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Expt. No.01 **Date: -**

Demonstration of media for cultivation of pathogens

1a). Mannitol Salt agar

Aim: - To prepare Mannitol Salt Agar medium.

Theory / Approach: -

MSA is generally used for the isolation of Staphylococci species. It is a selective medium for Staphylococci. This medium contains 7 % NaCl that is high enough to be inhibitory to many other bacteria. Staphylococci grow at this high concentration of salt. Colonies of pathogenic Staphylococci are surrounded by a yellow halo indicating mannitol fermentation.

Composition of medium: -

D. Water	100 ml
Mannitol	1 gm
Peptone	1 gm
NaCl	7 gm
Meat extracts	0.1 gm
Phenol red	2.5 mg
Agar Agar	2.5 gm
pH	7.4

Sterilization: - At 10 lbs/inch^2 (115 ^{0} C) for 30 minutes.

Procedure: -

All the ingredients are weighed accurately and are added in a definite amount of distilled water, mixed thoroughly and after the pH is adjusted to 7.4, Phenol red is added. Sterilization is done at 10 lbs/inch^2 (115 ^{0} C) for 30 minutes. Sterile Mannitol Salt Agar is displaced in sterile petriplates.

1b). Wilson and Blair's medium

Aim: - To prepare Wilson and Blair's medium.

Theory / Approach: -

Wilson and Blair's medium is used for the isolation of Salmonella species. It is a selective medium for Salmonella. Brilliant green makes the medium selective. It is also differential as *Salmonella paratyphi A* produce green coloured colonies while *Salmonella typhi and Salmonella paratyphi B* produce black coloured colonies by reducing sulphite to sulphate.

Composition of the medium: -

Solution A: - Bismuth sulphite Glucose Phosphate mixture

D. Water	100 ml
Bismuth Ammonio citrate	3 gm
Sodium sulphite	10 gm
$Na_2HPO_4 . 12 H_2O$	10 gm
Glucose	5 gm

Dissolve Bismuth Ammonio citrate in 25 ml boiling water and
Sodium sulphite in 50 ml boiling water. Mix two solutions and boil. Add the Disodium phosphate crystals while boiling the mixture. Cool. Add the glucose dissolved in 25 ml boiling water and cooled.

Solution B: - Iron Citrate Brilliant Green Mixture

Ferric Citrate solution (1% in sterile D/W)	200 ml
Brilliant Green solution (1% in sterile D/W)	25 ml

Mix together

Complete Medium: -

Sterile Nutrient Agar	100 ml
Solution A	20 ml
Solution B	4.5 ml

Procedure: -

Sterile nutrient agar is prepared and both solution A & B are added with sterile precautions and the plates are poured.

1 c) LOWESNSTEIN AND JENSON MEDIUM

It is used to inoculate ***Mycobacterium tuberculosis*** and other Mycobacteria. Malachite green present in the medium prevents the growth of the other organisms.

Formula and Preparation

Composition:

1) Homogenized whole egg (6-8 fresh hens egg) : 275ml
2) 1N HCl acid : 8 ml
3) Salt glycerol solution : 153 ml

(Salt glycerol solution is prepared by mixing 600 ml of distilled water and 12 ml glycerol, 6.3 gm of potassium di-hydrogen phosphate and 0.3 gm magnesium sulphate are then dissolved in glycerol and distilled water mixture.)

4) 2 % (w/v) Malachite green : 2.75 ml

(Prepare a 2 per cent solution of malachite green to sterile water with sterile precautions by dissolving the dye in the incubator for 1-2 hrs. This solution can be stored indefinitely and should be shaken before use.)

5) Pennicillin 'G' : 25,000 IU

Procedure:

i) Wash eggs and immerse in 70% (w/v) alcohol.

ii) Break the egg in sterile flask by using a sterile knife.

iii) Homogenize the contents.

iv) Add aseptically the ingredients 2,3,4,5 and mix well.

v) Adjust pH of the medium between 6.4-6.8.

vi) Dispense aseptically in sterile cap bottle (4ml of each).

vii) Slope the bottle or trey and aspirate at 80°C.

viii) Since the medium has been prepared with sterile precautions this heating is to solidify the medium, and not to sterilize it.

Quality control: Test the medium by inoculating 0.2 ml of sputum containing ***Mycobacterium tuberculosis.***

1 d). Corn Meal Agar medium

Aim: - To prepare Corn Meal Agar medium

Theory / Approach: -

This medium is used for the growth of yeasts *Candida albicans*. The appearance of chlamydospores is diagnostic.

Composition: -

D. Water	100 ml
Corn meal	4 gm
Agar Agar	2 gm
pH	6.8

Procedure: -

Corn meal is added in 100 ml water and heated at about 60 ^{0} C. Then it is filtered through filter paper. The volume again is made to 100 ml by adding D/W. Agar is added. The pH of the medium is adjusted to 6.8. Medium is sterilized at 15 lbs / inch^2 pressure for 15 minutes.

Expt. No. 02 **Date: -**

Staining Techniques

2a) ACID FAST STAINING

Aim: To perform the acid fast staining.

