Aim: To determine the bleeding time (BT).

Learning Objectives: Bleeding due to cut or injury is commonly known to everyone. However, when bleeding stops automatically within few minutes then there is no botheration, but when there is excessive and prolonged bleeding with small injuries, then it is of great concern to everyone. This may cause a suspicion of a disease and calls for a visit to a doctor thus indicating the clinical importance of determination of bleeding time.

Requirements:

Materials: Sterilized needle, cotton, stop-watch and filter paper/blotting paper.

Chemicals: Human blood, alcohol/spirit.

Principle: The average blood volume of adults is approximately 7 % of the body weight and is about 5 liters. This volume of blood is very important for normal functioning of circulatory system. If this volume decreases significantly due to loss of blood, called bleeding through accident/injury, it may affect the normal functioning of the body. The time required for cessation of bleeding from a deep cut/injury in the skin is one to five minutes and this is clinically very important. Spontaneous bleeding or excessive and prolonged bleeding may occur due to defects of platelets and blood vessel walls.

Procedure: Two methods are used to determine the bleeding time. These are **Duke's method** and **Ivy's method.** Duke's method is convenient and more commonly used in the laboratory and thus is described below:

1. Keep a piece of filter paper (about 4 inches long) or a Whatman filter paper ready in front.
2. Sterilize the tip of the ring finger with alcohol soaked in cotton. Dry it.
3. Prick the tip of the finger with a sterilized needle as shown in **Figure 1**, deep enough to ensure free flow of blood.
4. Immediately start the stop watch and note the time. The time of pricking of the finger is taken as the zero minute and counting of time begins from then only.
5. A drop of blood appears on the tip of the finger (**Figure 2**).
6. Dab/absorb this blood drop on top middle edge of the filter paper (slightly away from the edge).
7. Continue like this every 30 seconds, every time drying the blood drop on a fresh area on that filter paper in a row slightly away from the previous drop till the bleeding stops and no drop comes on the filter paper (**Figure 3**).
8. Stop the stopwatch at that moment and note the time taken for the bleeding to stop that is when there is no trace of blood spot on the filter paper. Encircle this spot and number all the spots from the beginning as 1 onwards.
9. Count the number of all the blood spots and express the results in minutes.

Observation: The blot of blood observed on the filter paper slowly decreases in size and finally no drop stains the filter paper. Each blot represents 30 seconds reading. Count each blot and multiply this by ½ to calculate the bleeding time in minutes as shown in **Figure 3.**

