Practical Immunology

3rd Level

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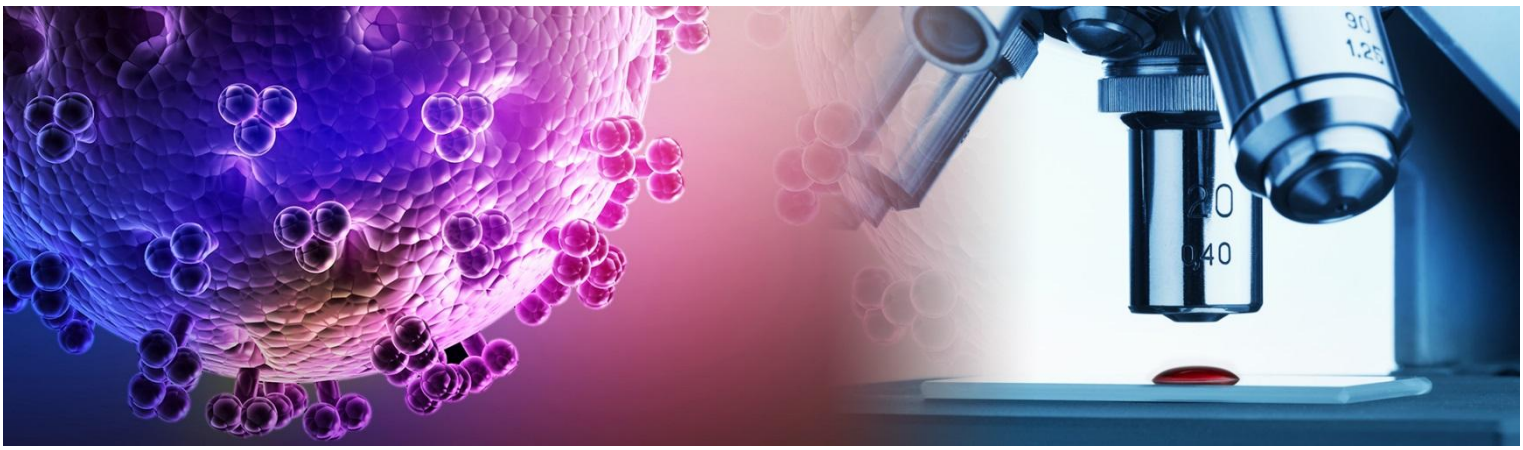


By: Assis. Lec. Ammar B. Al-Asadi

Introduction to immunity

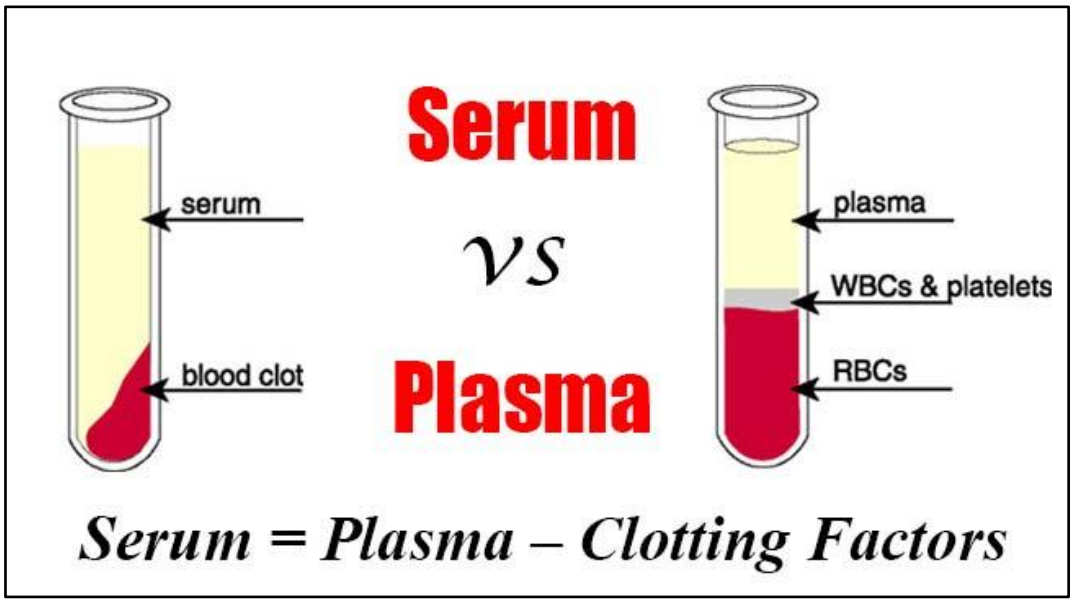
Immunity: body defense against foreign agent. The immune response is divided into cellular and humoral immunity, cellular immunity related to the activity of cells of the immune response, humoral immunity is related to action of some proteins, cytokines and immunoglobulin.

Lab. Samples: Blood (Serum – Plasma – RBCs), Urine and cerebrospinal fluid.

Blood: body fluid in humans and other animals that delivers necessary substances such as nutrients and oxygen to the cells and transports metabolic waste products away from those same cells. It is composed of blood cells suspended in blood plasma.

Plasma: It is the yellowish liquid part of the blood that carries cells and proteins (albumin, globulin and fibrinogen) throughout the body. It makes up about 55% of the body's total blood volume.

Serum: is the fluid and solute component of blood after clotting. it is the blood plasma not including the fibrinogens. Serum includes all proteins not used in blood clotting and all the electrolytes, antibodies, antigens, hormones, and any exogenous substances.



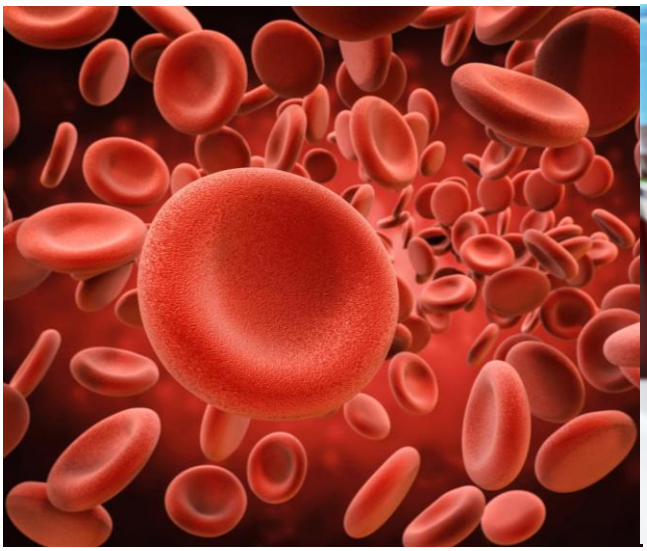
Plasma collection put the blood in tube with anticoagulant substance (EDTA, Sodium citrate)

Serum collection put the blood in tube without anticoagulant substance (Plain tube, Gel tube)

Preparation of erythrocyte:

1- Collect the blood by vein puncture method.
2- Add the blood into a container with an anticoagulant and then mixed.
3- Add an isotonic solution such as saline to the mixture.
4- Wash the RBCs suspension by centrifugation for 5 min. at 3000 rpm.
5- Discard the supernatant and again add saline to the precipitate, mix well and repeat the steps 4 and 5.
6- Re-suspend the precipitate again with saline and standardize it.

   Note: we can preserve the blood in saline for short time, and in Alsever 's solution for long time.



Saline:

It is normal or isotonic aqueous solutions for sodium chloride in 0.85% (8.5 gram of NaCl in 1 liter of D.W.). keep the cell as they are.

Alsever 's solution:

1- 2.05 g Dextrose (preservative)
2- 0.8 Sodium citrate (as an anticoagulant)
3- 0.42 g Sodium chloride (for controlling the osmotic pressure)
4- 0.054 Citric acid (pH control)

Animal marking

A) Temporary:

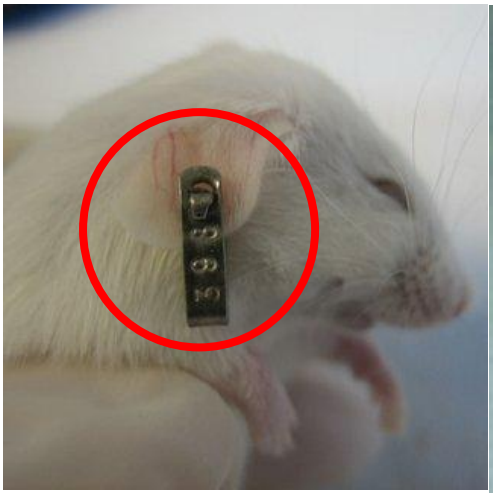
1- Cage marking
2- Dyes
3- Hair clip
4- Tagging

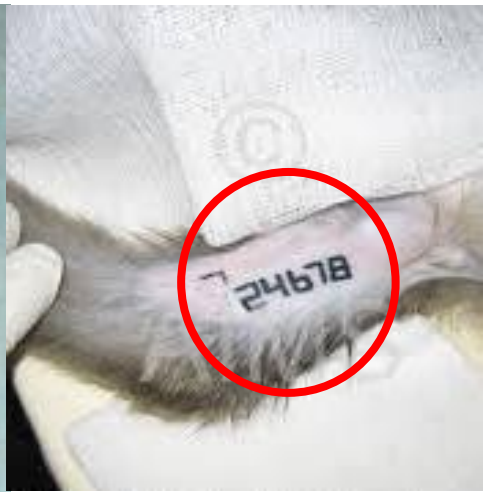
B) Permanent:

1- Natural marking
2- Branding
3- Ear punching
4- Tattooing

The marking method ultimately chosen **should be simple, easy to apply, easy to read, harmless to animal, and offer no potential interference with experimentation.** When selecting marking methods, careful consideration should be given to the following factors prior to making final judgment:

1- **Species of experimental animal used.** (marking methods differ significantly and often limit usage to certain animals).
2- **Number of animals in room** a group, or in a particular experiment (consideration for the limitations of a numbering system is important).
3- **Size and age of the animal.**
4- **Duration of the experiment.** (it is often very critical whether the experiment done in a short or long period, and if long, months or years?
5- **Nature of experiment.**

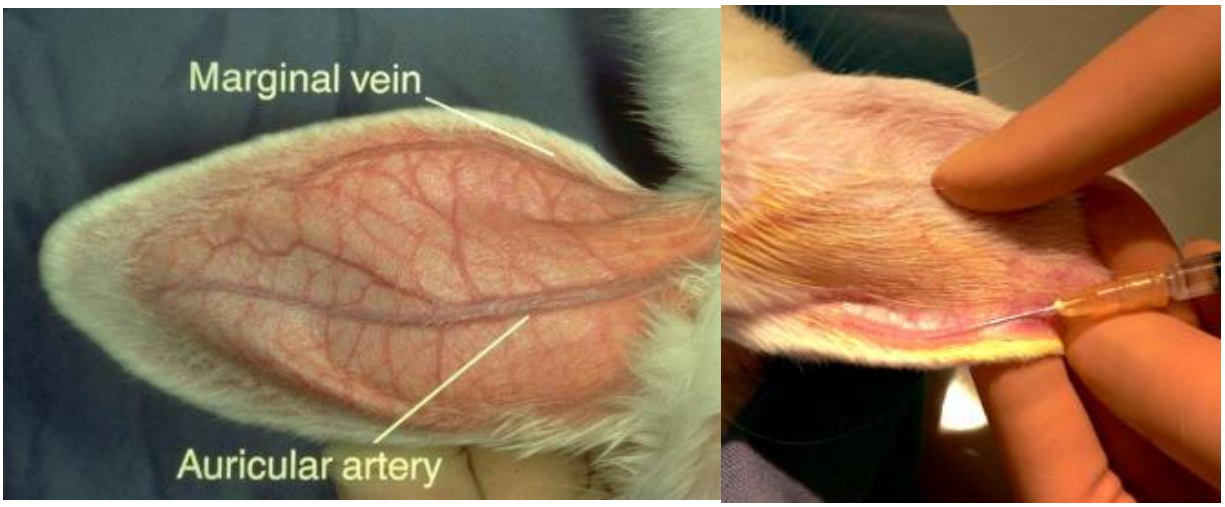




Route of injection

1- **Intravenous:** This procedure involves direct injection into blood stream of the animal. The ear vein is the most accessible and visible vein in the rabbit. Take the rabbit then:

- See the vein on the dorsal edge of either ear.
- Shave the hair away from the area.
- Clean the area with disinfectant (70% ethanol).
- For the ease injection, the diameter of the vein may be enlarged before injection by heating and by applying pressure over the vein at the base of ear.
- Hold the ear with one hand while supporting the ear from below with finger of the same hand.
- With vein clearly visible, insert the needle in the direction of the blood flow (toward the head of the rabbit). Slowly inject the inoculums (Ag) while watching for signs for dilution of the blood in the vein indicating that the injected material is passing into the vein. The blunder of the syringe should move down easily and no raised area in the surrounding tissue should be visible.
- When injection is complete with draw the needle and made pressure by finger to the injection site to stop bleeding and disinfectant with ethanol 70% the area by place a piece of cotton on site of injection.