Principle

The organism such as ***Mycobacterium tuberculosis*** **(**that cause tuberculosis) and ***Mycobacterium leprae*** (that cause leprosy) are extremely difficult to stain by ordinary methods because of the lipid containing cell wall. They bind Carbol-fuchsin tightly and resist de staining with strong decolorizing agent such as alcohol and strong acids. Acid -fast negative bacteria (other bacteria) radially lose the stain when treated with acid-alcohol solution. Heat is applied in the Ziehl-Nielsen hot stain method for the detection of ***Mycobacterium tuberculosis*** and cold stain method is used for the detection of ***Mycobacterium leprae*** and ***Nocardia asteroids*** (weakly acid-fast). All these methods gives Carbol-fuchsin as the primary stain and phenol as the mordant. Following the counter staining with methylene blue (or malachite green) the decolorized acid fast negative organisms and other cells take blue color in contrast with red colored acid fast organisms.

Requirements

1. Glass slides and cedar wood oil
2. Nichrome loop and Bunsen burner
3. Microscope

Reagents

1. Stock Carbol-fuchsin solution (saturated basic fuchsin)

Solution A

a) Basic fuchsin powder : 3 gm

b) 95 % (v/v) ethyl alcohol to 100ml

Dissolve the powder in alcohol by using mortal and pestle. If necessary heat it carefully in a boiling water bath.

Solution B

50 % (w/v) Phenol solution

2. Working Carbol-fuchsin staining solution:

Mix 10 ml of solution A with 90 ml of solution B. Keep at room temperature over night, filter and store in amber color dropping bottle.

3. 20 % (v/v) Sulphuric acid

4. Methylene blue counter stain (0.3 %, w/v)

a. Methylene blue : 0.3 gm

b. Distilled water to : 100 ml

Dissolve the powder in distilled water filter and store in amber colored dropping bottle.

All the reagents are stable at room temperature.

Specimen

Sputum containing ***M. tuberculosis***.

Procedure

1. Prepare smear from the sputum specimen on glass slide and fix it by heating on Bunsen burner flame.

2. **Staining**: Place the heat fixed slide on the staining rack or rods and flood the smear with working Carbol-fuchsin stain.

3. Heat gently by Bunsen burner flame, until steam rises. Avoid boiling and continue heating for about five minutes. Do not allow to dry the stain on the slide. Add more stain if necessary.

4. Wash the stain off the slide with water and continue rinsing until the water that runs off is colorless.

5. **Decolorization**: Cover the slide with 20 % Sulphuric acid for about one minute (yellow colored complex should be drained off completely).

6. Counter staining: Cover the slide with methylene blue stain for one minute.

7. Wash with tap water, allow water to drain, and allow it to dry in air or blot carefully.

8. **Microscopic examination**: Observe the slide under low power objective and then examine under oil immersion objective.

9. **Recording of results**:

1. Acid-fast organisms: Bright red bacilli on blue background

2. Other organisms: Dark blue

The positive findings are reported as follows

No. of Acid-fast bacteria in 10 fields	**Report**
None	Absent
1-2	Positive, +
2-10	Positive, ++
10-100	Positive, +++
Above 100	Positive, ++++

Date: -

2 b) Differential WBC count by Leishman's staining

Aim: - To stain the White Blood Cells by Leishman's staining method and differential count..

Theory: -

Blood is the red viscous fluid of the body that flows through the heart and blood vessels. It contains a colourless fluid called plasma, in which Red Blood Corpuscles (RBCs), White Blood Cells (WBCs) and Platelets (Thrombocytes) are suspended. The plasma has a protein called as **fibrinogen.**

During clotting of blood, fibrinogen is removed from plasma as fibrin and the clear fluid remained behind is called **serum.**

Plasma = Fluid obtained after centrifugation of blood after addition of anticoagulant (Contains fibrinogen)

Serum = Fluid obtained after centrifugation of blood without addition of anticoagulant (fibrinogen is absent).