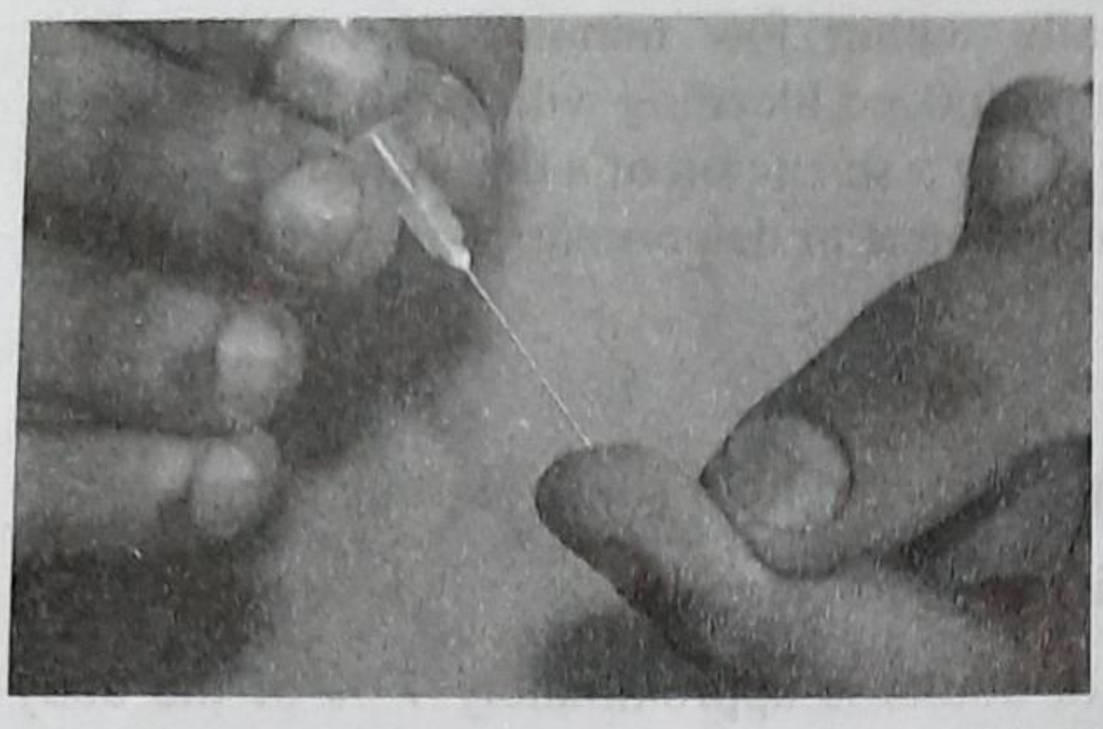

Figure 1: Pricking the tip of the ring finger with a sterilized needle.

Figure 2: Appearance of a blood drop at the pricking site on the finger tip.

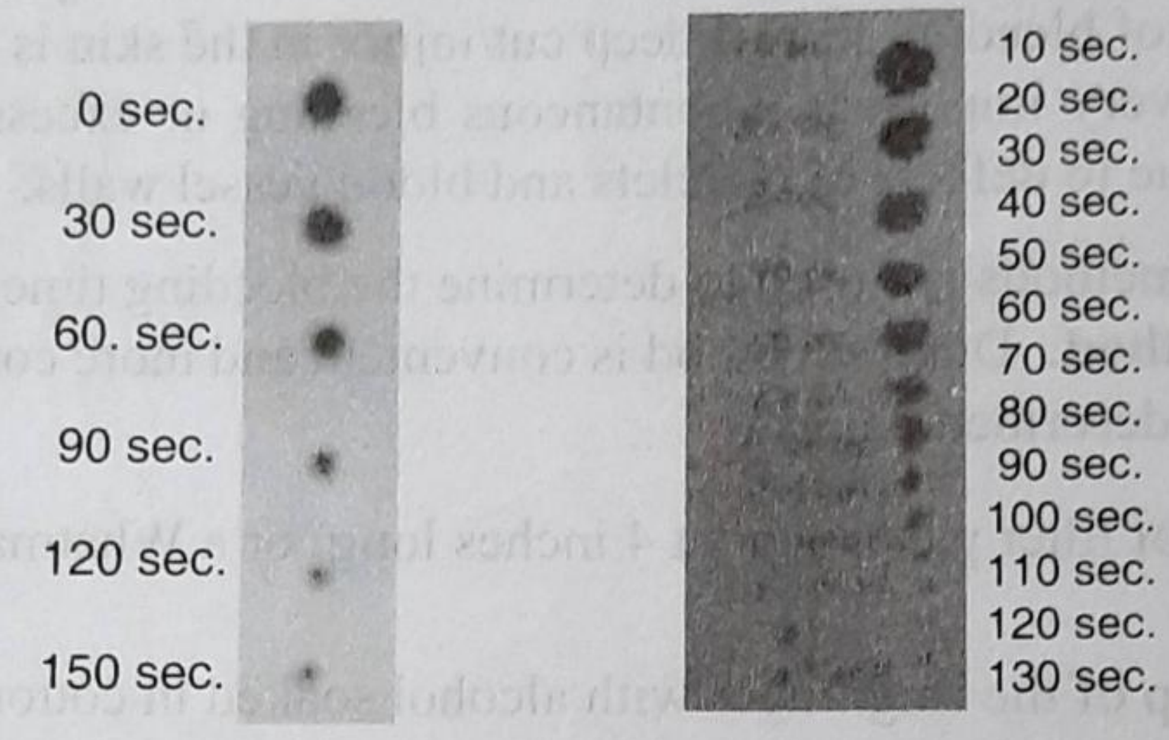

Figure 3: Filter paper showing recording of two observations of blood drops.