2- **Intraperitoneal (Mouse):** The injection of material into the peritoneal of abdominal cavity of the animal. The animal (Rabbit or mouse) should be place on its back.

- Anesthesia may be used for this injection.

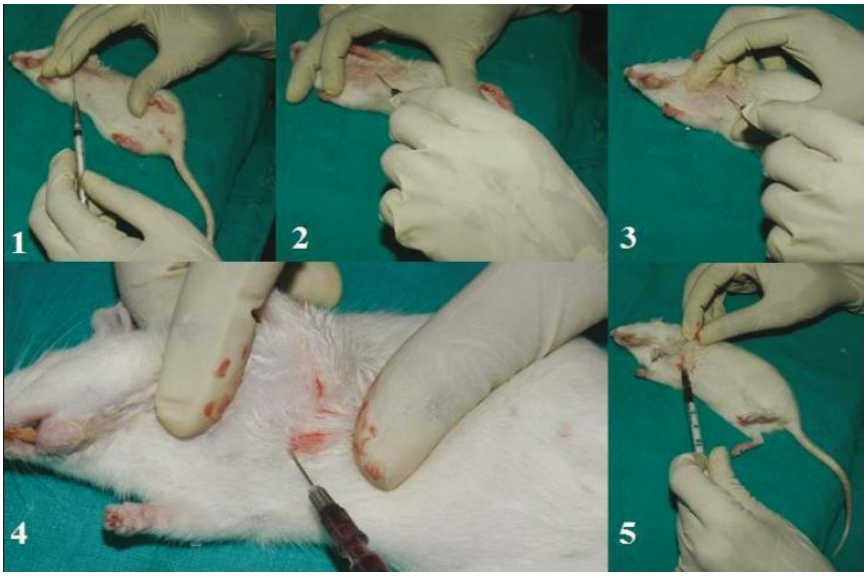
- Clip the hair away from the injection site and disinfect the site with 70% ethanol.
- Grasp the skin and peritoneum with finger.
- Insert the needle through the skin and peritoneum. The needle should be empty from the bubbles.
- Inject inoculums (Ag) carefully so as to prevent injection into the animal organs with practice, the procedure is relatively easy.
- Remove the needle with disinfect the area once again with alcohol.


3- **<u>Subcutaneous:</u>** This method involves the injection of material under the skin. Choose a site (usually the back).

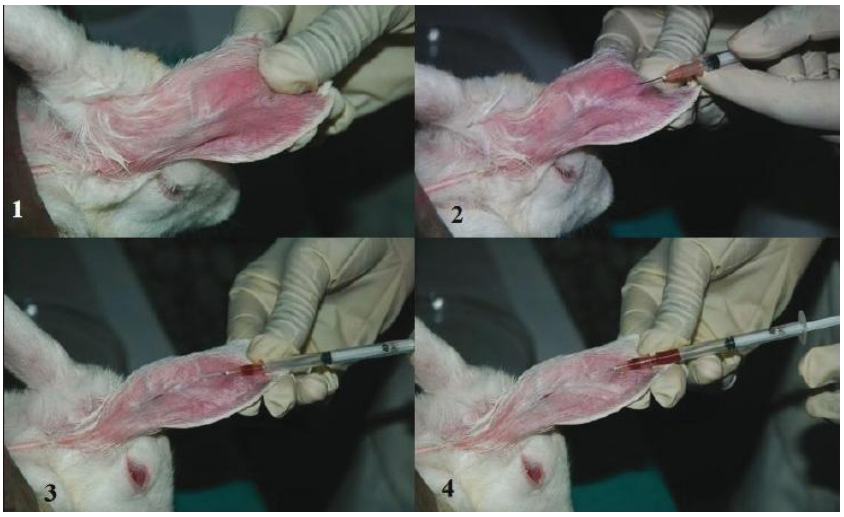
- Cline and shave the hair and disinfect the injection site with 70% ethanol.
- Load tuberculin syringe and insert the needle almost horizontal to the animal to a depth.
- Slowly inject the inoculums and observed the formation of a slightly raised area under the skin.
- Remove needle and disinfect the injection site once again with alcohol.


Blood sampling techniques

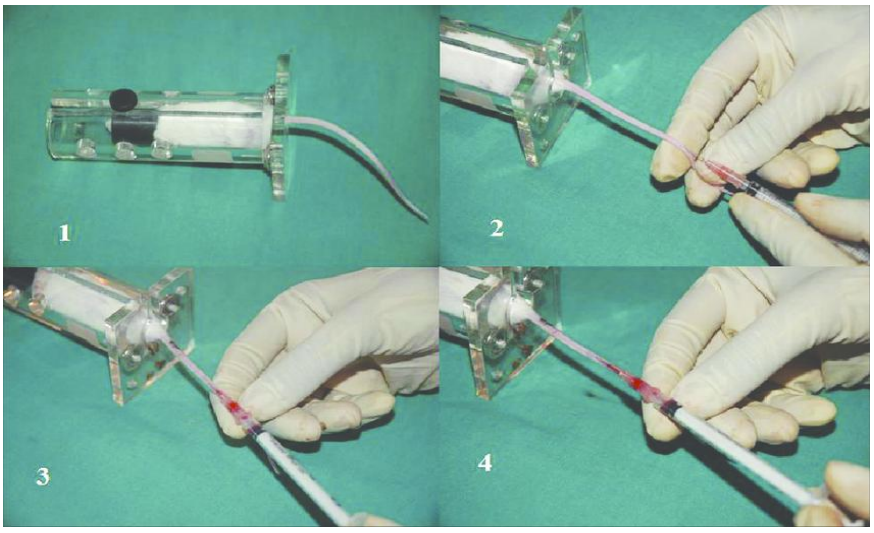
1- Cardiac puncture method



2- Auricular artery of rabbit



3- Blood collection from tai vein of mouse



Phagocytosis in vivo

Aim: study of phagocytosis in mice by intraperitoneal injection (I.P.).

Grater momentum: large fold of peritoneum has milky spots of macrophage collection.

Phagocytosis: process of cell or particulate matter engulfment by phagocytes e.g. monocyte, neutrophil (circulating) and macrophages (resident).

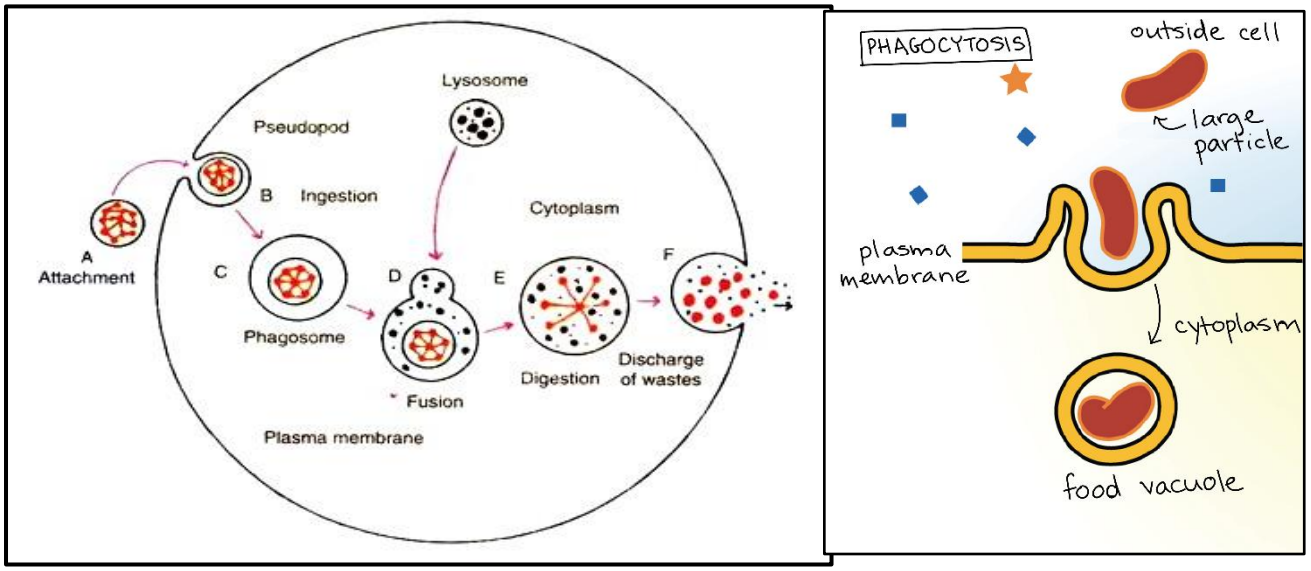
Phagocytic steps

1- **Chemotaxis:** Chemical attraction of phagocytes to microorganisms. The chemotactic chemicals which attract the phagocytes are the components of WBC, and damaged cells, peptides derived from complements and microbial products.

2- **Opsonization and Attachment:** Opsonin is any molecule that acts as a binding enhancer for the process of phagocytosis, for example, antibody and products of complement activation (C3b). Attachment: The plasma membrane of phagocyte gets attached to the surface of a microbe or foreign material.

3- **Ingestion:** After attachment the plasma membrane of phagocyte extends short projections known as pseudopods which engulf the microorganisms or foreign materials.

4- **Intracellular killing and Digestion:** phagosome fuses with lysosome to form phagolysosome then the process of digestion by hydrolytic enzyme.



Method

- Inject the mouse by 0.1 ml of *S. aureus* intraperitoneal.
- After 18-24 hrs. inject the mouse by 0.5 ml of saline.
- Make a slide of intraperitoneal fluid, liver, spleen and heart.
- Dry the slide, stain by wright stain for 5 min.
- Wash the slide by water.
- Examine 5-10 fields and count.

$$\text{P.I.} = \frac{\text{B}}{\text{A}} \leq \mathbf{1} \quad \boldsymbol{always}$$

A = No. of phagocytic cell.

B = No. of phagocytic cell that contain bacteria.

Peritoneal	Spleen	Liver	Heart
A	A	A	A
B	B	B	B
P.I.	P.I.	P.I.	P.I.

Compare P.I. for each organ then discuss the results.

Phagocytic index depends on:

- Route of injection.
- No. of injected bacterial cells.
- No. of phagocytic cells in the organ.
- Ability of phagocytic cells to engulf bacterial cells.
- Ability of bacterial cells to reach all organs.

Bactericidal effect of serum

Aim: Test the bactericidal effect of normal and heated serum.

