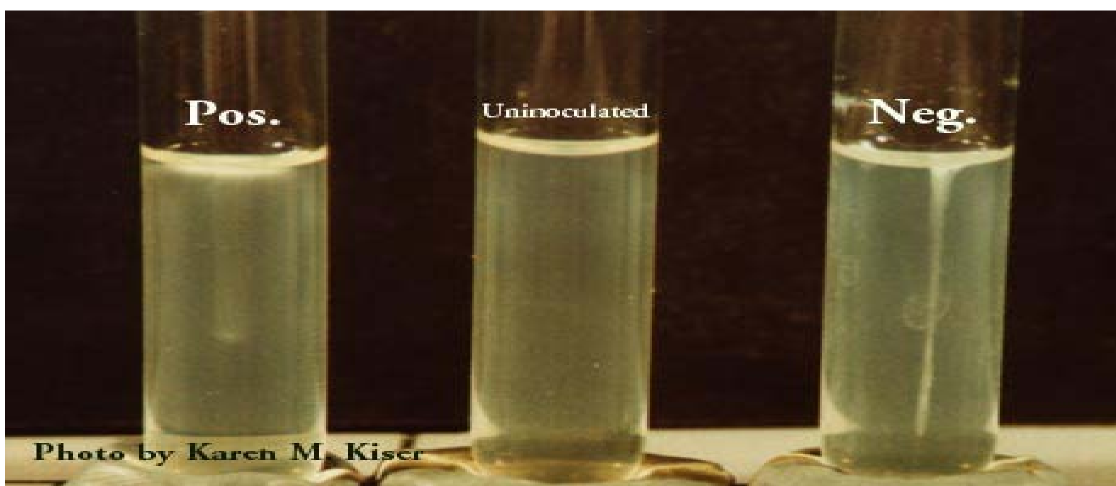
Motility media, nutrient broth or nutrient agar slant cultures, inoculating needle.

Using an inoculating needle and the stab technique, aseptically transfer each organism to a tube of motility medium.

Incubate 24-48 hours



Visible stab line, with cloudiness/pink color: motile
No distinct stab line, but with cloudiness/pink color elsewhere in the tube: motile
Visible stab line, clear media: non-motile



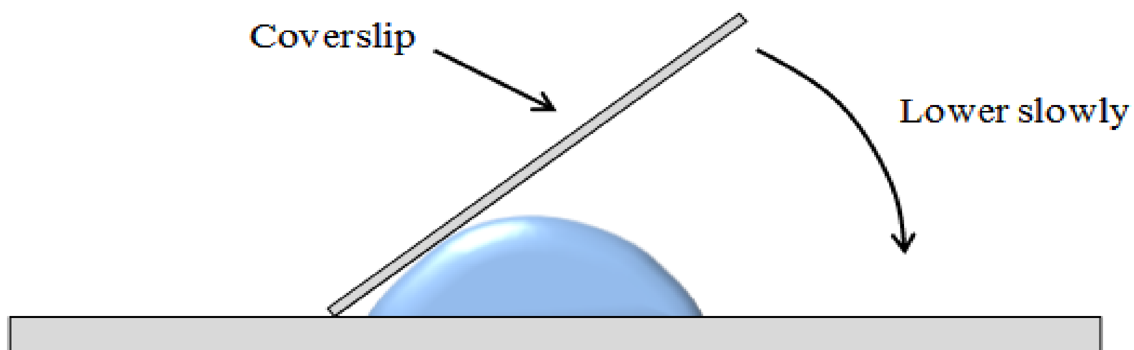
Wet mount

Materials:

Clean slides, cover slips, nutrient broth cultures, inoculating loop.

Procedure:

Use the vortex mixer to mix the broth culture.
Place several loopfuls of a pure bacterial culture
on a clean slide and cover with a cover slip.
Observe under the microscope. Reduce lighting to a
minimum for better contrast.
Begin focusing with the lowest power objective, as
always, and work up to the oil immersion objective.
If the organism is motile, you should see some of t
he bacteria darting about. In some cases, only a f
ew bacteria will be
moving, while the others are still. The organism m
ust still be considered motile



Student activity:

Test	Inoculating method	Growth
Indole		
KIA		
Urease		
Motility		
Citrate		

Antimicrobial Susceptibility Test

Kirby-Baur method for sensitivity testing

Requirements:

1. Test organism (overnight growth) because viability of bacteria.

2. Muller-Hinton agar :

3-Sterile normal saline or clear broth (peptone water)

4-McFarland standard (0.5 McFarland tube contain 0.05ml of 1.16% BaCl2+ 9.95 of 1% H2SO4 Turbidity equal to 1.5X108 CFU/ml).