Serum = Plasma – Fibrinogen

Functions of blood: -

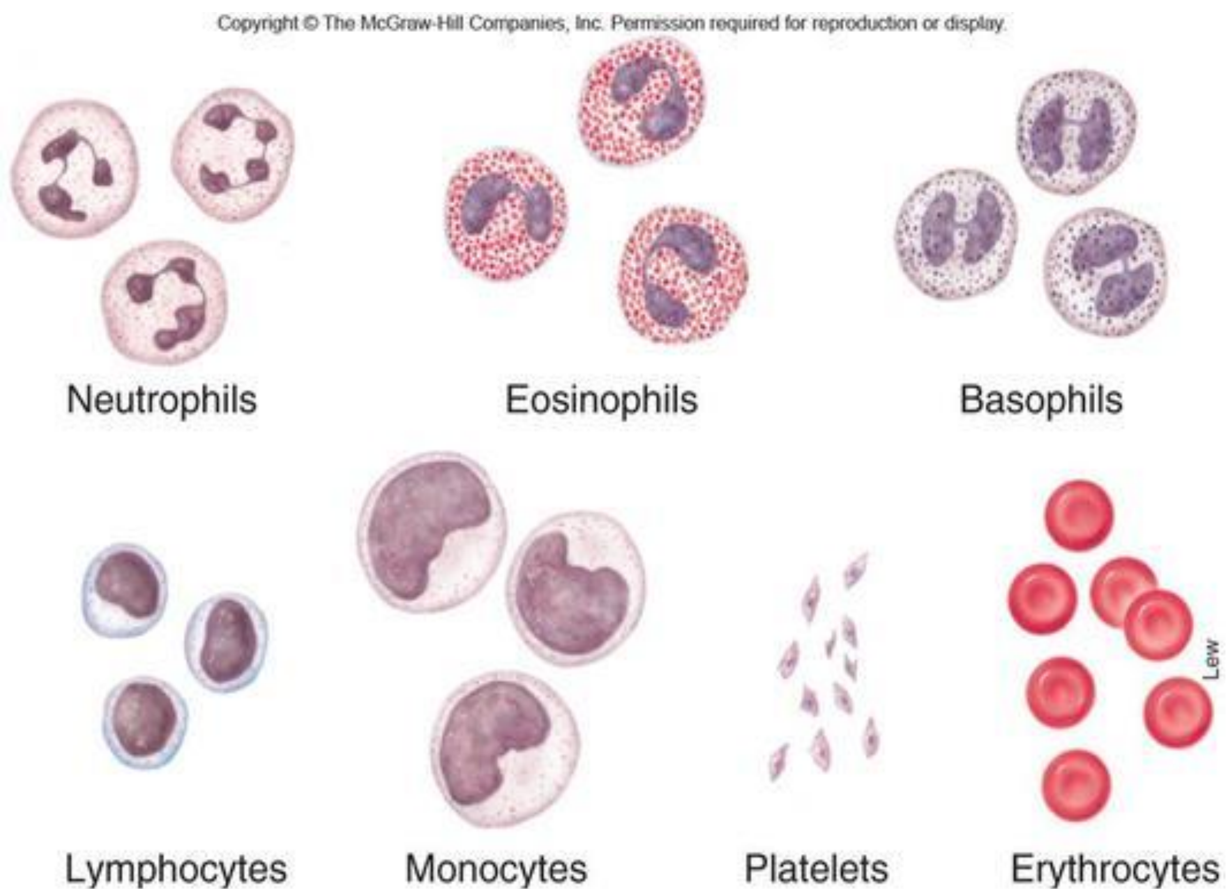
1. Distribution of oxygen and nutrients to all parts of the body
2. Removal of toxic material from body.
3. Maintenance of body temperature.
4. Defence mechanism of the body.
5. Prevention of excessive loss of blood during injury by clotting ability.

Blood staining is important for differential counting of White Blood Cells (WBCs), which is useful for diagnosis of diseases. The blood cells are of two types—

1. RBCs (Red Blood Corpuscles)
2. WBCs (White Blood Cells)

(Note: - RBCs are not cells in mature state because they do not contain nucleus. They contain haemoglobin molecule only.)

RBCs are more in number per cubic mm. WBCs are of two types mainly and are further classified as follows—



Name	**Granules**	**Nucleus**	**Normal %**	**Increase in number indicates**
Neutrophils	Purple coloured fine uniform	Multilobed (3 to 4). Deep purple	45 to 70	1. Septic Endocarditis 2. Pus formation in the body
Eosinophils	Pinkish to red coloured, Acidic in nature	Bilobed, violet coloured	1 to 5	1. Scarlet fever 2. Eosinophilia 3. Allergic infection
Basophils	Dark blue, larger, arranged at edge of cells	2 lobes or kidney shaped, light purple	0 to 1	1. Bacterial infection
Large lymphocytes	Absent	Small & cytoplasm large	20 to 45	1. Pulmonary tuberculosis
Small lymphocytes	Absent	Large & cytoplasm small		2. Whooping cough
Monocytes	Absent	Large kidney shaped,	2 to 8	1. Rocky mountain spotted fever

The basic components of the cell i.e. cytoplasm is stained by acidic dyes and acidic components of the cell i.e. nucleus is stained by basic dyes. In Leishman,s stain Acidic dye Eosin and Basic dye Methylene blue are present. Thus nucleus takes blue to purple colour and cytoplasm takes pink colour.

Requirements: -

1. Leishman's stain solution

Leishman's powder	150 mg
Methanol	100 ml

2. Absolute Ethanol

3. Lancet / Needle

4. Cotton, Grease free slides

5. Buffer of pH 6.8

Phosphate buffer

Sol. A → 0.2 M Solution of Monobasic Sodium Phosphate

(3.12 gm $NaH_2PO_4 . 2H_2O$ in 100 ml D/W)

Sol. B → 0.2 M Solution of Dibasic Sodium Phosphate

(2.83 gm Na_2HPO_4 or 7.17 gm of $Na_2HPO_4 . 12 H_2O$ in 100 ml of D/W)

Take 51 ml of solution A and 49 ml of solution B and dilute to 200 ml with D/W.

Procedure: -

1. Tip of finger is disinfected with absolute ethanol and punctured with the help of sterile lancet / needle.
2. First drop of blood is wiped out and second drop is taken at one end of clean, grease free slide.
3. Thin smear is prepared with the help of another slide.
4. Smear is air-dried but not heat fixed and flooded with Leishman's stain for 1 to 2 minutes.
5. Then buffer is added twice the amount of stain and allowed to react for 12 minutes.
6. Smear is gently washed with buffer, air dried and observed under oil immersion lens.
7. Count 100 WBCs sequentially and count types of WBCs.

Observation: -

As per diagrams and table.

Result: -

WE can see WBCs clearly by Leishman's staining method.

Conclusion: -

By blood staining we can observe cellular parts of blood like WBCs, RBCs. When we stain the blood, the parts of WBCs like nucleus, granules are clearly observed.

Expt. No. 03 **Date: -**

Haemoglobin estimation by Sahli's method

Aim: - Toestimate amount of haemoglobin in the blood by Sahli's method

Approach / Theory

Hemoglobin is the major constituent of the red cell cytoplasm, accounting for approximately 90% of the dry weight of the mature cell. It is comprised of heme and globin. Hemoglobin functions as the primary medium of exchange of oxygen and carbon dioxide. The commonly used methods are Sahli's/ acid hematin method.

