MICROBIAL GENETICS, MELECULAR BIOLOGY AND IMMUNOLOGY

PRACTICAL – I

(DMBL21)

(MSC MICROBIOLOGY)

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PRACTICAL PAPER III

IMMUNOLOY, MEDICAL AND CELLULAR MICROBIOLOGY

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Experiment No. 1

SEROLOGICAL DETERMINATION OF BLOOD GROUPING

Aim : To determine the blood groups by serological test

Principle : Blood group typing is determined by haemagglutination involving agglutination between the surface antigen on RBC with the corresponding antibody, because, human blood group typing (A, B, AB, or O) is based on surface antigens present on RBC cells.

Requirements : 70% alcohol, distilled water, disposable needles, cotton, clean glass slides.

Procedure :

Disinfect the middle figure of left hand with cotton moistened with 70% alcohol and allow it to dry.

Prick the middle finger with a sterile disposable needle, squeeze the blood and place a drop on each of the two glass slides.

Place a drop of anti-A serum over the drop of blood on one slide and anti-B serum on the other.

Mix them with clean toothpicks or glass rod.

Observe the slides for agglutination if any.

Results :. If agglutination was observed on the slide with anti A, the blood group is conformed as A. while that on slide with anti B is conformed as B group. If agglutination occur in both slides then it is conformed as AB group. Absence of agglutination in both the slides conforms the O group.

Experiment No. 2

SEPARATION OF SERUM PROTEINS

Aim : To separate both normal serum proteins by electrophoretic method.

Requirements : Agarose gel electrophoresis, conical flasks, beakers , cellophane tape, pipette, measuring cylinder,

Chemicals : Agarose, Na_2HPO_4, KH_2PO_4, KCl, NaCl, tris, glycine, methnol, acetic acid, Comassive Brillent Blue, lamelle buffer, etc.,

Preparation of reagents :

i) Phosphate buffered saline pH : 7.2

Di-sodium hydrogen orthophosphate	: 575 mg
Potassium di hydrogen phosphate	: 200 mg
Potassium chloride	: 200 mg
Sodium chloride	: 4.0 g

Dissolve the above chemicals in 450 ml of distilled water, and makeup the volume to 500 ml, after adjusting the pH to 7.2.

ii) Tris- glycine buffer pH 8.4 (30mM)

Tris	: 2.3 g
Glycine	: 1.42 g

Dissolve the above chemicals in 450 ml of distilled water, and makeup the volume to 500 ml, after adjusting the pH to 8.4.

iii) Staining solution

Comassive brillient blue : 100mg

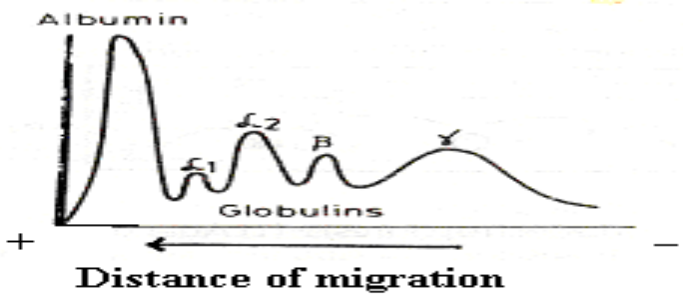
Methanol	: 50 ml
Acetic acid	: 7 ml
Dis. Water	: 43 ml

iv) De-staining solution

Acetic acid	: 14 ml
Methanol	: 40 ml
Distilled water	: 146 ml

Procedure :

Prepare 1 % molten agarose with phosphate buffer, and cool it to room temperature.
Take the boat of electrophoresis unit and seal the edges with cellophane tape.
Pour the molten agarose in to the boat and place the comb towards one edge,(0.5 cm away from the edge).
Allow it for hardening. After the agarose get hardened carefully remove the comb without damaging the wells.
Then remove the cellophane tape carefully without damaging the gel edges.
Place the boat on the platform of the electrophoretic unit with wells towards cathode side.
Pour the tris-glycine buffer in the buffer tank without trapping the air bubbles.
Then load 5 µl of serum along with 5 µl of lamelli buffer in to the well.
Apply the electric field initially at 50 mA/30 min later increase up to 100 mA/ 30 min.
After the sample reached the end of the gel, disconnect the current supply.
Observe the gel for the bands after staining with CBB and de-staining.



Observation :

Four thick bands corresponding to four serum proteins namely albumin, globulin proteins α globulin, β globulin, and γ globulins.

Experiment No. 3.1

OUCHTERLONY DOUBLE IMMUNO DIFFUSION

Aim : To determine the titre of Ag or Ab and to serological relationship between the given Ag and Ab by double immuno diffusion / Ouchterlony method.

Requirements :

Clean glass slides, glass plates, cork borer, dissection needles, pipettes, eppendorf tubes, microcentrifuge, micropipette, etc.,

Antigen and antibodies are commercially available.

Chemicals : Agarose, sodium azide, Na_2HPO_4, KH_2PO_4, KCl, NaCl, etc.,

Preparation of reagents :

i) Phosphate buffered saline pH : 7.2

Di-sodium hydrogen orthophosphate	: 575 mg
Potassium di hydrogen phosphate	: 200 mg
Potassium chloride	: 200 mg
Sodium chloride	: 4.0 g

Dissolve the above chemicals in 450 ml of distilled water, and makeup the volume to 500 ml, after adjusting the pH to 7.2.

ii) Staining solution

Comassive brillient blue	: 100mg
Methanol	: 50 ml
Acetic acid	: 7 ml
Dis. Water	: 43 ml

iii) De-staining solution

Acetic acid	: 14 ml
Methanol	: 40 ml
distilled water	: 146 ml

Procedure :

Prepare 1 % molten agarose with PBS and coat the glass slide after adding a pinch of sodium azide.

Place the slide in a refrigerator for 5 min. (for solidification).

Remove the agarose coated slide from the freezer after hardening of the gel and make the wells according to the template (6 peripheral wells and 1 central well) with a cork borer (about 4 mm diameter)

Remove the agarose plugs with the help of needle and seal the bottom of the well properly with molten agarose.

Prepare the antigen dilutions as ½, ¼,1/8, 1/16, 1/32, with PBS.

Prepare the antibody dilution (1/2) with PBS separately.

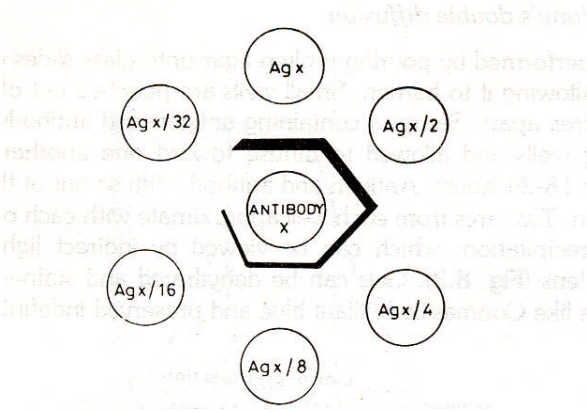
Add 20 µl of the antibody in the central well.