5. Cotton wool swab

6. Bacteriological loop

7. Antibiotic discs

8. Pair of forceps or sterile needle or automatic dispenser

9. Control (STD) strain :(ATCC American Type Culture Collection –National collection of type culture or called NCTC)

10. Ruler, caliper, template or video camera.

11. Chart called interpretative chart and chart for STD strain

Procedure

1-Preparation of inoculums (suspension):-

B. Direct colony technique :

Using a sterile wire loop, touch 3–5 well-isolated colonies of similar appearance to the test organism and emulsify in 3–4 ml of sterile physiological saline or nutrient broth. In a good light match the turbidity of the suspension to the turbidity standard (mix the standard immediately before use). When comparing turbidities it is easier to view against a printed card or sheet of paper.

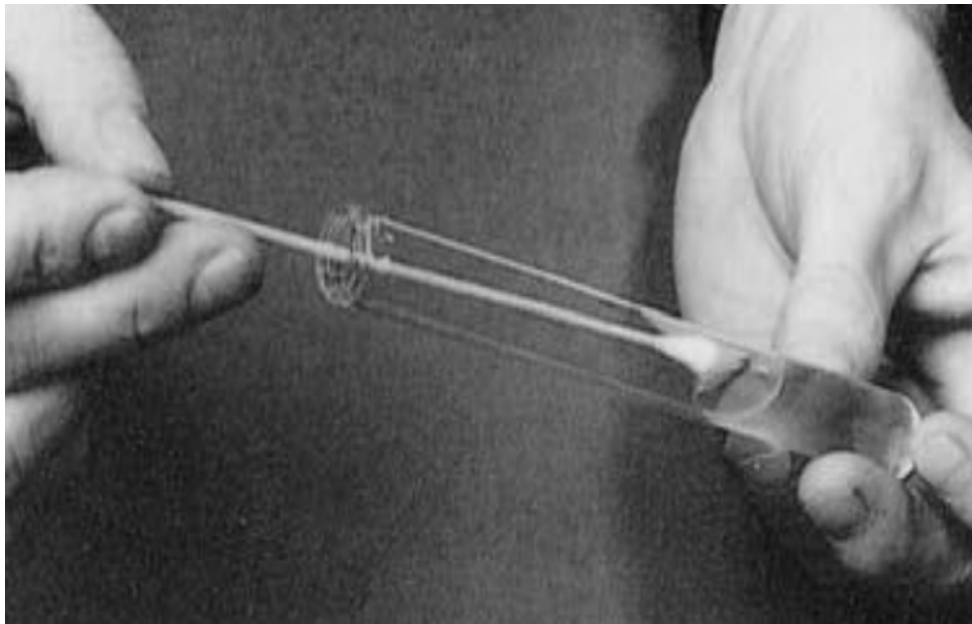
Mueller Hinton Agar Plate

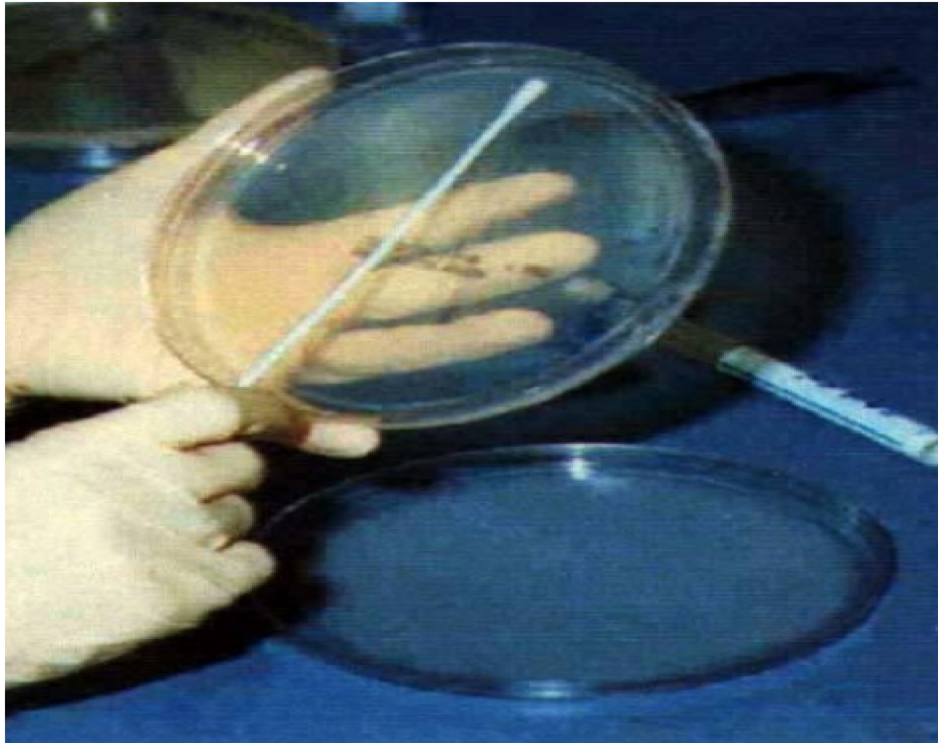
. Inoculation (seeding) or culture :

Using a sterile swab, inoculate a plate of Mueller Hinton agar. Remove excess fluid by pressing and rotating the swab against the side of the tube above the level of the suspension.