Results: The number of blots observed on the filter paper multiplied by half gives bleeding time in minutes. However, the bleeding time depends on the method used. Further, since the thickness of the skin of the fingertip varies from person to person, and is quite thick in some persons, a small cut in the skin of the earlobe with the corner edge of a sterilized blade gives better results.

Discussion: The time from the appearance of the drop of blood to the time when bleeding stops and the filter paper is no longer stained is taken as the bleeding time, and for a healthy person the normal bleeding time is 1 to 5 minutes when determined by the Duke's method. Gradual decrease in the size of the blood drop reaching to a stage when it does not stain the filter paper indicates the cessation of bleeding due to clotting of blood. Suspicion of a disease arises in cases of prolonged bleeding with minor cuts/injuries such as during cutting of nails, shaving or a fall etc. This test is required in such cases.

EXPERIMENT NO. 3

Aim: To determine the clotting time (CT) of human blood.

Learning Objectives: Blood clotting is a normal physiological process that occurs to cease bleeding to prevent loss of blood due to cut/injury etc. This exercise will enable the students to observe various steps involved in this phenomenon of fibrin formation.

Requirements:

Materials: Sterilized needle, cotton, capillary tube and stop watch.

Chemicals: Human blood, alcohol/spirit.

Principle: Bleeding takes place if the skin is cut, but after a few minutes blood coagulates to close the cut, thus preventing much loss of blood. Clotting of blood is a complicated process and several plasma factors and platelets are involved in this reaction. It is the time taken for blood to clot *in vitro* at a standard temperature, or it is the length of time from the moment blood is collected till the appearance of clotting. Normally it is 5 to 10 minutes.