Principle:

Blood is mixed with N/10 HCl resulting in the conversion of Hb to acid hematin which is brown in color. The solution is diluted till its color matches with the brown colored glass of the comparator box. The concentration of Hb is read directly.

Requirements:

1. Hemocytometer which consists of
   - i) comparator box which has brown colored glass on either side
   - ii) Hb pipette which is marked upto 20mm (0.02ml blood)
   - iii) Tube with markings of Hb on one side
   - iv) glass rod
   - v) dropper

2. Reagents required

i) N/10 HCl

ii) Distilled water

iii) Blood sample

Procedure

1. Add N/10 HCl into the tube upto mark 2g%
2. Mix the EDTA sample by gentle inversion and fill the pipette with 0.02ml (2 µl) blood.
3. Wipe the external surface of the pipette to remove any excess blood.
4. Add the blood into the tube containing HCl. Wash out the contents of the pipette by drawing in and blowing out the acid two to three times. Mix the blood with the acid thoroughly.
5. Allow to stand undisturbed for 10min.
6. Place the hemoglobinometer tube in the comparator and add distilled water to the solution drop by drop stirring with the glass rod till it's color matches with that of the comparator glass. While matching the color, the glass rod must be removed from the solution and held vertically in the tube.
7. Remove the stirrer and take the reading directly by noting the height of the diluted acid hematin and express in g%.

Observation

Result

Expt. No. 04 **Date: -**

ISOLATION AND STUDY OF NORMAL FLORA OF SKIN and THROAT

Aim: To isolate and study the normal flora of skin, nose and throat.

Introduction:

Microorganisms associated with the skin are: ***Staphylococcus epidermis***, ***Streptococcus pneumonia***, diphtheroids, bacilli, yeasts and fungi. The eye conjuctiva contains staphylococci, streptococci, diphtheroids and neisseriae. The upper respiratory tract has staphylococci, streptococci, diphtheroids, spirochaetes, ***Neisseria, Haemophilus*** and ***Branhamella***. The mouth and teeth contain spirochetes, anaerobes and microbes of dental caries, whereas intestinal tract possesses the members of the genera ***Bacteroids, Lactobacillus, Clostridium*** and ***Streptococcus*** besides small number of Proteus. ***Pseudomonas*** and ***Candida*** apecies. The human genitourinary tract has staphylococci, streptococci, lactobacilli, enteric bacilli, clostridia, spirochaetes, yeasts and protozoa of genus ***Trichomonas*** spp.

The environmental factor contributes a lot for the presence normal flora on specific area of the body. Different types of nutrient media are required for isolation of different types of microflora.

ISOLATION AND STUDY OF NORMAL FLORA OF HUMAN SKIN

Microorganisms associated with the skin are: ***Staphylococcus epidermis***, ***Streptococcus pneumonia***, diphtheroids, bacilli, yeasts and fungi. The resident flora of the skin is determined by using nutrient agar medium. Sabouraud agar plate for the isolation of yeast and molds are used. The upper respiratory tract has staphylococci, streptococci, diphtheroids, spirochaetes, ***Neisseria, Haemophilus*** and ***Branhamella***.

Requirements:

Nutrient agar plate

Sabouraud agar plate

Sterile saline test tubes

Gram staining set

Lacto phenol cotton blue

Procedure:

Prepare one plate each of nutrient agar and Sabouraud agar and label them accordingly.

Use sterile cotton swab moistened in sterile saline and rub on the skin near the wrist repeatedly.

From this swab streak on all the two plates at first corner and then streak with nichrome wire 3 quadrants for four-way streak inoculation.

Incubate the Sabouraud agar plate at 25 ^{o}C and the other plates at 37 ^{o}C for period of 48 hours.

***Examination of nutrient agar plate*:**

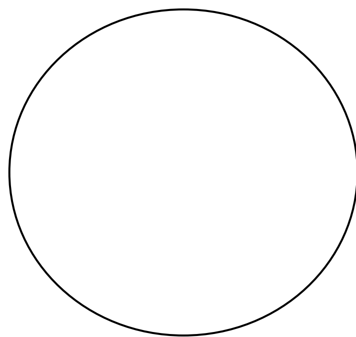
Observe the colony characters and prepare Gram-stained smears from each of the different colony.

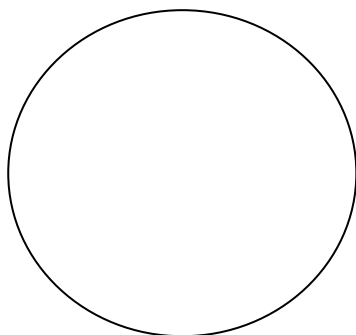
***Examination of Sabouraud agar plate*:** Cottony growth in scattered patches reveals the presence of mold.

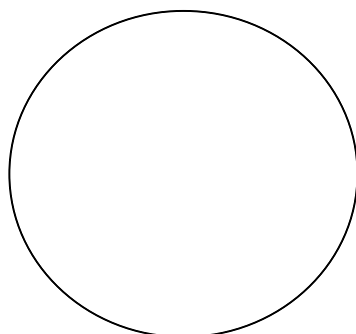
Prepare slides of fungus from the Sabouraud agar plate by using lacto phenol cotton blue.