Add the antigen dilutions in the peripheral wells (20 µl each).

Incubate the slides / glass plates at 4 ^{0}C for overnight for Ag and Ab diffusion

Stain the slides with CBB and de-stain with de-staining solution.

Observe the precipitin lines after 24 hours and record the pattern of precipitin lines.



Precautions :

Sodium azide (which is toxic) is used to prevent microbial contamination.
Seal the bottom of the wells properly to prevent seepage of samples.
Slides/glass plates should be kept in humid conditions to prevent the drying.
The size of wells and distance between them should chosen depending on the template.

Result :
The antigen is serologically related and it shows the line of identity.

Experiment No. 3.2

RADIAL IMMUNODIFFUTION (Mansini test)

Aim : To detect the concentration of antigen in unknown sample and to study the serological relationship.

Principle :

If antibody is placed in a stationary phase and the corresponding antigen in mobile phase, then the antigen diffuses radially and forms a precipitin ring surrounding the well at the zone of equivalence.

Requirements :

Clean glass slides, glass plates, cork borer, dissection needles, pipettes, eppendorf tubes, microcentrifuge, micropipette, etc.,

Chemicals: Agarose, Sodium azide, NaH_2PO_4, K_2HPO_4, NaCl, KCl, etc

Antigen and antibodies are commercially available.

Preparation of reagents:

i) Phosphate buffered saline pH : 7.2

Di-sodium hydrogen orthophosphate	: 575 mg
Potassium di hydrogen phosphate	: 200 mg
Potassium chloride	: 200 mg
Sodium chloride	: 4.0 g

Dissolve the above chemicals in 450 ml of distilled water, and makeup the volume to 500 ml, after adjusting the pH to 7.2.

ii) Staining solution

Comassive brilliant blue : 100mg

Methanol	:	50 ml
Acetic acid	:	7 ml
Dis. Water	:	43 ml

iii) De-staining solution

Acetic acid	:	14 ml
Methanol	:	40 ml
distilled water	:	146 ml

Procedure:

Prepare 1 % molten agarose with PBS and after adding a pinch of sodium azide, and antibodies, raised against the antigen to be tested, in 1/10 dilution.

Coat the glass slides with the agarose containing antibodies.

Place the slide in a refrigerator for 5 min. (for hardening).

Remove the agarose coated slide from the freezer after hardening of the gel and make the wells according to the template, 4 wells were made side by side with equal distance (about 4 mm diameter) in the center of the glass slide with a cork borer.

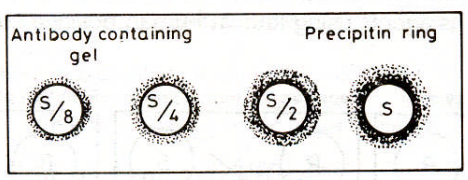
Prepare two fold dilutions of antigen (1/2 , ¼, 1/8) and load about 10 µl each in first four wells on the slide and 10 µl of the antibody to be detected in the fourth well.

Incubate the slide at 4 ^{0}C for overnight for Ag and Ab diffusion.

Stain the slides with CBB and de-stain with de-staining solution.

Observe the precipitin zones around each well after 24 hours and measure the diameter of the zone in all the 4 wells.

By comparing the diameter of the zone observed in the 4th well with those of the other wells, the concentration of the unknown antigen is estimated.



Precautions :

Sodium azide (which is toxic) is used to prevent microbial contamination.

Seal the bottom of the wells properly to prevent seepage of samples.

Slides/glass plates should be kept in humid conditions to prevent the drying.

The size of wells and distance between them should chosen depending on the template.

Observation / Result :

The radial spread of the unknown antigen is observed on agarose coated plate containing antibody.

The diameter of radial zone produced by unknown antigen is ---mm.

For example : If it is equivalent to that produced in the well with ¼ dilution , the concentration of the unknown is ¼ .

Experiment No. 4.

ROCKET GEL IMMUNO ELECTROPHORESIS

Aim : To determine the antigen relationships and its quantification in presence of electric field.

Principle :

If antibody is placed in a stationary phase and the corresponding antigen in mobile phase, in an electric field, the antigen diffuses from the well and move towards the anode side. While it is moving it precipitates the antibody along the zone of equivalence. Thus , the precipitation appears like rocket above the well. Height of the rocket is directly proportional to the concentration of the antigen.

Requirements : Submarine electrophoresis unit with power pack, clean (5 x 6cm) glass slides, cork borer, pipettes, eppendorf tubes, micropipette, whatmen No. 1 filter paper. Antigen and antibodies are commercially available.

Chemicals : Agarose (immunological grade), Tris, glycine, Na_2HPO_4, KH_2PO_4, KCl, NaCl, Sodium azide, CBB, Methanol, acetic acid etc.

Preparation of reagents :

i) Phosphate buffered saline pH : 7.2

Di-sodium hydrogen orthophosphate	: 575 mg
Potassium di hydrogen phosphate	: 200 mg
Potassium chloride	: 200 mg
Sodium chloride	: 4.0 g

Dissolve the above chemicals in 450 ml of distilled water, and makeup the volume to 500 ml, after adjusting the pH to 7.2.

ii) Tris- glycine buffer pH 8.4 (30mM)

Tris : 2.3 g

Glycine : 1.4 g

Dissolve the above chemicals in 450 ml of distilled water, and makeup the volume to 500 ml, after adjusting the pH to 8.4.

iii) Staining solution

Comassive brillient blue : 100mg

Methanol : 50 ml

Acetic acid : 7 ml

Dis. Water : 43 ml

iv) De-staining solution

Acetic acid : 14 ml

Methanol : 40 ml

Distilled water : 146 ml

Procedure :

Prepare 1 % molten agarose with PBS and after adding a pinch of sodium azide, and antibodies, raised against the antigen to be tested, in 1/10 dilution.

Coat the glass slides with the prepared agarose containing antibodies.

Place the slide in a refrigerator for 5 min. (for solidification).

Remove the agarose coated slide from the freezer after hardening of the gel and make 4 wells side by side with equal distance (about 4 mm diameter), 2 cm above from the edge. Then seal the bottom of the wells with molten agar.

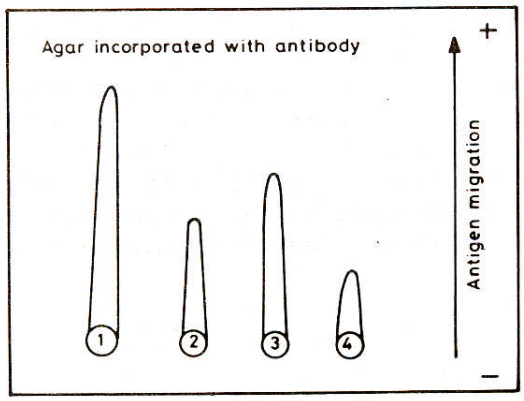
Place the agarose coated plate on the platform of the electrophoresis unit, in such a way that the wells should be placed towards cathode side.

