Semester – III

16MBP311 APPLICATION ORIENTED PRACTICAL – V 4H – 2C

Instruction Hours / week:L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 9 Hours

SCOPE

This practical is to provide the student with a basic knowledge of immunology and its significance to human disease.

OBJECTIVES

The general objectives of the lab will be to introduce immunology and basic serological techniques.

1. Separation of serum / plasma
2. ABO Blood grouping - Rh typing and cross matching. Estimation of hemoglobin content of human blood.
3. Agglutination tests.
   - WIDAL - slide and tube test
   - RA test.
   - RPR test.
   - ASO test.
4. Ouchterlony's Double Immunodiffusion test (ODD)
5. Counter immunoelectrophoresis (CIE)

REFERENCES

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Experiment – 1 SEPERATION OF SERUMA AND PLASMA FROM BLOOD

Aim

To separate serum and plasma from the collected blood sample.

Introduction

Serum is the liquid fraction of whole blood that is collected after the blood is allowed to clot. The clot is removed by centrifugation and the resulting supernatant, designated serum, is carefully removed using a Pasteur pipette. Plasma is produced when whole blood is collected in tubes that are treated with an anticoagulant. The blood does not clot in the plasma tube. The cells are removed by centrifugation. The supernatant, designated plasma is carefully removed from the cell pellet using a Pasteur pipette.

Serum Preparation

Collect whole blood in a covered test tube. If commercially available tubes are to be used, the researcher should use the red topped tubes. These are available from Becton Dickinson (BD). BD‟s trade name for the blood handling tubes is Vacutainer. 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.

The resulting supernatant is designated serum. Following centrifugation, it is important to immediately transfer the liquid component (serum) into a clean polypropylene tube using a Pasteur pipette. The samples should be maintained at 2–8°C while handling. If the serum is not analyzed immediately, the serum should be apportioned into 0.5 ml aliquots, stored, and transported at –20°C or lower. It is important to avoid freeze-thaw cycles because this is detrimental to many serum components. Samples which are hemolyzed, icteric or lipemic can invalidate certain tests.

Plasma preparation

Collect whole blood into commercially available anticoagulant-treated tubes e.g., EDTA-treated (lavender tops) or citrate-treated (light blue tops). Heparinized tubes (green tops) are indicated for some applications; however, heparin can often be contaminated with endotoxin, which can stimulate white blood cells to release cytokines. Cells are removed from plasma by centrifugation for 10 minutes at 1,000–2,000 x g using a refrigerated centrifuge. Centrifugation for 15 minutes at 2,000 x g depletes platelets in the plasma sample.

The resulting supernatant is designated plasma. Following centrifugation, it is important to immediately transfer the liquid component (plasma) into a clean polypropylene tube using a Pasteur pipette. The samples should be maintained at 2–8°C while handling. If the plasma is not analyzed immediately, the plasma should be apportioned into 0.5 ml aliquots, stored, and transported at –20°C or lower. It is

important to avoid freeze-thaw cycles. Samples which are hemolyzed, icteric, or lipemic can invalidate certain tests. There are other commercially available tubes for blood sample collection.

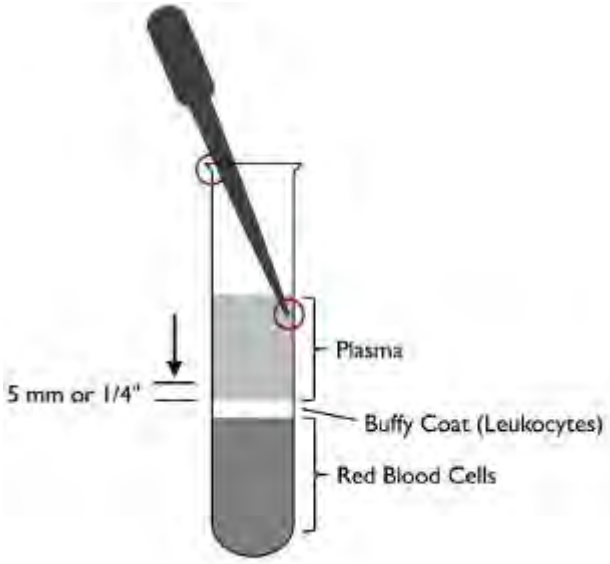


Figure 1. Separation of plasma from blood.

Table 1. Serum and plasma tubes

The commercially available serum tubes are as follows:	
Red	No anticoagulant.
Red with black	Treated with gel to help to separate the clot (not evaluated).
The commercially available plasma tubes are as follows:	
Lavender	Treated with EDTA.
Blue	Treated with citrate.
Green	Treated with heparin.
Grey	Treated with potassium oxalate/sodium fluoride (not evaluated).
Yellow	Treated with potassium oxalate/sodium fluoride (not evaluated).

Observations and Results

Experiment – 2 **ABO BLOOD GROUPING**

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:

- "A" group
- "B" group
- "AB" group
- "O" group

Table 1: ABO Blood Group System

ens 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
A and B	Neither Anti A nor Anti	AB	AB
Neither A nor B	Anti A, Anti B, Anti AB	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.