Colony Characters:

Colony characters	**Colony - 1**	**Colony - 2**	**Colony - 3**
Size			
Shape			
Colour			
Margin			
Elevation			
Opacity			
Consistency			
Gram nature			







Result:

From observation it can be said that there are different types of bacteria on the skin and throat.

Expt. No. 05 **Date: -**

Agglutination tests (slide tests)
5 a) Blood Grouping

Aim: -

To determine the blood group by agglutination test.

Theory / Approach: -

This practical is not directly connected to the study of microbiology. It demonstrates the serology based on antigen-antibody reactions. The *in vitro* reaction of antigen and antibody is called serology and this provides the basis of learning diagnostics, therapeutics, and epidemiological implications. The serological reactions are Agglutination, Precipitation, Complement fixation, ELISA, RIA etc.

For the determination of blood groups in human, agglutination test is used. In this test antigen is particulate that is present on RBCs and when specific antibodies combine with them, a lattice complex forms called agglutination, which can be seen by naked eye.

Depending upon antigens present in the blood, more than 15 different blood group systems have been discovered, but 2 major blood group systems are widely used.

1. **ABO blood group system**
2. **Rh blood group system.**

1. ABO blood group system (Classic blood group system): -

In 1900, Karl Land Steiner discovered this system. This system is based on presence or absence of 2 antigens, **Antigen A or Antigen B,** which are glycoproteins and are present on the cell membranes of **Red Blood Corpuscles (RBCs)(Erythrocytes).** These antigens are known as iso-antigens because they occur within the same species.

ABO system classifies human blood into 4 groups as –

Blood group A
Blood group B

Blood group AB
Blood group O

Blood group **A** people have **type A** antigen on their RBC surfaces.

Blood group **B** people have **type B** antigen on their RBC surfaces.

Blood group **AB** people have **both type A and type B** antigens on their RBC surfaces.

Blood group **O** people have **neither type A nor type B** antigen on their RBC surfaces.

The plasma of persons of **type A** antigen contains antibodies against **type B** antigen (anti-B antibodies).

The plasma of persons of **type B** antigen contains antibodies against **type A** antigen (anti-A antibodies).

The plasma of persons of **type AB** antigens does not contain antibodies against **type A or B** antigen.

The plasma of persons of **type O** antigen contains antibodies against both **type A & type B** antigens (anti-A & anti-B antibodies).

[Humans normally do not produce antibodies to a particular antigen until they are exposed to it; however, the ABO system is an exception. People routinely produce antibodies to those blood group antigens not present on their own RBCs without ever being directly exposed to them. It is thought that these antibodies are formed in response to antigens of the intestinal microbiota that are similar to the blood group substances. These antibodies could then cross react with antigens on the appropriate RBCs. It is for this reason that blood is typed before being transfused from one person to another.**]**

2. Rh blood group system: -

In 1940, Land Steiner and Wiener reported that rabbit sera containing antibodies against RBCs of the **Rhesus monkey** would agglutinate the RBCs of some human beings. The antigen was first designated as **Rh factor**. Later it was found to exist in the form of six antigens: - **C, c, D, d, E and e.** Among these 6 antigens, **antigen D** is responsible for the Rh-positive condition.

Rh blood group system is the second major classification of human blood types. Rh stands for Rhesus; this factor was first identified in the blood of Rhesus monkeys. Rh classification is based upon the presence or absence of the RhD antigen on RBCs. RhD-positive (RhD+) people have the RhD antigen while RhD-negative (RhD-) people do not. About 85% of all people in the United States are RhD+. Rh system classifies human blood into 2 types—

RhD-positive

RhD-negative

In contrast to the situation with the ABO system, RhD-negative people do not normally have anti-RhD antibodies in their plasma. They must be exposed to the RhD antigen to develop the antibodies.

Blood group	**Ags on RBCs**	**Abs**	**Can receive blood from**	**Can give**	**Remark**

	Ag A, B or RhD			**blood to**	
A Rh-posit.	A & RhD	Anti-B	A Rh-posit. A Rh-nega. O Rh-posit. O Rh-nega.	A Rh-posit. AB Rh-posit.	
A Rh negat.	A	Anti-B	A Rh-nega. O Rh-nega.	A Rh-posit. ABRh-posit. A Rh-nega. AB Rh-nega.	
B Rh Posit.	B & RhD	Anti-A	B Rh-posit. B Rh-nega. O Rh-posit. O Rh-nega.	B Rh-posit. AB Rh-posit.	
B Rh negat.	B	Anti-A	B Rh-nega. O Rh-nega.	B Rh-posit. AB Rh-posit. B Rh-nega. AB Rh-nega.	
AB Rh Posit.	A, B & RhD	Nil	A Rh-posit. A Rh-nega. B Rh-posit. B Rh-nega. AB Rh-posit. AB Rh-nega. O Rh-posit. O Rh-nega.	AB Rh-posit.	**Univer-sal Acceptor**
AB Rh negat.	A & B	Nil	A Rh-nega. B Rh-nega. AB Rh-nega. O Rh-nega.	AB Rh-posit. AB Rh-nega.	
O Rh Posit.	RhD	Anti-A Anti-B	O Rh-posit. O Rh-nega.	A Rh-posit. B Rh-posit. AB Rh-posit. O Rh-posit.	
O Rh negat.	Nil	Anti-A Anti-B	O Rh-nega.	A Rh-posit. A Rh-nega. B Rh-posit. B Rh-nega. AB Rh-posit. AB Rh-nega. O Rh-posit. O Rh-nega.	**Univer-sal donor**

Blood Transfusions and Rh incompatibility: -

Blood group typing is necessary before being transfused from one person to another. Donor and recipient must be compatible otherwise a severe hemagglutination may occur. For example, if type A blood is given to a type O person, the anti-A antibody in the recipient's blood combine with the A antigen on the surface of the donor's RBCs and cause them to agglutinate. These agglutinates can block blood vessels, causing serious circulatory problems.