Pour the tris-glycine buffer in the buffer tank of the unit.

Connect the glass plate with the buffer in the tank by placing the Whatmen No.1 filter paper wicks without trapping the air bubbles.

Prepare two fold dilutions of antigens (1/2 , ¼, 1/8) with PBS and load about 20 µl each in to the four wells on the slide and 20 µl of the antigen to be detected in the 4^{th} well.

Apply the electric field at the rate of 50 mA /hr and observe for the formation of rockets after proper staining and de-staining.



Observation / Result :

Measure the height of the rocket in all the 4 wells and construct a graph for concentration of Ag against height of the rocket.

From the standard graph, determine the concentration of the unknown antigen.

Experiment No : 5

ISOLATION OF NORMAL FLORA OF SKIN

Aim : To isolate the bacterial flora of the skin

Requirements : Manitol salt agar plates, sterile cotton swab, sterile saline in a test tube (10 ml, 0.85%), Hydrogen peroxide, gram staining reagents, clean glass slides, inoculation loop, spirit lamp, etc.,

Procedure :

Step I :

Moisten a sterile cotton swab in saline aseptically.

Remove the excess of saline by pushing the swab against the test tube wall.

Rub the swab over the skin surface of the nose, elbow or any other area.

Streak one – half of the manitol salt agar plate with the above swab.

Using a sterile inoculation loop, touch the swabbed area back and forth a few times

Streak the inoculating loop in other half of the manitol salt agar plate.

Incubate the plates at 35^0C in an inverted position for 24-48 hrs.

Step II

Test the colonies for the gram staining reaction.

Test the colonies for catalase production by adding H_2O_2 over the colonies.

Step III

With a sterile inoculating loop, transfer a catalase – positive and gram positive cocci on the second mannitol salt agar plate.

Results:

Members of the family Micrococcaceae usually form large opaque colonies. Appearances of yellow halo around the bacterial colony indicate a positive test for manitol fermentation. Release of the gas bubbles from the culture when H_2O_2 is added, indicates a positive test for catalase production.

Experiment No. 6

ISOLATION OF NORMAL FLORA OF ORAL CAVITY

Aim : To isolate bacteria present in oral cavity by isolating the micro flora of teeth crevices and that of saliva.

6.1 . Isolation of micro flora of teeth crevices:

Aim : To isolate the bacteria present in the crevice between the teeth after staining with the help of a dark-field microscope.

Requirements : Tooth picks, gram staining reagents, glass slides, distilled water, inoculation loop, microscope, etc.,

Procedure :

Remove a bit of material from the area between the teeth, using a toothpick.
Prepare two smears by keeping some of the material on each of the two clean slides.
Add a drop of distilled water to each slide.
Spread the material and prepare smear, approximately the size of a 25 paise coin.
Allow smears to air dry and heat fix in the usual manner.
Apply crystal violet or safranin to one smear.
Gram stain the second smear.

Observations and results:

Examine both the sides under oil immersion and draw the representative members observed in both the preparation. Organisms observed will belong to spirochetes, streptococci, micrococci, and small bacilli.

Precautions :

Dispose off the used toothpick in the container with disinfectant to avoid contaminations.

6.2 . Isolation of bacteria present in saliva

Aim : To isolate the bacteria present in saliva

Requirements :

Nutrient agar plate, blood agar plate, sterile test-tubes, 99 ml water blanks, sterile petri plates, sterile 1 ml pipette, sterile cotton tipped applicator sticks, Grams stain reagents, spirit lamp, etc.,

Procedure :

Collect 2 ml of saliva in to a sterile test tube.

Transfer 1 ml of saliva, using a sterile pipette, into one of the 99 ml water blanks to get 1/100 dilution (1×10^{-2}) and mix the contents thoroughly.

Similarly, transfer 1 ml of the suspension into another 99 ml water blank to get 1/1000 dilution (1×10^{-4}).

Transfer 0.1 ml suspension each onto a nutrient agar and blood agar plates.

Take a sterile cotton tipped applicator stick and spread the inoculum over the surface of nutrient agar and blood agar plate.

Incubate the plates at 37 ^{0}C for 48 hours in an inverted position.

Observe the blood agar plates for hemolytic reactions, if any.

Prepare smears of two distinct colonies which show distinct hemolytic reactions and gram stain them.

Observations :

On blood agar medium three types of hemolytic reactions may be seen.

Alpha (α) hemolysis - green discolouration surrounding the bacterial colonies.

Beta (β) hemolysis - appearance of clear zones surrounding the colonies.

Gamma (γ) hemolysis - no discoloration surrounding the colonies.

Gram staining is to compare the number of colonies appearing on two different media.

Experiment No. 7

TOTAL COUNT OF WBC OF BLOOD SAMPLE

Aim : To count the number of WBCs in blood using Haemocytometer.

Principle : The cell count is usually performed in a modified Fuchs- Rosenthal slide chamber, which has a film depth of 0.2 mm between the counting surface of the slide and the overlying cover-slip. The volume of the film overlying five large squares is 1 mm^3 and the count of the cells on five large square is thus the count per mm^3. When separate counts are to be made of the erythrocytes, 0.85% sodium chloride solution should be used as a diluents.

Requirements : Blood sample, 70% alcohol, WBC pipette, WBC diluting fluid :- Glacial acetic acid 15 ml, Distilled water, Disposable needles, Haemocytometer, 1% aqueous solution of Gentian violet.

Procedure :

Disinfect the middle figure of left hand with cotton moistened with 70% alcohol and allow it to dry.

Prick the end of the disinfected finger with a sterile needle to get blood.

Draw the blood by using a WBC pipette from fingertip up to the mark 0.5.

Dip the pipette immediately in WBC diluting fluid and suck the fluid up to the mark 11.

Mix the contents thoroughly.

Place the cover slip over the counting chamber and transfer the mixture slowly into the grooves of chamber.

Allow the cells to settle at the bottom of the chamber for 2 min.

Count the WBCs by using 10 x objective of the microscope in four corner chambers.

Calculate the number of cells in cubic millimeter of blood sample by using the formula

$$\text{The total no. of cells} = \frac{\text{No. of cells counted X the dilution factor X chamber depth}}{\text{Area counted}}$$

Observation /Result :

Experiment 8.

DIFFERENTIAL COUNT OF WBCs OF BLOOD SAMPLE

Aim : To count the number of different types of leukocytes in the given blood sample.

Principle : The blood smear is stained with Write's stain and observed under oil-immersion objective, to determine the relative percentages of each type of leucocytes.

Requirements : clean glass slides, 70% ethyl alcohol, sterile needle,etc.,

Procedure :

Disinfect the middle figure of left hand with cotton moistened with 70% alcohol and allow it to dry.

