وزارة التعليم
جامعة الملك سعود – كلية العلوم
قسم النبات والأحياء الدقيقة

Medical Bacteriology (460 MBIO) Practical Part

:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment numberIntroduction

:Experiment title laboratory safety

Top 10 laboratory safety rules:-

Follow the instructions .1

Whether it's listening to your instructor or lab supervisor or following a procedure in a book, it's critical to listen, pay attention, and be familiar with all the steps, from start to finish, before you begin. If you are unclear about any point or have questions, get them answered before starting, even if it's a question about a step later .on in the protocol. Know how to use all of the lab equipment before you begin

Keep snacks out of the lab .2

Food and drinks should never be consumed in a lab. There is a chance that they could become contaminated by the chemicals used in the lab. There is also a chance that the food and drinks could spill and contaminate an .experiment. If you need to eat or drink, make sure you do it before you enter a lab or wait until you leave

Don't sniff the chemicals .3

Not only should you not bring in food or drinks, but you shouldn't taste or smell chemicals or biological cultures already in the lab. Tasting or smelling some chemicals can be dangerous or even deadly. The best way to know what's in a container is to label it, so get in the habit of making a label for glassware before .adding the chemical

Dispose of waste properly .4

.Much of the waste created in a lab needs to be disposed of in something other than just the regular waste bin You also need to avoid dumping most chemicals down a drain since it could be bad for the plumbing system and potentially the environment. Make sure you know how to dispose of everything you plan on using in the .lab before you start your next experiment

Identify safety equipment .5

If something goes wrong while you're in the lab, you need to know where the safety equipment is located so ,that you can start using it right away. From the location of the fire extinguisher to the location of the eye wash .you should make sure the safety equipment is present and point out where it is before you begin an experiment

Think safety first .6

,If a chemical were to spill in the lab, what would you do? Or if you were injured while doing an experiment what would be your next move? It's impossible to eliminate all accidents from a lab, but you can take the right .steps to prepare yourself for one. It could prevent a small problem from turning into a larger one

Dress for the lab .7

From the moment you walk into a lab, you need to be dressed properly from head to toe. This means wearing long pants, a lab coat, safety goggles, covered shoes, and any other protective gear required by the lab. You should also put your hair up if you have long hair and wear gloves and hearing protection if the experiment .you are conducting calls for them

Don't play the mad scientist .8

Another important safety rule is to act responsibly in the lab. Don't play Mad Scientist, randomly mixing chemicals to see what happens. The result could be an explosion, fire, or release of toxic gases. Similarly, the .laboratory is not the place for horseplay

Leave Experiments at the Lab .9

It's important, for your safety and the safety of others, to leave your experiment in the lab. Don't take it home with you. You could have a spill or lose a specimen or have an accident. This is how science fiction films .start. In real life, you can hurt someone, cause a fire, or lose your job

Don't experiment on yourself .10

.The plot of many science fiction films starts with a scientist conducting an experiment on him or herself However, you won't gain superpowers or discover the secret to eternal youth. More than likely, whatever you .accomplish will be at great personal risk


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:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

Experiment number: first

:Experiment title Identification of Gram positive cocci (Catalase test)

:Brief introduction

Catalase test will help to differentiate those bacteria that produce the enzyme catalase such as staphylococci from non-catalase producing bacteria such as streptococci. This enzyme catalyses the release of oxygen from H2O2.

2 H2O2 …… Catalase …… 2H2O + O2

:Materials, tools and equipment used

- Agar plate Bacterial culture.
- Negative and positive control
- 3% H2O2
- Sterile wooden stick.
- Plastic wire loop or glass rod.
- Glass slide
- Tubes

Methods:

There are three methods:7

1. Slide method
2. Tube method

3. Plate method

Slide method

Using a sterile wooden stick, plastic wire loop or glass rod

1- Take a little amount of colony and place on a clean glass slide.

2- Put a drop of 3% H2O2 on to the colony.

3- Look for immediate bubbling.

Tube method

Collect 1 ml of 3% H2O2 in to a test tube.

Immerse the test colony in to the 3% H2O2 in to a test tube by using a sterile glass rod, plastic wire loop or a wooden stick.

- Look for bubbling.

Plate method

Flood or pour 3% H2O2 on to the test colony on the culture plate.

Look for bubbling. Do positive and negative controls with the test.

Result and interpretation:-

Active bubbling - Positive test

No release of bubbles - Negative test

Controls

Positive control - *Staphylococcus aureus*

Negative control - *Streptococcus pyogenes*

Caution:-

Blood agar , other blood containing media, and wire loop, are not suitable

for the test.

T:he reviewer

Resources

1. ASM Manual of Clinical Microbiology 2007.

2. Carbohydrate Fermentation Protocol by Microbe Library.

3. ELEK SD (November 1949). "The plate virulence test for diphtheria. J. Clin. Pathol. 2 (4): 250–8. doi:10.1136/jcp.2.4.250. PMC 1023322. PMID 15396422.

4. K.C. Chapin and T. Lauderdale. 2003. Reagents, stains, and media: bacteriology, p. 358. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), Manual of Clinical Microbiology, 8th ed. ASM Press, Washington, D.C.

5. Manual on Antimicrobial Susceptibility Testing, Dr. M.K. Lalitha

6. microbeonline.com

7. Professor of Microbiology Department of Microbiology Christian Medical College Vellore,

:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number2nd

:Experiment title **Identification of Gram positive cocci (Coagulase test)**

:Brief introduction

Coagulase is an enzyme mainly found in Staphylococcus aureus. Therefore this test will help to distinguish Staphylococcus aureus from other commonly isolated

Staphylococci. Two forms of coagulase exist. One is bound to the cell and other one is excreted from the cell as an enzyme. Bound coagulase is known as "clumping factor" which acts directly on the fibrinogen in plasma and convert it to fibrin to form a coagulum. When the coagulase is released as an enzyme, also called "free coagulase", reacts with the coagulase reacting factor (CRF) in plasma to form thrombin that then acts on fibrinogen in the plasma to form a fibrin clot.

Fibrinogen + Coagulase …… Fibrin

:Materials, tools and equipment used

• physiological saline.

• Rabbit plasma.

• Negative control - *Staphylococcus epidermidis* (ATCC)

• Positive control - *Staphylococcus aureus* (ATCC)

• Sterile wooden stick.

• Plastic wire loop or glass rod.

• Glass slide

• Tubes.

Methods:

Slide method

Place two separate drops of physiological saline on a clean slide.

1- Emulsify a colony and make two thick suspensions.

2- Add a drop of plasma to one of the suspensions and mix gently.

3- Look for clumping within 10 seconds in the plasma added suspension

Tube test

Method 1

Prepare 1:10 diluted plasma in physiological saline.

Add 0.5 ml of diluted plasma in a tube.

Add 0.1 ml of an 18-24 hour broth culture of the organisms.

Mix gently and incubate at 35-37°C.

Examine for formation of a coagulum after 1 hour; if no coagulum, examine at 2 and

6 hours.

Negative tubes should be left at room temperature overnight and re-examined.

Use negative and positive controls.

Method 2

Mix 0.5 ml undiluted plasma with an equal volume of an 18-24 hour broth culture.

Incubate at 37oC for 4 hours.

Examine after 1 and 4 hour for coagulum.

Negative tubes should be left at room temperature overnight and then re-examine.

Caution:

- EDTA blood is used. Citrated blood may give false positive result.

Result and interpretation:-

-Formation of coagulum - Positive test . (Due to released as an enzyme, also called "free coagulase", reacts with the coagulase reacting factor (CRF) in plasma to form thrombin that then acts on fibrinogen in the plasma to form a fibrin clot.)

-No coagulum - Negative test

T:he reviewer

Resources

1. ASM Manual of Clinical Microbiology 2007.

2. Carbohydrate Fermentation Protocol by Microbe Library.

3. ELEK SD (November 1949). "The plate virulence test for diphtheria. J. Clin. Pathol. 2 (4): 250–8. doi:10.1136/jcp.2.4.250. PMC 1023322. PMID 15396422.