If RhD positive blood is transfused to RhD negative person, anti-RhD antibodies develop in RhD negative person after several months but once this has happened, a second transfusion can result in a severe hemagglutination reaction.

The RhD factor is also important in pregnancies. A serious problem may occur when RhD negative mother and RhD positive father have RhD positive child. The child produces RBCs with the RhD antigen, which gains acess to the mother's circulation when the placenta ruptures during childbirth, or in some cases during an abortion, miscarriage, or any obstetrical procedure that may cause fetomaternal hemorrhage. Responding to the foreign RhD antigens, the mother produces anti-RhD antibodies that are able to cross the placenta. During any subsequent pregnancy, the RhD negative mother may produce enough anti-RhD antibodies to agglutinate and destroy the RBCs of the RhD positive child, producing **hemolytic disease of the newborn.**

Because of the hemolytic process, the liver and spleen enlarge. As these organs enlarge, their function decreases, leading to ascites (fluid accumulation), congestive heart failure and possibly death.

This disease can be prevented by injecting the RhD negative mother with anti-RhD antibodies within 72 hours after delivery of the child. These passively administered antibodies prevent the mother from developing antibodies against the RhD antigen of the RhD positive RBCs that have entered her circulation.

Requirements: -

1. Monoclonal antibodies against A, B and D antigens (Anti-A, Anti-B and Anti-D antibodies) (Anti-A are blue in colour, Anti-B are yellow in colour and Anti-D are colourless)
2. Slides
3. Lancet / needle
4. Cotton, absolute ethanol, matchsticks

Procedure: -

1. Tip of finger is disinfected with absolute ethanol and punctured with the help of sterile lancet / needle.
2. First drop of blood is wiped out and three drops are taken on clean, grease free slide at three different places.
3. Into the first drop anti-A antibodies, second drop anti-B antibodies and third drop anti-RhD antibodies are added.
4. Contents of each are mixed well by using separate matchsticks.
5. Agglutination is observed and recorded after 1 to 2 minutes.

Observation: -

1. Agglutination is observed / not observed in the drop in which anti-A antibodies are added.
2. Agglutination is observed / not observed in the drop in which anti-B antibodies are added.
3. Agglutination is observed / not observed in the drop in which anti-RhD antibodies are added.

Result: -

The blood group is ________________.

Conclusion: -

On the RBCs antigen-A / antigen-B / antigen-RhD are present / absent. By this serological method we can determine the presence of antigens on RBCs as per ABO and Rh blood group systems.

Expt. No. 5b **Date: -**

WIDAL TEST

Aim: To detect specific antibody produced in response to the stimulation by specific antigen of Salmonella species.

Principle:

The killed bacterial suspension of Salmonella caries specific 'O' and 'H' antigen. This will react with immunospecific antibodies which may be present in patient serum and agglutinate the antigen, and produce agglutination or clumps on the slide.

Clinical significance:

The organism Salmonella typhi is responsible for producing the disease in mankind which is known as enteric fever or typhoid fever, which is characterized generally by very high consistent fever, loss of appetite, transitory bacteraemia, round or oval shaped ulcer with smooth peritoneal surface of payer's patches and solitary lymphoid follicles of ileum etc. The organism possess 'O' antigen on the cell wall and 'H' antigen on its flagella, against which the host body produces immunospecific antibodies, to counteract the effect of corresponding antigens. On the other hand the paratyphoid fever caused by Salmonella paratyphi A or paratyphi B is characterized by milder course of disease. These organisms also possess somatic 'O' and flagellar antigen which is termed as A(H) and B(H) respectively.

Sample:

Fresh serum sample is preferred. In case of any delay the sample should be stored at 2°-8°C away from direct light. However the test is to be performed with in 24 hrs. of collection of sample.

Storage and stability:

All reagents are stable till expiry date mentioned on the table when stored at 2°-8°C away from direct light.

Procedure:

A. Rapid slide Test (Widal Screening test)

1. Clean the glass slide provided in the kit and wipes it.
2. Place one drop of undiluted serum to be tested in each on the first four circles (1-4).
3. Add one drop of antigen O, H, A(H), B(H) in circles 1,2,3,4 respectively.
4. Mix the contents of each circle with separate stick and spread to fill the whole circle area.
5. Rock the slide for one minute and observe for agglutination.
6. If agglutination is visible within one minute then proceed for quantitative estimation.

B. Quantitative Slide Test:

Clean the glass slide supplied in the kit and proceeds as follows.

Circle	Serum Volume	Appropriate Antigen Drop		Titre
1	0.08 ml	1 Drop	Mix and rotate for one minute and observe agglutination	1:20
2	0.04 ml	1 Drop		1:40
3	0.02 ml	1 Drop		1:80
4	0.01 ml	1 Drop		1:160
5	0.005 ml	1 Drop		1:320

Repeat above procedure for visible agglutination.

Titer is the highest dilution observed in rapid slide screening test which gives visible agglutination.