Prick the end of the disinfected finger with a sterile needle to get blood.

Place a drop of blood towards one edge of a clean glass slide by gently squeezing the finger.

Take a fresh clean slide, place it against the drop of blood and allow the blood to spread along the edge of the applied slide.

Hold the spreader at an angle greater than 45^0.

Move and push the spreader slide towards the opposite end of the first slide rapidly to form a thin smear.

Allow the smear to air dry.

Stain the smear with wright's stain for 4 min.

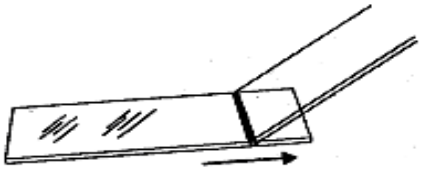
Add equal amount of distilled water to the smear and allow the mixture for 10 minutes.

Gently wash the slide under running tap water for 30 seconds.

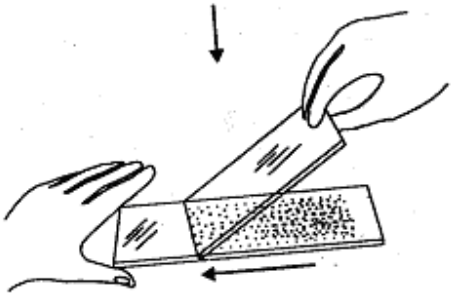
Blot dry the slide with blotting paper and examine under oil immersion objective after placing a drop of immersion oil over the smear.

Count 100 white blood cells and record each type of leukocytes encountered (by referring to standard permanent slides for identification of types of leukocytes). And estimate the percentages of each type of leukocytes.

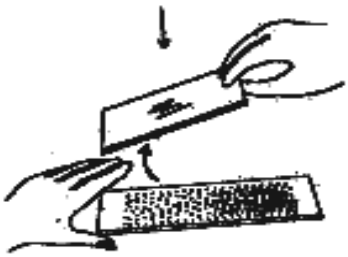
Place a drop of blood towards one end of the slide



Place another slide against the drop of blood and allow the drop to spread along the edge of the applied (top) slide.



Push the top slide to the left at an angle of 30 0 along the entire surface of the bottom



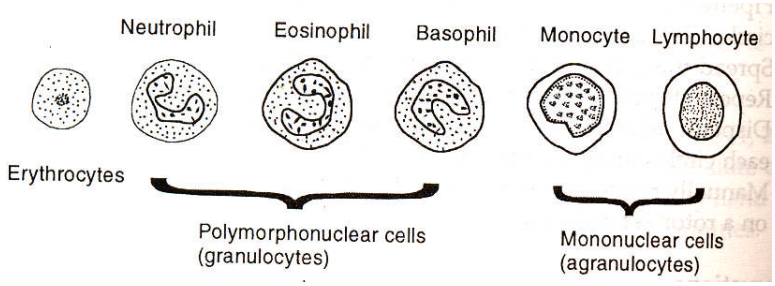
Remove the top slide and allow the lower slide to air dry

Preparation of blood smear

Precautions :

Examine the smear for uniform thickness with naked eye and microscopically under oil-immersion objective.

Always choose an area on the slide, ideal for counting.



Results :

Blood smear consists of five types of leukocytes (white blood cells, WBCs) : neutrophils, lymphocytes, monocytes, eosinophils and basophils in addition to platelets and erythrocytes. Among them neutrophils are more abundant (50-70%) followed by lymphocytes (20-30%), monocytes (2 –6%), eosinophils (1-5%) and basophils (less than 1%).

Experiment No. 9.

OBSERVATION OF SYMPTOMS OF VARIOUS INFECTIOUS DISEASES

Aim : To study the symptoms of some important infectious diseases. (from photographs).

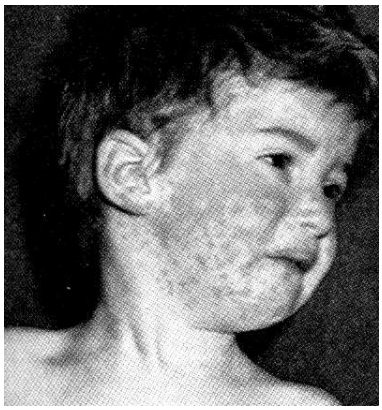
Tetanus

Tetanus is caused by *Clostridium tetani*.

The disease is characterized by convulsive contraction of voluntary muscles.

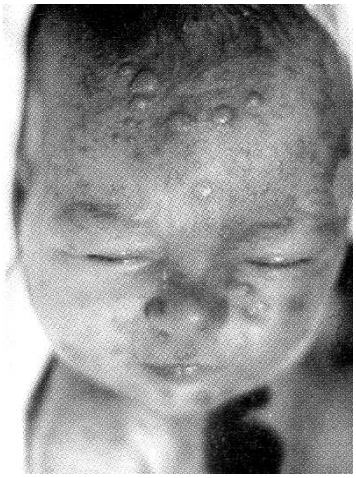
Next the muscles of jaw are affected and it leads to ‘lock jaw’ symptoms, which unable the patient to open his mouth.

Finally , voluntary muscles also involved resulting in bending of the body backwards like a bow.



Measles

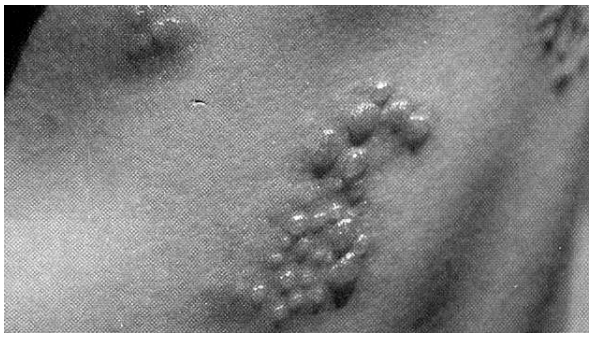
Measles is a febrile (accompanied by fever) disease with a rash caused by the rubeola virus.
The infection is acquired by inhalation of droplet nuclei.
Symptoms appear in 9 to 11 days in children and 21 days in adults, after entry in to the body.
Koplik's spots, white spots with central bluish specks appear on the upper lip and cheek mucosa.
After 2-3 days, symptoms like fever, conjunctivitis, and cough appears.
These symptoms followed by a rash spread from forehead to the upper extremities, trunk, and lower extremities and disappears in the same order after several days.



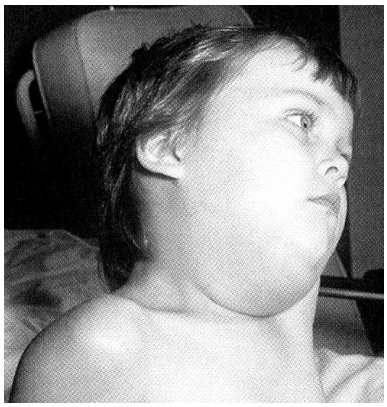
Chicken pox and ***Shingles***

Chicken pox and shingles are caused by a herpes virus – varicella-zoster virus (VZV).
Chicken pox is highly contagious disease that causes skin lesions and occurs mostly in children.
The infection is acquired by inhalation of droplet nuclei. From upper respiratory tract it enters the blood and causes fever and malaise.