4. K.C. Chapin and T. Lauderdale. 2003. Reagents, stains, and media: bacteriology, p. 358. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), Manual of Clinical Microbiology, 8th ed. ASM Press, Washington, D.C.

5. Manual on Antimicrobial Susceptibility Testing, Dr. M.K. Lalitha

6. microbeonline.com

:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number 3ed

:Experiment title **Identification of Gram positive cocci (*Staphylococcus.spp*) 1-Deoxyribonuclease (DNase) test**

:Brief introduction

Deoxyribonuclease hydrolyses DNA. The test organism is cultured on a DNA containing medium. After overnight incubation, the colonies are tested for DNase production by flooding the plate with a 1N HCl solution. The acid precipitate unhydrolyzed DNA. DNase producing colonies are surrounded by clear areas indicating DNA hydrolysis

:Materials, tools and equipment used

• Petri plates.
• A medium containing DNA (DNAse Agar).
• 1 N solution of HCl.
• Loops.
• Incubator (370 C).
• Bacterial Strain (ATCC).
• negative control (*Staphylococcus epidermidis*) ATCC.
• positive controls (*Staphylococcus aureus*) ATCC.

Methods:

• A loop full of bacteria is streaked by one line over the surface.

• Plates were incubated at 37 C for 24 h.

• After incubation, Cover the surface of the plate with 1 N solution of HCl.

:Results and observations

- Look for clearing around the colonies within 5 minutes. (Due to degradation of DNA around bacterial growth by DNAse enzyme)

: **Conclusion**

Clearing around the colonies within 5 minutes. (Due to degradation of DNA around bacterial growth by DNAse enzyme).

:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number4th

:Experiment title **Identification of Gram positive cocci (*Streptcoccus.spp*) Hemolysis Test and Preparation of Blood, Chocolate Agar Medium**

:Brief introduction

Hemolysis (from Greek which means blood breakdown) is the breakdown of red blood cells. The ability of bacterial colonies to induce hemolysis when grown on blood agar is used to classify certain microorganisms. This is particularly useful in classifying streptococcal species. A substance that causes hemolysis is a hemolysin.

*complete hemolysis (β-hemolysis), incomplete hemolysis (α-hemolysis), non hemolytic (γ-hemolysis).

:Materials, tools and equipment used

• Blood Agar Base (BAB).

• Sheep Blood.

• Fresh bacterial culture (ATCC).

• Sterile normal saline.

• Incubator.

• Autoclave.

• Petri Plates.

• Filters.

• Beta haemolysis (β-hemolysis) bacteria (*Streptococcus agalactiae*)

• Alpha Haemolysis (α-hemolysis) bacteria (*Streptococcus pneumoniae*)

• Non Haemolysis (γ-hemolysis) bacteria (*Enterococcus faecalis*)

Methods:

1. Prepare a BAB medium and autoclaved at 121 for 20 min.

2. Adding 5% of blood to blood agar base after autoclaving at 95 C for chocolate agar.

3. Adding 5% of blood to blood agar base after autoclaving at 50 C for Blood Agar.

:Results and observations

• Beta haemolysis (β-hemolysis) the area arrownd colony appears lightened (color less) and transparent (Streptococcus agalactiae).

• Alpha Haemolysis (α-hemolysis) the area arrownd colony is unchanged(*Streptococcus pneumoniae*).

• Non Haemolysis (γ-hemolysis) bacteria (*Enterococcus faecalis*)

Hemolysis	appearance	Designation	Example
.Complete	colorless, clear, Sharply defined zone	Beta(β)	S. pyogenes
.Partial streptococci	Greenish discoloration (reduced hemoglobin)	Alpha(α)	Viridans
.None	No change	Gamma(δ)	Enterococci

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:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number5th

:Experiment title **Identification of Gram positive cocci (*Streptcoccus.spp*) Optochin sensitivity test [Ethylhydrocuprein hydrochloride (EHCH) sensitivity test]**

:Brief introduction

Optochin (ethylhydrocupreine hydrochloride) is a chemical (quinine derivative) that is used in the presumptive identification of alpha-hemolytic Streptococcus pneumoniae, which is optochin sensitive. Other alpha-hemolytic Streptococcal species are resistant to optochin. (Mnemonic: OVRPS: Optochin- Viridians Resistant and Optochin- Pneumococcus sensitive). Optochin is completely soluble in water.

It use to detected ***Streptococcus pneumonia*** bacteria.

:Materials, tools and equipment used

- Optochin disc.
- blood agar.
- Positive control *Streptococcus pneumonia*.
- Negative control *Enterococcus faecalis*.
- Incubator.
- Autoclave.

Methods:

:First: Morphologically

1- Place a disc impregnated with EHC 0.05 mg optochin on a surface of a blood agar plate inoculated with test organism.

2- Incubate at 35°C for overnight in 5-10% CO2 and examine for inhibition zone.

:Results and observations

Zone of inhibition of 14 mm or more around a 6 mm disc or 16 mm or more around a 10mm disc, presumptively identifies the test organism as *Streptococcus pneumoniae*.

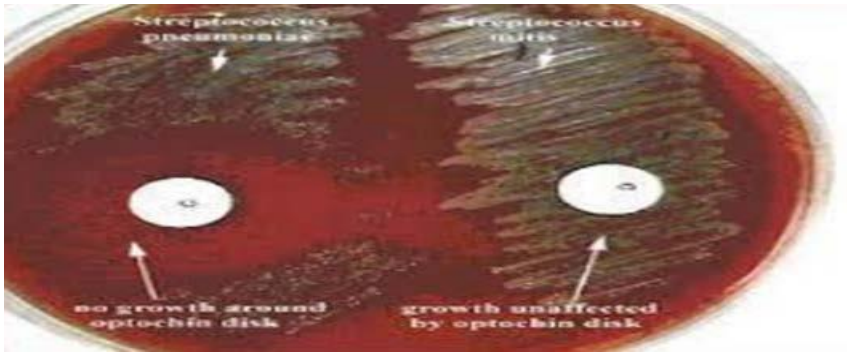
Zones smaller than these should be identified using bile solubility test. If the organism is soluble in bile, it is identified as *Streptococcus pneumoniae*.

No zone of inhibition – *Viridans* group *Streptococci*.

Controls

Positive control - *Streptococcus pneumoniae.*

Negative control – *Enterococcus faecalis*



positive and a negative Optochin test

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:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number6th

:Experiment title **Identification of Gram positive cocci (*Streptcoccus.spp*) Bile solubility test**

:Brief introduction

The bile solubility test is a qualitative procedure for determining the ability of bacterial cells to lyse in the presence of bile salts under specific conditions of time and temperature. The test is primarily used to differentiate bile soluble *Streptococcus pneumoniae* from bile insoluble alpha-hemolytic streptococci.

The working mechanism of the test is not clearly understood, however, one theory is that the bile salts facilitate lysis of pneumococcal cells by activating an intracellular autolytic enzyme.

:Materials, tools and equipment used

- 10% sodium deoxycholate.
- Primary culture plate.
- Incubator.
- Normal saline.
- Tubes.
- Vortex.
- A suitable rack to hold tubes.

Methods:

There are 2 methods:-

1. Plate method

2. Tube method

Test Tube Method:

1. Dispense 1ml of sterile 0.85% saline into a small test tube.

2. Prepare a heavy suspension of the organism in the saline. Shake or vortex to form a uniform suspension.

3. Divide the suspension into two tubes (0.5ml each), one labeled "TEST," the other labeled "CONTROL."

4. Dispense 10 drops of Bile Spot Reagent into the tube marked "TEST". Add 0.5ml of saline to the tube marked "CONTROL". Gently mix each tube.

5. Incubate the tubes for 3 hours at 35ºC, checking hourly.

Direct Plate Method:

1. Place a drop of Bile Spot Reagent near a suspected 18-24 hour old colony, gently roll the drop over several representative colonies. Take care not to dislodge the colonies.