Interpretation of result:

A: Rapid slide test:

Granular agglutination in case of ‘O’ and flocculating agglutination in case of H or A(H), or B(H) indicates the positive reaction.

B: Quantitative slide test:

A diagnostic titre of 1:80 suggest positive reaction

Expt. No. 05 c **Date: -**

RPR TEST (Rapid Plasma Reagin) for syphilis

Aim: To diagnose suspected case of syphilis by RPR test.

Principle:

Rapid Plasma Reagin (RPR) refers to a type of test that looks for non-specific antibodies in the blood of the patient that may indicate that the organism (*Treponema pallidum*) that causes syphilis is present. The term "reagin" means that this test does not look for antibodies against the actual bacterium, but rather for antibodies against substances released by cells when they are damaged by *T. pallidum*. The RPR test is an effective screening test, as it is very good at detecting people without symptoms who are affected by syphilis.

This test is based on the relationship between the lipid extract of tissue i.e. cardiolipin-cholesterol-lecithin. These lipids act as antigen against a non-specific antibody (reagin) which develops in the blood serum of syphilis affected persons. RPR is useful as a rapid screening test for syphilis patients. In this test only small amount of serum or plasma is used. Prior inactivation of serum or dilution of antigen is not required.

RPR test is modified version of Wasserman's reaction in which the antigens coated with the particle are allowed to react with the sample and if the antibodies for syphilis are present, the flocculation will occur on the slide due to aggression of carbon particles will form. Aggregate in the center of slide will be formed to give appearance of button which indicates negative reaction.

Introduction:

Syphilis is caused by the organism ***Treponema pallidum***. This organism is an obligate parasite of mankind. It is delicate spiral organism with terminal flagella. It is very difficult to study them as they cannot be stained by ordinary aniline dye neither can be grown in laboratory on artificial medium. Syphilis caused by ***Treponema pallidum*** is a contagious veneral disease marked by lesions on the skin and other organs of the body. By the mode and transmission the disease is classified as acquired or congenital.

In the primary stage i.e. after 2-6 weeks of incubation a hard sore is developed at the site of infection, where as in the secondary stage i.e. after 8-12 weeks of incubation malaise, moderate fever, lesion on skin or mucous membrane, sore throat, lymphnode enlargement, affection of bones, eyes or

other organs and large number of organism in serous secretions can be seen. The tertiary stage of the disease which may soon follow secondary stage or be delayed for many years can be characterized by commonest lesions of many internal organs and skin.

Requirements:

Reagent 1 – R.P.R. Antigen

Reagent 2- Positive control

Reagent 3- Negative control

Accessories- Plastic droppers, mixing sticks, rubber teat, delivery dropper, plastic slides.

Procedure: Screening Test

1. Place one drop of serum/plasma (50 µl) on the slide with disposable serum dropper.
2. After gently mixing R.P.R. antigen suspension, place one drop (10-20 µl) by antigen dropper.
3. Mix well and spread out the liquid o entire area of the circle by using disposable mixing stick.
4. Rock the slide gently for 6 minutes and observe under good light source for appearance of carbon particle clumping.

Observation: -

Clumping observed / not observed

Result: -

Patient is suffering / not suffering from syphilis.

Expt. No. 6 **Date: -**

PRICIPITATION TESTS

6a) SINGLE RADIAL IMMUNODIFFUSION

Aim: To perform radial immunodiffusion and estimation of antigen concentration.

Principle:

` Single radial immunodiffusion is used extensively for the quantitative estimation of antigens. The antigen-antibody precipitation is made more sensitive by the incorporation of antiserum in the agarose. Antigen (Ag) is then allowed to diffuse from wells cut in the gel in which the antiserum is uniformly distributed. Initially, as the antigen diffuses out of the well, its concentration is relatively high and soluble antigen-antibody adducts are formed. However, as Ag diffuses further from the well, the Ag-Ab complex reacts with more amount of antibody resulting in a lattice that precipitates to from a precipitin ring.

Thus, by running range of known antigen concentrations on the gel and by measuring the diameters of their precipitin rings, a calibration graph is plotted. Antigen concentrations of unknown samples, run on the same gel can be found by measuring the diameter of precipitin rings and extra polating this value on the calibration graph.

Duration of the experiment:

Experiment is carried out over a span of two days, approximate time taken on each day is indicated below

Day I: 1 hour (preparation of gel and loading of antigen samples)

Day II: 30 min. (observation and interpretation)

Requirements:

1. Agarose
2. 10 X Assay buffer
3. Standard antigens (A,B,C, and D)
4. Test antigens (1 and 2)
5. Antiserum
6. Gel puncture syringe
7. Glass plate
8. Template

9. Alcohol
10. Distilled water
11. Glass wares : Conical flask, measuring cylinder
12. Others: Micropipette, tips, moist chamber.