After two weeks, small, irregular, rose coloured skin lesions appear.
The fluid in them becomes cloudy.
They first start on the scalp and trunk and spread to the face and limbs.
Sometimes to the mouth, throat, and vagina.
They dry and crust over in a few days.



In Shingles, painful lesions like those of chickenpox usually are confined to a single region supplied by a particular nerve.
Infection produce clusters of skin lesions distinguishable from that of chickenpox.
Symptoms range from mild itching to continuous, severe pain and can include headache, fever and malaise.
Lesions often appear on the trunk in a gridle like pattern (*zoster*, girdle) but can infect the face and eyes.
New vesicles consistently erupt, whereas old ones fail to heal.



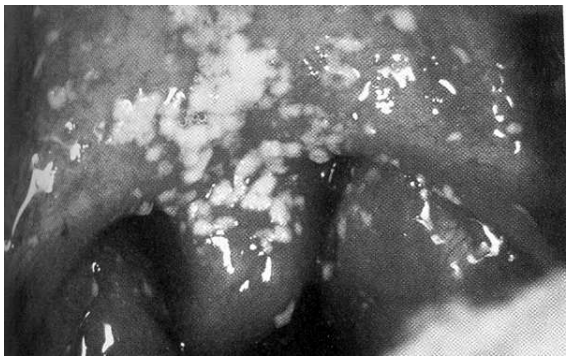
Mumps

It usually affects the children of the age 5 – 15 years.
The mumps virus belongs to the family paramyxoviridae.
Parotidis is the characteristic symptom – inflammation and enlargement of parotid salivary glands.
The enlarged perotid glands obscure the angle of the mandible and may elevate the ear lobe.
It results in difficulty in opening of the mouth. Pain is common at this stage.

Candidiasis

Candida albicans, one among the normal flora of the digestive and urenogenital tracts, causes candidiasis in debilitating individuals.

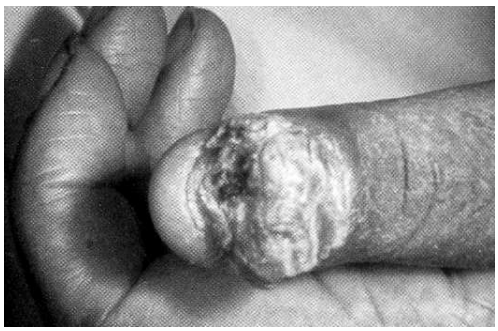
Superficial candidiasis appears as thrush, milky patches of inflammation on oral mucous membrane.
Most common in infants, diabetics, debliating patients. This is referred to as oral candidiaisis.



Oral candiaisis.

Cannery workers whose hands are in water for long periods sometimes develop skin and nail lesions - Candidiasis of nails.

It is common opportunistic organism causing infections in patients with diseases such as tuberculosis, leukemia and AIDS.

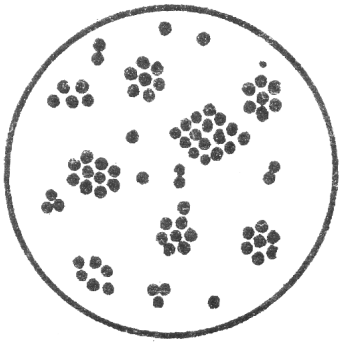


Candidiasis of nails.

Experiment No. 10

MICROSCOPIC OBSERVATION OF PATHOGENIC BACTERIA

Aim : Observation of morphology of some important pathogenic bacteria (permanent sides)



Staphylococcus aureus

Staphylococci cells are gram positive coccoid forms.

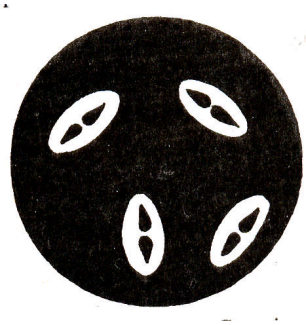
They appear like grape like bunches.

Each cell measure 0.7 –1.0 μm in diameter. They arranged in bunches like grapes.

The cells are capsulate and non spore forming.

It is aerobic or facultative anaerobic, and grows well on common media under aerobic conditions.

Colonies on nutrient agar or blood agar medium are small (2-4 mm),smooth, shining, opaque, golden yellow (sometimes yellow to white) domes resembling small drops of glass paint.



Streptococcus pneumoniae

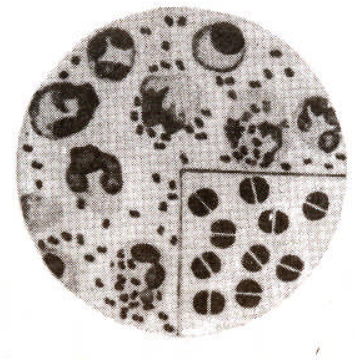
The bacterium is a gram positive, non spore forming and nonmotile coccoid form.

The cells are typically present in pairs in vivo or in short chains in culture, and are surrounded by a large conspicuous capsule.

Each coccoid cell is some what elongated and pointed at one end but rounded at the other (i.e. lanceolate type) and the two members of the pair are arranged with their long axis in line with each other, approximately 1 μm long and the pair point away from each other.

A prominent polysaccharide capsule is present surrounding each pair of cells.

On blood agar medium, the colonies are small (0.5-1 mm), dome shaped, glistening with an area of greenish discoloration around them.



Meningococci in cerebrospinal fluid.
Inset – enlarged view to show flat adjacent sides of the cocci

Neissiria meningitides

This bacteria is commonly called as meningococci.

The bacterium is gram negative, aerobic, coccoid form which are typically arranged in pairs.

These cocci are non sporulating , non motile and oxidase positive.

The cocci are oval or semispherical , measuring 0.6 –0.8 μm and arranged in pairs with adjacent sides flattened.

On agar medium colonies appear like small 1 mm in diameter, translucent, round, convex, bluish grey with a smooth glistening surface and smooth margin.

Salmonella typhi

It is an exclusive human pathogen causing enteric fever.
It is a gram negative rod shaped bacterium measuring 2- 4 x 0.5 um.
It is actively motile with numerous long peritrichous flagella and does not possess a capsule.
On nutrient agar medium, colonies appear as grayish white, circular, dome shaped, smooth, translucent and 2-3 mm in diameter.
On McConkey agar, the differential medium for lactose fermenters and non lactose fermenters, *S. typhi* appears as colorless colonies (non lactose fermenter)
It is negative for indole production and Voges- Proskauer test but positive for citrate utilization and methyl red reduction tests.