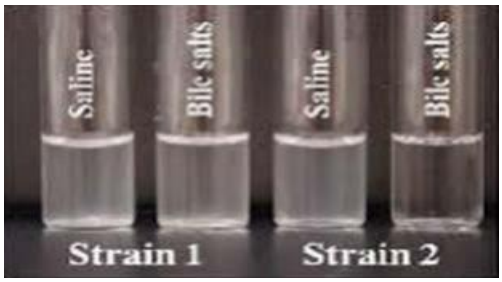
2. Keep the plate right side up and incubate at 35ºC for 30 minutes.

Results and observations:-

Test Tube Method: Examine the "TEST" tube for clearing or loss of turbidity as compared with the "CONTROL" tube. Bile solubility is demonstrated as a clearing or loss of turbidity, relative to the "CONTROL" tube within three hours.

Direct Plate Method: Bile solubility is demonstrated as a disintegration of the colony and/or the appearance of a hemolytic zone in the medium at the sig

colony was located within thirty minutes. Insolubility is demonstrated when there is no change in the integrity of the colony within 30 minutes.


Saline
Bile salts
Saline
Bile salts
Strain 1
Strain 2

:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number7th

:Experiment title **Identification of Gram positive cocci (*Streptcoccus.spp*): Bacitracin sensitivity test**

:Brief introduction

The bacitracin susceptibility test is used to distinguish Group A *streptococci*, from other *streptococci*. When grown on blood agar, Group A *streptococci* are sensitive to (killed by) the antibiotic bacitracin. A sterile disk impregnated with bacitracin is placed on the first sector of an isolation plate before incubation. A zone of inhibition (area with no growth) will be seen around the disk after incubation if the organism is a Group A beta-hemolytic *Streptococcus*. Other beta-hemolytic *streptococci* are resistant to (not killed by) bacitracin. Their colonies will thus grow right up to the disk of bacitracin.

:Materials, tools and equipment used

- A disks of (0.04 IU Bacitracin).
- 5% Sheep Blood Agar plates.
- Sterile inoculating loops.
- 37 C Incubator

Methods:

1. With a sterile inoculating loop, obtain a portion of an isolated colony of the strepcococci being tested.
2. Streak the plate for isolation.
3. With sterile forceps, obtain a bacitracin disk and place on the inoculated agar at the intersection of the primary and secondary streaks. Tap the disk with

forceps to ensure adherence to the agar surface

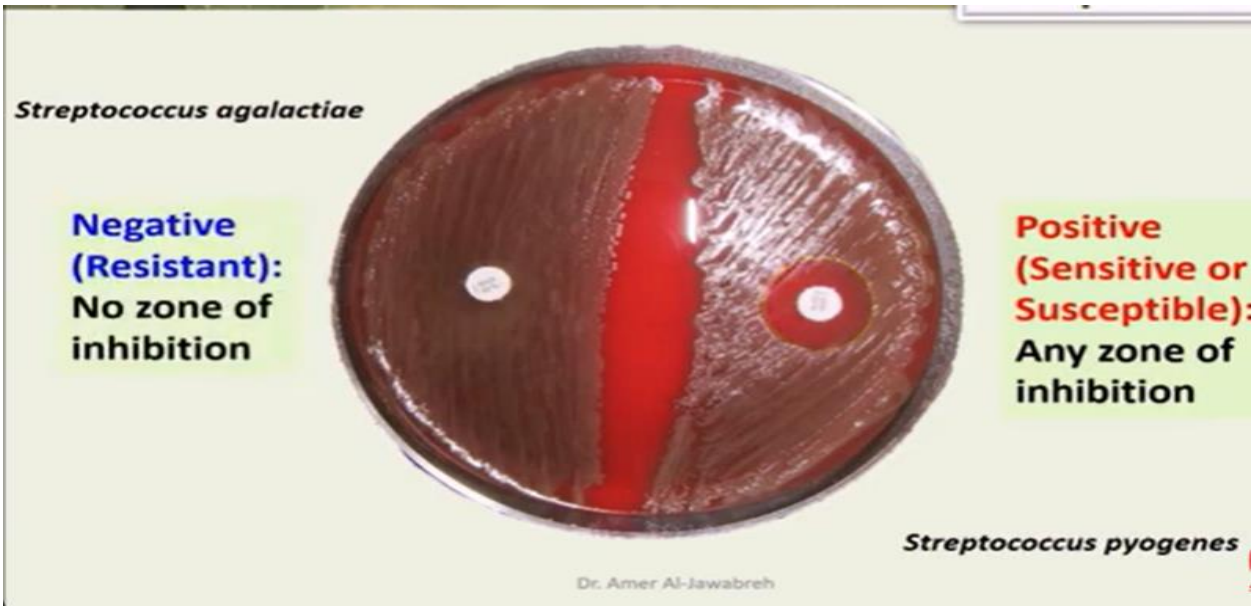
4. Incubate the blood agar plate in ambient air at 35°C for 18 to 24 hours.

5. After the incubation period, examine the blood agar for a zone of growth inhibition around the bacitracin disk.

Result and interpretation:-

Susceptible= any zone of growth inhibition

Resistant=no zone of growth inhibition



Bacitracin sensitivity test

:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number8th

:Experiment title **Identification of Gram positive cocci (CAMP test)**

:Brief introduction

The **CAMP test** (Christie-Atkinson-Munch-Peterson) is a test to identify group B β- hemolytic *streptococci* (*Streptococcus agalactiae*) based on their formation of a substance (CAMP factor) that enlarges the area of hemolysis formed by the β-hemolysin elaborated from *Staphylococcus aureus*

*The CAMP test can be used to identify *Streptococcus agalactiae*

:Materials, tools and equipment used

• 37 C Incubator.

• Sterile inoculating loops.

• Bunsen burner.

- **Media:** Blood Agar
- **Reagent:** Beta-lysin reagent.
- Culture of aureus
- Commercial reagents: Disks containing beta-lysin of aureus.
- Sterile wooden applicator sticks or bacteriologic loops
- Distilled water
- Petri dish and slide

• Beta-lysin–producing strain of *Staphylococcus aureus* (ATCC)

• *Streptococcus agalactiae* (positive control) (ATCC)

- *Streptococcus pyogenes* (Negative control) (ATCC)

Methods:

1. Streak a beta-lysin–producing strain of *S.aureus* down the center of a sheep blood agar plate.

2. The test organism streak should be 3 to 4 cm long.

3. Streak test organisms across the plate perpendicular to the S. aureus streak

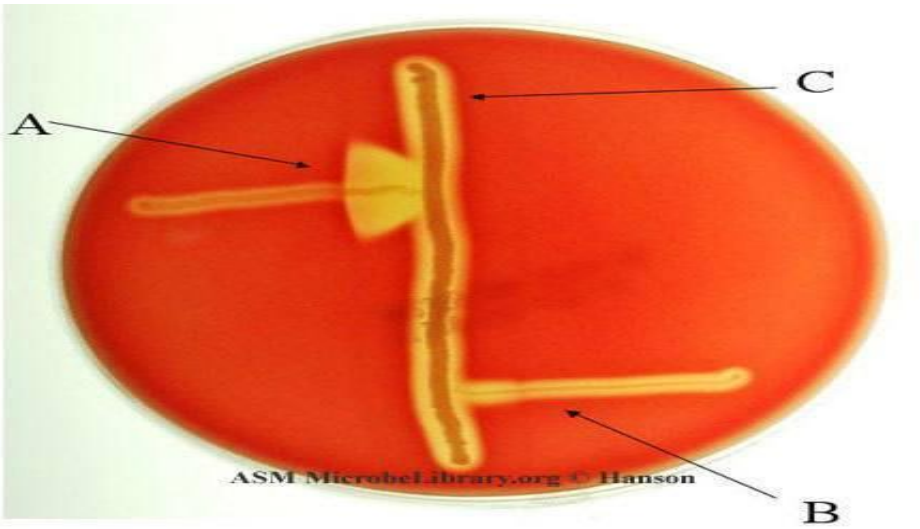
within 2 mm. (Multiple organisms can be tested on a single plate).

