THE NEOTIA UNIVERSITY

ज्ञानम् आत्म प्रदीपाय

Department of Biotechnology
School of life Sciences

Practical Manual
Course: **Immunology-I**

Course Code:CC-BTP506

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Experiment 1: Blood group determination

Aim: To determine the blood group and Rh factor of an individual.

Introduction: Blood grouping is the classification of blood based on the presence or absence of two inherited antigenic substances on the surface of red blood cells (RBCs). The ABO and Rh are the major, clinically significant and the most important of all the blood group systems. The ABO blood group system was first discovered by Karl Landsteiner in 1900. The human ABO blood group system is divided into the following four major groups depending on the antigen present on the surface of their red blood cells:

1. "A" group
2. "B" group
3. "AB" group
4. "O" group

ABO Blood Group System

Antigens on the surface of Red Blood Cells	Antibodies in the Serum	ABO Blood Group	Genotype
A	Anti-B	A	AA or AO
B	Anti-A	B	BB or BO
AB	Neither Anti A or Anti B	AB	AB
Neither A or B	Anti A and Anti B	O	OO

The associated Anti A and Anti B antibodies usually belong to IgM class of immunoglobulins. The Rhesus system (Rh) is the second most important blood group system in humans. The most significant and immunogenic Rhesus antigen is the RhD antigen. The individuals carrying the Rh antigen are considered to have positive blood group whereas those individuals that lack this antigen are considered to have negative blood group.

Principle: The ABO and Rh blood grouping system is based on agglutination reaction. When red blood cells carrying one or both the antigens are exposed to the corresponding antibodies they interact with each other to form visible agglutination or clumping. The ABO blood group antigens are O-linked glycoproteins in which the terminal sugar residues exposed at the cell surface of the red blood cells determine whether the antigen is A or B. Blood group A individuals have A antigens on RBCs and anti-B antibodies in serum.

Similarly, blood group B individuals have B antigens on RBCs and anti-A antibodies in serum. Blood group AB individuals have both A and B antigens on RBCs and neither anti-A nor anti-B antibodies in serum. Whereas, blood group O individuals have neither A antigens nor B antigens, but possess both anti-A and anti-B antibodies in serum. The Rh antigens are transmembrane proteins in which the loops exposed on the surface of red blood cells interact with the corresponding antibodies.

Materials required:

Anti-A Sera

Anti-B Sera

Anti RhD Sera

Glass Slide

Blood Lancet

Disposable mixing stick

70% Alcohol/ Spirit

Cotton

Storage: Anti A Sera, Anti B Sera and Anti RhD Sera should be stored at 2-8^{o} C.

Procedure:

1. Dangle the hand down to increase the flow of blood in the fingers.
2. Clean the fingertip to be pierced with spirit or 70% alcohol.
3. Add one drop each of Anti-A and Anti-B serum on two separate places on the slide
4. With the help of the sterile lancet, pierce the fingertip and place one drop of blood on Anti-A and Anti-B.
5. Mix each blood drop and the antiserum using a fresh mixing stick.
6. Observe agglutination in the form of fine red granules within 30 seconds.

Anti RhD takes slightly longer time to agglutinate compared to Anti A and Anti B.

Observation/Results: Determination of blood group and Rh factor based on agglutination

Anti-A	Anti-B	Anti-RhD	Blood Group
√	×	√	A+
×	√	√	B+
√	√	√	AB+
×	×	√	O+

Interpretation:

If agglutination is observed when blood is mixed with Anti A reagent, then the individual is said to have blood group "A".

If agglutination is observed when blood is mixed with Anti B reagent, then the individual is said to have blood group "B".

If agglutination is observed when blood is mixed with Anti A and Anti B reagent, then the individual¬ is said to have blood group "AB".

If no agglutination is observed when blood is mixed with Anti A and Anti B reagent, then the¬ individual is said to have blood group "O".

If agglutination is observed when blood is mixed with Anti RhD reagent, then the individual is said to¬ have "+ve" Rh factor.

If no agglutination is observed when blood is mixed with Anti RhD reagent, then the individual is¬ said to have "-ve" Rh factor.

Safety Precautions:

1. Always wear gloves while performing the experiment.
2. Ensure the slide is clean and dry prior to use.
3. Do not allow the antisera reagent dropper to touch the blood sample.
4. The result of the reaction should be interpreted immediately after mixing.
5. Avoid intermixing of the antisera reagents while performing the experiment as it may give false result.
6. Used lancets, cottons and slides should be disposed carefully.

Experiment-2: Identification of blood cells of immunological importance (leucocytes)

A. Preparation of blood smear

Aim: To identify blood cells of immunological importance through microscopic observation

Introduction: Erythrocytes are the dominant (99%) but not the only type of cells in the blood. We also have *leukocytes* (WBC) and *blood platelets*. Erythrocytes, leukocytes and blood platelets are also being referred to as the *formed elements of the blood*. Erythrocytes do not contain a nucleus. They do contain haemoglobin, which fills almost the entire cytoplasm. Leukocytes can be further subdivided into granular leukocytes, i.e. neutrophils, basophils and eosiniphils, and non-granular leukocytes, i.e. monocytes and lymphocytes

Normal differential leukocyte count :

~60% neutrophils (50% - 70%)

~ 3% eosinophils (>0% - 5%)

~ 0.5% basophils (>0% - 2%)

~ 5% monocytes (1% - 9%)

~ 30% lymphocytes (20% - 40%)

Granular leukocytes :

Granular leukocytes are all approximately the same size - ~ 12-15 μm in diameter. Their nuclei form lobes, and nucleoli cannot be seen. The number of nuclear lobes varies according to cell type. The term granulocytes refers to the presence of granules in the cytoplasm of these cells.

Neutrophil

Eosinophil

Besophil

Materials required:

Glass Slide

Blood Lancet

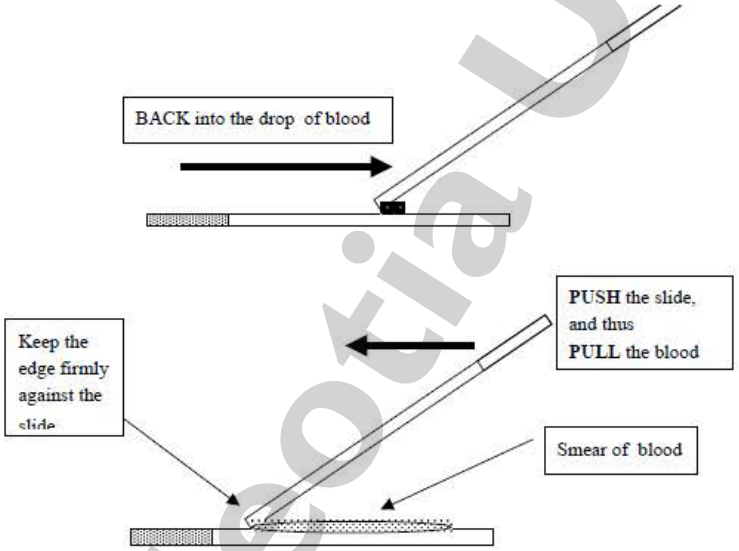
70% Alcohol/ Spirit

Cotton

Procedure:

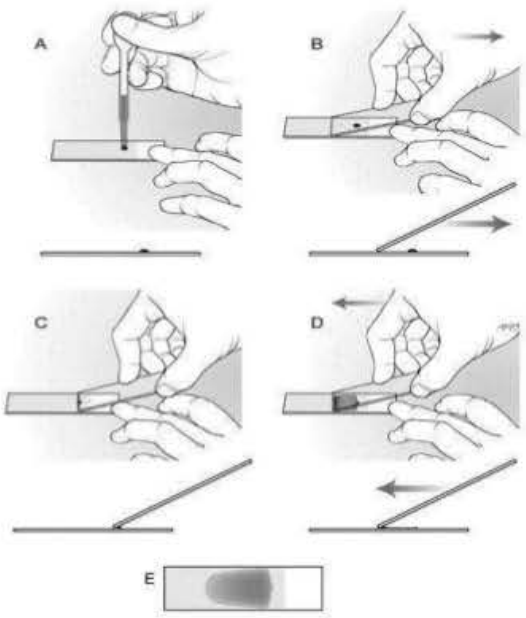
It is easiest to use microscope slides with a frosted end, so that identifying information can be written there with pencil.