The protein in the serum called globulins (albumin, alpha-1 globulin (α-1), alpha-2 globulin (α-2), beta globulin (β) and gamma globulin (γ).

The immune system consists of two parts:

1- Cellular immune response (Reticuloendothelial system)
2- Humoral immune response (e.g. Antibody, properdin, Beta-lysin, lysozymes, complement)

Antibody (Ab) = Immunoglobulin (Ig):

Gamma globulin protein, Y- shaped protein produced by B-cells, use by immune system to identify and neutralize foreign objects like bacteria and viruses.

Properdin:

Gamma globulin protein, activates complement, effects on G-ve and G+ve bacteria

Beta-lysin (β-lysin):

Amino acid produced by platelets during blood coagulation (clotting), cause lysis of G+ve bacteria

Lysozymes:

Enzyme that damage bacterial cell wall (especially G+ve), abundant in secretion such as tears, saliva, human milk and mucus.

Complement:

β-globulin protein (10% of globulins), present in blood and tissue fluids of human and animals (guinea pig is a rich source of complement), consist of 9 types labeled as (C1, C2, C3, C4, C5, C6, C7, C8, C9), thermo-labile (serum heating in water bath to 56 ^{o}C for 30 min. leads to complement inactivation, cause G-ve destruction.

Function of Complement

1- **Cell lysis:** rupturing membranes of foreign cells like bacteria, viruses, and RBCs by MAC (membrane attack complex)
2- **Opsonization:** enhancing phagocytosis of antigen (e.g. C3b).
3- **Chemotaxis:** attracting macrophages and neutrophils to the site of infection (e.g. C5a)
4- **Clearance of immune complexes:** Ag-Ab complexes are removed to the liver and spleen.

Method

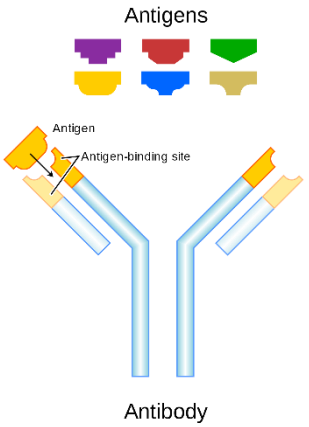
1- Studied bacterial isolates cultured (*E. coli* & *S. aureus*) in nutrient broth (N.B.) incubated at 37 ^{o}C for 18 hr. and then adjusted to 10^4 CFU/ml with N.B. medium as initial inoculum size.
2- Pooled normal human serum (PNHS).
3- Test tube (A): 0.4 ml PNHS + 0.1 ml bacterial inoculum.
4- Test tube (B): 0.4 ml PNHS (heated at 56 ^{o}C for 30 min.) + 0.1 ml bacterial inoculum.
5- Control tube: 0.4 ml saline + 0.1 ml bacterial inoculum.
6- All tubes are incubated at 37 ^{o}C for 1 hr.
7- Standard plate counting was performed for each tube (A, B and control) over Muller-Hinton agar plates using sterile cotton swab.
8- The percentage of bactericidal activity was calculated as following:

$$\% \text{ Bactericidal activity of normal serum} = 100 - 100 \left[\frac{\text{No CFU/ml of normal serum}}{\text{No CFU/ml of control}} \right]$$

$$\% \text{ Bactericidal activity of heated serum} = 100 - 100 \left[\frac{\text{No CFU/ml of heated serum}}{\text{No CFU/ml of control}} \right]$$

Antigen preparation

Antigen (Ag): is macromolecule that induced to formation of antibody (Ab), which react specifically with the Ag.



Main characteristics of the antigen:

1- High molecular weight ≥ 10000 Dalton.
2- Foreignness
3- Chemical complexity

Vaccine: any substance when introduce artificially to human or animal body produces an immune resulting Ab.

Salmonella antigen

Somatic (O) antigen	Flagellar (H) antigen
1- Found on the surface of the cell	1- Flagella
2- Lipid complex (it is polysaccharide)	2- Protein
3- Stable at 100 ^{o}C and in alcohol	3- Inactivated at more than 60 ^{o}C

The properties of formal-saline (formaldehyde + saline):

1- Kill microorganisms.
2- Fix the flagella on the cell wall.
3- Keep it for a long time without deterioration.
4- Mask the body bacterial cell.

Method

Somatic (O) antigen preparation:

1- Streaking a few typical *Salmonella* colonies from the Salmonella-Shigella agar (S.S agar) plate onto brain heart infusion agar plates using a sterile cotton swab.

2- Plates were incubated overnight at 37 ^{o}C.

3- Sterile normal saline was used to harvest the lawns and a bottle containing 250 ml of bacterial suspension was immersed in a boiling water bath for 3.5 hr.

4- It was left to stand at room temperature overnight and again immersed in boiling water for an additional 1.5 hr. on the next day.

5- The now killed bacteria were washed 3 times with normal saline.

6- The resulting product had the appearance of skim milk.

7- It was judged sterile by its failure to cause turbidity in brain heart infusion broth tubes after prolonged incubation at 37 ^{o}C, and its failure to produce growth on S.S agar plates.

8- Storage was in 0.3% formal saline at 4 ^{o}C.

Flagellar (H) antigen preparation:

1- Inoculated a few typical *Salmonella* colonies from the (S.S agar) plate onto a 250 ml brain heart infusion broth.

2- Broth was incubated overnight at 37 ^{o}C.

3- Then, 250 ml of 0.6% formal saline was added and the mixture was allowed to stand at room temperature for 5 d. sterility test were performed.

4- Washing killed bacteria and discard the supernatant.

5- Re-suspend the precipitate with 0.3% formal saline and storage at 4 ^{o}C.

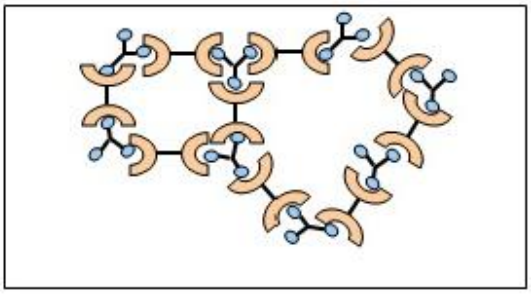
Precipitation reaction

One of the easiest of serological tests, relies on fact that antigen and antibody mixed in the proper proportion from large macromolecular complexes called **precipitates**. Correct proportions are important to create precipitation, two techniques determine optimal antibody and antigen concentrations:

- Immunodiffusion
- Immunelectrophoresis

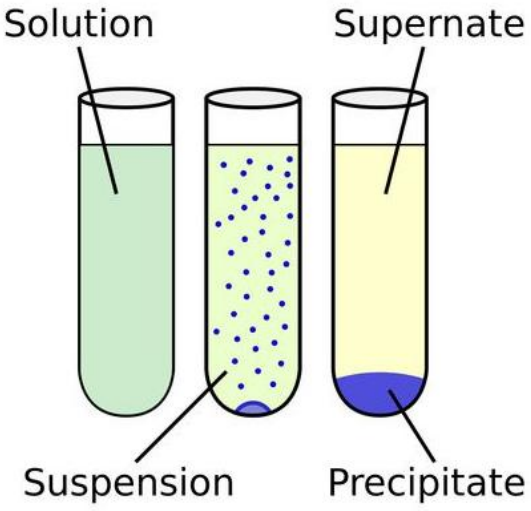
- Occur best when antigen and antibody are present in optimal proportions (Equivalence).

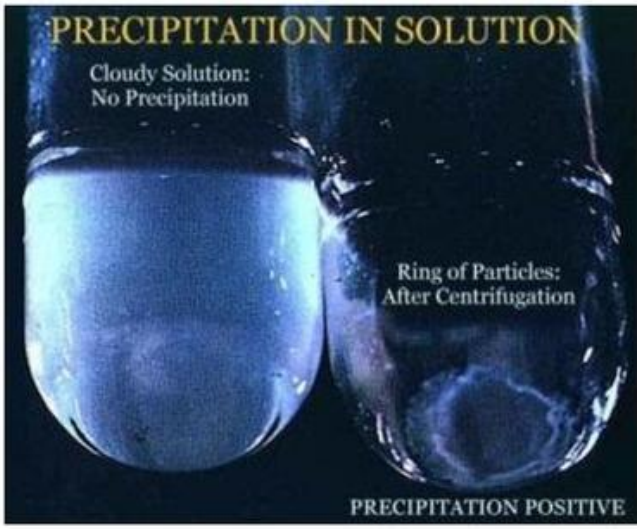
- Antibody that aggregate soluble antigens are called **Precipitins**.



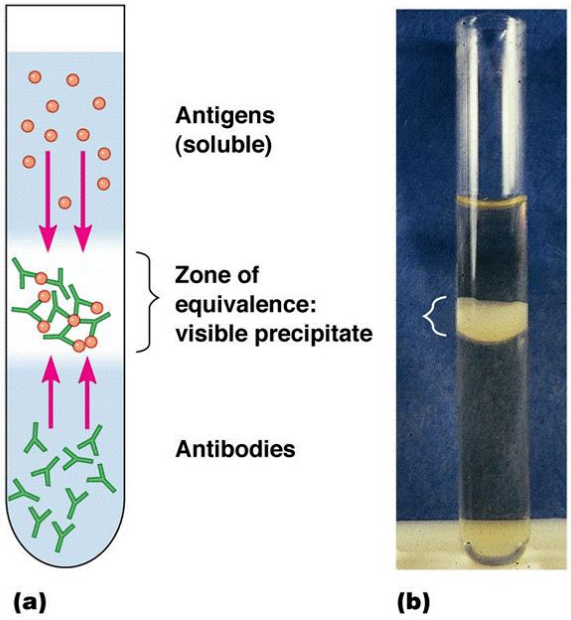
1) Precipitation in solution

A) Bottom precipitate: occur when soluble Ag interact with soluble Ab and form a visible precipitate that give bottom precipitate after centrifugation.

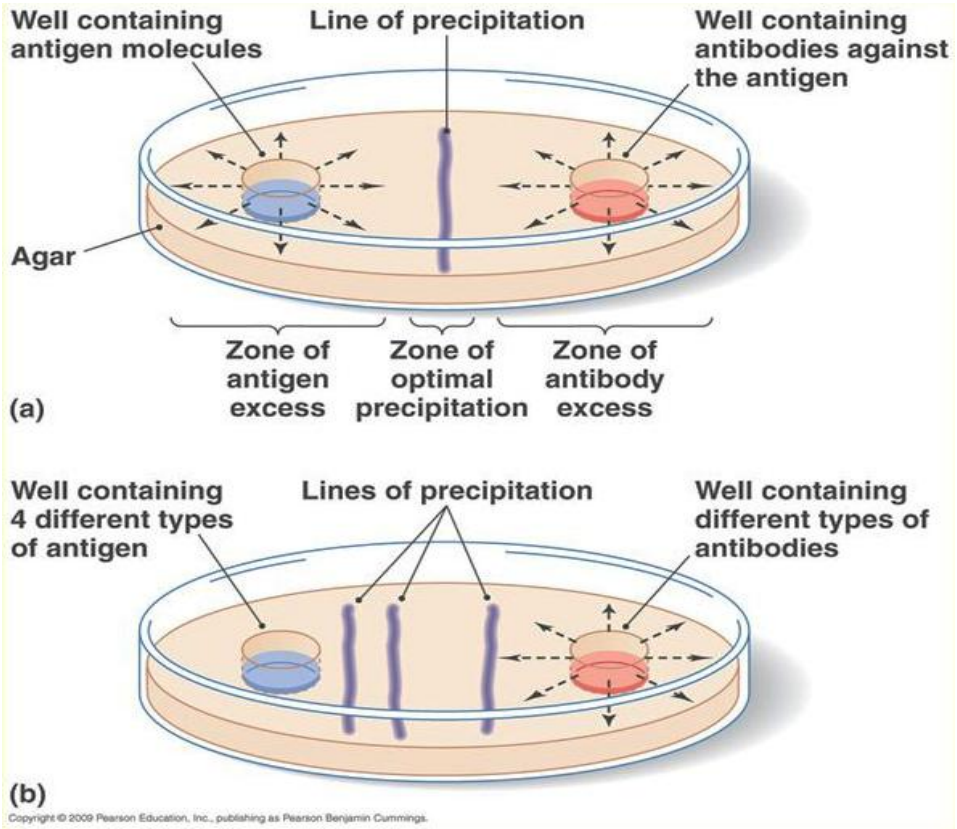




B) Ring precipitate (Ring test) (Tube Precipitation test / Oudin Precipitation): involve soluble antigens with antibodies in tubes (test or capillary tubes), layer Ag over Ab, precipitate occurs at the interface of the two reagents, forming a ring, simplest and qualitative test, used for express-diagnosis of infection disease (ex: Anthrax).