4. Incubate at 35°-37°C in ambient air for 18-24 hours.

5. Wedge shaped pattern radiating from the test organism near the S. aureus indicates positivity

:Results and observations.

The area of increased hemolysis occurs where the beta hemolysin secreted by the staphylococcus and the CAMP factor secreted by the group B streptococcus intersect . (See the figure)



CAMP Test

A. Test Organism: Group B streptococci (*Streptococcus agalactiae*)

B. Negative control: Group A streptococci (*Streptococcus pyogenes*)

C. *S. aureus*

CAMP test (Christie, Atkins & Munch-Petersen test)

Principle

The CAMP factor produced by Group B streptococci lyses sheep red blood cells pretreated with β-toxin of *Staphylococcus aureus.*

Procedure

Use layered 10 % sheep blood agar plates.

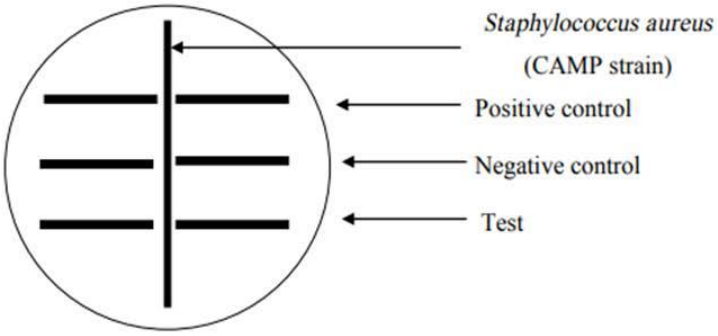
Streak *Staphylococcus aureus* (NCTC 7428) in a line across diameter of the plate.

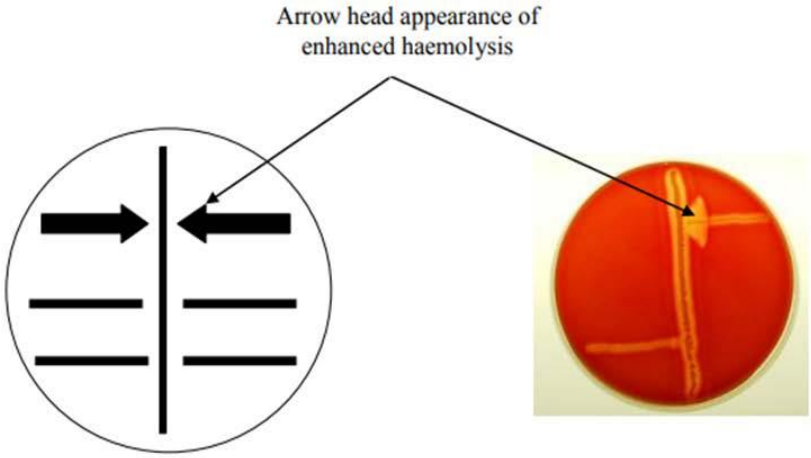
At right angles to the *Staphylococcus aureus* inoculum streak the test cultures without touching the staphylococcal inoculum.

Similarly, streak the positive and the negative controls.

Incubate the plates at 35^0C aerobically or in the presence of 5% CO_2.

Incubate for 24 hours and observe the results.





Controls

Positive control – *Streptococcus agalactiae*

Negative control – *Enterococcus faecalis*

:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number9th

:Experiment title **Identification of Gram positive bacilli Identification of *Bacillus SPP (*Spore Stain).**

:Brief introduction

When vegetative cells of certain bacteria such as *Bacillus* spp and *Clostridium* spp are subjected to environmental stresses such as nutrient deprivation, they produce metabolically inactive or dormant form-endospore. Formation of endospore circumvent the problems associated with environmental stress and helps them to survive.

:Materials, tools and equipment used

• Malachite green (primary stain).

• Acid alcohol (decolorizer).

• Safrinin stain (counter).

• Stain rack.

• Hot plate.

• Classes Slides.

• Paper towel (cut the size of the slide).

• Spore producer bacteria (*Bacillus* sp)

Methods:

1. Prepare smears of organisms to be tested for presence of endospores on a clean microscope slide and air dry it.

2. Heat fix the smear.

3. Place a small piece of blotting paper (absorbent paper) over the smear and place the slide (smear side up) on a wire gauze on a ring stand

4. Heat the slide gently till it starts to evaporate (either by putting the slide on a staining rack that has been placed over a boiling water bath or via bunsen burner).

5. Prepare smears of organisms to be tested for presence of endospores on a clean microscope slide and air dry it.

6. Heat fix the smear.

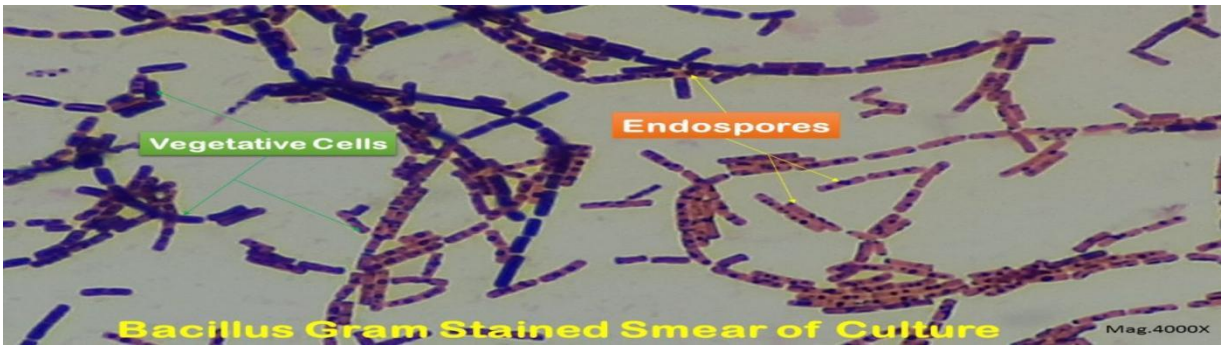
7. Place a small piece of blotting paper (absorbent paper) over the smear and place the slide (smear side up) on a wire gauze on a ring stand.

8. Heat the slide gently till it starts to evaporate (either by putting the slide on a staining rack that has been placed over a boiling water bath or via bunsen burner).

:Results and observations

***Observe the bacteria under 1000X (oil immersion) total magnification.**

Results: The vegetative cells will appear pink/red (counter stain) and the spores will appear green (primary stain)



:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number10th

:Experiment title **Identification of Gram positive Bacilli Identification of *Bacillus SPP (*Test for the detection of toxigenicity of Corynebacterium diphtheriae (Elek plate test))**

:Brief introduction

also known as the immunodiffusion technique, is an *in vitro* virulence test performed upon *Corynebacterium diphtheriae*. It is used to test for toxigenicity of *C. diphtheriae*. It was characterized in 1949 by american microbiologist Stephen Dyonis Elek (1914–1992). A strip of filter paper impregnated with diphtheria antitoxin is buried just beneath the surface of a special agar plate before the agar hardens. Strains to be tested are streaked with known positive and known negative toxigenic strains on the agar's surface in a line across the plate, and at a right angle to the antitoxin paperstrip.

After incubation for 24 hours at 37 degrees Celsius, plates are examined with

transmitted light for the presence of fine precipitin lines at a 45-degree angle to the streaks. The presence of precipitin lines indicates that the strain produced toxin that reacted with the antitoxin.

:Materials, tools and equipment used

• Nutrient agar

• Horse serum.

• Strip of anti-toxin.

• Sterile petri dish.

• Rotate

• Forceps.

• Slides.

• *Toxinogenic strain of C. diphtheriae*

• Paper towel (cut the size of the slide).