Shigella dysenteriae

Shigella are gram negative, non spore forming, non motile, non capsulate, aerobic/ facultative aerobic bacteria.
Each bacterial cell is rod shaped measuring 1 –3 x 0.5 um in size. Fimbriae may be present.
On culture media, colonies appears like small (2 mm), smooth convex, circular and translucent.

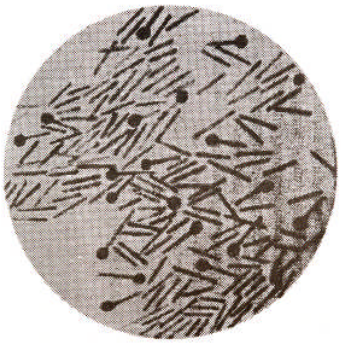
Esherichia coli

It is a gram negative, non sporulating, straight rod measuring 1 – 3 X 0.4-0.7 um.
It may be motile with peritrichous flagella or non motile.

On nutrient agar medium, colonies are large, thick, grayish white, smooth , opaque or partially translucent.
It is positive for indole production and methyl red reduction tests, but negative for Voges-Proskauer test and citrate utilization test.

Vibrio cholerae

These are curved or comma shaped rods with round or slightly pointed ends.
These are gram positive, non- capsulate, non spore forming strongly aerobic/ weakly facultative anaerobic organisms.
They measure about 1.5 – 3.0 x 0.5 um in size.
The cells are actively motile with a single polar flagellum.
On nutrient agar medium colonies appear like mist, translucent, 1 –2 mm in diameter, showing a characteristic bluish colour in transmitted light.

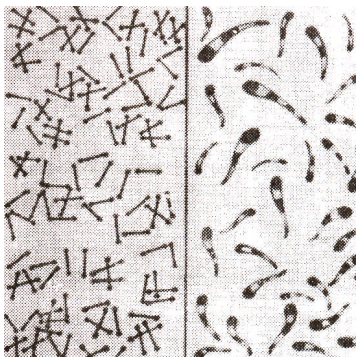


(some cells with spores and some without spores)

Clostridium tetani

C. tetani cells are gram positive, slender straight rods
measuring 2.5 – 5 x 0.5 – 1 μm in size.
The cells produce motile with peritrichous flagella.
They possess no capsules. The organism is a strict anaerobe.
C. tetani produces conspicuous and highly resistant endospores.

The endospores are spherical and formed at the end of the cell giving the cell a 'Drumstick' appearance.
On solid media the colonies are thin, translucent, spreading with finger –like projections.



Unseparated irregularly arranged cell (left)
Typical club shaped cells with strongly stained parts (right)

Corynebacterium diphtheriae

It is a gram positive, aerobic/facultative anaerobic, non spore forming.
It is non capsular, nonmotile, straight or curved rod that tapers with characteristic club shaped swelling at one or both ends.
They measure 3 – 6 μm in length and 0.6 – 0.8 μm in width.
The bacilli are arranged in a characteristic fashion in smears.
They are usually seen in pairs or small groups, the bacilli being at various angles to each other, resembling the letters V or L or like Chinese letters.

Mycobacterium tuberculosis

The cells are straight or slightly curved delicate long rods that measure 3.0 x 0.3 μm
They occur singly or in pairs or as fragile filaments.
It is a strict aerobe and cells are non capsulate, non motile and non spore forming
The filaments in virulent strains appear as coiled bundles called 'serpentine cords'.
The cells are gram positive and also acid fast.

Experiment No. 11

DETERMINATION OF MINIMAL INHIBITORY CONCENTRATION (MIC) OF ANTIBIOTIC

Aim : To determine the lowest concentration of antibiotic required to inhibit the growth of the organisms *in vitro*. This concentration is referred at as the MIC.

Principle : The effect of chemotherapeutic agent varies with the target species. Some idea of the effectness of a chemotherapeutic agent against a pathogen can be obtained from minimal inhibitory concentration (MIC). The MIC is the lowest concentration of a drug that prevents the growth of a particular pathogen. A pathogen should have a MIC value low enough to be destroyed by the drug. A pathogen with too high MIC value is resistant to the agent at normal body concentrations.

Requirements : Test antibiotics (penicillin and tetracycline), physical balance, nutrient broth, inoculation loop, pipettes, spirit lamp, test tubes, young bacterial cultures of *Streptococcus aureus* and *Pseudomonas aeruginosa*.

Procedure :

1. Prepare different concentrations of antibiotics (10 ppm, 20 ppm, 50ppm, 100ppm, 500 ppm, 1000 ppm, 2000 ppm) using sterile distilled water.
2. Prepare nutrient broth and transfer 4.9 ml each in to 8 test tubes and then sterilize.
3. After cooling transfer aseptically 0.1ml of each of dilutions of antibiotic compound in to nutrient broth test tubes (the final volume is 5.0ml in each tube)
4. To each of the tubes add 0.1ml of bacterial culture broths. Maintain 1 set for *S. aureus* and another for *P. aeriginosa*.
5. Shake the tubes such that the inoculum is uniformly mixed with the broth.
6. Maintain 2 controls.
    i) Blank –A, with only the antibiotic and broth.
    ii) Blank –T , which contains the broth and bacterial inoculum.

These controls help in ensuring the aseptic conditions under which the experiment is conducted.

7. Incubate the tubes at 37 ^{0}C for 24 hrs.
8. Observe the tubes for turbidity changes after 24 hours.

Antibiotic	Organism	Blak T	10 ppm	20 ppm	50 ppm	100 ppm	500 ppm	1000 ppm	2000 ppm
Streptomycin	*S. aureus*		+	+	+	+	_	_	_
	P. aeriginosa								
Tetracycline	*S. aureus*								
	P. aeriginosa								

'+' indicates growth '-' indicates no growth

Observations and results :

Turbidity appearance indicates the growth of the organism and indirectly inactivity of the antibiotic ; while no turbidity indicates the inhibition of microorganism by the test antibiotic. If necessary measure the turbidity changes in a colorimeter at 600 nm wave length. Note down the maximum concentration at which turbidity did not appear (no growth of the organism), and that will be the minimum inhibitory concentration of that antibiotic for that microorganism under given set of conditions.

Experiment No. 12

COMPARISON OF ANTIBACTERIAL ACTIVITY OF PENICILLIN AND TETRACYCLINE BY DISC METHOD

Aim: To compare the antibacterial activity of tetracycline and penicillin by disc diffusion method.

Requirements: Fresh bacterial culture (*Bacillus subtilis*), Petri plates, Nutrient agar medium, Inoculation chamber, Forceps, selected antibiotic discs.