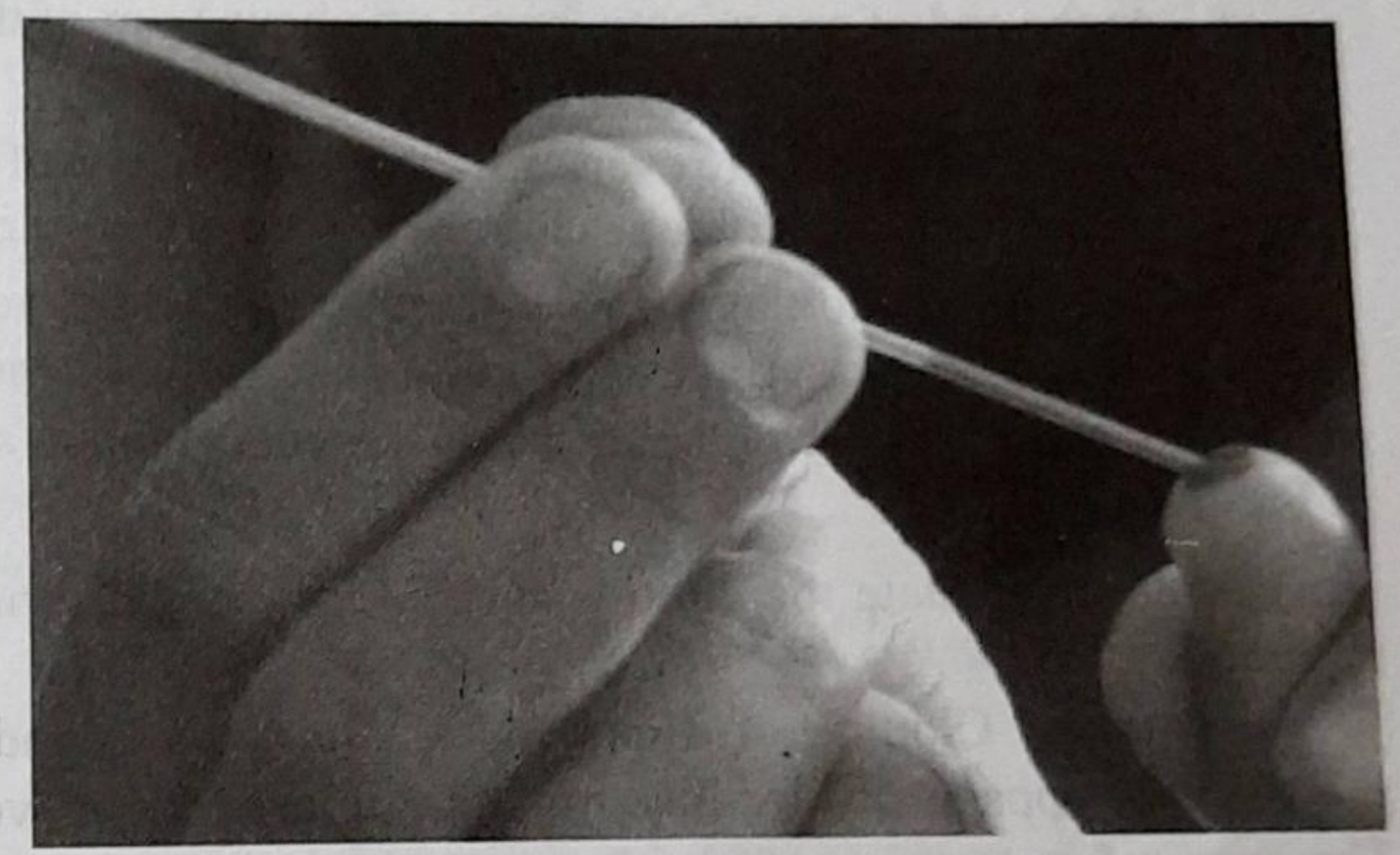

Figure 1: Filling of capillary tube with blood.