Place a drop of blood approximately 4 mm in diameter on the slide (near the end of the slide). Spread the drop by using another slide (called here the "spreader"), placing the spreader at 30-45° angle and BACKING into the drop of blood. The spreader catches the drop and it spreads by capillary action along its edge. To make a short smear, hold the spreader at a steeper angle, and to make a longer smear, hold it closer to the drop. Now, push the spreader across the slide; this PULLS the blood across to make the smear. Do not push the blood by having it ahead of the smearing slide! It should take about one second to smear the drop. A smooth action is required, with the edge of the spreader held against the slide. This will yield a nice, even smear.

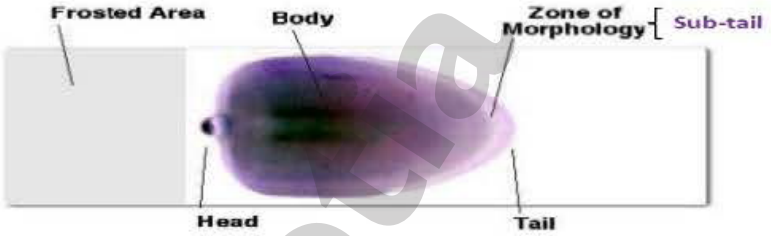


Smears should be air-dried, and then dipped into 100% methanol. A coplin jar with a screw top is best for this. *Be sure the alcohol does not reach the frosted end of the slide.* After one minute, the slides are removed and placed on end to drain the alcohol. They can then be placed into a plastic slide box for complete drying.

Slide to slide Method



Different zones of a blood smear:



Safety Precautions:

1. Always wear gloves while performing the experiment.
2. Ensure the slide is clean and dry prior to use.
3. Do not allow the antisera reagent dropper to touch the blood sample.
4. The result of the reaction should be interpreted immediately after mixing.
5. Avoid intermixing of the antisera reagents while performing the experiment as it may give false result.
6. Used lancets, cottons and slides should be disposed carefully.

B. Preparation of stains

Aim: to prepare the different type of stains required for staining of blood smear to make the blood cells visible under microscope

Introduction:

Romanowsky stain:

Romanowsky staining is a prototypical staining technique that was the forerunner of several distinct but similar methods, including **Giemsa**, Jenner, Wright, Field, and **Leishman** stains, which are used to differentiate cells in pathologic specimens. It was named after the Russian physician Dmitri Leonidovich Romanowsky (1861–1921), who invented it in 1891.

A Romanowsky stain is any stain combination consisting of **eosin Y** or **eosin B** with **methylene blue** and/or any of its oxidations products. Such stains produce the typical purple coloration of leukocyte nuclei and neutrophilic granules as well as the numerous blues and pinks found in other cell types. Methyl alcohol is used as both a solvent and fixative in this procedure.

The remarkable property of the Romanowsky dyes of making subtle distinctions in shades of staining, and of staining granules differentially, depends on two components: azure B (trimethylthionine) or methylene blue (basic or cationic dye) and eosin Y (tetrabromofluorescein) (acidic or anionic dye).

Azure B is bound to anionic molecules, and eosin Y is bound to cationic sites on proteins. Thus the acidic groupings of the nucleic acids and proteins of the cell nuclei and cytoplasm of primitive cells determine their uptake of the basic dye azure B, and, conversely, the presence of basic groupings on the haemoglobin molecule results in its affinity for acidic dyes and its staining by eosin.

The granules in the cytoplasm of neutrophil leucocytes are weakly stained by the azure complexes. Eosinophilic granules contain a spermine derivative with an alkaline grouping that stains strongly with the acidic component of the dye, whereas basophilic granules contain heparin, which has an affinity for the basic component of the dye.

Because the aqueous dye solutions were unstable, methanol was introduced as a solvent, and William Boog Leishman and James Homer Wright advocated its use as a **fixative** prior to staining.

Leishmans stain/ Geimsa stain/ Wrights stain

Among the Romanowsky stains now in use, Jenner is the simplest and Giemsa is the most complex. Leishman stain, which occupies an intermediate position, is still widely used in the routine staining of blood films, although the results are inferior to those obtained by the combined May–Grünwald–Giemsa, Jenner–Giemsa, and azure B–eosin Y methods.

Wright stain, which is widely used in North America, gives results that are similar to those obtained with Leishman stain, whereas Wright–Giemsa gives results that are similar to those obtained with May–Grünwald–Giemsa.

A pH to the alkaline side of neutrality makes the azure component more prominent at the expense of the eosin and vice versa. A pH of 6.8 is usually recommended for general use.

To achieve a uniform pH, 50 ml of 66 mmol/l Sörensen phosphate buffer may be added to each litre of the water used in diluting the stains and washing the films.

Preparation of Leishman stain

Weigh out 0.2 g of the powdered dye, and transfer it to a conical flask of 200–250-ml capacity. Add 100 ml of methanol and warm the mixture to 50 °C for 15 min, occasionally shaking it. Allow the flask to cool and filter the solution. It is then ready for use, but it will improve on standing.

Make up 50 ml of 66 mmol/l Sörensen phosphate buffer of the required pH to 1 litre with water at a pH of 6.8.

Preparation of Geimsa stain:

Weigh 1 g of the powdered dye and transfer it to a conical flask of 200–250-ml capacity. Add 100 ml of methanol and warm the mixture to 50 °C; keep at this temperature for 15 min with occasional shaking, then filter the solution. It is then ready for use, but it will improve on standing for a few hours.

Buffer preparation:

The **alkaline stock** is Sodium phosphate, dibasic anhydrous, **N2HPO4**, Mix 9.5 gm with distilled water to make 1000 mL.

The **acid stock** is Potassium phosphate monobasic anhydrous, **KH2PO4** , mix 9.07 gm with distilled water to make 1000 mL.

***Working buffer**: Mix 39 mL of acid stock with 61 mL of the alkaline stock, and 900 mL of distilled water. Check pH, and adjust to pH 7 or 7.2 by adding the acid buffer stock to lower pH or alkaline to raise pH. Just a very few mL should be necessary to reach the required pH.*

Preparation of Wright's stain:

Weigh 1gm of Wrights stain and transfer to a dry brown bottle, add a few glass beads and add 400 ml of methanol, mix well at intervals till the dye completely dissolved. Warming at 37C may help the dye to dissolve. Filter the stain and store at room temperature and at dark. Prepare 3-5 days before using.

Materials required:

Conical flask

Beaker

Glass beads

Wright stain powder dye

Geimsa stain powder dye

Leishman's stain powder dye

Methanol

Na-Phosphate di basic anhydrous

Potassium phosphate monobasic anhydrous

Sorenson phosphate buffer

Water bath

Fliter paper

Funnel

C. Staining blood smear

Aim: To stain the blood smears to make the blood cells observable under microscope

Protocol for staining with Leishman's stain:

1. Use smears that are as thin as possible and air-dried. Place the slide on a staining rack ensuring the smear is upper most. Air dry.

2. Fully cover the smear (flood the slide) with Leishman's Stain solution. Stain for 2 minutes.

3. Add twice the amount of buffered distilled water and mix by swirling or rocking the rack . Incubate for at least 10 min. Ensure that there is sufficient diluted stain on the slide to prevent its drying out during this period. If not add more stain and buffered water.

4. Rinse thoroughly with buffered distilled water and allow it to remain on the slide for ~ 1 min. Until the smear has a piniksh tinge.