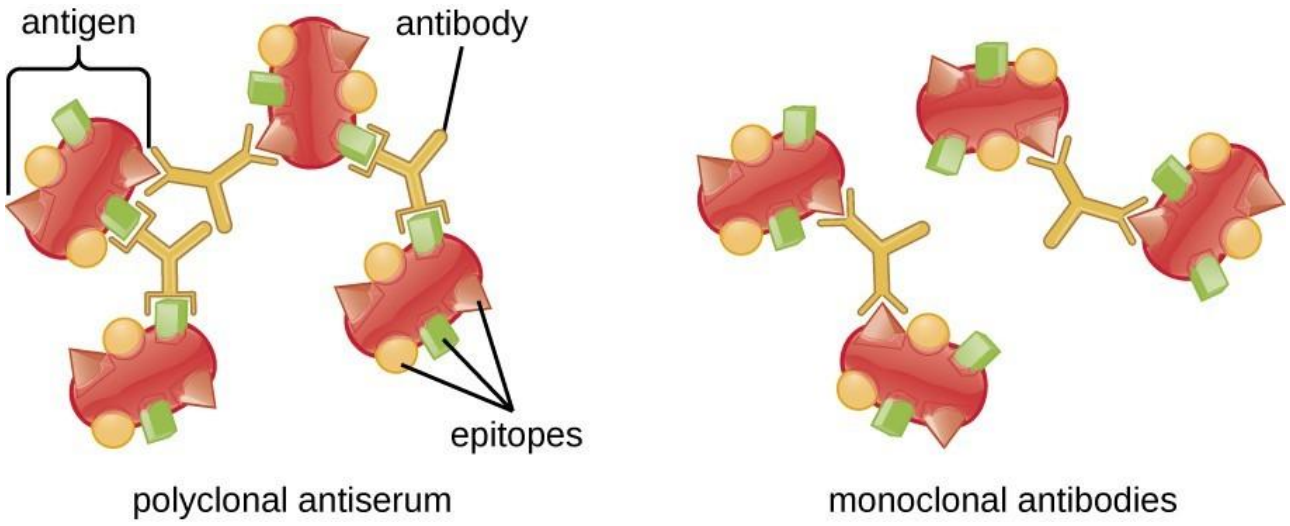


2) Precipitation in gel



Antigen-Antibody precipitation reactions:

Polyclonal antibodies can form lattices, or large aggregates, monoclonal antibody can link only two molecules of antigen and no precipitate is formed.



<u>Ag / Ab precipitation reaction</u>

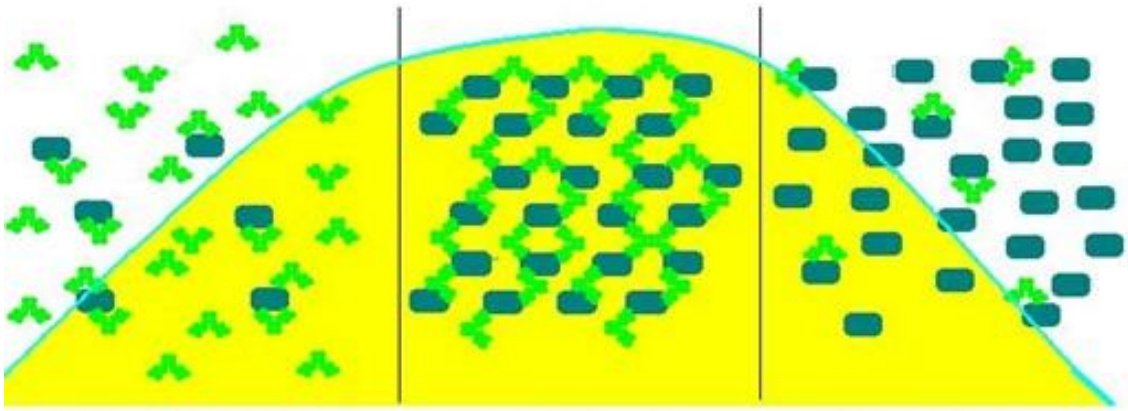
- All antigen-antibody bindings are reversible according to low or mas action

- free reactants are in equilibrium with bound reactants ⟶ Equivalent zone

- Excess antibody ⟶ Prozone

- Excess antigen ⟶ Postzone

- These zones are demonstrated in the **precipitation curve**



Simple Immunodiffusion (ID)

Immunodiffusion procedures are carried out in an agar gel medium, the precipitate is easily seen in gels visible precipitin lines but no visible precipitate forms in regions of Ab or Ag excess.

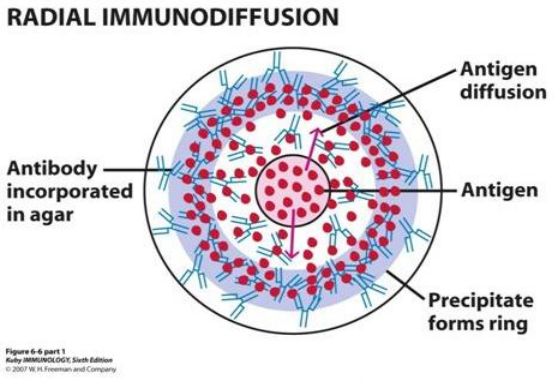
A) Single Radial Immunodiffusion (Mancini Reaction)

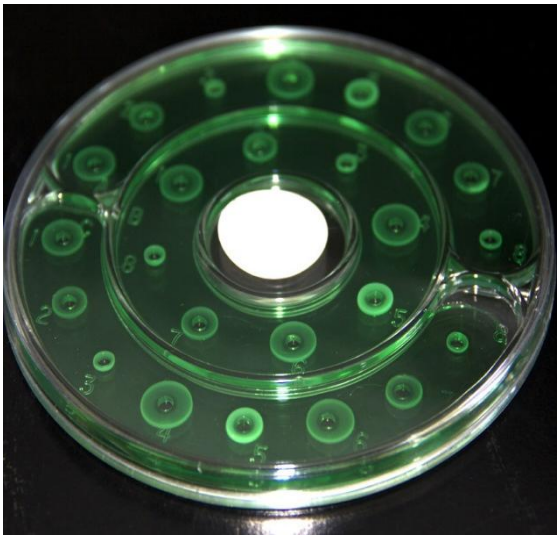
- Ab is put into a gel and Ag is put in a well cut into the gel and a precipitin ring formed when Ag diffuses out in all directions.
- The technique is quantitative
- Is based upon the reaction between an Ag and specific Ab during a diffusion period
- The Ag-Ab interaction is manifested by a well-defined ring of precipitation around the Ag well.

Interpretation: Diameter of ring is proportional to the antigen concentration.

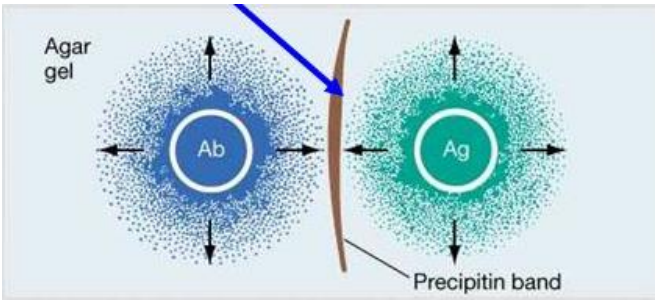
B) Double Immunodiffusion (Ouchteriony Reaction)

- Both antibody and antigen diffuse from wells into a gel medium.
- As the materials diffuse toward one another, precipitate line form the Ag-Ab interactions (Qualitative).

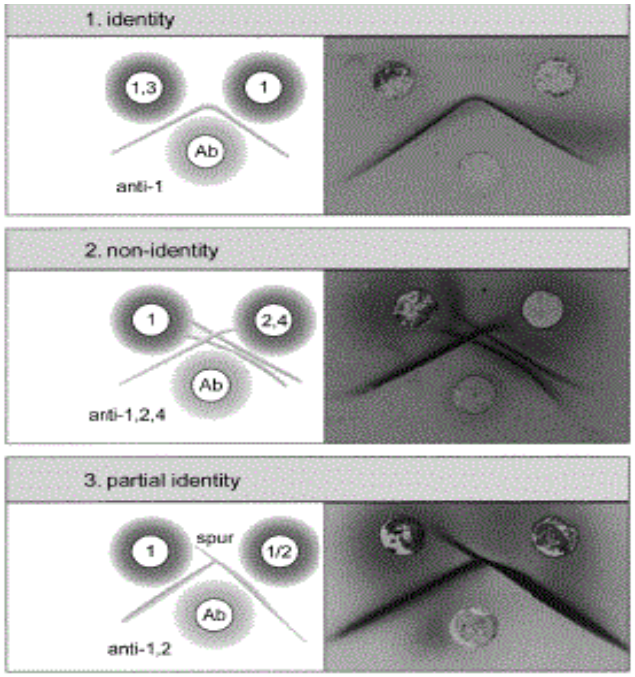


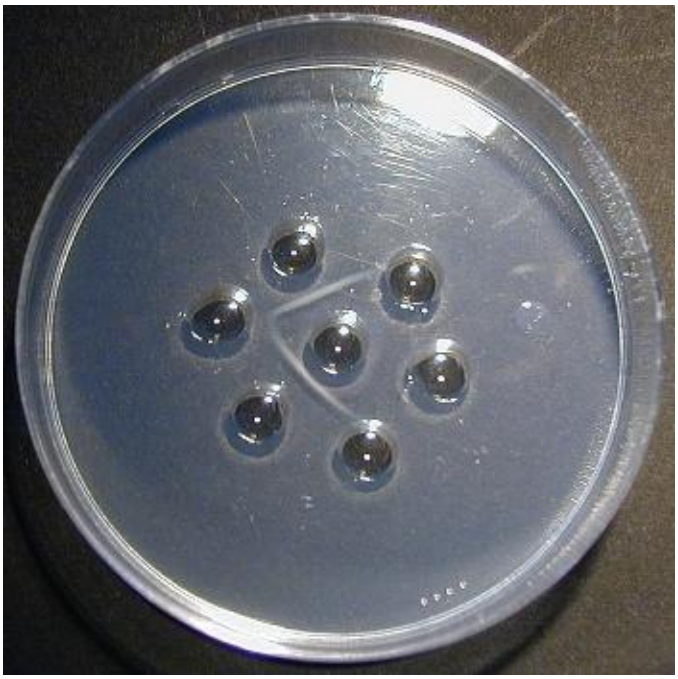


- Precipitation reactions in gels yield visible precipitin lines; no visible precipitate forms in regions of Ab or Ag excess.



- If multiple wells of Ag are positioned around an Ab well on the same plate, several patterns of reactivity may be observed:





1- Precipitation appears as a continuous line in the form of an arc between the two outer wells and the center well. There are no spurs at the angle and this type of reaction is termed a band of identity.