Methods:

1. Mix a tube of melted nutrient agar with 2 ml of sterile horse serum.
2. Rotate the tube to mix the serum and agar. Do not shake the tube.
3. Pour the mixture into a sterile petri dish.
4. Using lightly flamed forceps, lay the strip of anti-toxin impregnated filter paper across the center of the petri dish allowing it to sink beneath the agar surface.
5. Allow the agar to set, then lift one corner of the lid and let the plate dry for 30-45 min in the incubator.
6. When dry inoculate with a toxinogenic strain of *C. diphtheriae* by streaking a single line of inoculum across the plate and paper strip at right angles to the strip.
7. Repeat this about 1 inch away from the C. *diphtheriae* inoculum with a test strain.
8. Incubate the plate for 24 hrs and observe the results

:Results and observations

After 24 hours of incubation at 37°C, plate is examined with transmitted light for the presence of fine precipitin lines at 45 degree angle to the streaks.

Positive Test: Precipitin lines form at zone of equivalence, test organism is toxigenic.

If toxin is produced by the test strain, it diffuses sideways from the streak. The

antitoxin diffuses from the filter paper and where the toxin and the antitoxin meet (at zone of equivalence) a precipitin line formed.

:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number11th

:Experiment title **Identification of Gram Identification of Gram negative cocci (Carbohydrate fermentation test)**

:Brief introduction

The identification of some bacteria is based on that what nutrients the bacteria can utilize and the end products produced in the process. These characteristics are controlled by the enzymes which the bacteria have. The pattern of sugars fermented

may be unique to a particular species or strain as the type of enzyme(s) produced by bacteria is genetically controlled. Fermentation products are usually acid (lactic acid,acetic acid etc.), neutral (ethyl alcohol etc.), or gases (carbon dioxide, hydrogen,etc.).

:Materials, tools and equipment used

- Phenol Red Glucose Broth
- Phenol Red Lactose Broth
- Phenol Red Maltose Broth
- Phenol Red Mannitol Broth
- Phenol Red Sucrose Broth

Methods:

Phenol Red Carbohydrate Broth is commonly used in carbohydrate fermentation test. The carbohydrate source can varies based on your test requirements.Common broth media are:

1. Phenol Red Glucose Broth
2. Phenol Red Lactose Broth
3. Phenol Red Maltose Broth
4. Phenol Red Mannitol Broth
5. Phenol Red Sucrose Broth

Preparation and Composition of the media

Get specific Phenol Red Carbohydrate Test media from the commercial suppliers or

Phenol Red Broth Base and add specific carbohydrate source based on your test requirements, or you can prepare media mixing the following ingredients.

Composition of Phenol Red Carbohydrate Broth

- Trypticase or proteose peptone No. 3: 10 g
- Sodium Chloride (NaCl): 5 g
- Beef extract (optional): 1 g
- Phenol red (7.2 ml of 0.25% phenol red solution): 0.018 g
- Carbohydrate source: 10 g

A. Preparation of the media

- Prepare broth media by mixing all ingredients in 1000 mL of distilled/deionized water and heating gently to dissolve it *(Note: Use single carbohydrate source based on your requirements).*

- Fill 13 x 100 mm test tubes with 4-5 ml of phenol red carbohydrate broth.
- Insert a Durham tube to detect gas production.
- Autoclave the prepared test media (at 121°C for 15 minutes) to sterilize. The sterilization process will also drive the broth into the inverted Durham tube. *(Note: When using arabinose, lactose,maltose, salicin, sucrose, trehalose, or xylose, autoclave at 121°C for only 3 minutes as these carbohydrates are subject to breakdown by autoclaving)*

The prepared broth media will be a light red color and the final pH should be 7.4 ± 0.2 Alternatively, prepare Phenol Red Broth Base, heat sterilize and cool to 45°C. Prepare specific carbohydrate solution separately, filter the solution using membrane filter (pore size: 0.45 μm). Add carbohydrate solution to the broth base and mix it.

The preferred carbohydrate concentration is 1%.

B.Inoculation and Incubation

- Aseptically inoculate each test tube with the test microorganism using an inoculating needle or loop. Alternatively, inoculate each test tube with 1-2 drops of an 18- to 24-hour brain-heart infusion broth culture of the desired organism .
- Incubate tubes at 35-37°C for 18-24 hours. Longer incubation periods may be required to confirm a negative result.

:Results and observations

1. **Acid production:**

1. **Positive:** After incubation the liquid in the tube turns yellow *(indicated by the change in the color of the phenol red indicator)*. It indicates that there is drop in the pH because of the production of the acid by the fermentation of the carbohydrate (sugar) present in the media.

NOTE:**If you are using other pH indicators please refer to Table 1 for their corresponding colors in particular pH.*

2. **Negative:** The tube containing medium will remain red, indicating the bacteria cannot ferment that particular carbohydrate source present in the media.

2. **Gas Production**

1. **Positive:** A bubble (small or big depending up the amount of gas produced) will be seen in the inverted Durham tube.

2. **Negative:** There won't be any bubble in the inverted Durham tube i.e. bacteria does not produce gas from the fermentation of that particular carbohydrate present in the media i.e. anaerogenic organism.

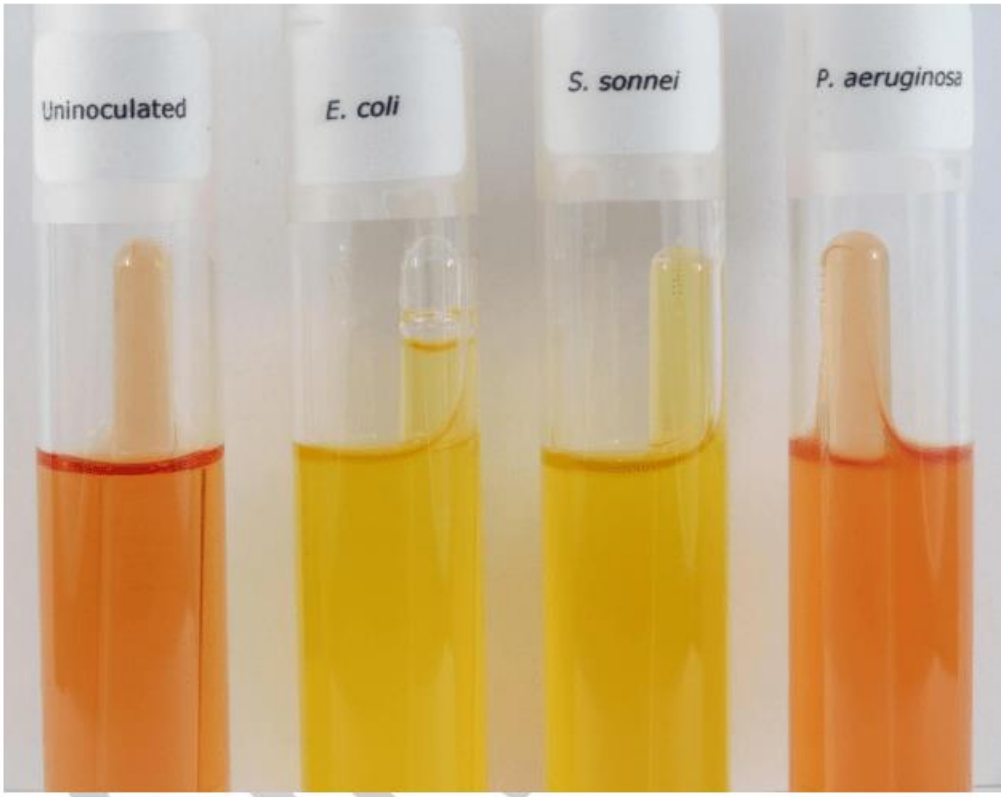


Figure 1. Peptone media with phenol red indicator. From left to right: Uninoculated tube; glucose fermenter with gas production (visible air bubble in the inverted Durham tube); glucose fermenter without gas production (no visible air bubble in the inverted Durham tube); non-fermenter.

:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number12th

:Experiment title **Identification of Gram Identification of Gram negative bacilli Identification of Enterobacteriaceae**

(Oxidase test)

:Brief introduction

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the **cytochrome coxidase** oxidizes the reagent **(tetramethyl-p**

phenylenediamine) to **(indophenols)** purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless.