5. Dry the slides by standing the slide upright on piece of using blotting paper and air-dry.

Expected results:

Erythrocytes: light pink to brown
Cores of lymphocytes: deep, dark blue to blue-violet
Cytoplasm of lymphocytes: light blue
Nuclei of neutrophil, polymorphonuclear leukocytes: a deep blue to blue-violet
Granules of neutrophilic polymorphonuclear leukocytes: red
Cores of eosinophil leukocytes: blue violet

Protocol for staining with Geimsa stain:

1. Fix the smear with methanol for 30 seconds.

2. Take 94 ml of PBS buffer (pH 7.2) or buffered distilled water (pH 7.2) and add 6 ml of Geimsa stock solution (i.e. 6% solution)

3. Filter the stain

4. Stain slides with this stain for 45 minutes.

5. Wash slides with running tap water

6. Air dry keeping in upright position and wipe it with tissue paper..

7. May dry at 37C incubator for few minutes

Expected Results:

Red blood cells will be seen as pinkish grey,

Platelets deep pink

Lymphocytes, neutrophils and monocytes with a purple– blue nucleus and a pale cytoplasm.

Eosinophils have coarse, bright purple–red granules in the cytoplasm,

Neutrophils have finer, purple granules.

Basophilic stippling in uninfected red blood cells is blue.

Staining with Geimsa + Wright's stain

Principle:

Acidic element in the cell (nucleus protein and nucleic acid of the cell) + Azure B (basic dye) ------- **Blue colouration**

Basic elements (such as Haemoglobin) + Acidic dye (Eosin Y) ------ **Red coloration**

Preparing staining buffer

Stock buffers (two)

The alkaline stock is Sodium phosphate, dibasic anhydrous, **Na2HPO4**, Mix 9.5 gm with distilled water to make 1000 mL.

The acid stock is Potassium phosphate monobasic anhydrous, **KH2PO4**, mix 9.07 gm with distilled water to make 1000 mL

Working buffer: Mix 39 mL of acid stock with 61 mL of the alkaline stock, and 900 mL of distilled water. Check pH, and adjust to pH 7 or 7.2 by adding the acid buffer stock to lower pH or alkaline to raise pH. Just a very few mL should be necessary to reach the required pH.

Take 94 ml buffer solution pH 7.2. Then add 6 ml of Giemsa stock solution.Wash slide thoroughly with buffered distilled water and air dry.

Protocol:

1. Fix the slide in methanol for 5 minutes

2. Air dry.

3. Apply Wright's stain for 2-5 minutes

4. Shake gently during incubation

5. Add same volume of distilled water for 2 minutes

6. Rinse slide with running water till the edges shows light pinkish-red colour

7. Apply undiluted Geimsa stain for 1 minute

8. Shake gently during incubation

9. Rinse slide with running water

10. Wash with 0.5% acetic acid solution (2-3 dips)

11. Air Dry

Expected Results:

Nuclei : Blue

Basophilic cytoplasmic : Blue

Components

Neutrophilic granules: Lilac

Eosinophilic granules :Orange

Mastcell granules: Deep blue -violet

Nucleoli :Blue-violet

Red cells :Pink

Cytoplasm of mature monocytes Grey blue

D. Observation of blood smears under microscope

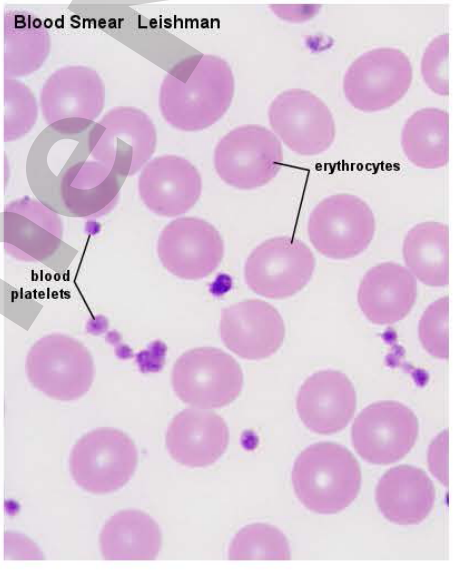
Aim: To identify immunologically important cells under the microscope

Introduction: Differential count is the percent distribution of various white blood cells in the peripheral blood. It is determined from a blood smear stained with polychromatic stain and after examination of stained smear by using oil immersion objective (1000X magnification). The number of each type of white cells is then expressed as a percentage of the total number of cells. The staine3d blood smear also helps to study abnormal morphology of RBC and leukocytes. Study of blood smear also helps in diagnosis of various anaemia , leukaemia, and detection of blood parasites including malarial parasites.

Where to look for cells in a blood smear

The density of cells varies across the smear. Cells will be "heaped and piled" close to the point were the drop of blood was placed on the slide. There are fewer cells close to the tip of the smear. In this region, white blood cells are sometimes damaged and erythrocytes may be deformed. The best area to look at is between these two regions. Where it is located exactly and how wide it is will depend on the smear, but the middle of the smear is a good starting point.

In a suitable region of the blood smear, the **erythrocytes** are rarely forming clumps or rows. Instead, they are more or less evenly spaced and occasionally forming groups of maybe 2 or 3 cells. Due to the biconcave shape of the erythrocytes, their center will look lighter than their periphery. Depending on the way in which the blood smear was prepared, blood platelets may be found singly, in small groups or large clumps.

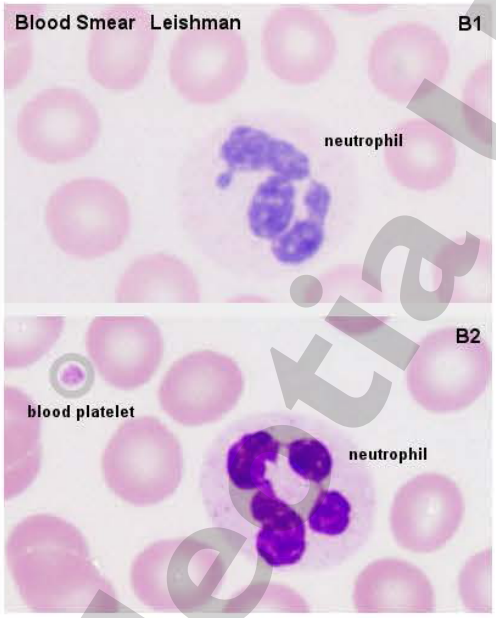


Observation:

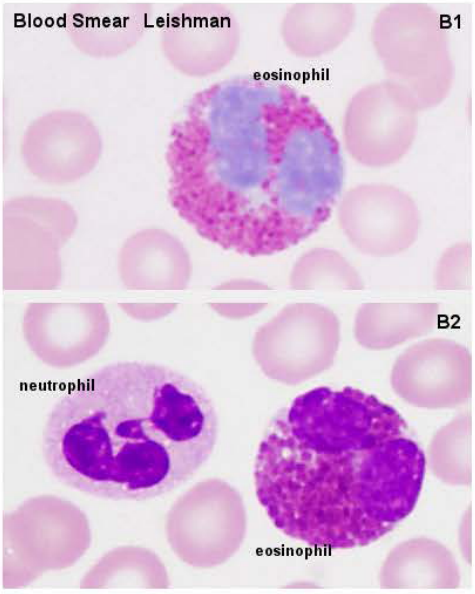
Granulocytes:

These are the cells which contains granules and a polymorphic nucleus in the cytoplasm. Three types of granulocytes derived their names from the staining reaction of the granules present in the cytoplasm. Granular leukocytes are all approximately the same size - ~ 12-15 μm in diameter. Their nuclei form lobes, and nucleoli cannot be seen. The number of nuclear lobes varies according to cell type.