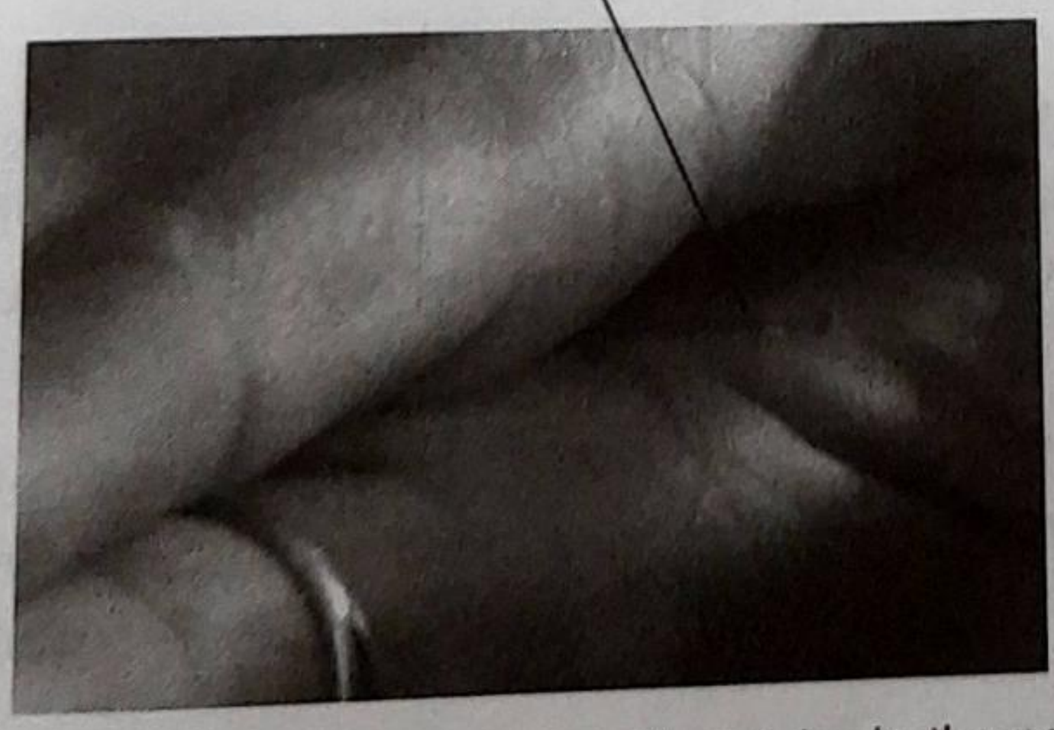

Figure 2: Holding the capillary tube In the palm of the hand.

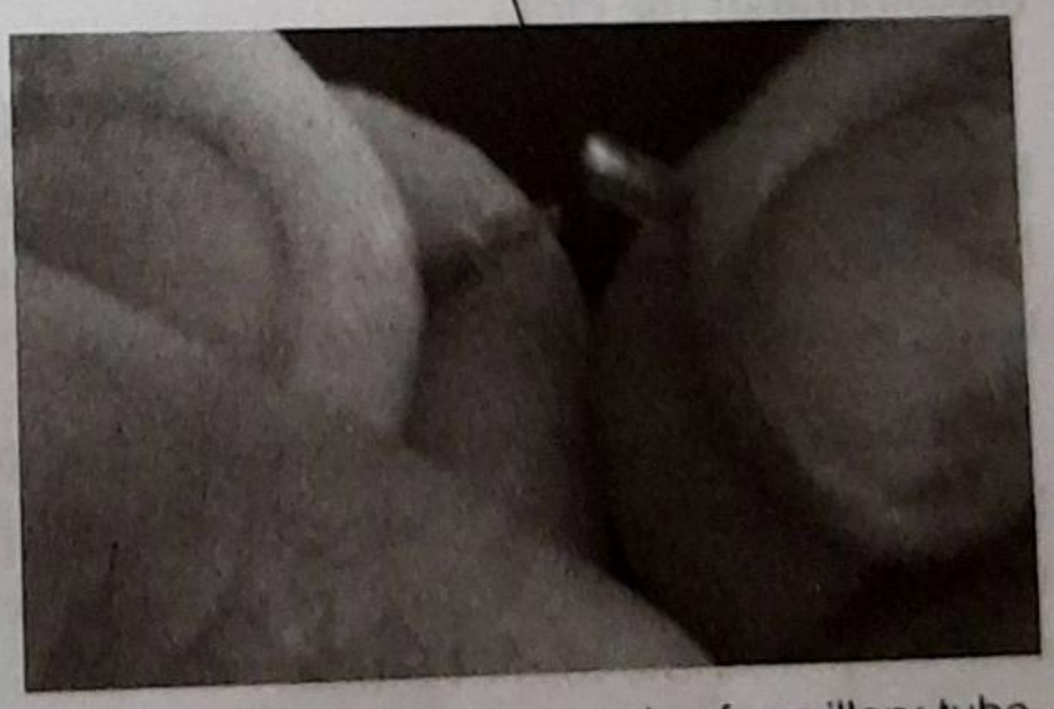

Figure 3: Two broken ends of capillary tube showing fibrin thread.