Procedure:

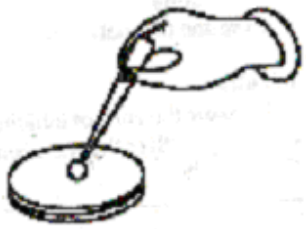
1. Preparation of bacterial suspension: Take the bacteria from pure slant culture with the help of a inoculation loop and mix it with suitable amount of sterile distilled water, in a test tube, in an inoculation chamber. Mix the contents thoroughly until a suspension was formed. (Take any antibiotic sensitive strains of bacteria, for better results. E.g. *Bacillus subtilis*).
2. Preparation of medium seeded plates : Prepare the nutrient agar medium as per the following composition

   Peptone – 5.0 g
   Beef extract – 3.0 g
   NaCl – 5.0 g
   Agar agar – 20.0 g
   Distilled water – 1000 ml
   pH – 7.0

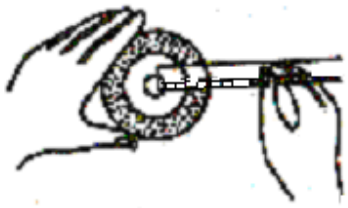
   Sterilize the medium by autoclaving at 121^{o}C temperature and 15 lbs pressure for 15 minutes.
3. Cool the medium to room temperature and add sufficient amount of the prepared bacterial suspension to it. Mix the contents thoroughly.
4. Disperse 20 ml aliquots of the inoculated medium into each petri plate.
5. Allow the plates to solidify.
6. After solidification, place the selected antibiotic disc at the center of the plate with the help of sterilized forceps and gently press it towards the medium surface.

7. Incubate the plates in inverted position for 24 hours at 37^{o}C temperature.
8. Observe the plates after incubation, for the formation of clear zone around the disc.
9. Measure the radius (r) of the clear zone and calculate the area of clear zone by using the formula πr^2 and compare the zones produced by the two types of antibiotic discs used.

Result : Depending on the bacteria used, the antibacterial activity of the antibiotic used will differ. For *B. subtilis,* tetracycline will produce wide zone than pencillin.



Placing the antibitic disc onto the seeded medium plate



Measurenent of clear zone around the antibiotic disc

Experiment No. 13

IMVIC TESTS FOR *E.coli* and *Bacillus* sp.

a) Test for indole formation (indole test)

Aim : To test the ability of ***E.coli*** **and** ***Bacillus*** **sp.** for the metabolic formation / accumulation of indole.

Principle : Several bacteria are proteolytic. They utilize different amino acids derived from hydrolysis of proteins by their catabolic degradation for growth. Some amino acids are catabolized half way and such products are accumulated in medium. Tryptophan present in growth medium broth is degraded incompletely resulting accumulation of 'indole'. Presence of indole in fermented medium indicates its inability to degrade.

Indole is tested for its presence, if present, the test is given as indole positive.

Requirements :

Indole test medium broth tubes, Test organism, Kovac's reagent, etc.,

Procedure :

Prepare actively growing cell suspension of test organism in broth or saline.
Inoculate in to indole test broth tube (s) and incubate at optimum temperature for 48- 96 hrs.
Add 0.5 ml of kovac's reagent to fermented tube (s) and shake gently.
Formation of red colour in alcohol layer indicates positive for indole.
Observe the tubes and note result.

Observation / result : ***E.coli*** **is positive and** ***Bacillus*** **sp is negative.**

b)Methyl Red Test

Aim : To identify ***E.coli*** **and** ***Bacillus*** **sp.** for fermentation of glucose to form high acid concentration buffered medium, by testing with methyl red indicator.

Principle : Several bacteria ferment glucose in well buffered growth and form acid to decrease pH beyond buffering capacity of medium. Accumulation acid decreases medium pH value below 4.5 which is tested with methyl red acid or base indicator. Presence of acidic pH is indicate by red colour with methyl red and taken as methyl red (MR) positive.

Requirements : Glucose phosphate, peptone water tubes, methyl red indicator solution, test bacterial culture, other routine lab requirements.

Procedure :

Prepare suspension culture of actively growing bacterial test organism.
Inoculate in to glucose phosphate peptone water tube (S) and incubate for 48 hrs, at optimum growth conditions
Add about 5 drops of methyl red indicator solution to each tube, mix and observe immediately.
Bright red colour indicates positive test for methyl red and yellow colour indicates negative

Observation / result : ***E.coli*** **is positive and** ***Bacillus*** **sp is negative.**

c) Voges-Perskauer Test

Aim : To test the ability of ***E.coli*** **and** ***Bacillus*** **sp.** to ferment glucose to form acetyl methyl carbinol in buffered medium.

Principle : Some bacteria ferment glucose in buffered medium but do not decrease pH as they do not form and accumulate sufficient acid. Instead a fermentation product acetyl methyl carbinol or its reduction product, 2,3 – butylene glycol is formed which does not change pH. By oxidizing it to di-acetyl with the addition of oxidizing agent (KOH) and shaking in air, the presence of this product is tested. Di-acetyl reacts with α- naphthol to give pink colour becoming crimson later. This test is called as Voges- Perskauer (VP) test. Generally enterobacteria are tested for this reaction. An organism is positive for MR and negative for VP are vise versa.

Requirements : Glucose phosphate peptone water tubes, 40% KOH solution, 5% α-naphthol solution in absolute ethanol, test culture, others

Procedure :
Prepare culture suspension of actively growing test organism
Inoculate in to glucose phosphate peptone water tubes and incubate for 48 hrs at optimum growth conditions.
Add 1 ml of 40 % KOH solution and 3 ml 5% of α - naphthol in alcohol solution to each tube and shake vigorously to ensure maximum aeration.

Positive reaction is indicated by the development of pink colour in 2-5 min. and becoming crimsom in 30 min.
Observe the tubes at different times and note the results.

Observation / Result : ***E.coli*** **is negative and** ***Bacillus*** **sp is positive**

d) Citrate utilization test

Aim : To test the ability of ***E.coli*** **and** ***Bacillus*** **sp.** for its ability to utilize citrate as carbon source in defined growth medium

Principle : Majority of bacteria are able to utilize citrate as carbon source and ammonium salt as nitrogen source in the growth medium. Some are not able to do this. The ability of a given organism to use citrate and ammonium as nutrient sources in defined medium is tested. Citrate, if utilized, concentration decreases and there will be increase in medium pH. This is indicated by change in colour of acid base indicator. Generally, enterobacteria are identified by this test. If an organism is able to use citrate a s carbon source, it is identified as citrate positive.

Requirements : Koser's liquid citrate medium (pH 6.8), Simmons citrate agar (pH 6.8), bromothymol blue indicator solution, test culture, and others.

Procedure :
Prepare a saline suspension of actively growing test organism.
Inoculate into Koser's liquid citrate medium tubes and Simmon's citrate agar slants.
Incubate for 96 hrs at optimum growth conditions.
Observe turbidity (growth in Koser's medium and change of colour (from light green to blue) in Simmon's citrate agar slants which indicate positive for citrate positive utilization.
A sub culture may be done in the same media to eliminate false positives
Identify the given culture for its citrate utilization based on the test result.