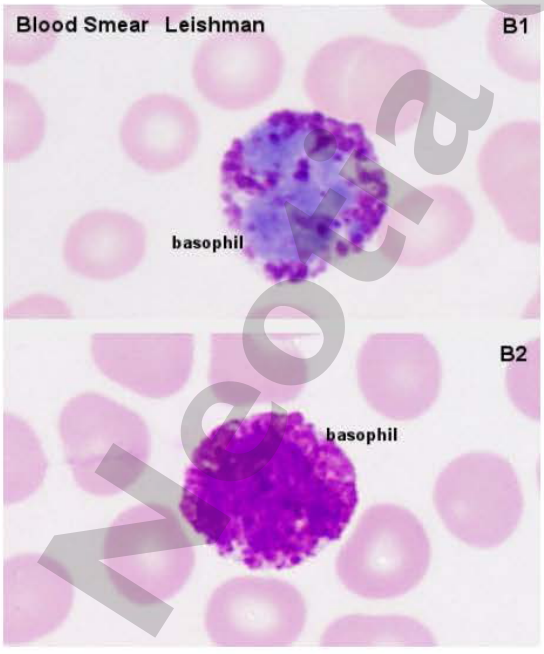
Neutrophil have a very characteristic nucleus. It is divided into 3-5 lobes which are connected by thin strands of chromatin. The number of lobes increases with cell age. Up to 7 lobes can be found in very old neutrophils (*hypersegmented cells*). Neutrophils staines when stained with basic as well as acid dyes.



Eosinophils: Their nucleus usually has only two lobes. Almost all of the cytoplasm appears filled with the specific granules of the eosinophils. As the term "eosinophil" indicates, these granules are not neutral but stain red or pink when eosin or a similar dye is used in the staining process.



Basophilis have a 2 or 3 lobed nucleus. The lobes are usually not as well defined as in neutrophilic granulocytes and the nucleus may appear S-shaped. Contains fewer coarse granules and stained with basic dyes. Specific granules of basophils are stained deeply bluish or reddish-violet.

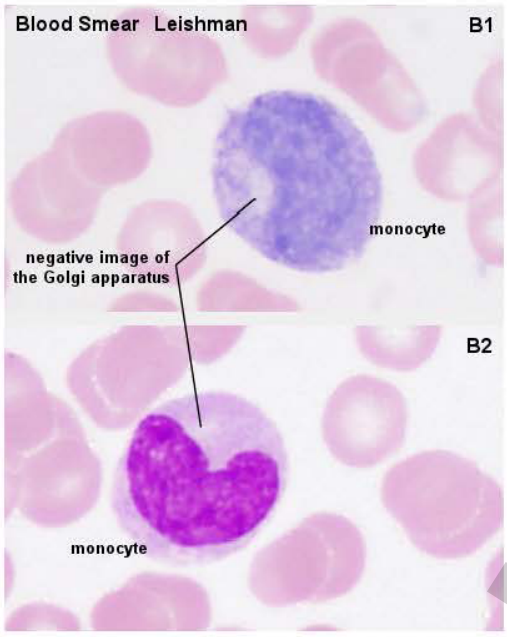


Agranulocytes:

Leukocytes that lacks granules and consists of two types of cells:

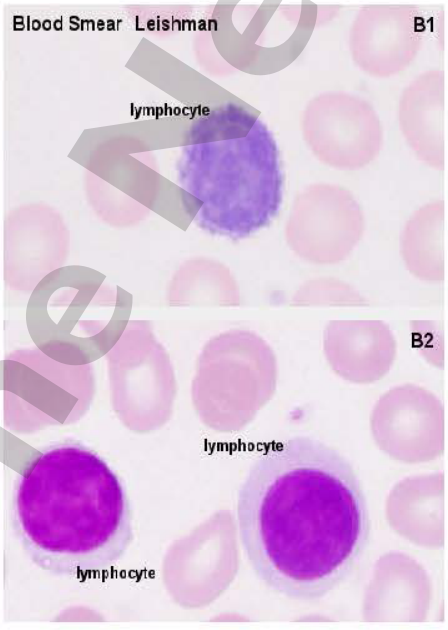
Monocytes: these cells can be slightly larger than granulocytes (~ 16-22 µm in diameter). Their cytoplasm stains usually somewhat stronger than that of granulocytes, but it does not contain any structures which would be visible in the light microscope using most traditional

stains (a few very fine bluish gains may be visible in some monocytes). The "textbook" monocyte has a C-shaped or kidney shaped nucleus.



Lymphocytes: two forms observed: small and large lymphocytes.

The smallest lymphocytes may be smaller than erythrocytes while the largest may reach the size of large granulocytes. In small ones, which are the majority of lymphocytes in the blood, the nucleus may appear to fill the entire cell. Large lymphocytes have a wider rim of cytoplasm which surrounds the nucleus. Both the nucleus and the cytoplasm stain blue.



Experiment-3: Serum isolation from goat/chicken blood

Aim: To isolate serum from fresh goat/ chicken blood

Introduction:

Serum is the liquid fraction of whole blood that is collected after the blood is allowed to clot. Serum is that part of blood which is similar in composition with plasma but exclude clotting factors of blood. The components of plasma and serum are similar as both contain hormones, glucose, electrolytes, antibodies, antigens, nutrients and certain other particles except clotting factors which are present only in plasma.

So we can say that **Plasma – clotting factors = Serum**

Materials required:

Covered plastic tube

Cold centrifuge

Microcentifuge tube

Pasteur pipet

Micropipet/tips

Protocol:

Collect whole blood in a covered tube. After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 15-30 minutes. Remove the clot by centrifuging at 1,000-2,000 x g for 10 minutes in a refrigerated centrifuge.

Plasma or serum can be separated from whole blood ***without centrifugation*** *by allowing the blood to just let stand. By gravity all the cells will settle down in due course of time.*

Preparation of Serum:

1. Allow fresh blood to stand at room temperature for 1 hour.
2. Store blood overnight at 4oC to allow clot formation.
3. Pour off serum, the majority of clot should remain in bottom of flask.

4. Centrifuge at 3000 rpm for 10 min. Transfer supernatant to new centrifuge tube and repeat centrifugation. If supernatant is not cleared of contaminating red blood cells, repeat centrifugation until homogeneous supernatant obtained; serum.

Note: The serum usually has a slight pink tinge due to presence of hemoglobin from lysed red blood cells.

If you simply let a tube sit, the red cells will settle as they are denser. The time it takes to settle is, in fact, a clinical test called the erythrocyte sedimentation rate (ESR).

The negative charges (zeta potential) that hold red cells apart are from sialic acid. Elevated protein levels help neutralize this charge, hence the more rapid sedimentation rate when you are sick and have an elevation of acute phase reactants in the blood.

Experiment 4: Widal Test (Slide)

Aim: To detect the presence of *Salmonella* genus which causes enteric or Typhoid Fever by using qualitative slide agglutination test.

Introduction: Widal test is a serological method to diagnose enteric fever or typhoid which is caused by the infection with pathogenic microorganisms like *Salmonella typhi*,*Salmonella paratyphi* A, B and C. The method of this diagnostic test is based upon a visible agglutination reaction either in a test tube or on a slide between antibodies of patient serum and antigens specifically prepared from *Salmonella sp.*

Principle: Enteric fever or typhoid is a life threatening disease usually occurs due to the infection of pathogenic microorganisms, e.g. *Salmonella typhi*, *Salmonella paratyphi* A, B and C. These microorganisms are transmitted to human body through food and drinks contaminated with fecal matter.Early diagnosis and treatment for this fever are essential to avoid serious clinical complications. During the course of infection antibodies are produced against Salmonella antigens. Widal test, a serological method for the detection of *Salmonella sp.*, was developed by F Widal in 1896. During this test a visible agglutination is formed due to the reaction in a test tube or on a slide between antibodies present in the infected person's blood sample and specific antigens of *S. typhi*and*S. paratyphi*. Forthe slide agglutination test, stained *Salmonella* antigens are used to detect the presence ofspecific agglutinin in the patient's serum. The slide agglutination test is used as a primaryscreening procedure.

The organisms causing enteric fever possesses two major antigens namely somatic antigen, O and a flagellar antigen, H along with another surface antigen, Vi. During infection antibodies are produced in patient's sera against *Salmonella typhi* O and H and *Salmonella paratyphi* AH and BH antigens. During infection antibodies are produced in patient's sera against these antigens. Antigens specifically prepared from this organism are used in the agglutination test to detect the presence of antibodies in patients' sera which are elucidated in response to infection by these bacteria. There are some agglutinins that are produced in the patient's serumduring the fever period, which react with somatic antigen O of *Salmonella typhi*, A or Bof *Salmonella paratyphi* and then with flagellar antigen H which is common in most ofthe *Salmonella* species.