Procedure: Two methods are used for determining the clotting time of blood. These are **Capillary glass tube method** and **Lee and White method.** However, capillary glass tube

method is convenient, easy to perform and is more commonly used in the undergraduate laboratories. It is described below:

1. Sterilize the tip of the ring finger with alcohol soaked in cotton. Let it dry.
2. Prick this tip with a sterilized needle deep enough to ensure free flow of blood as shown in **Experiment No. 2.**
3. Quickly start the stop watch. The time of pricking of the finger is taken as the zero minute and counting of time begins immediately.
4. When a large drop of blood appears on the tip of the finger, introduce one end of the glass capillary tube into the blood drop (**Figure 1**). Blood flows rapidly into the capillary tube and fills it.
5. Hold the capillary tube filled with blood in the palm of the hand (**Figure 2**) to maintain it at the body temperature (37°C).
6. At the end of one minute, break off about 1 cm of the capillary tube from one end and observe the flow of blood from its tip.
7. Repeat the procedure every 30 seconds till thread of fibrin connects the two broken ends of the capillary tube (**Figure 3**). Note the time and stop the stopwatch.

Observations: Clotting time is the time interval between the entry of blood into the glass capillary tube or a syringe and formation of fibrin threads. Appearance of a fine thread of fibrin of about 5 mm length indicates blood coagulation and formation of fibrin as seen in **Figure 3**.

Results: Calculate the clotting time according to fibrin formation and express it in minutes.

Discussion: Clotting or **Hemostasis** (Haema = blood; stasis = halt) refers to the process of stoppage of bleeding from the injured blood vessels as shown in **Figure 4.** The term refers to tying of bleeding vessels during a surgical operation by the surgeon. Hemostasis is a homeostatic mechanism occurring to prevent loss of blood as a result of a complex natural physiological response involving four inter-related steps. These are vasoconstriction, platelet plug formation, formation of a blood clot and fibrinolysis i.e. dissolution of the clot.

Normal clotting time is 5-10 minutes. Many methods have been devised for determining blood clotting time accurately as it is an important physiological process for cessation of bleeding. Since it varies widely, measurements of the clotting factors themselves are done using sophisticated chemical procedures.

Clinical Significance: Since the clotting time is the time taken from the puncture of the blood vessel to the formation of a fibrin thread, it is of great clinical significance in understanding the physiology of a patient who has to undergo a minor or major surgery so as to minimize the loss of blood.

EXPERIMENT NO. 6

Aim: To determine the blood groups and Rh factor.

Learning Objectives: With the help of this experiment, the student will learn various types of blood groups and the importance of matching the blood groups of the recipient and donor in blood transfusion cases.

Requirements:

Materials: Sterilized pricking needle, glass slides, dropper, cotton, compound microscope and needles/applicator sticks (or tooth picks).

Chemicals: Human blood, antiserums –Anti A, Anti B and Rh or antisera D and alcohol or spirit. Preparation of antiserum is not required as these come in the form of small kit which can be purchased as seen in **Figure 1** and are ready for use.

Principle: In 1900 an Austrian scientist, Karl Landsteiner first reported the presence of two blood antigens, A and B on the surface of human blood cell. Based on this, he divided human red blood cells into three groups. These were A, B and O. These form the basis of blood groups. Now, more than thirty blood group specific antigens can be recognized on the membrane of human RBCs. These antigens enable the blood group of individuals different from each other. The chief blood groups are the following in which the first two, ABO and Rh are the main systems of blood groups.

1. The classical ABO blood groups,
2. Rh blood groups, and
3. M and N blood groups.

The RBCs contain group specific substances known as agglutinogens and the plasma/serum contains agglutinins. These are A and B. The human beings are divided into four groups depending on the basis of presence or absence of these as blood group A, B, AB and O. On the other hand Rh system is of two types. The persons who possess Rh agglutinogens on their RBCs are called Rh +ve persons while those who do not have Rh agglutinogens are called Rh –ve persons. The Rh blood group system was discovered by Landsteiner and Weiner in 1940. Since Rh factor was first reported in RBCs of rhesus monkey, it is designated like that. Approximately 85 % human population is Rh +ve and 15 % is Rh –ve.

In blood transfusion cases, blood group of recipient and donor must be matched otherwise agglutination or clumping of RBCs occurs. The phenomenon of agglutination is due to the interaction between the factors agglutinogen present on the RBCs membrane and agglutinin present in the plasma.