Table 2: Enlists the materials required with their quantity and recommended storage

S. No.	Materials Provided	Quantity 100 expts	Storage
1	Anti A Sera	5 ml	2-8^{o}C
2	Anti B Sera	5 ml	2-8^{o}C
3	Anti RhD Sera	5 ml	2-8^{o}C
4	Cavity slide	10 Nos.	RT
5	Mixing stick	300 Nos.	RT
6	Blood Lancet	100 Nos.	RT

Materials Required

Reagents: 70% Alcohol/ Spirit
Other requirements: Cotton

Storage

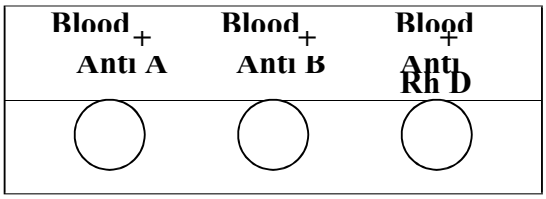
Store the Anti A Sera, Anti B Sera and Anti RhD Sera at 2-8^{o}C. Other contents can be stored at room temperature (15-25^{o}C).

Important Instructions

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

Procedure

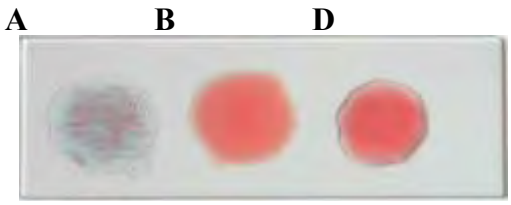
1. Dangle the hand down to increase the flow of blood in the fingers.
2. Clean the fingertip to be pierced with spirit or 70% alcohol (usually ring or middle finger).
3. With the help of the sterile lancet, pierce the fingertip and place one drop of blood in each of the cavities.
4. Add one drop of antiserum into each cavity as shown below:



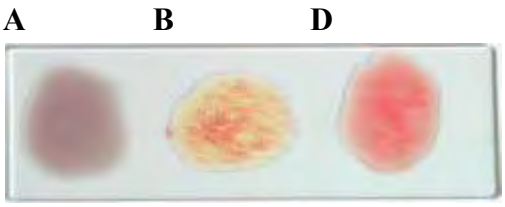
1. Mix each blood drop and the antiserum using a fresh mixing stick.
2. Observe agglutination in the form of fine red granules within 30 seconds. Anti RhD takes slightly longer time to agglutinate compared to Anti A and Anti B.

3. Note: Proper care should be taken while disposing the lancet and mixing sticks.

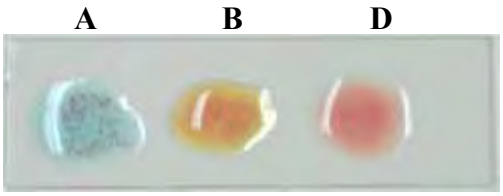
Observations and Result



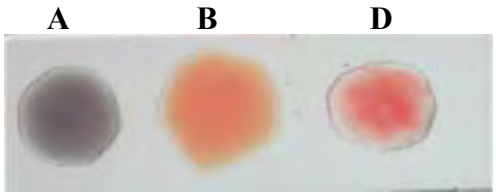
Slide 1



Slide 2



Slide 3



Slide 4

Table 3: Determination of blood group and Rh factor based on agglutination seen

Sr. No.	Anti A	Anti B	Anti RhD	Blood Group
Slide 1	π	X	π	**A**
Slide 2	X	π	π	**B**
Slide 3	π	π	π	**AB +ve**
Slide 4	X	X	π	**O**

π: Agglutination
X: No agglutination

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.

Experiment No – 3 **WIDAL TEST**

Aim

To detect the titre value of antibodies present in test serum due to the infection of *Salmonella* genus causing enteric or Typhoid Fever by using quantitative tube 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*. The tube agglutination test is a quantitative method which is used for the determination of titre values of antibodies present in the patient serum.

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 serum during the fever period, which react with somatic antigen O of *Salmonella typhi*, A or B of *Salmonella paratyphi* and then with flagellar antigen H which is common in most of the *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, as shown in Figure 1.

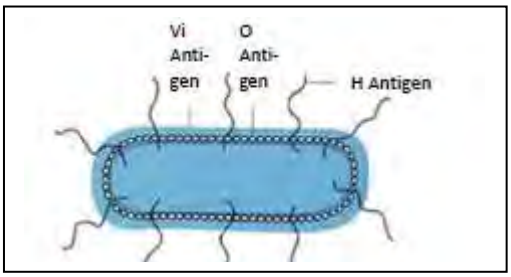


Figure 1. Antigenic structure of *Salmonella*

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 anti-sera.

Vi antigens- 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.

H Antigens- This is a heat 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 (Tube Test) - utilizes the principle for visible agglutination of Salmonella antigen suspension upon reaction with anti-salmonella antibodies present in test serum.

Table 1- Enlists the materials provided with their quantity and recommended storage

S. No.	Materials Provided	Quantity 10 Exp	Storage
1	Salmonella typhi „O‟ Antigen	4 ml	2-8°C
2	Salmonella typhi „H‟ Antigen	4 ml	2-8°C
3	Salmonella paratyphi „AH‟ Antigen	4 ml	2-8°C
4	Salmonella paratyphi „BH‟ Antigen	4 ml	2-8°C
5	Test Sample	4 ml	2-8°C

Materials Required

Glass wares: Sterile test tubes with caps.
Reagent: 0.85% sterile saline.
Other requirements: Incubator, Micropipettes, Tips.

Important Instructions:

- Widal Tube Test reagents should be done according to the kit instructions.

- Allow all reagents to reach room temperature before use.
- Do not dilute any of the kit reagents.
- Do not intermix the reagents.
- Do not freeze any of the kit reagents.
- Wear masks and gloves while handling the reagents.