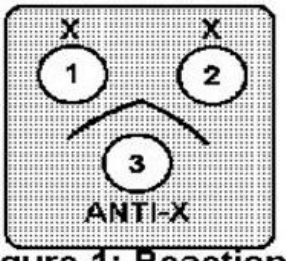


Figure 1: Reaction of Identity

2- If a solution with antigen X and Y is placed in well 1, a solution with antigen X only is placed in well 2, and antiserum containing antibodies specific for both X and Y is placed in well 3, a reaction similar to that appearing in Fig. 2 will occur. Notice that there is a spur reaction towards the XY well. This indicates that the two antigenic materials in wells 1 and 2 are related, but that the material in well 1 possesses an antigenic specificity not possessed by the material in well 2. Such a reaction with spur formation indicates partial identity.

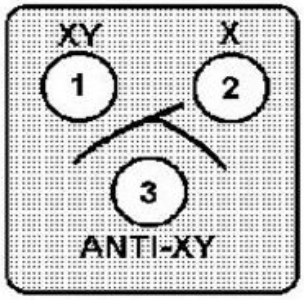


Fig. 2: Reaction of Partial Identity

3- If the material in wells 1 and 2 do not possess common antigens and the antiserum in well 3 possesses specificities for both materials, the reaction will appear as two crossed lines.

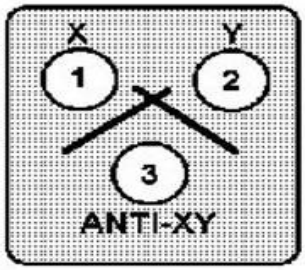


Fig. 3: Reaction of Non-Identity

Counter Immunoelectrophoresis (CIE):

This method not only depends entirely on diffusion of antigen and antibody in a gel, but also uses electrophoresis for their rapid movement (Fig. 22.23). By using this method protein can be separated within an hour. CIE is useful for the diagnosis of bacterial meningitis and the other diseases.

The principle of CIE is based on the movement of antigens and antibodies to opposite poles after applying electric current in buffers of correct electric strength and pH, because some of the antigens and antibodies have the opposite charges. If a reaction occurs, a precipitation line appears within an hour.

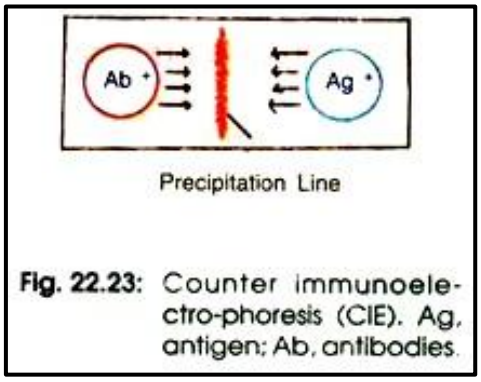


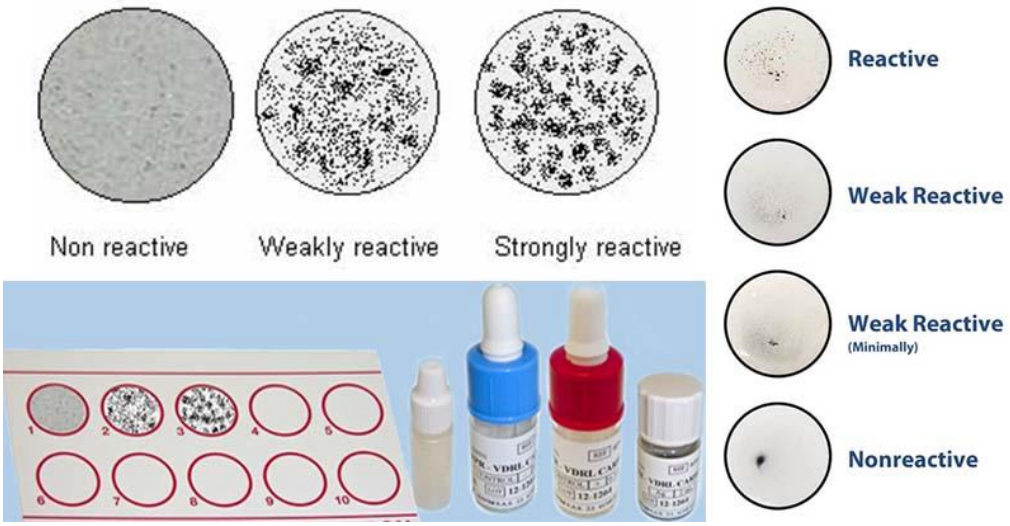
Fig. 22.23: Counter immunoelectro-phoresis (CIE). Ag, antigen; Ab, antibodies.

Flocculation test

interaction of soluble antigens with antibodies, producing a precipitate of fine particles that can be seen with the naked eye or by microscope.

Example:

- VDRL slide flocculation
- RPR card test
- Kahn 's test for syphilis



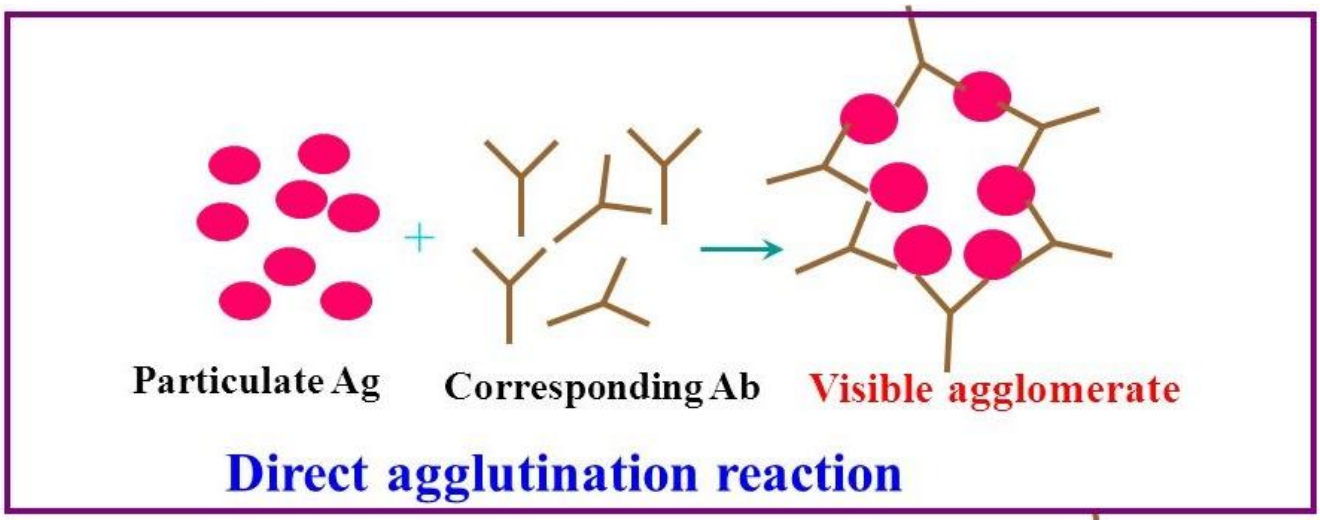
Agglutination Reactions

Agglutination is the process of linking together of antigens by antibodies and formation of visible aggregates. Agglutination reactions involve particulate antigens i.e. soluble antigens adhering to particles. Agglutination reactions are very sensitive, readable and available in several varieties.

It is of two types, direct and indirect agglutination tests:

1. Direct agglutination test:

Combination of an insoluble particulate antigen (cellular Ag) with its soluble antibody, forms antigen-antibody complex. The binding of antibodies to surface antigens results in visible clumps. This test used for antigen detection. For example, sero-typing and sero-grouping of *Vibrio cholera* and *Salmonella* spp.



2. Indirect (passive) agglutination test:

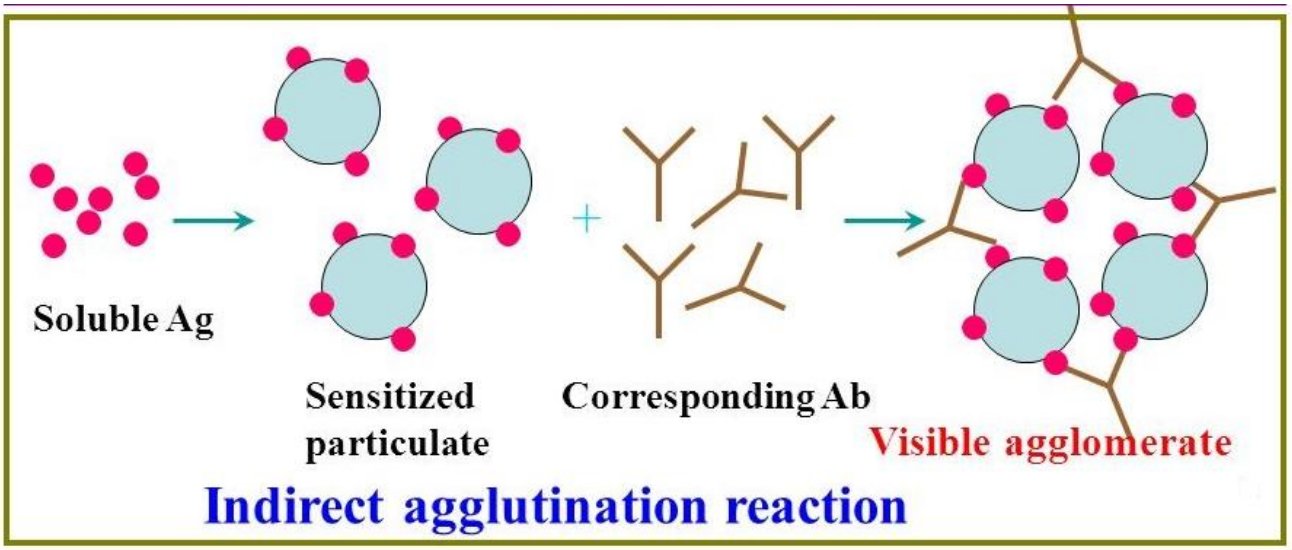
antigens are adsorbed onto particles (e.g. RBCs, latex, gelatin and bentonite), soluble antigens can respond to agglutination test. Antibody reacts with the soluble antigen adhering to the particles.

Examples of types:

- Latex agglutination

- Co-agglutination
- Passive hemagglutination (treated red blood cells made resistant)

Examples of tests: agglutination for leptospirosis – widal test (typhoid fever)



3- Hemagglutination (Phenomenon of clumping of RBCs)

Uses erythrocytes as the biological carriers of bacterial antigens, and purified polysaccharides or proteins for determining the presence of corresponding antibodies in a specimen.

ABO Blood grouping

Blood group antigens are actually sugars attached to the red blood cell, this Ag are built onto the red cell, individuals inherit a gene which codes for specific sugar to be added to the red cell, the type of sugar added determines the blood group.

ABO blood group system is based on the presence or absence of the A and B Ag on RBCs

Blood Group	Ag on RBCs	Ab in serum	Genotypes
A	A	Anti-B	AA or Ai
B	B	Anti-A	BB or Bi
AB	A and B	-	AB
O	-	Anti-A & Anti-B	ii

Hemolysis: if an individual is transfused with an incompatible blood group destruction of the red blood cells will occur, this may result in the death of the recipient.