Observation / Result : ***E.coli*** **is negative and** ***Bacillus*** **sp is positive**

Experiment No. 14

DIAGNOSIS OF TYPHOID FEVER – WIDAL TEST

Aim : To detect the presence of *Salmonella* antigen in the serum of the patient blood

Principle : *Salmonella* possesses the following antigens based on which they are classified and identified – I) flagellar antigen – H ii) somatic antigen O iii) a surface antigen Vi. WIDAL test is conducted for the measurement of O agglutinin for typhoid bacilli in the patient's sera.

Requirements : Saline solution, serum prepared from patients blood, O antigen test tubes, test tube stand, etc.,

Procedure :

1. Preparation of serial dilutions of patients serum :

a) Take ten serological test tubes and label them as 1 –10, and place them in a test tube stand.

b) Pipette 1 ml of patient serum in to the first test tube.

c) Add 0.5 ml of saline to each of the remaining test tubes (no. 2-10)

d) Transfer carefully, 0.5 ml of serum from 1^{st} test tube into 2^{nd} test tube, shake the contents well.

e) Repeat the process by transferring 0.5 ml from 2^{nd} tube to the 3^{rd} tube, and from 3^{rd} tube to 4^{th} and so on up to the last test tube.

2. Transfer 0.5 ml of antigen to each test tube, using separate pipette.
3. Shake all the test tubes in the test tube stand, and incubate them at 37 ^{0}C in a water bath for 30 min.

4. Centrifuge the test tubes at 2000 rpm for 7 minutes, and observe for the presence of flakes (agglutinations).
5. Record the antibody titer, that gives the concentration of antibodies in the patient's serum. E.g. if the titer is 1/640 , it will be interpreted that the patients serum contains 640 units of antibodies.

Note : Usually, the antibody conc. increases with increase in disease severity.

Titer is the highest dilution, up to which a positive reaction be observed/recorded.

Experiment No. 15

VENERIAL DISEASE REASREACH LABORATORY TEST

Aim : Detection Syphilis infection by VDRL test (diagnosis of syphilis**). Detection of syphilis antibodies in patients serum.**

Principle : This test was developed by venereal disease research laboratory, USPHS, New York. It is a non-specific standard test for Syphilis (STS). In VDRL test, the inactivated serum (inactivated by heating at 56 0C for 30 min) is mixed with cardiolipin antigen on a special slide and rotated for 4 minutes. Cardiolipin remains as uniform crystals in normal serum but forms a visible clumps on combining with reagin antibody. The reaction is read under a low power microscope. By testing serial dilutions, the antibody titer can be determined.

Requirements : VDRL antigen, (Cardiolipin 0.03 % ; lecithin- 0.21% and Cholesterol 0.9% these three should be with buffer), reactive serum (positive control) – heat inactivated serum (incubated for 30 min at 56 ^{0}C), heat inactivated non-reactive serum (negative control), clean glass slide with depressions (cavity slides) or paraffin rings with 15 mm inside diameter.

Procedure :

1. Number 3 rings on the cavity glass slide. 1st one is called as test ring, 2nd is positive control ring and the 3rd is negative control ring.
2. Take 0.05ml of patients serum on the test ring, 0.05 ml of known (positive) serum in positive control ring and 0.05ml of known negative serum in (-ve) control ring.
3. Add one drop (1/60 ml) of antigen to the three rings, then rotate the slide on VDRL rotator for 4 min.

4. Observe the slide under low power magnification of the microscope, for the flocculation or precipitation.

Observations and results :

- Formation of large clumps – presumptive positive test for syphilis.
- Small clumps – weakly positive reaction.
- No clumps – a negative test for syphilis.

Experiment No. 16

EXERCISES IN CELLULAR MICROBIOLOGY

The following are some of the problems (solved) related to cellular microbiology.

EXERCISE – I

1. Match the following adhesions with their respective receptors.

Adhesins	**Receptors**	
(a) Lipoteichoic acid	(i) Galactose	(c)
(b) Glyceraledehyde 3 – (P) dehydrogenase	(ii) Vitronectin	(d)
(c) M – Protein	(iii) Fibronection	(a)
(d) Vitronection binding proteins	(iv) Myosin	(b)
(e) Invasin	(v) Heparin sulphate proteoglycan	(f)
(f) ActA	(vi) α-5-β, integrin	(e)

2. Find out the habitats for the following invasive pathogens.

(i) *Salmonella typhimurium* --- Intra vacuolar nich

ii) *Listeria monocytogenes* -- Cytosol nich

iii) *Shigella* species -- Cytosol nich

iv) *Yersenia* species -- Extra vacuolar nich

v) *Lagieonella pneumophilia* -- Intra vacuolar nich

vi) *Mycobacterium tuberculosis* -- Intra vacuolar nich

3. Abbreviate the following

i) EGFR - Epidermal growth factor receptor

ii) ICAM - Intercellular adhesion molecule

iii) HUVEC - Human Umblical Vascular Endothelial Cells

iv) Jak - Janus Kinase

v) MAPK - Mitogen activated Protein Kinase

vi) VCAM - Vascular Cell Adhesion Molecule

vii) Sifs - *Salmonella* induced filaments

viii) STAT - Signal transducers & activators of transcription

ix) MRSA - Methicillin resistant *Staphylococcus aureus*

x) FAK - Focal adhesion kinase

4. Match the following toxins with their activities :

i) Streptolysin O	ADP ribosylation of Ras	(viii)
ii) TSST -1	Cleavage of ε – cadhesin	(x)
iii) DT	Cell membrane permeabilisation	(i)
iv) Shiga toxin	receptor for intimin	(vii)
v) PT	ADP ribosylation of Gs	(ix)
vi) Anthrax EF	binding to MHC class II mol. s	(ii)
vii) Tir	ADP ribosylation of Gi	(v)
viii) Exos	N- glycosidase activity on 28 s rRNA	(iv)
ix) LT	ADP ribisylation of EF_2	(iii)
x) *Bacteriodes fragilis* endotoxin	binding to calmodulin	(vi)

5. Match the following first messengers with their molecule type

	First messengers	**Molecule type**	
i)	Insulin	Amino acid	(iii)
ii)	Vasopressin	Fatty acid	(iv)
iii)	Ephinephrin	Polypeptide	(i)
iv)	Testosterone	peptides	(ii)

EXERCISE II

1. A mutant gene (Ras) is found in many cancers of the lung, pancreas and colon and may be responsible for the altered growth and metabolism of tumor cells. Ras protein, a product of Ras gene is a mutated G- protein that lacks GTase activity. How does the absence of activity effected the adenylate cyclase signaling pathway.

Ans : G- proteins are molecular switches with two inter-convertable forms, an active GTP bound form and an inactivate GDP bound form. In the normal G – proteins GTPase activity converts the G – protein which is in active form to the inactivate form. Because the Ras protein lacks the GTPase activity it cannot be inactivated. So the result is continuous activation of adenylate cyclase and prolonged responses to certain extracellular signals.

Dr. M. RAGHU RAM