* Oxidase test is most helpful in screening colonies suspected of being one of

the **Enterobacteriaceae** (all negative).

:Materials, tools and equipment used

• Moist filter paper

• Wubstrate (1% tetramethyl-p-phenylenediamine dihydrochloride),

• Wooden wire or platinum loop.

Methods:

1. Take a filter paper soaked with the substrate tetramethyl-p-phenylenediamine

Dihydrochloride.

2. Moisten the paper with a sterile distilled water.

3. Pick the colony to be tested with wooden or platinum loop and smear in the filter paper.

4. Observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds.

Precaution to be taken while performing oxidase test:

1. Do not use Nickel-base alloy wires containing chromium and iron (nichrome) to pick the colony and make smear as this may give false positive results

2. Interpret the results within 10 seconds, timing is critical

:Results and observations

1- Positive: Development of dark purple color (indophenols) within 10 seconds.

2- Negative: Absence of color.

Bacterial genera characterized as oxidase positive

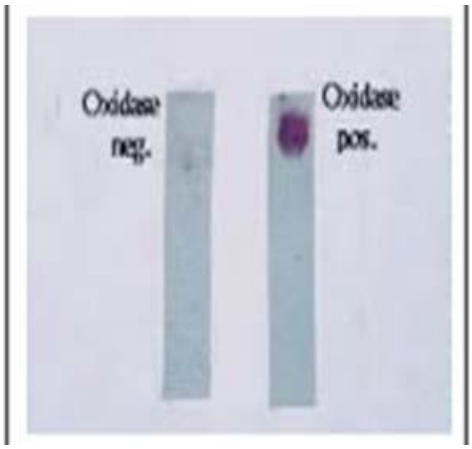
include *Neisseria* and *Pseudomonas etc*. Genera of the **Enterobacteriaceae** family are

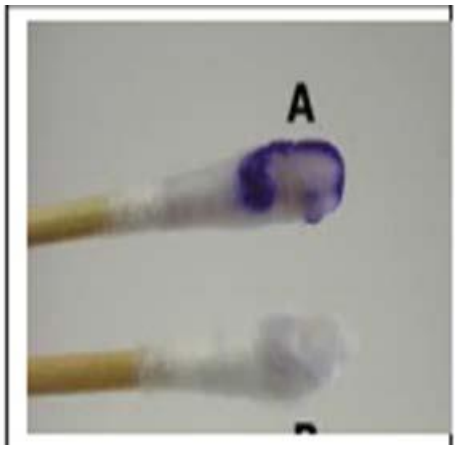
characterized as **oxidase negative.**

Controls

A Positive control - Pseudomonas aeruginosa

B Negative control - Escherichia coli


Oxidase neg.
Oxidase pos.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:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number13th

:Experiment title **Identification of Gram Identification of Gram negative bacilli Identification of *Enterobacteriaceae***

(Bacterial Motility)

:Brief introduction

Bacterial motility is a very useful character when speciation of bacteria is done. There are several ways that we can demonstrate the motility of bacteria.

1- Detection of motility on semi-solid agar

2- Detection of motility by microscopy

3- Hanging drop preparation

:Materials, tools and equipment used

1. Glass slides (glass slide with depression)
2. Paraffin wax
3. Loop
4. Coverslip
5. Microscope
6. Bunsen burner
7. Young broth culture of motile bacteria (e.g. *Proteus mirabilis*)
8. Nutrient agar

Methods:

Detection of motility on semi-solid agar

1. Prepare a semisolid agar medium in a test tube.
2. Inoculate with a straight wire, making a single stab down the center of the tube to about half the depth of the medium
3. Incubate under the conditions favoring motility.
4. Incubate at 37°C
5. Examine at intervals, e.g. after 6 h, and 1 and 2 days (depends on generation time of bacteria) . Freshly prepared medium containing 1% glucose can be used for motility tests on anaerobes.

Hanging drop preparation:

1. Take a clean glass slide and apply paraffin ring, adhesive tape ring to make circular concavity. (This step is not needed if a glass slide with depression is available).
2. Hold a clean coverslip by its edges and carefully dab Vaseline on its corners using a toothpick.
3. Place a loopful of the broth culture to be tested in the center of the prepared coverslip.
4. Turn the prepared glass slide or concavity slide upside down (concavity down) over the drop on the coverslip so that the vaseline seals the coverslip to the slide around the concavity.
5. Turn the slide over so the coverslip is on top and the drop can be observed hanging from the coverslip over the concavity.
6. Place the preparation in the microscope slide holder and align it using the naked eye so an edge of the drop is under the low power objectives.
7. Turn the objective to its lowest position using the coarse adjustment and

CLOSE THE DIAPHRAGM.

8. Look through the eyepiece and raise the objective slowly using the coarse adjustment knob until the edge of the drop is observed as an irregular line crossing the field.

9. Move the slide to make that line (the edge of the drop) pass through the center of the field.

10.Without raising or lowering the tube, swing the high dry objective into position (Be sure the high dry objective is clean).

11.Observe the slide through the eyepiece and adjust the fine adjustment until the edge of the drop can be seen as a thick, usually dark line.

12.Focus the edge of the drop carefully and look at each side of that line for very small objects that are the bacteria. The cells will look either like dark or slightly greenish, very small rods or spheres. Remember the high dry objective magnifies a little less than half as much as the oil immersion objective.

13.Adjust the light using the diaphragm lever to maximize the visibility of the cells.

14.Observe the cells noting their morphology and grouping and determine whether true motility can be observed.

15.Brownian movement should be visible on slides of all the organisms, but there should also show true motility.

16.Wash the depression slide and after soaking in lysol buckets or discard the prepared glass slide.

Detection of motility by microscopy:

1. Mix a loopful of growth from a nutrient agar subculture in a drop of sterile distilled water on one end of a slide. On the other end of the slide, mix another

loopful of growth in a drop of peptone water.

2. Cover each preparation with a cover glass.

3. Examine microscopically using the 40objective.

:Results and observations

:on semi-solid agar

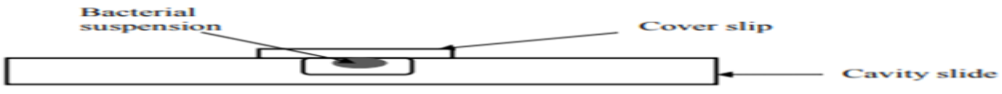
1. **Non-motile bacteria** generally give growths that are confined to the stab-line, have sharply defined margins and leave the surrounding medium clearly transparent.

2. **Motile Bacteria** typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque.

: On Hanging drop

While examining living organism for the property of active locomotion, it is essential to distinguish true motility, whereby the organism move in different directions and change their positions in the field, from either

- Passive drifting of the organisms in the same direction in a convectional current in the fluid or
- Brownian movement, which is an oscillatory movement about a nearly fixed point possessed by all small bodies suspended in fluid and due to irregularities in their bombardments by molecules of water



:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number14th

:Experiment title **Identification of Gram negative bacilli**

Identification of Enterobacteriaceae (Triple Sugar Iron Agar (TSI))

:Brief introduction

Whenever you see the name of this test i.e. Triple Sugar Iron Agar, you have to remember that it's a test which has **three sugar** (Lactose, Sucrose, and Glucose) and also iron; and it contains Agar as solidifying agent (TSI is a solid media

having slant and butt). TSI is similar to Kligler's iron agar (**KIA),** except that Kligler's iron agar contains only two carbohydrates: glucose and lactose.

:Materials, tools and equipment used

Beef extract 3.0 g

Yeast extract 3.0 g

Peptone 20.0 g

Glucose 1.0 g45

Lactose 10.0 g

Sucrose 10.0 g

Ferrous sulfate or ferrous ammonium sulfate 0.2 g

NaCl 5.0 g

Sodium thiosulfate 0.3 g

Phenol red 0.024 g

Agar 13.0 g

Distilled water 1,000 mL

Methods:

- Combine the ingredients, and adjust the pH to 7.3
- Boil to dissolve the agar and dispense into tubes.
- Sterilize by autoclaving at 121°C for 15 minutes
- Cool in a slanted position to give a 2.5 cm butt and a 3.8 cm slant.