Procedure: This procedure is called the **"Rapid Slide Test".**

1. Take 3 clean grease free glass slides.
2. Lay them on the filter paper after labeling as A, B and D with the help of glass marker.
3. Sterilize the finger tip by wiping it with alcohol soaked in cotton.

4. Prick the finger tip with sterilized needle.
5. Take 3 drops of blood separately on these three slides as shown in the **Figure 2.**
6. Quickly, take 1 drop each of anti A serum, anti B serum and anti Rh with the help of separate droppers and put anti A serum on the blood drop of slide marked as A, anti B serum on the blood drop of slide marked as B and anti Rh serum on the blood drop of slide marked as D respectively (**Figure 2**).
7. Immediately mix drop of blood and antiserum with the help of separate applicator sticks.
8. Observe/look for agglutination or clumping of blood cells after 2-3 minutes. Observe the slides also under the microscope. If there is no agglutination, then RBCs remain separated and evenly distributed but if agglutination occurs, then RBCs are massed together in clumps and lose their outline as shown in **Figure 3.**

Observations: Agglutination of RBC by a reagent indicates +ve test while absence of agglutination indicates –ve test (**Figure 3**). Observations of the tests performed on the slide are given in **Table 1.**

Figure 1: Bottles of antisera A, B & D.

Results: No agglutination with either of the antiserum, that is A or B indicates that the blood sample has blood group 'O'. Blood group O has no antigen on the blood cells but has antibodies to both A and B in their plasma and thus shows no agglutination. Further, blood is Rh+ if it shows agglutination with Rh antiserum or antisera D.

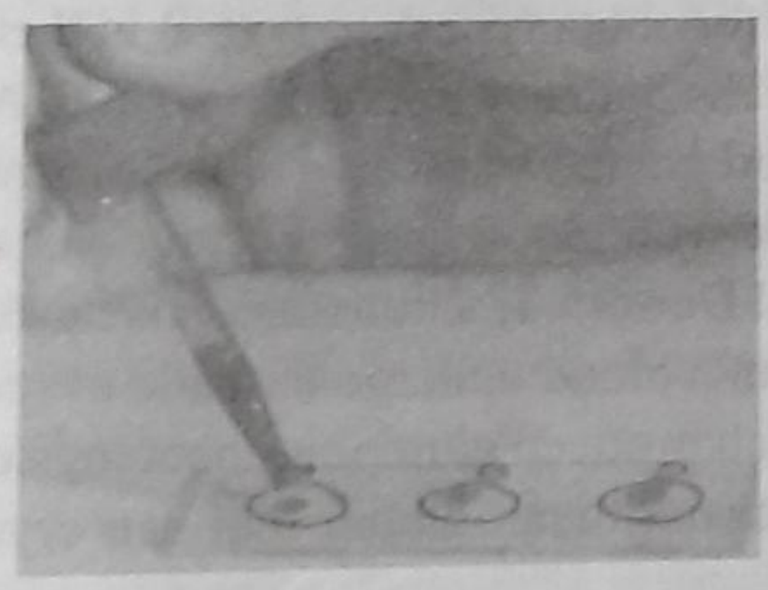

Figure 2: Glass slide with three drops of blood. To circle marked "A" antiserum A is added.

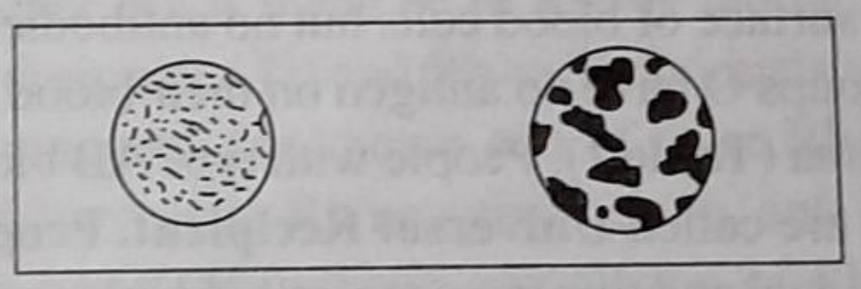

Figure 3: The left circle shows that RBCs are well separated and there is no agglutination. However the right pattern shows that RBCs are massed together and there is agglutination.