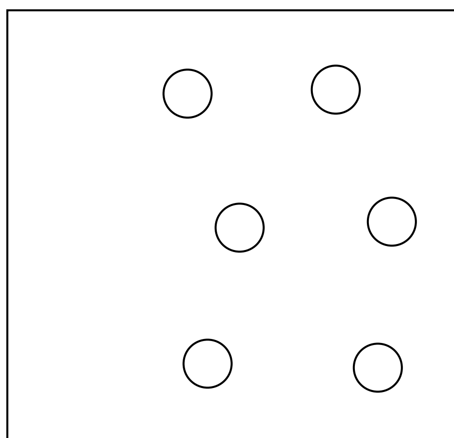
Procedure:

1. Prepare 10 ml of 1.0 % Agarose (0.1 gm/10ml) in 1 X assay buffer by heating slowly till agarose dissolves completely. Take care not to scorch of froth the solution.
2. Allow the molten agarose to cool at 55°C.
3. Add 120 µl of antiserum to 6 ml of agarose solution. Mix thoroughly for uniform distribution of antibody.
4. Pour agarose solution containing the antiserum on to a grease free glass plate set on a horizontal surface. Leave it undisturbed to form a gel.
5. Cut wells using a gel puncture using the template provided..
6. Add 20 µl of the given standard antigens and test antigens to the wells as shown in figure..
7. Keep the gel plate in a moist chamber. (Box containing wet cotton) and incubate over night at room temperature.
8. Mark the edges of the circle and measure the diameter of the ring. Note down your observations as shown in observation table.
9. Plot a graph of diameter of ring (on Y-axis) verses concentration of antigen (on X-axis) on semi-log graph sheet.
10. Determine the concentration of unknown by reading the concentration against the ring diameter from the graph.

Observation table:

Sr. No.	Standard antigen concentration (in mg/ml)	Ring diameter (in mm)
1	0.25	
2	0.50	
3	1.0	
4	2.0	
5	Test sample 1	
6	Test sample 2	

Figure: Template



6b). IMMUNOELECTROPHORESIS

Aim: To learn the technique of immunoelectrophoresis.

Principle:

Immunoelectrophoresis is a powerful technique to characterize antibodies. The technique is based on the principles of electrophoresis of antigens and immunodiffusion of the electrophoresed antigens with a polyspecific antiserum to form precipitin bands.

Electrophoresis:

During electrophoresis, molecules placed in an electric field acquire a charge and move towards appropriate electrode. Mobility of the molecule is dependent on a number of factors:

- It is proportional to field strength and net charge of molecule.
- Inversely proportional to frictional coefficient of the molecule which is dependent on size/shape of the molecule and viscosity of the medium.
- Heat generated by high ionic strength buffers.
- Change in pH of buffer due to electrolysis of water.
- Endosmosis: The agarose matrix absorbs hydroxyl ions on the surface during electrophoresis, resulting in a net increase in positive ions, which migrate towards the negative electrode with a solvent shell. This net solvent flow is referred to as endosmosis. Sample molecules migrating against these ions meet resistance and are hindered in their movement, whereas sample molecules migrating along with the ions move faster.
- Thus when antigens are subjected to electrophoresis in an agarose gel, they get separated according to their acquired charge, size and shape, by migrating to different positions.
-

Immunodiffusion:

Antigens thus resolved by electrophoresis are subjected to immunodiffusion with antiserum added in a trough cut in the agarose gel. Due to diffusion, density gradient antigen and antibody are formed and at the zone of equivalence, antigen-antibody complex precipitates to form an opaque arc shaped line in the gel The precipitin line indicates the presence of antibody, specific to antigen. If the antibody is homogeneous only one precipitin line is visible. Presence of more than one precipitin line establishes

the heterogeneity of antibody, while the absence of precipitin line indicates that the antiserum does not have antibody to any of the antigens separated by electrophoresis.

Duration of experiment: Experiment is carried out over a span of two days, approximate time taken on each day is indicated below:

Day 1: 3 Hours 30 minutes (Electrophoresis and Immunodiffusion)

Day 2: 30 Minutes (Observation and interpretation)

Requirements:

1. Agarose	1.0 gm.
2. 5X Electrophoresis buffer	200 ml.
3. Antigen	0.1 ml.
4. Test Antiserum-A	1.5 ml.
5. Test Antiserum-B	1.5 ml.
6. Glass ware:	Conical flask, Measuring cylinder.
7. Reagent:	Distilled water.
8. Other:	Micropipette, Tips, Moist chamber (box with wet cotton)

Procedure:

Preparation of gel plates

1. Prepare 10ml of 1.5% agarose (0.15 g/10ml) in 1X electrophoresis buffer by heating slowly till agarose dissolves completely. Take care not to scorch/froth the solution.
2. Mark the end of a glass plate that will be towards negative electrode during electrophoresis.
3. Place the glass plate on a horizontal surface. Pipette and spread 10 ml of agarose solution onto the plate. Take care that the plate is not disturbed and allow the gel to solidify.
4. Place the glass plate on the template holder and fix the template. Punch a 3 mm well with the gel puncher towards the negative end.
5. Cut two troughs with the gel cutter, but do not remove the gel from the trough.

Electrophoresis

6. Add 12-25 µl of antigen to the well.
7. Place the glass slide in the electrophoresis tank such that the antigen well is at the anode/negative electrode. Pour 1X electrophoresis buffer such that it covers the gel.
8. Set the voltage to 50-100 V, electrophorese until the blue dye travels 3-4 cm from the well. Do not electrophorese beyond 3 hours, as it is likely to generate heat.

Immunodiffusion

9. Remove gel from both the troughs and keep the plate at room temperature for 15 min. Add 250 micro lit. of antiserum-A in one of the troughs and antiserum-B in the other.
10. Place the plate in a moist chamber and allow diffusion to occur at room temperature, overnight.

Interpretation:

- Presence/absence of precipitin line indicates that the presence/absence of antibody specific to the antigen
- Presence of more than one line indicates the heterogeneity of the antiserum to the antigen.
- Presence of single precipitin line indicates homogeneity of the antiserum to the antigen.