Observations and Result

Experiment No – 4 **RA TEST**

Aim

To detect the presence of Rheumatoid Factors (RF) which are produced during Rheumatoid arthritis (RA).

Introduction

Rheumatoid arthritis or RA is a chronic and inflammatory disease of flexible (synovial) joints. During this disorder the synovial lymphocytes produce abnormal IgG and in response to this the host immune system generates IgM and these are called Rheumatoid factors (RF). These are autoantibody as they are generated against one's own tissue and for this reason RA is considered as a systematic autoimmune disease. Through a blood test the presence of RF can be detected and consequently RA is diagnosed.

Principle

Agglutination is a reaction of clumping together of antigen-bearing cells, microorganisms or particles in the presence of specific antibodies (agglutinins) in a suspension. Reaction time for agglutination to occur is shorter compared to other antigen-antibody interactions. Latex agglutination makes use of latex particles which are built from different organic materials to a desired diameter, and may be functionalized with chemical groups to facilitate attachment of molecules. Latex agglutination tests have been in use since 1956 to detect a wide range of analytes in the clinical laboratory. The first description of a test based on latex agglutination was the „Rheumatoid Factor Test" proposed by Singer and Plotz in 1956. In this method the patient's blood sample is mixed with tiny latex beads covered with human antibodies (IgG). The latex beads clump or agglutinate if rheumatoid factor (IgM RF) is present.

Table 1: Enlists the materials provided in this kit with their quantity and recommended storage

S. No.	Materials Provided	Quantity	Storage
		20 expts	
1	Latex reagent	1.5 ml	2-8^{O}C
2	Positive control	0.2 ml	2-8^{O}C
3	Negative control	0.2 ml	2-8^{O}C
4	Disposable Agglutination	10 Nos.	R
5	Disposable Mixing Sticks	120 Nos.	R

Materials Required But Not Provided

Test Serum Sample, Micropipettes, Tips, Gloves and Masks.

Storage

. On receipt, store Latex reagent, Positive control and Negative control at 2-8°C.

Important Instructions

- RA Test Teaching Kit should be used according to the kit instructions.
- Allow all reagents to reach room temperature before use.

- Do not dilute any of the kit reagents.
- Do not intermix the reagents.
- Do not freeze any of the kit reagents.
- Ensure the Agglutination card is clean and dry prior to use.
- Wear masks and gloves while handling the reagents.

Procedure

- Before starting the experiment, bring all reagents to room temperature and mix well.
- Take 10µl of test serum sample on one of the latex disposable slide circle.
- Take 10 µl of Positive and Negative control each on other circles.
- Add 25 µl of Latex reagent to all these three circles. Do not let the dropper tip touch the liquid on the slide.
- Using disposable mixing sticks mix all the contents uniformly over the entire circles of slide.
- Rock the slide gently, back and forth for 2 minutes. Observe for agglutination.

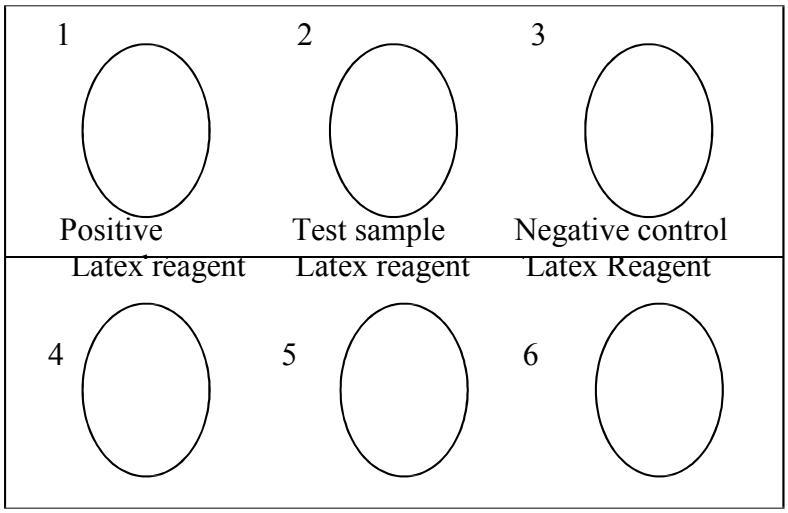


Figure 1. Diagrammatic representation of RA test

Observations and Result

After mixing the Latex reagent with Positive control, Negative control and Test sample separately observe for the agglutination reaction.

Figure 2. Agglutination reaction for Latex reagent with Positive, Negative control and Test sample

Circle 1: Positive Control; **Circle 2:** Test sample; **Circle 3:** Negative Control

Experiment No – 5 **RPR TEST**

Aim

To perform the RPR Test for the detection of syphillis.

Introduction

The Rapid Plasma Reagin (RPR) is a macroscopic flocculation test which detects the presence of reagin antibody in the serum of a patent suffering from syphilis. During this test method reagins form visible flocculants upon reacting with the carbon-containing RPR antigen.

Principle

Syphilis is a sexually transmitted (venereal) disease caused by the spirochete *Treponema pallidum*. It is a Gram-negative spirochaete bacterium with subspecies that cause treponemal diseases like syphilis, bejel, pinta and yaws. Two types of antibodiesare produced in response to this infection – the host forms specific anti-treponemal antibodies to *Treponema pallidum* and anti-lipid antibodies in response to the lipoidal material released from the infected host cell which are referred to as reagins. In a serum sample containing reagin antibody, flocculation occurs due to agglutination of the carbon particles of the RPR antigens, which appear as black clumps against the white background of the card. This agglutination is observed macroscopically. The non-reactive specimens show an even light grey colour. 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*.