In this test four specific antigen suspensions are used e.g. H,*Salmonella typhi* (O antigen), *Salmonella paratyphi* - A and *Salmonella paratyphi* - B. If agglutination occurs with O antigen then it is considered positive for *Salmonella typhi*. If agglutination occurs in A or B antigen then it is confirmed as positive for *Salmonella paratyphi*. Agglutination will occur in H antigen circle for all the cases of antigens like O, A, and B. *Salmonella* species are characterized by three antigens present on the cell.

O Antigen: This is a somatic antigen and is present on the outer membrane of the cell. Its specificity is determined by the nature of the repeating units in the outer O-polysaccharide chain. Somatic antigens are heat stable, alcohol resistant and forms compact and granular clumps when mixed with O antisera.

Vi antigens:This is a virulence antigen which is a capsular polysaccharide that overlays the O antigen. This capsule is not necessary for infection but it increases the infectivity by making it less detectable by the body's immune system.It is heat labile and can be detected using Vi antisera. Vi antigen can interfere with O antigen testing.

HAntigens: This is aheat labile flagellar antigen which is inactivated both by boiling and alcohol. H antigens rapidly form fluffy clumps when treated with the corresponding antisera. H antigen induces rapid formation of corresponding antibodies as it is strongly immunogenic. Widal Test Teaching Kit (Slide Test) utilizes the principle for rapidslide agglutination to detect the presence of *Salmonellatyphi* and *paratyphi*.The kit gives direct results in form of visible agglutination.

Materials Required:

1 Salmonella typhi 'O' Antigen
2 *Salmonella typhi* 'H' Antigen
3 *Salmonella paratyphi* 'AH' Antigen
4 *Salmonella paratyphi* 'BH' Antigen
5 Positive control
6 Negative control
7 Test Serum Sample
8 Glass Slide
9 Disposable Mixing Sticks

10 Micropipettes, Tips,

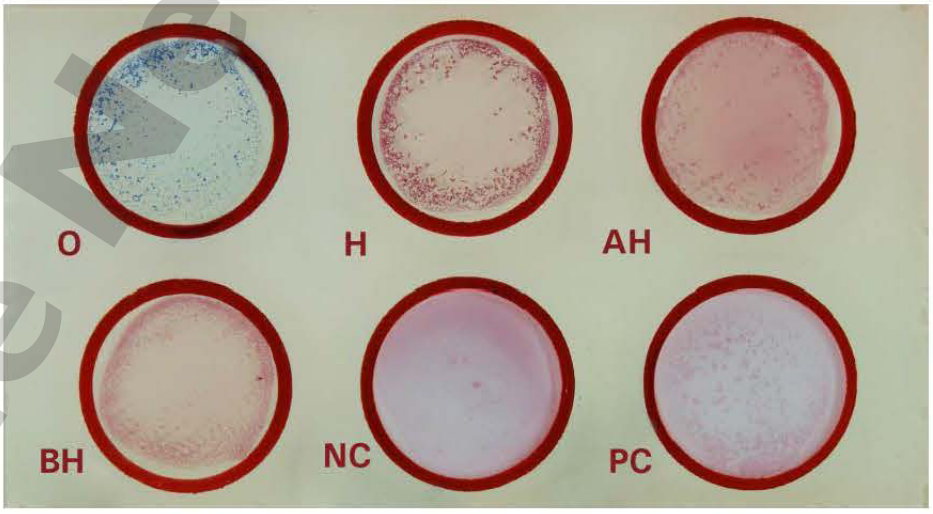
11. Gloves and Masks.

Procedure:

1. Before starting the experiment, bring all reagents to room temperature and mix well.
2. Mark the circles of slides as PC (Positive control), NC (Negative control), O, H, AH, BH as per antigen solutions used for testing .
3. Add 1 drop of positive control (25µl) into the circle marked as PC of given glass slide.
4. Then add 1 drop of negative control (25µl) into the reaction circle marked as NC.
5. Add 1 drop of test sample (25µl) into each reaction circle labeled as O, H, AH, BH according to given antigen solution.
6. Add 1 drop of Antigen solution of *Salmonella typhi* 'H' into PC and NC circle each. Mix well with using new mixing stick for each circle.
7. To circles labeled as O, H, AH, BH in which test samples has been added, add antigen solutions of *Salmonella typhi* 'O', *Salmonella typhi* 'H', *Salmonella paratyphi* 'AH' and *Salmonella paratyphi* 'BH', respectively.
8. Mix the content of each reaction circle uniformly with separate mixing stick.
9. Rock the glass slide gently (approximately for one minute) and observe for agglutination.

Observation and Result:

After mixing the test sample with Antigen Solution, Positive control, Negative control separately observe for the agglutination reaction.



Interpretation:

During the infection of Salmonella the human body responds to the antigenic stimulus and as a result corresponding antibodies are produced. When the test sample is treated with colored and attenuated Salmonella antigen suspensions, the antibodies present in the sample react with the antigen suspension to give clearly visible agglutination which can be seen through naked eye. Therefore, formation of agglutination indicates positive result which means the presence of anti-salmonella antibodies in the test sample. No agglutination is a negative test result which indicates the absence of anti-salmonella antibodies.

Safety precautions: Wear masks and gloves while handling the reagents

Experiment 5: Widal Test (Tube)

Aim: To detect the presence of *Salmonella* genus which causes enteric or Typhoid Fever by using qualitative slide agglutination test.

Introduction: Widal test is a serological method to diagnose enteric fever or typhoid which is caused by the infection with pathogenic microorganisms like *Salmonella typhi*,*Salmonella paratyphi* A, B and C. The method of this diagnostic test is based upon a visible agglutination reaction either in a test tube or on a slide between antibodies of patient serum and antigens specifically prepared from *Salmonella sp.*

Principle: Enteric fever or typhoid is a life threatening disease usually occurs due to the infection of pathogenic microorganisms, e.g. *Salmonella typhi*, *Salmonella paratyphi* A, B and C. These microorganisms are transmitted to human body through food and drinks contaminated with fecal matter.Early diagnosis and treatment for this fever are essential to avoid serious clinical complications. During the course of infection antibodies are produced against Salmonella antigens. Widal test, a serological method for the detection of *Salmonella sp*., was developed by F Widal in 1896. During this test a visible agglutination is formed due to the reaction in a test tube or on a slide between antibodies present in the infected person's blood sample and specific antigens of *S. typhi*and*S. paratyphi*. Forthe slide agglutination test, stained *Salmonella* antigens are used to detect the presence ofspecific agglutinin in the patient's serum. The slide agglutination test is used as a primaryscreening procedure.

The organisms causing enteric fever possesses two major antigens namely somatic antigen, O and a flagellar antigen, H along with another surface antigen, Vi. During infection antibodies are produced in patient's sera against *Salmonella typhi* O and H and *Salmonella paratyphi* AH and BH antigens. During infection antibodies are produced in patient's sera against these antigens. Antigens specifically prepared from this organism are used in the agglutination test to detect the presence of antibodies in patients' sera which are elucidated in response to infection by these bacteria. There are some agglutinins that are produced in the patient's serumduring the fever period, which react with somatic antigen O of *Salmonella typhi*, A or Bof *Salmonella paratyphi* and then with flagellar antigen H which is common in most ofthe *Salmonella* species.

In this test four specific antigen suspensions are used e.g. H,*Salmonella typhi* (O antigen), *Salmonella paratyphi* - A and *Salmonella paratyphi* - B. If agglutination occurs with O

antigen then it is considered positive for *Salmonella typhi*. If agglutination occurs in A or B antigen then it is confirmed as positive for *Salmonella paratyphi*. Agglutination will occur in H antigen circle for all the cases of antigens like O, A, and B. *Salmonella* species are characterized by three antigens present on the cell.