Streak the swab evenly over the surface of the medium in three directions, rotating the plate approximately 60 to ensure even distribution

With the Petri dish lid in place, allow 3–5 minutes (no longer than 15 minutes) for the surface of the agar to dry.





3-Antibiotic disc application

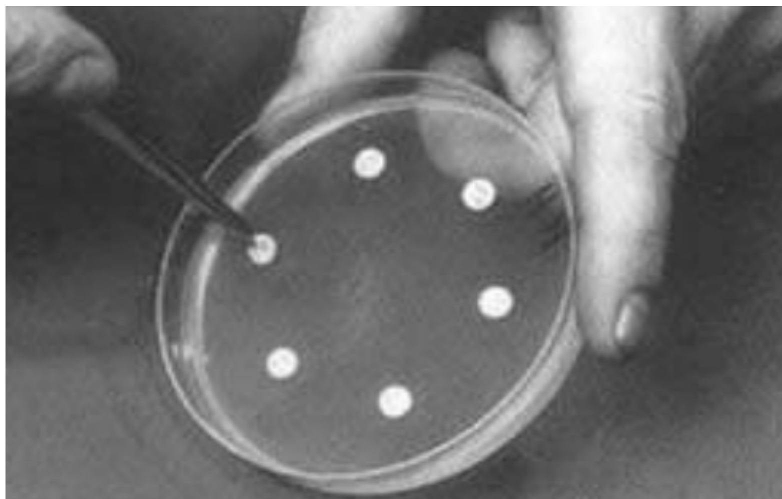
Using sterile forceps, needle mounted in a holder, or a multidisc dispenser,

place the appropriate antimicrobial discs, evenly distributed on the

inoculated plate. Using a template as shown in Plate will help to ensure the

discs are correctly placed.

Note: The discs should be about 15 mm from the edge of the plate and no closer than about 25 mm from disc to disc. No more than 6 discs should be applied (90 mm dish).



4 –Pre-incubation on R.T for few min. to make drying , because wetting lead to distorted zone (allow surface of the media to dry)

5-Incubation time(overnight) Within 30 minutes of applying the discs, invert the plate and incubate it aerobically at 35 C for 16–18 h (temperatures over 35 C invalidate results for oxacillin).