Table 1- Enlists the materials provided with their quantity and recommended storage

S. No.	Materials Provided	Quantity	Storage
		20 exp	
1	RPR Antigen	1.5 ml	2-8°C
2	Positive control	0.5 ml	2-8°C
3	Negative control	0.5 ml	2-8°C
4	Disposable Slides	10 Nos.	RT
5	Disposable Mixing Sticks	120 Nos.	RT

Materials Required

Test Serum Sample, Micropipettes, Tips, Gloves and Masks.

Storage

On receipt, store RPR Antigen, Positive control and Negative control at 2-8 °C.

Important Instructions

1. RPR Test reagents should be used according to the kit instructions.
2. Allow all reagents to reach room temperature before use.
3. Do not dilute any of the reagents.
4. Do not intermix the reagents.
5. Do not freeze any of the kit reagents.
6. Ensure the Agglutination card is clean and dry prior to use.

Procedure

1. Before starting the experiment, bring all reagents to room temperature and mix well.

2. Take 25µl of the test sample, positive control and negative control onto three different reaction circle of the disposable slide provided in kit.

3. Add 25µl of well mixed RPR reagent to test sample, positive control and negative control circles.

4. Do not touch the dropper tip to liquid on the slide.

5. Using a mixing stick, mix the test sample and RPR reagent thoroughly by spreading uniformly over the entire circle.

6. Repeat step 4 for Positive and Negative controls.

7. Rotate the slide gently and continuously for 8 mins. to observe flocculation.

Observations and Result

After mixing the RPR antigen with Positive control, Negative control and Test sample separately observe for visible flocculation.

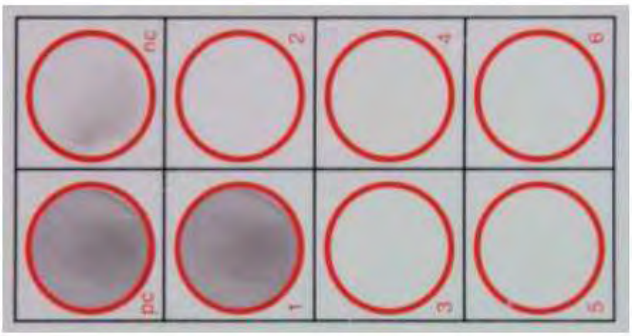


Figure 2 - Flocculation for RPR antigen with Positive control, Negative control and Test sample

PC : Positive Control

NC : Negative Control

CIRCLE 1 : Test Sample

Experiment No – 6 **ASO TEST**

Aim:

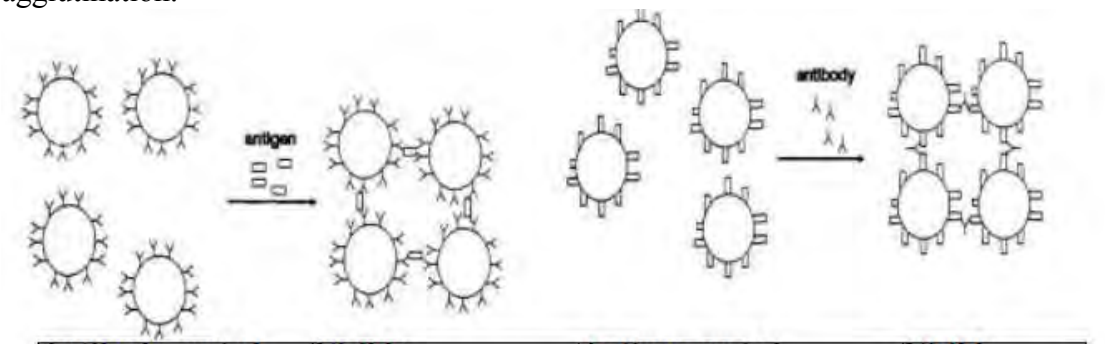
To learn the technique of latex agglutination.

Introduction

Agglutination is a reaction of clumping together of antigen-bearing cells, microorganisms or particles in the presence of specific antibodies (agglutinins) in a suspension. Reaction time for agglutination to occur is shorter compared to other antigen antibody-antibody interactions. Latex agglutination makes use of latex particles which are built from different organic materials to a desired diameter, and may be functionalized with chemical groups to facilitate attachment of molecules. Latex agglutination tests have been in use since 1956 to detect a wide range of analytes in the clinical laboratory. The first description of a test based on latex agglutination was the „Rheumatoid Factor Test" proposed by Singer and Plotz in 1956. It can be used for detection of both antigen and antibody.

Principle:

In latex agglutination, antibodies are adsorbed to the latex particles (under appropriate ionic and alkaline pH conditions) by binding to the Fc region of antibodies leaving Fab region free to interact with antigen present in the applied specimen. The use of smaller latex particle has improved the sensitivity and reagent longevity of latex agglutination.



Antibody coated latex particles	Visible clumping	Antigen coated latex particles	Visible clumping

Applications of Latex Agglutination Tests

- Latex agglutination tests are very popular in clinical laboratories.
- These tests are applied
- To the detection of many infectious diseases.
- To detect microbial and viral infections, autoimmune diseases, hormones, drugs and serum proteins.
- To check for certain antibodies or antigens in a variety of bodily fluids including saliva, urine, cerebrospinal fluid, and blood.