O Antigen: This is a somatic antigen and is present on the outer membrane of the cell. Its specificity is determined by the nature of the repeating units in the outer O-polysaccharide chain. Somatic antigens are heat stable, alcohol resistant and forms compact and granular clumps when mixed with O antisera.

Vi antigens:This is a virulence antigen which is a capsular polysaccharide that overlays the O antigen. This capsule is not necessary for infection but it increases the infectivity by making it less detectable by the body's immune system.It is heat labile and can be detected using Vi antisera. Vi antigen can interfere with O antigen testing.

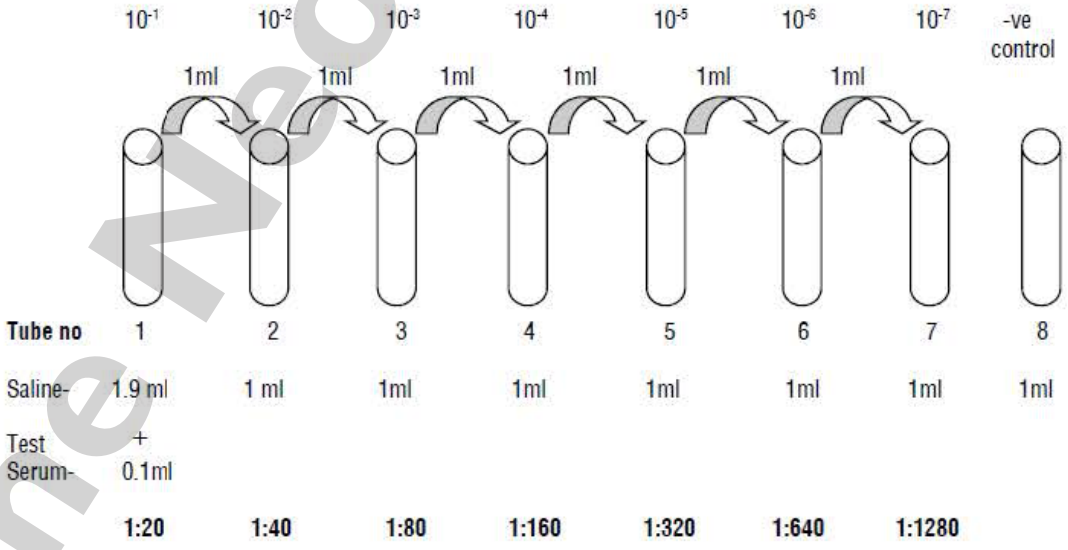
HAntigens: This is aheat labile flagellar antigen which is inactivated both by boiling and alcohol. H antigens rapidly form fluffy clumps when treated with the corresponding antisera. H antigen induces rapid formation of corresponding antibodies as it is strongly immunogenic. Widal Test Teaching Kit (Slide Test) utilizes the principle for rapidslide agglutination to detect the presence of *Salmonellatyphi* and *paratyphi*.The kit gives direct results in form of visible agglutination.

Materials Required:

1 Salmonella typhi 'O' Antigen
2 *Salmonella typhi* 'H' Antigen
3 *Salmonella paratyphi* 'AH' Antigen
4 *Salmonella paratyphi* 'BH' Antigen
5 Test Samples
6 Sterile test tubes with cap
7 Sterile Saline
8 Micropipet / tips
9 Incubator
10. Gloves and Masks

Procedure:

1. Before starting the experiment, bring all reagents to room temperature and mix well.
2. Prepare 4 sets of test tubes for individual antigen. Each set contains 1- 8 tubes.
3. Add 1.9 ml of 0.85% sterile saline to tube no. 1 of each antigen set.
4. To tube no. 2-8 of all sets add 1 ml of physiological saline.
5. To tube No. 1 of all sets add 0.1 ml of test sample to be tested and mix well.
6. Transfer 1 ml of the diluted serum sample from tube No. 1 to tube No. 2 and mix well.
7. Transfer 1 ml of the diluted serum sample from tube No. 2 to tube No. 3 and mix well. Continue this serial dilution till tube No. 7 in each set of antigen.
8. Discard 1.0 ml of the diluted serum from tube No.7 of each set.
9. So the dilutions of the serum sample from tube No. 1 to 7 respectively in each antigen set are 1:20, 1:40,1:80, 1:160, 1: 320, 1:640, 1: 1280.
10. Tube no. 8 is negative control with 0.85% sterile saline.
11. To one set i.e. from tube no.1- 8 add 50 µl of Salmonella typhi 'O' antigen.
12. In second set i.e. from tube no.1- 8 add 50 µl of Salmonella typhi 'H' antigen.
13. Respectively for third and fourth sets, add Salmonella paratyphi 'AH' and Salmonella paratyphi 'BH' to all tubes from 1-8.
14. Mix well, cover and incubate these tubes overnight at 370C (approximately 18 hours).
15. After incubation dislodge the sediment and observe for agglutination.



Observation and Result:

After incubation dislodge the sedimented button of every tube very gently and observe for agglutination macroscopically. Note down the titre value of the antibody in the given test sample for all the antigens.

Interpretation:

During the infection of Salmonella the human body responds to the antigenic stimulus and as a result corresponding antibodies are produced. When the test sample is treated with colored and attenuated Salmonella antigen suspensions, the antibodies present in the sample react with the antigen suspension to give clearly visible agglutination which can be seen through naked eye. The antibody titre of the test sample is its highest dilution that gives a visible agglutination. Agglutinin titre greater than 1:80 is considered as significant infection and low titres indicate absence of infection.

Safety precautions: Wear masks and gloves while handling the reagents

Experiment-6: Radial Immuno diffusion

Aim:

To study the immunodiffusion technique by Single Radial Immunodiffusion.

Introduction:

Single Radial Immunodiffusion, also known as Mancini technique, is a quantitative immunodiffusion technique used to detect the concentration of antigen by measuring the diameter of the precipitin ring formed by the interaction of the antigen and the antibody at optimal concentration. In this method the antibody is incorporated into the agarose gel whereas the antigen diffuses into it in a radial pattern.

Principle:

Single Radial Immunodiffusion is used extensively for the quantitative estimation of antigen. Here the antigen-antibody reaction is made more sensitive by the addition of antiserum into the agarose gel and loading the antigen sample in the well. As the antigen diffuses into the agarose radially in all directions, it's concentration continuously falls until the equivalence point is reached at which the antigen concentration is in equal proportion to that of the antibody present in the agarose gel. At this point ring of precipitation ('precipitin ring') is formed around the well. The diameter of the precipitin ring is proportional to the concentration of antigen. With increasing concentration of antigen, precipitin rings with larger diameter are formed.

The size of the precipitin rings depend on

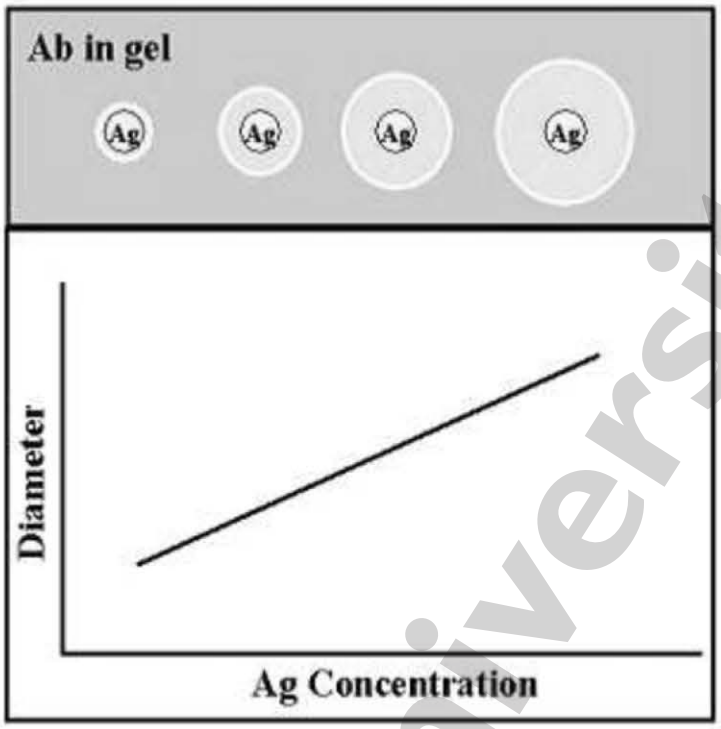
_ Antigen concentration in the sample well

_ Antibody concentration in the agarose gel

_ Size of the sample well

_ Volume of the sample

Thus, by having various concentrations of a standard antigen, standard curve can be obtained from which one can determine the amount of an antigen in an unknown sample. Thus, this is a quantitative test. If more than one ring appears in the test, more than one antigen/antibody reaction may have occurred. This could be due to a mixture of antigens or antibodies.