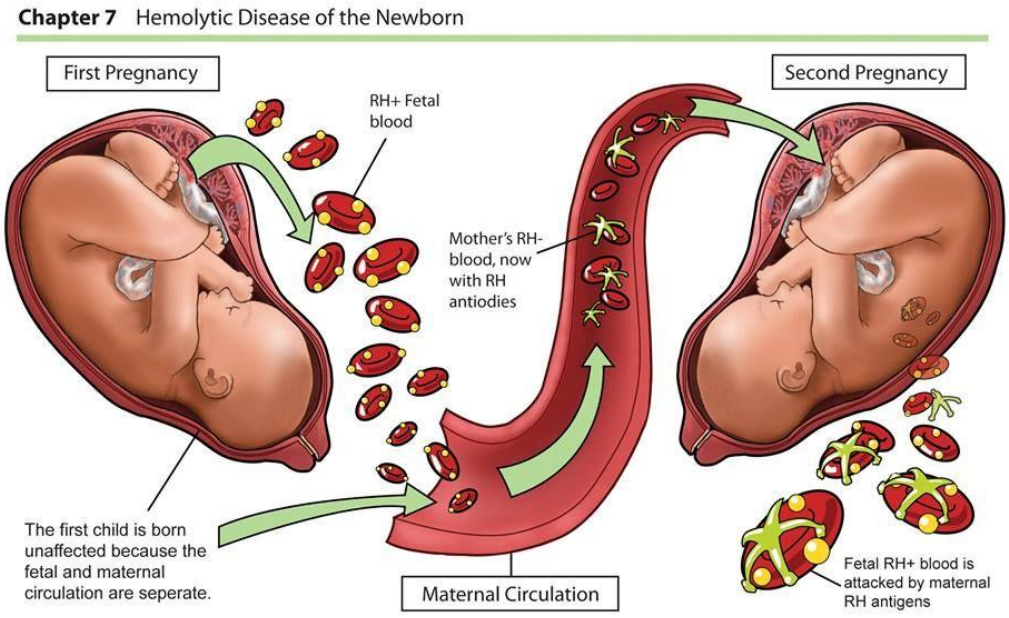
Rh (D) Antigen: refers to the presence or absence of the D antigen on the red blood cell.

Unlike ABO blood group system, individuals who lack the D antigen do not naturally make it, production of antibody to D requires exposure to the antigen, the D antigen is very immunogenic for this reason all individuals are typed for D, if negative must receive Rh (D) negative blood.

Hemolytic disease of the Newborn

- Child is Rh positive
- During pregnancy fetal Rh positive RBCs escape into maternal circulation
- Mother produces antibodies to Rh (D) antigen
- Second pregnancy with Rh (D) positive child results in destruction of fetal D positive RBCs

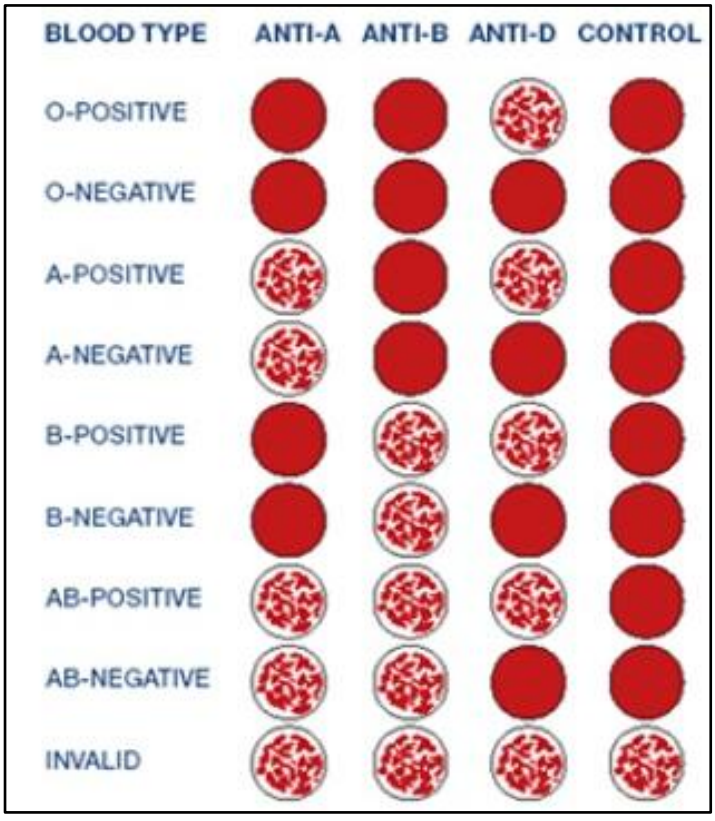
If a woman has Rh negative and gives birth to a child, or if she has a miscarriage or abortion, she is given an injection of anti-Rh antibodies called anti-Rh gamma globulin or RhoGAM. The antibodies bind to the fetal Rh antigens and inactivates them if they crossed the placenta during birth, and the mother 's immune system does not respond by producing antibodies.

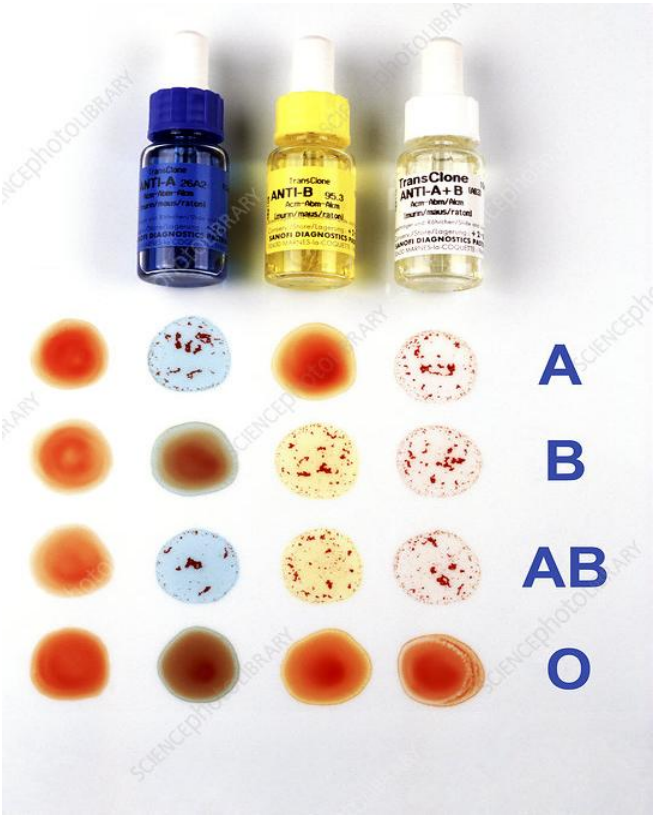


Method

The principle is based on the Antigen – Antibody reaction and show agglutination. Agglutination test are usually carried out in tubes or on slides for ABO and Rh grouping.

- Put 3 drops of the blood on a clean – dry glass slide (these drop represent the Ag).
- Add drop of anti-A (represent the Ab) to the first drop
  Add drop of anti-B (represent the Ab) to the second drop
  Add drop of anti-D (represent the Ab) to the third drop
- Move the slid in circular motion
- Slid most read within 5 min. to avoid the evaporation
- If agglutination occurs in the first drop it mean that the blood group is A
  If agglutination occurs in the second drop it means that the blood group is B
  If agglutination occurs in the both drops it means that the blood group is AB
  If there is no agglutination in the both drops it mean that the blood group is O
  If agglutination occur in the third drop it means that the blood Rh + if no it is Rh –

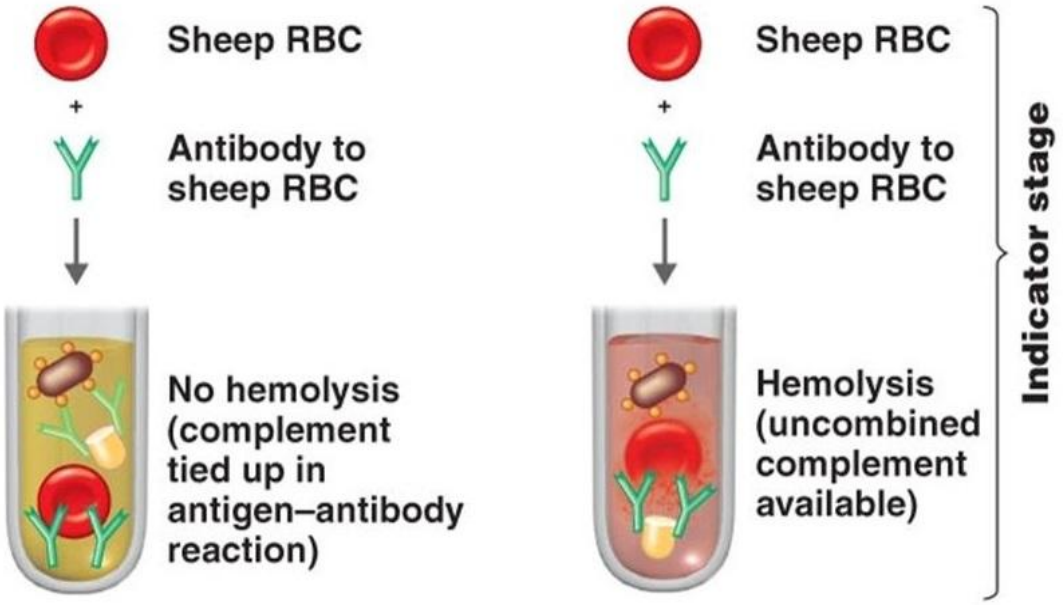




Complement Fixation

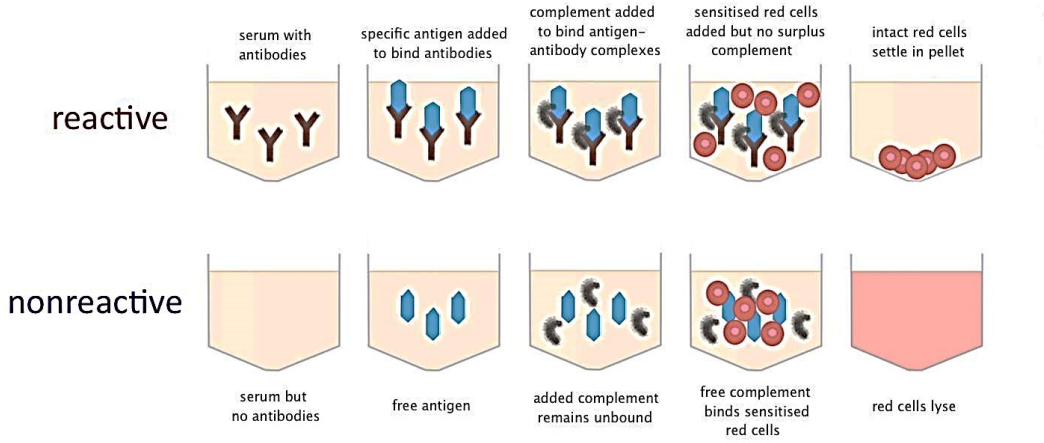
Complement fixation is a classic method for demonstrating the presence of antibody in patient serum. The complement fixation test consists of two components.

The first component is an indicator system that uses combination of sheep red blood cells, complement-fixing antibody such as immunoglobulin G produced against the sheep red blood cells and an exogenous source of complement usually guinea pig serum. When these elements are mixed in optimum conditions, the anti-sheep antibody binds on the surface of red blood cells. Complement subsequently binds to this antigen -antibody complex formed and will cause the red blood cells to lyse.