The test involves three solutions i.e. Antigen solution, Negative control & Positive control. The Antigen solution (preserved with 0.099% sodium azide) contains inactivated antigens reactive with Test reagent and non-reactive with Control reagent. The Negative control contains latex particles coated with non-specific antibodies preserved in 0.099% sodium azide. The Positive control contains latex particles coated with specific antibodies preserved in 0.099% sodium azide.

Table 1. Enlists the materials provided with their quantity and recommended storage

S. No.	Materials Provided	Quantity	Storage
		20 Expts	
1	Antigen Solution- Solution A	1.08 ml	2-8°C
2	Negative Control- Solution B	0.54 ml	2-8°C
3	Positive Control- Solution C	0.54 ml	2-8°C
4	Disposable Agglutination Cards	7 Nos.	RT
5	Disposable Mixing Sticks	46 Nos.	RT

Storage

On receipt, store the Antigen solution, Positive control and Negative control at 2- 8◦C.

Important Instructions:

1. Latex Test Kit should be used according to the kit instructions.
2. Allow all reagents to reach room temperature before use.
3. Do not dilute any of the kit reagents.
4. Do not intermix the reagents.
5. Do not freeze any of the kit reagents.
6. Ensure the agglutination card is clean and dry prior to use.
7. Wear gloves while handling the reagents.

Procedure

1. Before starting the experiment, gently mix all the bottles provided in this kit.
2. Add 1 drop of Solution A (25µL) into the circles marked as 1 and 2 of a clean dry agglutination card.
3. Add 1 drop of Solution B (25µL) into circle 1.
4. Add 1 drop of Solution C (25µL) to circle 2.
5. Spread the drops over the area of both the circles using fresh mixing stick for each circle.
6. Rock the card gently (approximately two to three minutes) and observe for agglutination. An agglutination reaction is indicated by visible aggregation of the latex particles.
7. The circles marked as 3, 4 and 5, 6 can be used similarly.
8. After performing the experiment, discard the slides and mixing sticks.

Observations and Result

After mixing the Antigen Solution with Positive control and Negative control separately observe for the agglutination reaction.

1: Corresponds to Negative result 2: Corresponds to Positive result

Interpretation

The results can be interpreted as follows:

Circle	Test	Interpretation
1	Negative	No Agglutination
2	Positive	Agglutination seen

- For circle 1, the inactivated antigens of the Antigen solution do not bind to the latex particles coated with non-specific antibodies of the Negative control. Hence, no agglutination is seen.
- For circle 2, the inactivated antigens of the Antigen solution bind to the latex particles coated with specific antibodies of the Positive control. Hence, agglutination is seen.

Experiment No – 7 OUCHTERLONY'S DOUBLE IMMUNO DIFFUSION TEST

Aim

To study the reaction pattern of an antigen with a set of antibodies by Ouchterlony
Double Diffusion method.

Introduction

Immunodiffusion in gels encompasses a variety of techniques, which are useful for the analysis of antigens and antibodies. Gel immunodiffusion can be classified into two groups:

- Single Immunodiffusion
- Double Immunodiffusion

In the Ouchterlony double diffusion, both the antigen and the antibody diffuse toward each other in a semisolid medium to a point till their optimum concentration is reached. Ab and of precipitation occurs at this point. The qualitative Ouchterlony Test can simultaneously monitor multiple Antibody-Antigen system and can be used to identify particular antigens in a preparation. This procedure was developed by Örjan Ouchterlony.

Principle

When soluble antigen and antibody samples are placed in adjacent wells in agarose gel, they diffuse radially into the agarose gel and set up two opposing concentration gradients between the wells. Once the gradients reach to an optimal proportion, interactions of the corresponding molecules occur and a line of precipitation will form. Using such a technique, the antigenic relationship between two antigens can be analyzed. Distinct precipitation line patterns are formed against the same anti-sera depending on whether two antigens share all antigenic epitopes or partially share their antigenic epitopes or do not share their antigenic epitopes at all. The Ouchterlony test also can be used to estimate the relative concentration of antigens. When an antigen has a relatively higher concentration, the equivalent zone will be formed a little bit away from the antigen well. When an antigen has a relatively lower concentration, the equivalent zone will be formed a little bit closer the antigen well. The pattern of lines that form can be interpreted to determine the relationship between the antigens and antibodies.