Materials required:

1. Agarose
2. 10X Assay buffer
3. Antiserum
4. Standard Antigen
5. Standard Antigen
6. Standard Antigen
7. Standard Antigen
8. Test Antigen
9. Test Antigen
10. Glass plate
11. Gel puncher
12. Template
13. Measuring cylinder
14. Conical flask
15. Beaker
16. Alcohol
17. Water
18. Incubator
19. Microwave oven

20. Vortex mixture

21. Micropipet/tip

22. Moist chamber

Procedure:

1. Prepare 10 ml of 1% agarose gel (as give in the important instructions). Take 6 ml of this gel solution in a clean test tube.

2. Allow the solution to cool down to 55-60oC and add 80 ml of antiserum to 6 ml of agarose solution. Mix well for uniform distribution of the antibody.

3. Pour agarose solution containing the antiserum on to a grease free glass plate placed on a horizontal surface. Allow the gel to set for 30 minutes.

4. Place the glass plate on the template provided.

5. Punch wells with the help of gel puncher corresponding to the markings on the template. Use gentle suction to avoid forming rugged wells.

6. Add 10 µl of the given standard antigen and test antigen samples to the wells.

A. Standard Antigen A (3.75 mg/ml)

B. Standard Antigen B (7.5 mg/ml)

C. Standard Antigen C (15 mg/ml)

D. Standard Antigen D (30 mg/ml)

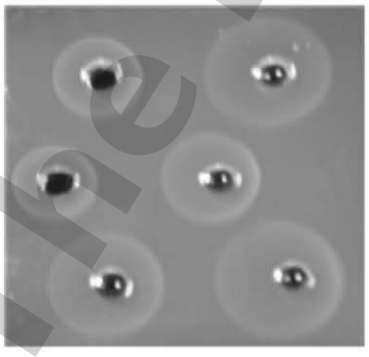
E. Test Antigen 1

F. Test Antigen 2

7. Incubate the glass plate in a moist chamber overnight at 37oC.

Observation and Result:

Observe for precipitin rings surrounding the antigen wells. Mark the edges of the precipitin rings and measure the diameter of the rings.

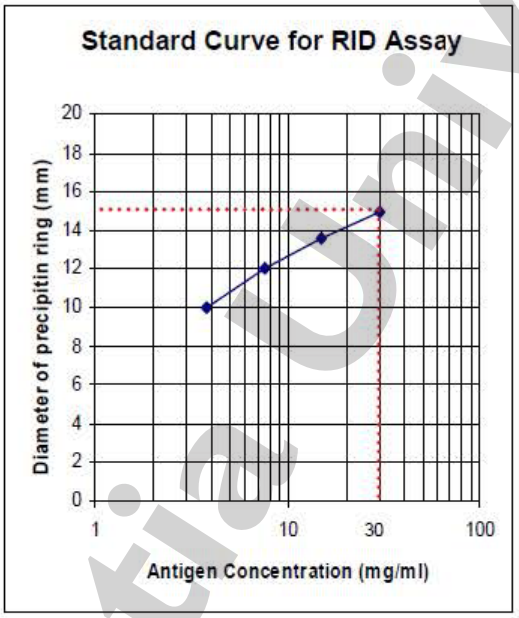


Sample	Standard Antigen Concentration (in mg/ml)	Ring Diameter (in mm)
A	3.75	
B	7.5	
C	15	
D	30	
E	Test antigen-1	
F	Test antigen-2	

Plot a graph of the diameter of the precipitin ring (on Y-axis) versus the concentration of antigen (on X-axis) on a standard graph sheet. Determine the concentration of the unknown antigen from the graph by finding the concentration against the ring diameter.

Interpretation:

The diameter of the precipitin ring depends upon the concentration of antigens loaded in the wells. By plotting the graph of concentration of antigens versus diameter of the corresponding precipitin ring one can calculate the concentration of any test antigen.



Experiment 7: Ouchterlony Double Diffusion (Antigen-Antibody Pattern)

Aim: To learn the technique of Ouchterlony double diffusion (Antibody Titration).

Introduction: Interaction between antigen and antibody at the molecular level forms the basis for several techniques that are useful in modern day scientific studies and in routine clinical diagnosis. These techniques are either based on the use of labeled reagents, a tracer or immunoprecipitation. Ouchterlony double diffusion (ODD) or double immunodiffusion technique is one of the simplest techniques extensively used to check antisera for the presence of antibodies for a particular Ag and to determine its titre.

Principle: Precipitation of soluble antigen and antibody complexes as visible lattices in agarose can be utilized to find the 'Titre' value' of the given antiserum. In Ouchterlony Double Diffusion technique a standard, uniform concentration of the antigen is subjected to diffusion with serially diluted antiserum samples. This is achieved when antigen sample is applied to a single central well, surrounded with suitable placed wells with serially diluted antiserum samples. Also, the same can be done in parallel rows of wells with constant antigen samples in all the wells of a row with corresponding rows of wells being loaded with the serially diluted antiserum samples. This later arrangement has the advantage with three rows of wells in a same agarose slide where, two sets of antisera samples can be tested against a common antigen. The highest dilution where the reaction between antigen and antibody stops is termed as the 'Titre Value' of the antiserum.

Materials required:

1. Antiserum
2. Assay buffer
3. Antiserum
4. Antigen
5. Glass plate
6. Moist chamber

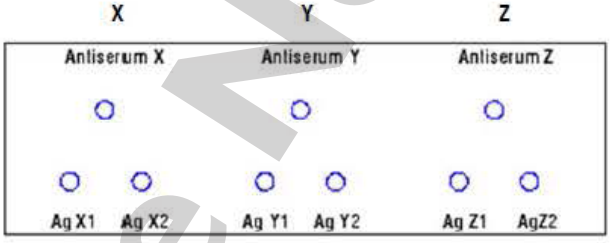
7. gel puncher
8. Measuring cylinder
9. Beaker
10. Conical flask
11. Microwave oven
12. Micropipet
13. Incubator
14. Vortex mixer

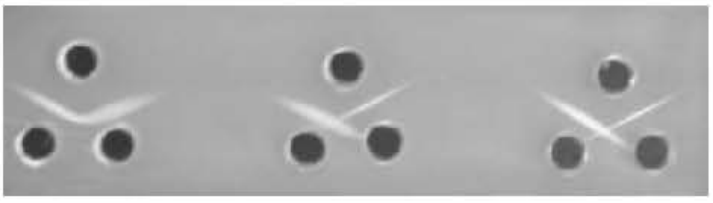
Procedure:

1. Prepare 10 ml of 1% agarose (as given in important instructions).
2. Cool the solution to 55-60oC and pour 5 ml/plate on to grease free glass plates placed on a horizontal surface. Allow the gel to set for 30 minutes.
3. Place the glass plate on the template provided.
4. Punch wells with the help of the gel puncher corresponding to the markings on the template. Use gentle suction to avoid forming of rugged wells.
5. Add 10 µl each of the antiserum and the corresponding antigens to the wells
6. Keep the glass plate in a moist chamber overnight at 37oC.
7. After incubation, observe for opaque precipitin lines between the antigen and antiserum wells

Observation and Result:

Observe for presence of precipitin lines between antigen and antisera wells. Note the pattern of precipitin line observed in each case.