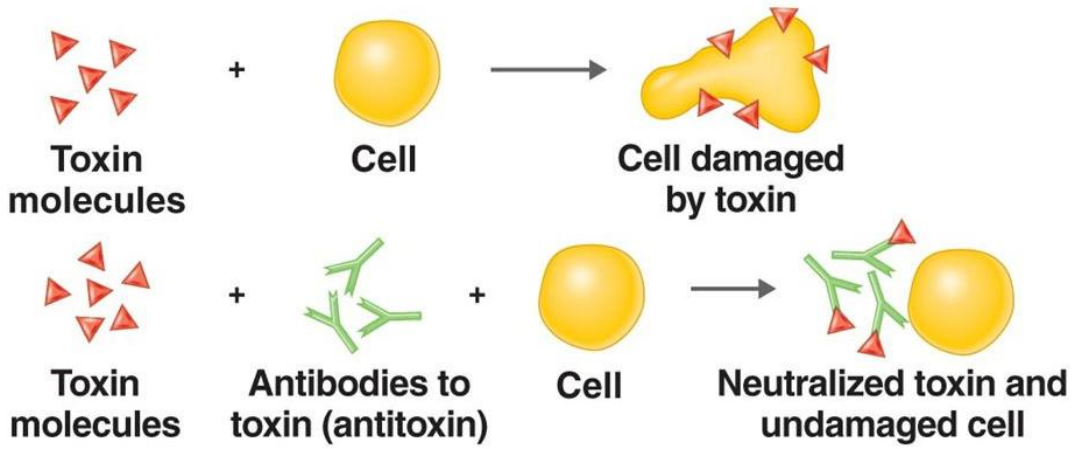
The second component is a known antigen and patient serum added to a suspension of sheep red blood cells in addition to complement. These two components of the complement fixation method are tested in sequence. Patient serum is first added to the known antigen, and complement is added to the solution. If the serum contains antibody to the antigen, the resulting antigen-antibody complexes will bind all of the complement. Sheep red blood cells and the anti-sheep antibody are then added. If complement has not been bound by an antigen-antibody complex formed from the patient serum and known antigens, it is available to bind to the indicator system of sheep cells and anti-sheep antibody. Lysis of the indicator

sheep red blood cells signifies both a lack of antibody in patient serum and a negative complement fixation test. If the patient's serum does contain a complement-fixing antibody, a positive result will be indicated by the lack of red blood cell lysis.



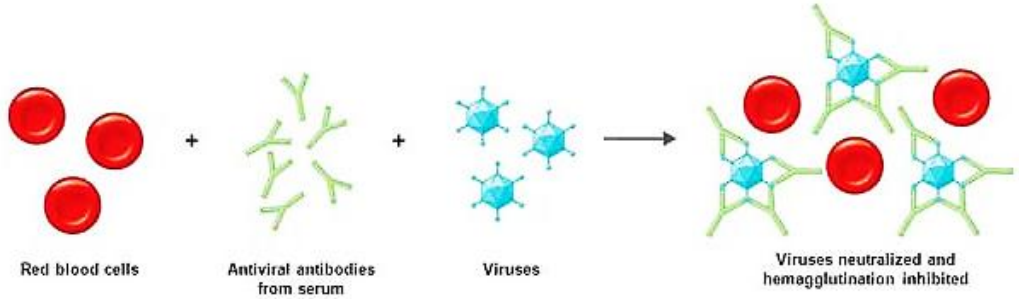
Neutralization Reactions

The neutralization reactions are the reactions of antigen- antibody that involve the elimination of harmful effects of bacterial exotoxins or a virus by specific antibodies. These neutralizing substances i.e. antibodies are known as antitoxins. This specific antibody is produced by a host cell in response to a bacterial exotoxin or corresponding toxoid (inactivated toxin). The antitoxin reacts with exotoxin and neutralizes it. These antitoxins can be artificially induced in animals such as horses. Thus, the antitoxin of animal sources in turn can be injected into human which provides a passive immunity against a toxin present in human body produced by the pathogens causing diphtheria, tetanus, etc.



1. Diagnosis of Viral Infections:

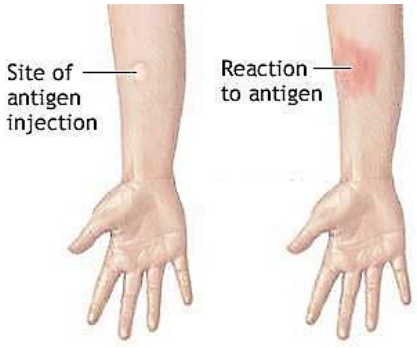
Neutralization test is very useful in diagnosis of viral infections in humans. After introduction of a virus, antibodies are produced in response and bind to receptor sites present on the viral surfaces. After binding of antibodies, viral particles fail to reach to the cells. Thereafter, the virus is destroyed. When serum contains antibodies against a particular virus, the antibodies will not allow the virus to infect the cell in cell culture; consequently, the cells will not be damaged.

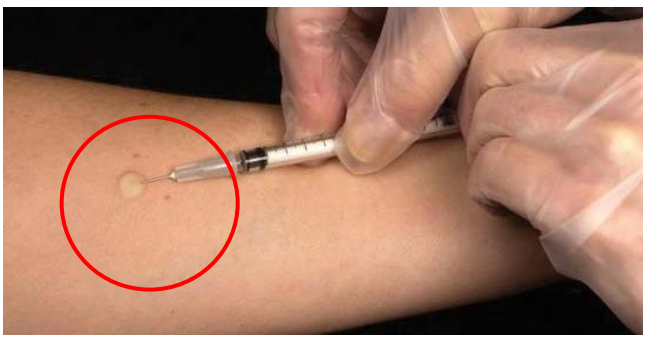


2. Schick Test:

Schick test measures the level of immune system of a person to the infection of diphtheria. When testing the status of immunity, a small amount of diphtheria exotoxin is inoculated in the skin of a person. Depending on ability and quantity of antitoxin, positive or negative responses develop.

If serum antitoxin in body would be in sufficient amount to neutralize the exotoxin, no visible reaction will occur. In control, when serum antitoxin is in insufficient amount the exotoxin will damage the tissues at the site where incision was made, and will produce a swollen and reddish area which is converted into brown within 4 or 5 days. This shows that the immune response is not present to a satisfactory level.





Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is a plate-based assay designed for detecting and quantifying substance such as peptides, proteins, antibodies, antigens and hormones.

ELISA system major components:

1- Antibody: allows for specific detection of substance of interest, e.g. IgG.
2- Solid phase (sorbent): allow one to wash away all the material that is not specifically captured.
3- Enzymatic amplification: allows to turn a little capture into a visible color change that can be quantified using an absorbance plate reader, e.g. Horse Radish Peroxidase (HRP) or alkaline phosphatase.
4- Substrate: the enzyme acts as a catalyst to oxidize substrate in the presence of hydrogen peroxide to produce a blue color, reaction stopped with dilute acid to cause complex to turn yellow, e.g. Tetramethylbenzidine (TMB).

Application:

1- Measure antibody levels (allergies, vaccines).
2- Detect viruses (Hepatitis, HIV, Venereal diseases).
3- Detect hormonal changes (Pregnancy).
4- Detect circulatory inflammatory markers (Cytokine).

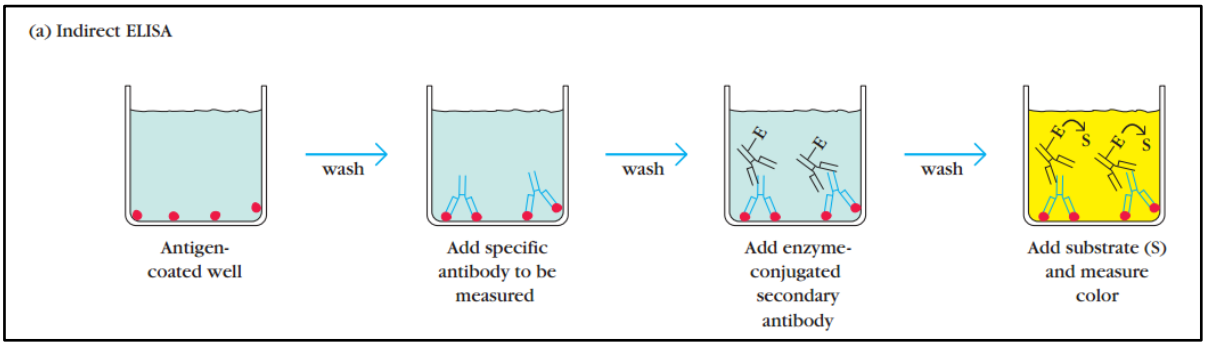
Types of ELISA

A- Indirect ELISA:

It is used to measure **antibody**. Known antigen is coated on the plastic lining of the wells of microtiter plate which is made up of polystyrene latex. Then added patient serum (contain Ab1) to the wells. If the patient's serum contains antibody specific to antigen, the antibody will bind to the absorbed antigen otherwise not.

After incubation the wells are washed and the antibody bound to the antigen is detected by adding an enzyme-conjugated secondary antibody (Ab2) that binds to Ab1. Any free Ab2 is again washed away, and a substrate for the enzyme is added, which is hydrolyzed by the enzyme and develops a color. Varying concentrations of antibody in serum shows changes in the intensity of color.

This color was measured using a specialized plate reader and compared with the amount of product generated when the same set of reactions is performed using a standard curve of known Ab1 concentrations.

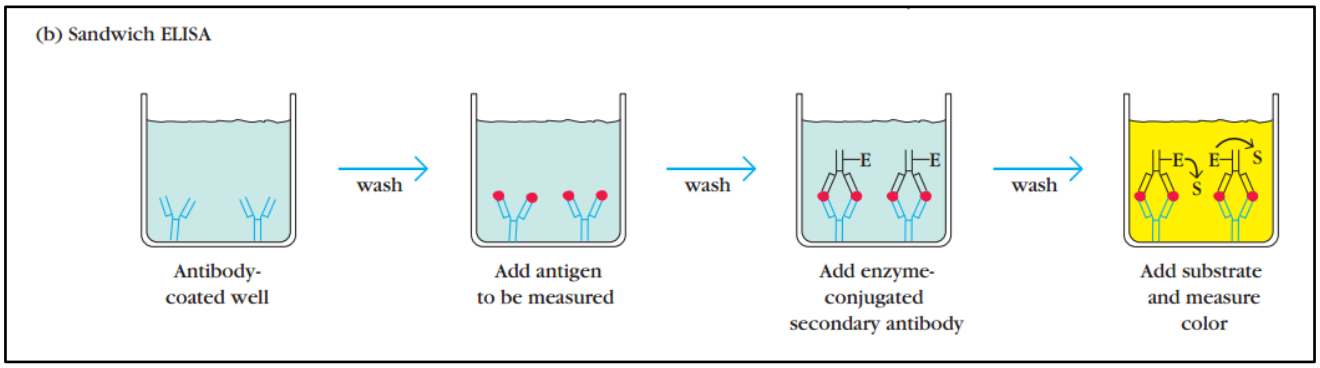


(A direct ELISA assay would detect the amount of antigen on the plate using enzyme coupled antibodies, and is rarely used). This type of ELISA used to detect the presence of serum antibodies against HIV, the causative agent of AIDS.

B- Sandwich ELISA

Antigen can be detected by a sandwich ELISA. The antibody is immobilized on a microtiter well. A sample containing unknown amounts of antigen is allowed to react with the immobilized Ab.

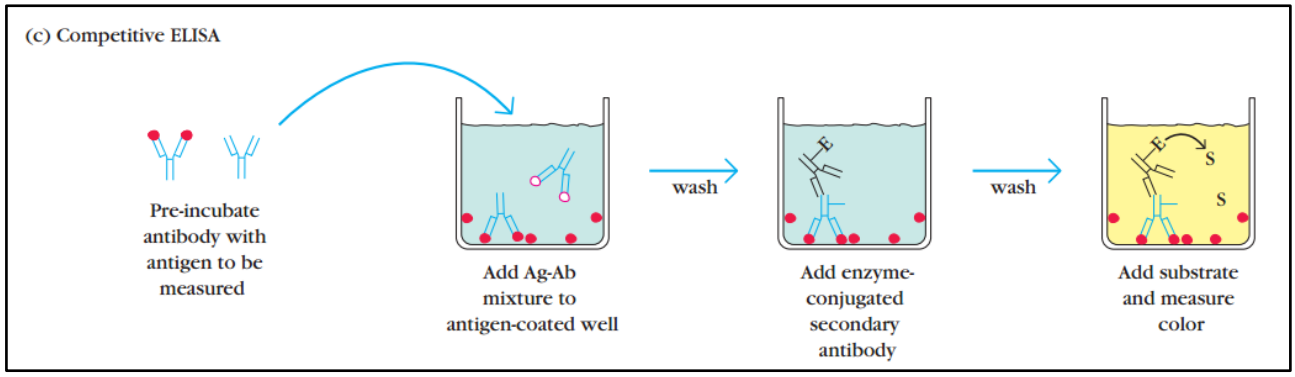
After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen. After any free second antibody is removed by washing, substrate is added, and the colored reaction product is measured.