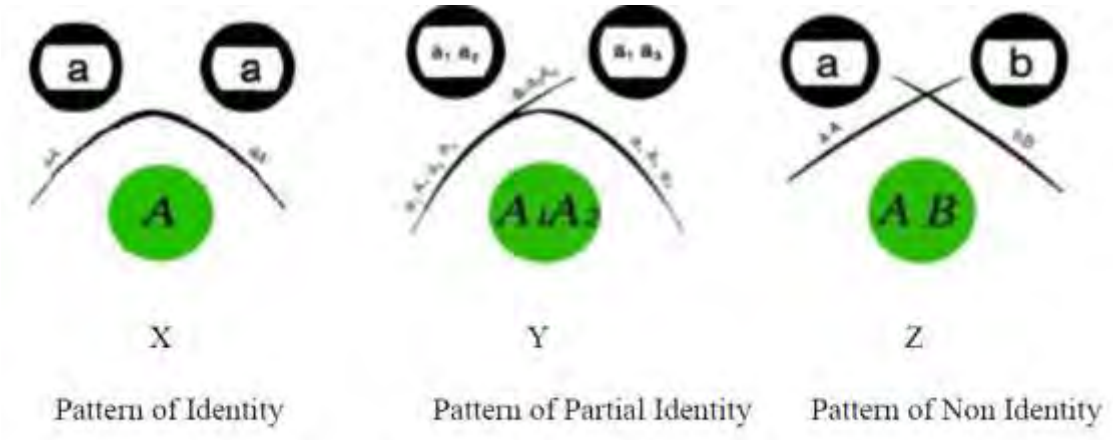


Figure 1. Antigen- Antibody Patterns formed in Ouchterlony Double Diffusion

Pattern of Identity X

Pattern of identity occurs when the antigens in the two wells are identical and specific for the antibody in the antiserum present in the third well. The concentration of the two antigens been the same, they will diffuse at the same rate resulting in a smooth line of precipitate. The antibodies cannot distinguish between the two antigens i.e. the two antigens are immunologically identical as shown in Figure 1.

Pattern of Partial Identity Y

Pattern of partial identity occurs when the antigens in the two wells share some epitopes which are same for both, yet each of the two antigens also have unique epitopes. In this case antiserum contains polyclonal antibodies specific for each epitope. When one of the antigens has some of the same epitopes compared to other, the polyclonal antibody population will respond differently to the two antigens and the precipitin line formed for each antigen will be different. The „spur‟ is thought to result from the determinants present in one antigen but lacking in the other antigen (refer to Figure 1). A similar pattern of partial identity is observed if the antibodies are cross reactive with an epitope on one of the antigen that is similar, but not identical to that present on the other antigen.

Pattern of Non-Identity: Z

Pattern of non-identity occurs when the antigens in the two wells are totally different. They are neither cross reactive, nor do they have any epitopes which are same. In this case the antiserum containing the antibodies is heterogeneous as some of the antibodies react with antigen in one well while some react with antigen present in the other well. So the two antigens are immunologically unrelated as far as that antiserum is concerned (refer to Figure 1).

Table 1. Enlists the materials provided with their quantity and recommended storage

S. No.	Materials Provided	Quantity 10 expts	Storage
1	Agarose	1.2 g	R T
2	10X Assay buffer	12 ml	2-8 °C
3	Antiserum X	0.1 ml	2-8°C
4	Antiserum Y	0.1 ml	2-8°C
5	Antiserum Z	0.1 ml	2-8°C
6	Antigen X1	0.1 ml	2-8°C
7	Antigen X2	0.1 ml	2-8°C
8	Antigen Y1	0.1 ml	2-8°C
9	Antigen Y2	0.1 ml	2-8°C
10	Antigen Z1	0.1 ml	2-8°C
11	Antigen Z2	0.1 ml	2-8°C
12	Glass plate	4 Nos.	R T
13	Gel puncher	1 No.	R T
14	Template	4 Nos.	R T

Materials Required

Glass wares: Measuring cylinder, Beaker

Reagents: Alcohol

Other requirements: Incubator (37 °C), Microwave or Bunsen burner, Vortex mixer, spatula, Micropipettes, Tips, Moist chamber (box with wet cotton)

Storage

Store 10X Assay buffer, Antisera and Antigens at 2-8oC. Other kit contents can be stored at room temperature (15-25°C).

Important Instructions

- Before starting the experiment the entire procedure has to be read carefully.
- Always wear gloves while performing the experiment.
- Preparation of 1X Assay Buffer: To prepare 10 ml of 1X Assay Buffer, add 1 ml of 10X Assay buffer to 9 ml of sterile distilled water.
- Preparation of 1% Agarose gel: To prepare 10 ml of agarose gel, add 0.1g of agarose powder to 10 ml of 1X Assay Buffer, boil to dissolve the agarose completely.
- Wipe the glass plates with cotton; make it grease free using alcohol for even spreading of agarose.
- Cut the wells neatly without rugged margins.
- Ensure that the moist chamber has enough wet cotton to keep the atmosphere humid.

Procedure

- Prepare 10 ml of 1% agarose (as given in important instructions).
- Cool the solution to 55-60°C and pour 5 ml/plate on to grease free glass plates placed on a horizontal surface. Allow the gel to set for 30 minutes.
- Place the glass plate on the template provided.
- 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.
- Add 10 µl each of the antiserum and the corresponding antigens to the wells as shown in Fig 2.
- Keep the glass plate in a moist chamber overnight at 37°C.
- After incubation, observe for opaque precipitin lines between the antigen and antiserum wells.

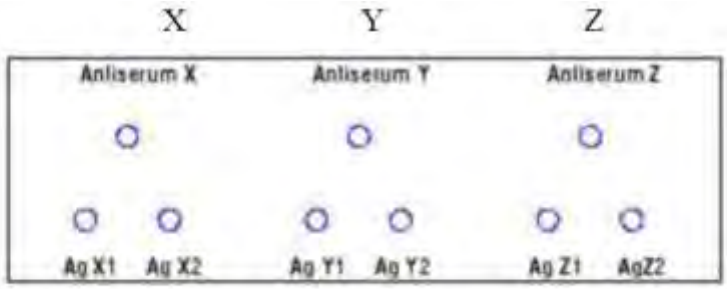


Figure 2. Template for addition of antiserum and antigen to their respective wells

Observations and Result

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

Figure 3. Diagram showing pattern of precipitin line.

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.
- If pattern X or pattern of identity is observed between the antigens and the antiserum, it indicates that the antigens are immunologically identical.
- If pattern Y or pattern of partial identity is observed, it indicates that the antigens are partially similar or cross-reactive.
- 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 unrelated.