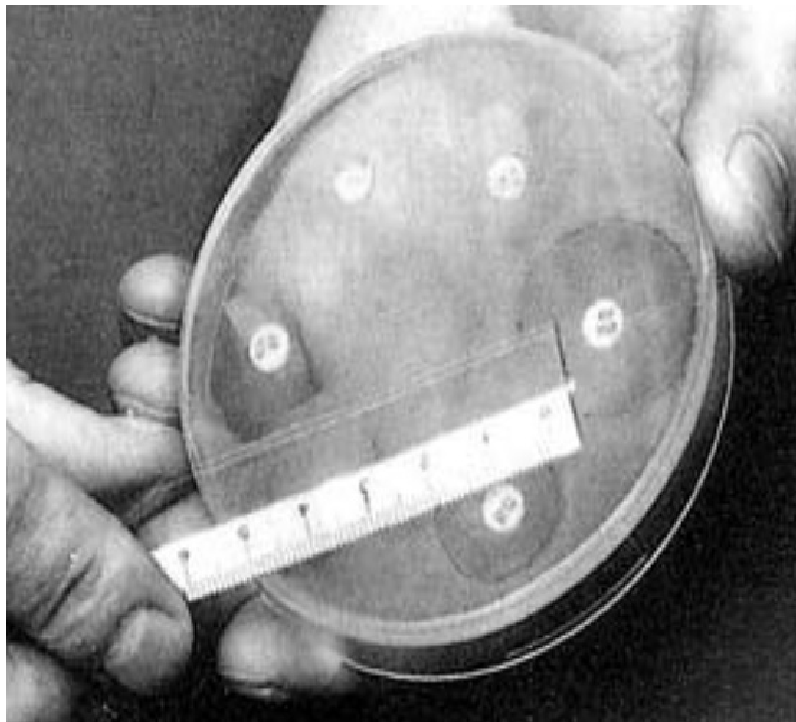
6 -Reading

After overnight incubation, examine the control and test plates to ensure the growth is confluent or near confluent. Using a ruler on the underside of the plate measure the diameter of each zone of inhibition in mm, or use template for reading , caliper or video camera (the endpoint of inhibition is where growth starts).

7-Interpretation

as: Sensitive , resistant , intermediate

Intermediate included inside resistant , but if increase dose (in case of safe antibiotic) become sensitive while if it is not safe not increase dose. Comparable with interpretative chart



Antibiotics used	Zone diameter measured (mm)		
	Sensitive	Intermediate	Resistant
Ampicillin, 10 µg	≥10	N/A	≤9
Chloramphenicol, 30 µg	≥23	12 - 22	≤11
Clindamycin, 2 µg	≥18	16 - 17	≤15
Ciprofloxacin, 5 µg	≥24	19 - 23	≤18
Erythromycin, 15 µg	≥19	16 - 18	≤15
Gentamicin, 10 µg	≥23	N/A	≤22
Kanamycin, 30 µg	≥18	14 - 17	≤13
Nalidixic acid, 30 µg	≥15	N/A	≤14
Streptomycin, 10 µg	≥15	12 - 14	≤11
Tetracycline, 30 µg	≥33	16 - 32	≤15
Vancomycin, 30 µg	-	-	-

Identification of Staphylococcus aureus

Morphology

……………………………………………………….

……………………………………………………….

Culture

Blood agar

……………………………………………………….
……………………………………………………….

Mannitol salt agar:

………………………………………………………
……………………………………………………….

Biochemical tests

S. aureus is:

Coagulase …………………..

DNA-ase ……………………

Catalase …………………….

Staphylococcus saprophyticus:

Blood agar

…………………………………………………….
…………………………………………………….

Mannitol salt agar:

……………………………………………………
……………………………………………………

Biochemical tests

Coagulase …………………..

DNA-ase ……………………

Catalase …………………….

Staphylococcus epidermidis:

Blood agar

…………………………………………………….

MacConkey agar

……………………………………………………

Mannitol salt agar:

……………………………………………………

Biochemical tests

Coagulase …………………..
DNA-ase ……………………
Catalase …………………….

Review Questions

1. Mention 5 Virulence factors of *S.aureus*

1)……………………………………

2)……………………………………

3)……………………………………

4)……………………………………

5)……………………………………

2. Mention one causative agent of the following:

1. Toxic Shock Syndrome……………………….
2. Cystitis………………………………………
3. Bacteremia following canula……………….
4. Impetigo……………………………………..

3. Normal Habitat of S.aureus ………… ……