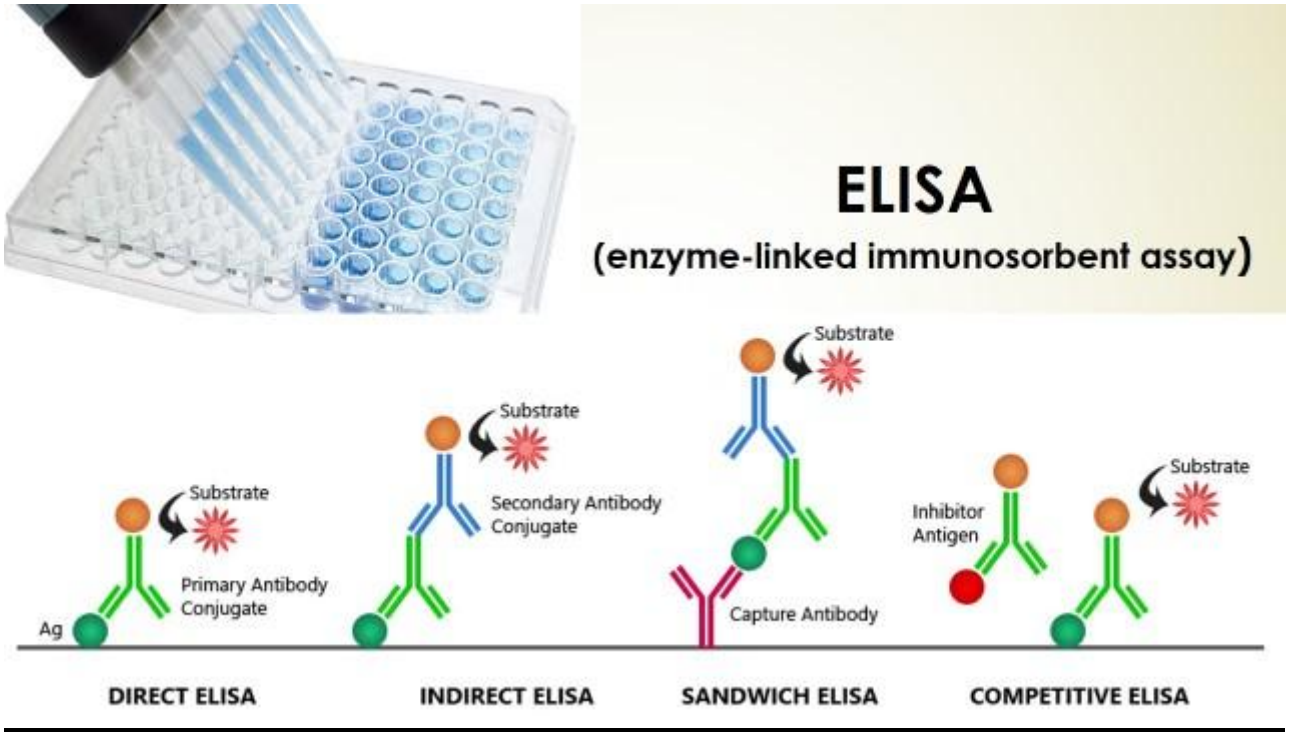


Sandwich ELISAs used to measure soluble cytokine concentrations in tissue culture supernatants, as well as in serum and body fluids.

C- Competitive ELISA

The competitive ELISA provides another extremely sensitive variation for measuring amounts of antigen. Antibody is first incubated in solution with a sample containing antigen. The antigen-antibody mixture is then added to an antigen-coated microtiter well. The more antigen presents in the initial solution-phase sample; the less free antibody will be available to bind to the antigen-coated well. After washing off the unbound antibody, an enzyme-conjugated Ab2 specific for the isotype of the Ab1 can be added to determine the amount of Ab1 bound to the well. In the competitive assay, the higher the concentration of antigen in the original sample, the lower the final signal.

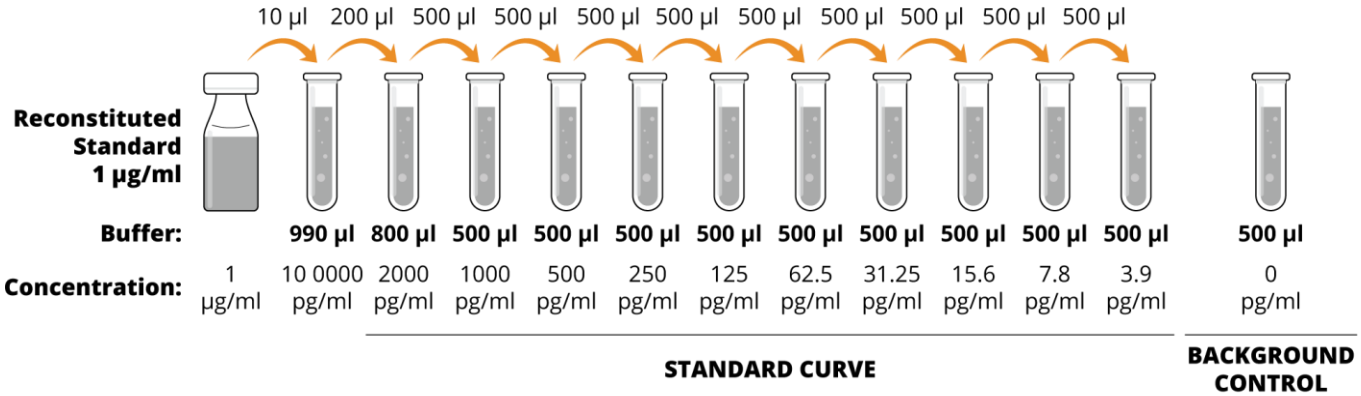




Method

For example, of sandwich ELISA determination of TNF-α by R&D Quantitative kit.

- All reagents, samples and working standards for TNF-α were prepared



- Excess microplate strips was removed from the plate frame
- A volume of 0.2 ml of standard, control, and sample was added per well. Then it was covered with the adhesive strip provided. It was incubated for 2 hours at room temperature.
- Each well was aspirated and washed, the processes was repeated three times for a total of four washes. It was washed by filling each well with wash buffer (400 µl) by

autowasher (complete removal of liquid at each step is important to good performance).

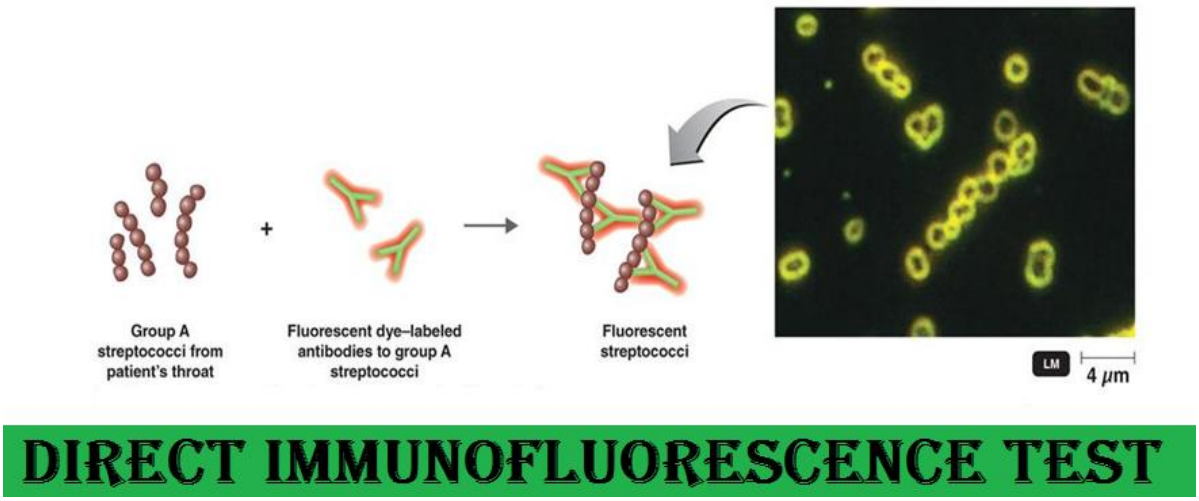
- After the last wash, any remaining wash buffer was removed by aspirating or decanting. The plate was inverted and it was blotted against clean paper towels.
- Two hundred µl of human TNF-α conjugate was added to each well, covered with a new adhesive strip and then incubated for two hours at room temperature.
- The aspiration / wash was repeated as in step 5 and 6.
- Two hundred µl of substrate solution was added to each well, incubated at room temperature for 30 minute (with protection from light).
- Fifty µl of stop solution was added to each well. The color in the wells was changed from blue to yellow.
- The optical density of each well was determined within 30 minutes, by using a microplate reader at wavelength 570 nm

Immunofluorescence

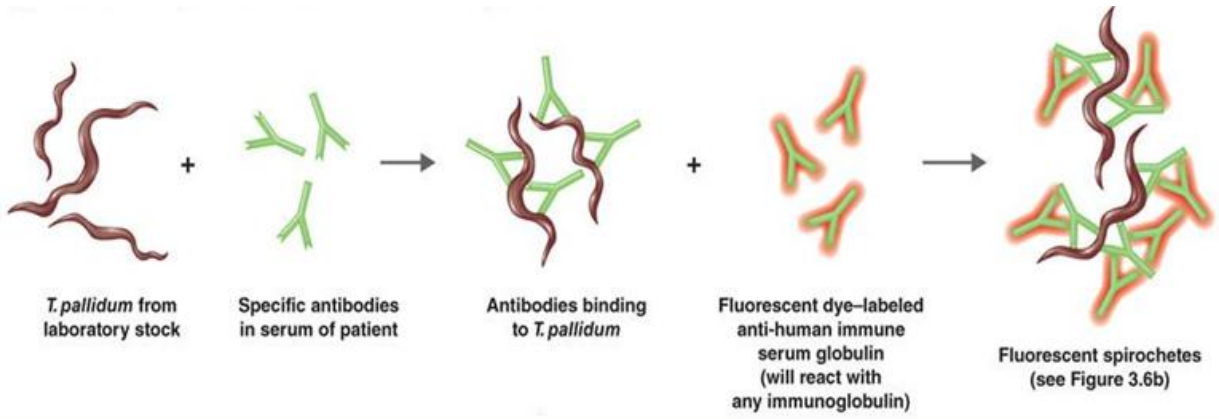
Immunofluorescence (IF) is a common laboratory technique, which is based on the use of specific antibodies which have been chemically conjugated to fluorescent dyes. These labeled antibodies bind directly or indirectly to cellular antigens.

Fluorescent – Antibody (FA) techniques:

Direct (FA) test to identify group A *Staphylococci* and used dye FITC (Fluorescein Isothiocyanate) associated with antibody.



Indirect (FA) test, such as that used in the diagnosis syphilis, the fluorescent dye FITC is attached to anti-human gamma globulin, which reacts with any human immunoglobulin (such as the *Treponema palladium* specific antibody) that has previously reacted with antigen.



INDIRECT IMMUNOFLUORESCENCE TEST