Interpretation: When antigen and antibody meet in optimal proportions a precipitation line is formed. In Ouchterlony Double Diffusion (Antigen Antibody Pattern), three patterns of precipitin lines can be observed.

1. If pattern X or pattern of identity is observed between the antigens and the antiserum, it indicates that the antigens are immunologically identical.
2. If pattern Y or pattern of partial identity is observed, it indicates that the antigens are partially similar or cross-reactive.
3. If pattern Z or pattern of non-identity is observed, it indicates that there is no cross-reaction between the antigens. i.e. the two antigens are immunologically

Experiment 8: Ouchterlony Double Diffusion (Antibody titration)

Aim: To learn the technique of Ouchterlony double diffusion (Antibody Titration).

Introduction: Interaction between antigen and antibody at the molecular level forms the basis for several techniques that are useful in modern day scientific studies and in routine clinical diagnosis. These techniques are either based on the use of labeled reagents, a tracer or immunoprecipitation. Ouchterlony double diffusion (ODD) or double immunodiffusion technique is one of the simplest techniques extensively used to check antisera for the presence of antibodies for a particular Ag and to determine its titre.

Principle: Precipitation of soluble antigen and antibody complexes as visible lattices in agarose can be utilized to find the 'Titre' value' of the given antiserum. In Ouchterlony Double Diffusion technique a standard, uniform concentration of the antigen is subjected to diffusion with serially diluted antiserum samples. This is achieved when antigen sample is applied to a single central well, surrounded with suitable placed wells with serially diluted antiserum samples. Also, the same can be done in parallel rows of wells with constant antigen samples in all the wells of a row with corresponding rows of wells being loaded with the serially diluted antiserum samples. This later arrangement has the advantage with three rows of wells in a same agarose slide where, two sets of antisera samples can be tested against a common antigen. The highest dilution where the reaction between antigen and antibody stops is termed as the 'Titre Value' of the antiserum.

Materials required:

1. Antiserum
2. Assay buffer
3. Antiserum
4. Antigen
5. Glass plate
6. Moist chamber
7. gel puncher
8. Measuring cylinder
9. Beaker

10. Conical flask

11. Microwave oven

12. Micropipet

13. Incubator

14. Vortex mixer

Procedure: 1. Prepare 10 ml of 1% agarose (as given in important instructions).

2. Cool the solution to 55-600 C and pour 6 ml of the agarose solution on to grease free glass plate placed on a horizontal surface. Allow the gel to set for 30 minutes.

3. Place the glass plate on the template provided.

4. Punch wells with the help of gel puncher corresponding to the markings on the template. Use gentle suction to avoid forming rugged wells.

5. Serially dilute the test antiserum up to 1:32 dilution as

a. of 1X assay buffer in each of the five vials

b. Add 10 µl of test antiserum into the first vial and mix well. The dilution of antiserum in thisµb. Add 10 vial is 1:2.

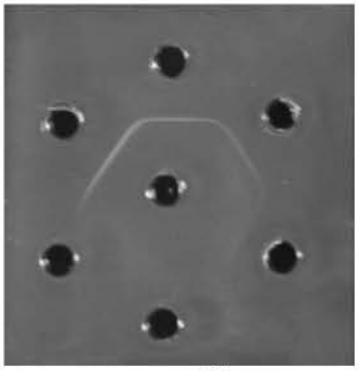
c. Transfer 10 µl of 1:2 diluted antiserum from the first vial into the second vial. The dilution isµc. Transfer 10 this is 1:4.

d. Repeat the dilutions up to fifth vial

6. l each of undiluted, 1:2, 1:4, 1:8, 1:16, 1:32 dilutions ofµl of the antigen to the center well and 10 µ6. Add 10 antiserum into the surrounding wells

7. Keep the glass plate in a moist chamber overnight at 37o C. 8. After incubation, observe for opaque precipitin lines between the antigen and antisera wells. 9. Note down the highest dilution at which the precipitin line is formed. This is the titre value of the antiserum.

Observation and Result: Observe for presence of precipitin lines between the centre antigen well and the corresponding serially diluted antisera wells and note done the titre value of the test antiserum.



Interpretation: Precipitin lines will be observed between the centre antigen and corresponding antiserum wells. The dilution of the antiserum where the precipitin line is no longer observed is the titre value of the antiserum.

Experiment 9: Immuno electrophoresis

Aim:

To learn the technique of Immunoelectrophoresis.

Introduction:

Immunoelectrophoresis is a powerful qualitative technique for the characterization of any antibody. In this method one antigen mixture is electrophoresed in an agarose gel that allows the separation of its different components based on their charge along the gel slide, followed by the lateral diffusion of the serum or monoclonal antibody within the gel. Antibodies specific to the antigens form white precipitation arcs which can be seen against a dark background. This technique is useful in determining the blood levels of three major immunoglobulins: IgM, IgG and IgA. The process combines antigen separation technique of electrophoresis and immunodiffusion of the separated antigen against an antibody. It is used extensively to check the presence, specificity and homogeneity of the antibodies and can detect relatively high antibody concentrations.

Principle:

In immunoelectrophoresis, the antigen mixture is first electrophoresed to separate its constituents by charge. The antiserum containing the antibodies added into the troughs diffuses with a plane front to react with the antigens. Due to diffusion, density gradient of the antigen and antibody are obtained and at a specific antigen/antibody ratio (equivalence point) huge macromolecules are formed. They form a visible white complex which precipitates as arcs in the gel. The arc is closer to the trough at the point where the antigen is in highest concentration. The method is very specific and highly sensitive because distinct zones are formed. In this method it is important that the ratio between the quantities of antigen and antibody be proper (antibody titer).

Materials required:

1. Agarose
2. Electrophoresis buffer
3. Antiserum
4. Antigen

5. Glass plate

6. gel puncher

7. Measuring cylinder

8. Beaker

9. Conical flask

10. Microwave oven

11. Micropipet

Procedure:

1. Prepare 10 ml of 1.5% agarose (as given in important instructions).

2. Mark the side of the glass plate that will be towards negative electrode during electrophoresis.

3. Cool the solution to 55-60oC and pour 6 ml/plate on to grease free glass plate placed on a horizontal surface. Allow the gel to set for 30 minutes.

4. Place the glass plate on the template provided.

5. Punch a well with the help of the gel puncher corresponding to the markings on the template at the negative end. Use gentle suction to avoid forming rugged wells.

6. Cut two troughs with the help of the gel cutter, but do not remove the gel from the troughs.

7. Add 10 ml of the antigen to the well and place the glass plate in the electrophoresis tank such that the antigen well is at the cathode/negative electrode.

8. Pour 1X Electrophoresis buffer into the electrophoresis tank such that it just covers the gel.

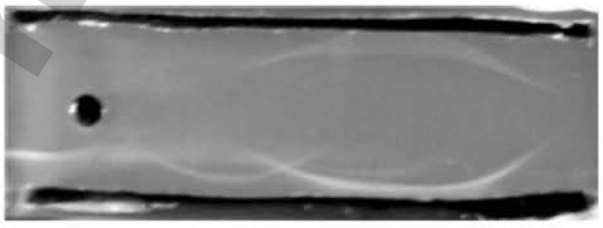
9. Electrophorese at 80-120 volts and 60-70 mA, until the blue dye travels 3-4 cms from the well. Do not electrophorese beyond 3 hours, as it is likely to generate heat.

10. After electrophoresis, remove the gel from both the troughs and keep the plate at room temperature for 15min. Add 80 ml of antiserum A in one of the trough and antiserum B in the other.

11. Place the glass plate in a moist chamber and incubate overnight at 37oC.

Observation and Result:

Observe for precipitin lines between antiserum troughs and the antigen well.



Interpretation:

The formation of precipitin line indicates the presence of antibody specific to the antigen.

1. Homogeneity of the antiserum to the antigen is denoted by presence of a single continuous precipitin line

2. Heterogeneity of the antiserum to the antigen is denoted by presence of more than one precipitin line which not only gives an indication of the number of immunodominant epitopes, but also the non identical nature of such epitopes.