Experiment No – 8 COUNTER CURRENT IMMUNO ELECTROPHORESIS TECHNIQUE

Aim

To learn the method of Counter Current Immunoelectrophoresis to rapidly check any antisera for the presence and specificity of antibodies for a particular antigen.

Introduction

Counter current immunoelectrophoresis is a modification of immunoelectrophoresis in which antigen and antibody migrate towards opposite directions and form a visible white precipitate in the area between the wells. It is also known as voltage facilitated double immunodiffusion because the migration of antigen and antibody through the agarose gel is due to the applied voltage rather than simple double immunodiffusion. In this method, the antigen and antibody are placed in parallel wells and under the influence of an electric field move towards one another. A precipitin band appears where they meet in the appropriate ratio. This qualitative technique is much faster and more sensitive than the double diffusion technique.

Principle

In this method, immunoprecipitation occurs when antigen at the cathode negative pole) is caused to migrate in an electric field through a suitable medium of diffusion against a stream of antibody migrating backward from the anode (positive pole) because of endosmotic flow. When an electrical current is applied through the alkaline buffer, the negatively charged antigen molecules migrate toward the positive electrode and thus towards the wells filled with antibody and the positively charged antibodies are migrated toward the negative electrode. At some point between the wells, a zone of equivalence occurs and the antigen-antibody complex precipitates as a visible white line.

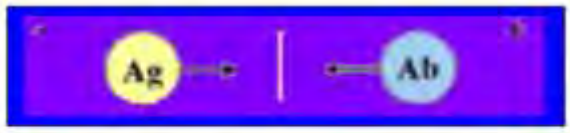


Figure 1. Counter Current Immunoelectrophoresis

Table 1. Enlists the materials provided with their quantity and recommended storage

S. No.	Materials Provided	Quantity		Storage
		10 expts	20 expts	
1	Agarose	1.8 g	3.45 g	RT
2	50X Electrophoresis Buffer	72 ml	138 ml	RT
3	Positive Control (Antiserum)	0.1 ml	0.2 ml	2-8^{o}C
4	Test Antiserum 1	0.1 ml	0.2 ml	2-8^{o}C
5	Test Antiserum 2	0.1 ml	0.2 ml	2-8^{o}C
6	Test Antiserum 3	0.1 ml	0.2 ml	2-8^{o}C
7	Antigen	0.4ml	0.8 ml	2-8^{o}C
8	Glass plate	2 Nos.	4 Nos.	RT
9	Template	2 Nos.	4 Nos.	RT
10	Gel puncher	1 No.	1 No.	RT

Materials Required

Glass wares: Conical flask, Measuring cylinder, Beaker

Reagents: Sterile distilled water, alcohol

Other requirements: Incubator (37°C), Microwave or Bunsen burner, Vortex mixer, spatula, Micropipettes, Tips, Moist chamber (box with wet cotton)

Storage

Store the Antigen, Test Antiserum and Positive Control at 2- 8°C.

Important Instructions

1. Before starting the experiment the entire procedure has to be read carefully.
2. Always wear gloves while performing the experiment.
3. **Preparation of 1X Electrophoresis Buffer:** To prepare 300 ml of 1X Electrophoresis Buffer, add 6 ml of 50X Electrophoresis Buffer to 294 ml of sterile distilled water.
4. **Preparation of 1.5% Agarose gel:** To prepare 10 ml of agarose gel, add 0.15 g of agarose powder to 10 ml of 1X Electrophoresis Buffer, boil to dissolve the agarose completely.
5. Wipe the glass plates with cotton; make it grease free using alcohol for even spreading of agarose.
6. Cut the well and troughs neatly without rugged margins.
7. Ensure that the moist chamber has enough wet cotton to keep the atmosphere humid.

Procedure

1. Prepare 10 ml of 1.5% agarose (as given in important instructions).
2. Mark the end of the slide that will be towards negative electrode during the 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 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 antigen sample to the wells that will be placed towards the negative electrode and 10 µl of antiserum samples to the wells towards the positive electrode as shown in figure 2.

Tank Preparation

1. Pour 1X Electrophoresis buffer into the electrophoresis tank such that it just covers the gel.
2. Electrophorese at 80-120 volts and 55-60 mA, until precipitin lines are observed.
3. Place the glass plate in a moist chamber and incubate overnight at 37°C.

Observations and Result

Observe for precipitin lines between the antigen and corresponding antiserum wells.