TSI agar is also available commercially

Results and observations

1. Alkaline slant/no change in butt (K/NC) i.e Red/Red = glucose, lactose and

sucrose non-fermenter.

2. Alkaline slant/Alkaline butt (K/K) i.e Red/Red = glucose, lactose and sucrose non-fermenter .

3. Alkaline slant/acidic butt (K/A); Red/Yellow = glucose fermentation only, gas (+

or -), H_2S (+ or -).

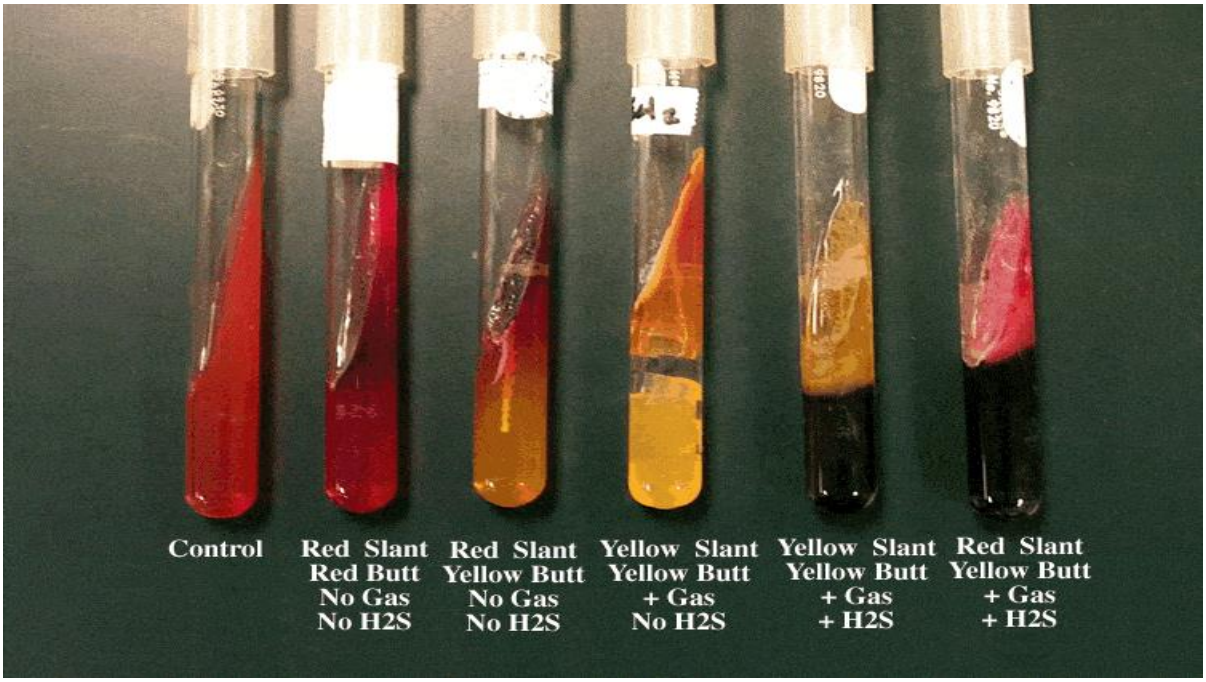
4. Acidic slant/acidic butt (A/A); Yellow/Yellow = glucose, lactose and/or sucrose fermenter gas (+ or -), H_2S (+ or -).

1. If lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator **yellow** both in butt and in the slant. Some organisms generate gases, which produces bubbles/cracks on the medium.

2. If lactose is not fermented but the small amount of glucose is, the oxygen deficient butt will be **yellow** (remember that butt has comparatively more glucose than slant i.e. more media more glucose), but on the slant the acid produced (less acid produces in slant as media in slant is less) will be oxidized to carbon dioxide and water by the organism and the slant will be **red** (alkaline or neutral pH).

3. If neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red. The slant can become a deeper **red-purple** (more alkaline) as a result of production of ammonia from the oxidative deamination of amino acids (remember peoptone is a major constituent of TSI agar).

4. if H_2S is produced, the **black** color of ferrous sulfide is seen.



https://microbeonline.com/triple-sugar-iron-agar-tsi-principle-procedure-andinterpretation/

:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number15th

:Experiment title **Identification of Gram negative bacilli Identification of Identification of *Salmonella* and *Shigella* Sero-typing of Salmonella species.**

:Brief introduction

Salmonella species are classified according their somatic antigens (O) and flagellar antigens (H). This is known as the Kauffmann-White classification. This classification is used in the sero-typing of Salmonella. Diagnostic antisera can be prepared in the laboratory by immunizing rabbits with the appropriate antigen. Antisera to somatic antigens are available as polyvalent containing serogroups A to G or A to S and also as group A (O2), B (O4), C1 (O 6, 7), C2 (O8), D (O9), E (O3, 10, 15,19), F (O11), and G (O13, 22). Antisera to flagellar antigens are also available as polyvalent and mono valent. Antisera commercially available and

should be kept in a refrigerator. Antisera should be at room temperature when they are used for the test.

:Materials, tools and equipment used

1- Glass slides (glass slide with depression).

2- Loop

3- Microscope

4- Bunsen burner

5- Young culture of bacteria.

6- Anti Salmonella *Typhi*, *paratyphi* A-B-C, Vi Sera.

7- Salmonella 0 Antiserum Group E.

Methods:

- Confirm that you are handling an isolate of *Enterobacteriaceae* by doing an oxidase

(negative) from the KIA tube.

- Take a clean glass slide and prepare two even suspensions of organisms taken from

the KIA slant on a drop of terile physiological saline.

- Mix the polyvalent O anti-serum thoroughly and put a drop carefully on to one of the

suspensions of organisms. Use the other suspension as a control to see whether

there is any auto-agglutination.

- Using a sterile wire loop mix the contents gently and rotate for about 2 minutes.

- Look for agglutination against a dark background in the suspension where the anti

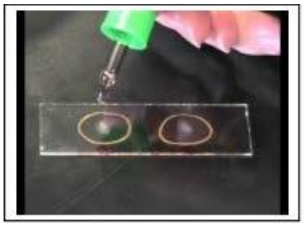
serum was added.

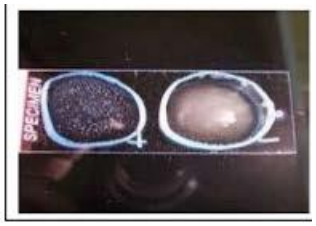
- If you see the agglutination, do similarly with the polyvalent H antiserum.

- If no agglutination, no further testing with anti-sera.

- If you see agglutination with that too, do the same procedure with the other O

antisera groups according to the prevalence of the species in the country.





Results and observations

Sero-typing of Shigella species:-

Shigella species are divided in to 4 sero-groups depending on their somatic antigens.

Sero-group A (*Shigella dysenteriae*), sero-group B (*Shigella flexneri*), sero-group C

(*Shigella boydii*) and sero-group D (*Shigella sonnei*).

Diagnostic antisera can be prepared in the laboratory like for Salmonella by

immunizing rabbits with the appropriate antigen. Antisera to somatic antigens are

available as polyvalent containing sero-groups A, B, C and D. Further monovalent antisera are available depending on the sero-group.

Procedure is similar to that of Salmonella sero-typing. If the *Shigella* like organisms do not give a positive agglutination test with the anti-sera, suspect the presence of a masking K antigen. Make a suspension (about 109 bacteria/ml) of the culture in 0.5 ml of saline, heat at 1000C for 1 hour, cool, centrifuge, re-suspend in fresh saline and re-test with the range of anti-sera.