Discussion: ABO blood groups are determined by a gene I (Isoglutinin). There are 4 alleles of this gene viz. I^A, I^B, I^O. The proteins produced by A and B alleles are antigens. ABO blood group antigens are glycoprotein and glycolipids of unknown function. These are basically oligosaccharide chains that project above the RBC surface. These chains are attached to the proteins and lipids that lie in the RBC plasma membrane. The ABO locus has three main allelic forms- A, B, and O **(Table 1).** The A allele encodes a glycosyltransferase that produces the A antigen (N-acetyl galactosamine in its immunodominant sugar), and the B allele encodes a glycosyl transferase that creates the B antigen (D galactose is its immune dominant sugar). The O allele encodes an enzyme with no function and therefore, neither A nor B antigen is produced, leaving the underlying precursor (the H antigen) unchanged.

The immune system forms antibodies against whichever ABO blood group, antigens are not found on the individual RBCs. Thus, a group A individual will have anti B antibodies and a group B individual will have anti A antibodies. Blood group O is common and individual with this blood type will have both anti A and a ıti B in their serum. Blood group AB is the least common, and these individuals will have neither anti A nor anti B in their serum **(Table 1).** ABO antibodies (usually IgM and IgG type) in the serum are formed naturally. Their production is stimulated when the immune system encounters the "missing" ABO blood group antigens in foods or in micro-organisms. This happens at an early age because sugars that are identical to or very similar to the ABO blood group antigens are found throughout nature.

ABO blood types are also found in some animals such as apes-chimpanzees and gorillas.

Table 1: Characteristics of ABO Blood Groups with Genotypes and Phenotypes for ABO Locus in Humans.

Blood Group	Antigens on RBCs	Antibodies in Serum	Red cell type agglutinated	Transfusions accepted from	Pheno-types	Genotypes
A	A	Anti B	B, AB	A or O	A	I^{AA} or I^{AO}
B	B	Anti A	A, AB	B or O	B	I^{BB} or I^{BO}
AB	A and B	Neither	Neither	A, B, AB or O	AB	$I^{A}I^{B}$
O	Neither	Anti A & B	A, B & AB	O	O	$I^{O}I^{O}$

Thus, individual with blood group type A have Antigen 'A' on the surface of RBC and antibodies to type 'B' blood in plasma. Blood group B have Antigen 'B' on blood cells and antibodies to type 'A' blood in their plasma. Blood group AB has Antigens for both A and B on the surface of blood cells but no antibodies for either type A or type B in their plasma. Blood groups O have no antigen on their blood cells but have antibodies to both A and B in their plasma (**Table 1**). People with type AB blood group can receive blood of any type and therefore are called **Universal Recipient.** People with blood group O can donate blood to any individual and therefore are called **Universal Donors.** If a transfusion is made between an incompatible donor and recipient, the recipient's blood undergo cascade of reactions of antigens on its cell and antibodies in plasma which produce clumping that clogs capillaries **(Table 2 and Figure 4)**). Other cells burst releasing hemoglobin that can crystallize in kidneys and cause kidney failure.

Table 2: Reactions of matching different blood groups of Donors and Recipients indicating that People with AB Blood Group are Universal Recipient while with O Blood Group are Universal Donors.

Antibodies in Receiver's Plasma ↓	Antigens on donor's red blood cells →			
	Group A	Group B	Group AB	Group O
Group A	**Will mix**	Agglutination	Agglutination	**Will mix**
Group B	Agglutination	**Will mix**	Agglutination	**Will mix**
Group AB	**Will mix**	**Will mix**	**Will mix**	**Will mix**
Group O	Agglutination	Agglutination	Agglutination	**Will mix**