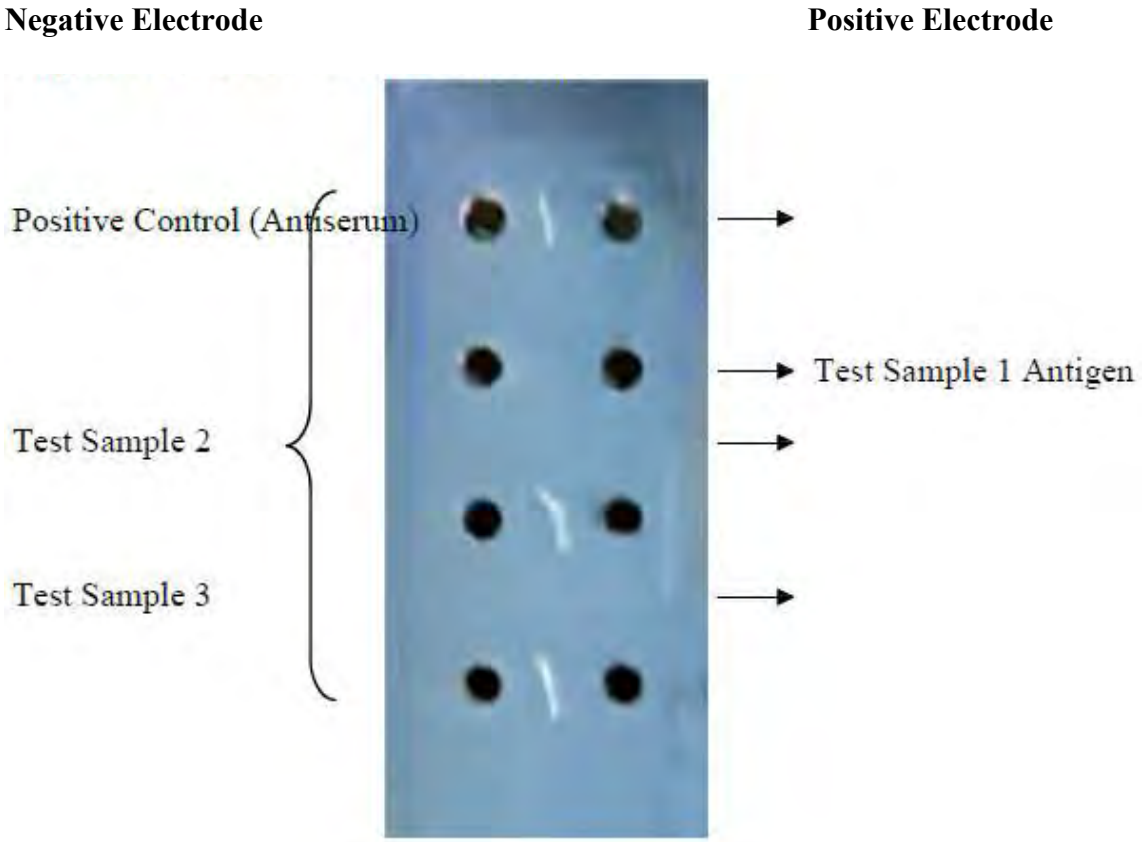


Figure 2. Precipitin lines observed in Counter Current Immunoelectrophoresis

Interpretation

The precipitin line indicates the presence of antibody specific to the antigen while the absence of precipitin line indicates absence of corresponding antibody in the test antiserum to the given antigen. The presence of more than one precipitin line indicates the heterogeneity of the antibody for the antigen in the test sera.

KARPAGAM ACADEMY OF HIGHER EDUCATION
DEPARTMENT OF MICROBIOLOGY

(Deemed University Established Under Section 3 of UGC Act, 1956)
Eachanari PO, Coimbatore -641 021, India.

I –B.Sc Microbiology (Batch 2017-2020)

Possible Viva Questions

1. Define Glycocalyx
2. Comment on endospore staining.
3. Discuss on the enumeration of microbes using colony count and turbidimetric method.
4. Write any two ecological significance of the archae bacteria.
5. What is meant by thermophiles?.
6. What are cyanobacteria?
7. Define halophile
8. What are methanogens?
9. What is meant by biological cycle?
10. How are archae bacteria classified? Explain.
11. Write a brief note on halophiles
12. Write in detail about the general characteristics about archae.
13. Write short notes on barophiles and pshyrophiles.
14. Explain in detail about cultivation of anaerobic bacteria.
15. Define culture media
16. Define growth.
17. Define generation time. How are bacterial growth classified?
18. Define simple and complex media.
19. Describe about turbidostat and chemostat.
20. Write short notes on factors affecting microbial growth.
21. Distinguish between moist and dry heat sterilization with apt examples.
22. Define protoplasts and sphaeroplasts.
23. Draw a neat diagram on the structure of flagella.
24. Distinguish between prokaryotes and eukaryotes.
25. Comment on the archae bacterial cell wall.
26. Discuss about the structure of the gram positive cell wall.
27. Describe the stages involved in the formation of endospore.
28. Discuss on the role of endospore in the bacterial sustainability.
29. Comment on the action of penicillin on the bacterial cell wall.
30. Discuss the structure and function of pili.
31. Write a brief note on the capsule stain.
32. Explain the principle behind the acid fast stain.
33. Write short notes on Gram stain.
34. Explain about the principle behind pure culture techniques.
35. Define Stain.
36. What is meant by mordant?
37. Distinguish between eubacteria and archae bacteria.
38. Write short notes on the concept of species, taxa, and strain.
39. What is meant by 16S rRNA gene sequencing.

P. SRINIVASAN
Assistant Professor
Department of Microbiology

40. Comment on the mechanism of gene sequencing and ribotyping.
41. Discuss in detail about the signature sequences.
42. Discuss in detail about reverse sequencing methods.
43. What is meant by evolutionary chronometers?.
44. Comment on acidic and basic dyes.
45. Write short notes on the use of chemical methods for controlling microbes.
46. Write short notes bacterial motility and cell count techniques.
47. Write short notes on calculation of specific growth rate.
48. Define taxonomy.
49. Define genus and species
50. Write any two points about principle of taxonomy.
51. What is meant by monophasic and polyphasic taxonomy.
52. Discuss on RAPD.
53. Write about the archae bacterial cell wall.
54. How are cyanobacteria cultured in lab?
55. Explain in detail about the role of phytoplankton in microbial kingdom.

P. SRINIVASAN
Assistant Professor
Department of Microbiology