:Course number and code460 MBIO

:Course Name**Medical Bacteriology**

:Experiment number16th

:Experiment title **Identification of *Mycobacterial* species Acid Fast Stain (ZN) (THE ZIEHL-NEELSEN ACID-FAST STAIN)**

:Brief introduction

Members of the bacterial genus *Mycobacterium* contain large amount of lipids (fatty) substances within their cell walls. These fatty waxes resist staining by ordinary methods.

When these organisms are stained with a concentrated solution of a basic dye such as carbol fuchsin, while applying heat, the stain can penetrate the lipid cell wall and reach the cell cytoplasm. Once the cytoplasm is stained it resists decolourization even with harsh agents such as acid alcohol which cannot dissolve and penetrate beneath the mycobacterial lipid wall. Under these conditions of staining the mycobacteria are said to be *acid fast*. Other bacteria of those, cell

walls do not contain high concentration of lipid are readily decolorized by acid alcohol after staining with carbol fuchsin and are said to be *nonacid fast*.

The original technique for applying carbol fuchsin with heat is called the Ziehl

Neelsen stain named after two bacteriologists who developed it in the late 1800s.

:Materials, tools and equipment used

• Microscope slides.

• Diamond marking pen.

• Alcohol.

• Wire loop.

• Forceps with (denaturised)

• Bunsen burner or spirit lamp, for heating the fuchsin dye.

• Filter paper.

• Funnel.

• Staining rack to hold smear slides.

• Microscope, immersion oil.

• Acid Fast Stain (set).

sputum sample.

• Pure water: distilled or filtered. Tap water, or other water, often contains

saprophytic mycobacteria,

which cannot be distinguished microscopically from pathogen mycobacteria.

• Denaturised alcohol

• Dettol

REAGENTS

• Carbol - Fuchsin : must be of the best quality. The best concentration is of 1 %

Fuchsin in 5 – 6 % phenol

• Acid-Alcohol for the decoloration : a very strong decolorizer : sulphuric acid 20 – 25

% in water or HCl 3

% in alcohol 70 % which may be of technical quality.

• Methylene blue 0,1 % in water

Methods:

1- Put the fixed slides on horizontal bars. Avoid contact between the slides in order to avoid cross contamination.

2- Then cover the slide with filtered alkaline carbol – fuchsin. The whole smear must be covered with the dye solution Heat each slide slowly until it is steaming (white vapours ascending), but avoid boiling at any time.

Repeat this intermittent heating two or three times.

3- Let stain for 5 minutes. A longer time intensifies the colouration but the dye may not dry during the process since this causes disturbing fuchsin deposits.

4- Gently rinse the slide with running water.

5- Decolorise by flooding the slide with acid-alcohol solution. Everything, except the mycobacteria (= Acid-Fast Bacilli =AFB) will loose the red colour. When, after rinsing, the smear still shows visual red spots, unstain again until the red colour has disappeared. The mycobacteria can hardly be decolorised !

6- Gently rinse the slide with running water.

7- Flood the slide with the counter stain. The very fine, and generally rarely found,

AFB are hardly

visible and require a different background. Therefore, an optimal contrasting colour, methylene blue is chosen.

8- Let react for 2 minutes.

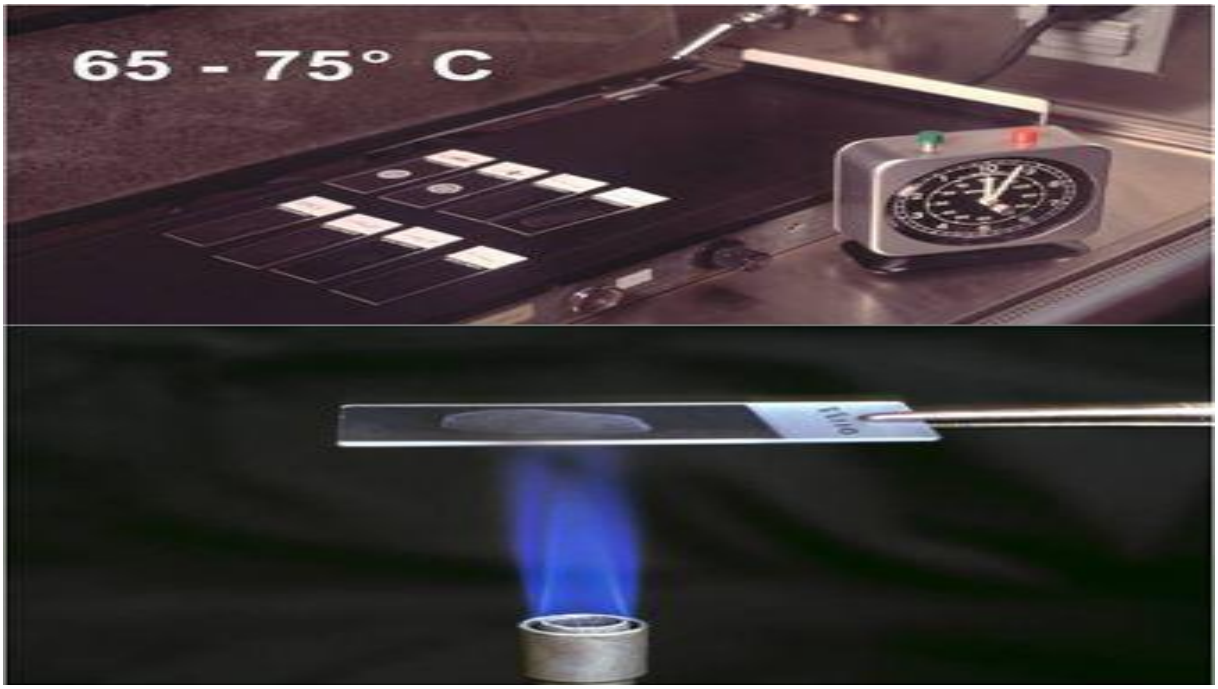
9- Gently rinse the slide with running water.

10- Wipe the back of each slide clean with a new tissue, and place them in a slide rack for the smears to air-dry but avoid direct sunlight.

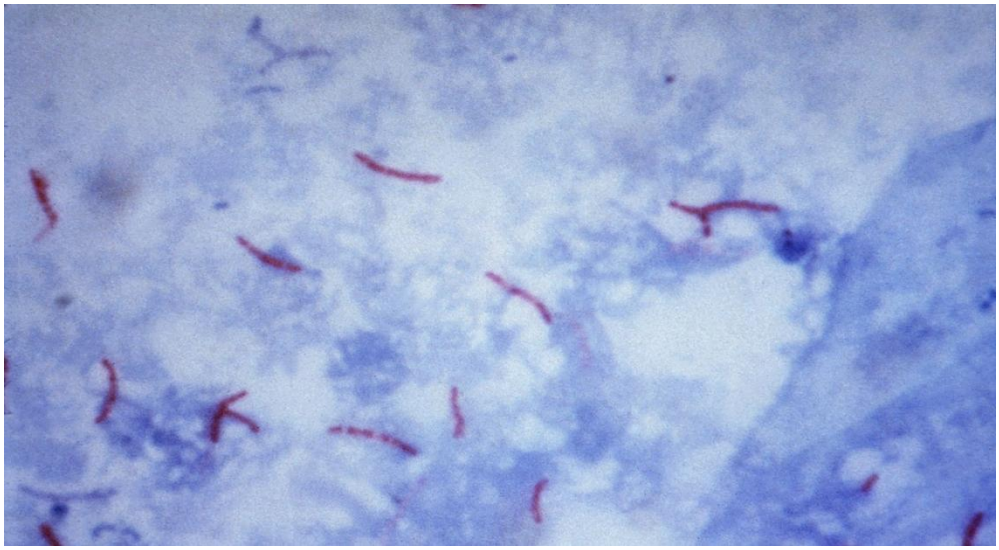
11- Examine the slide under the microscope (immersion oil, objective 100x, eye piece 10x).

Results and observations

Acid-fast cells stain reddish-purple; nonacid-fast cells stain blue or the color of the Counter stain if a different one is used.



Heat Fixation of smear (Upper: using electric heater, lower: using burner)



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