

LECTURE: 26

Title **SIMPLE SEROLOGICAL LABORATORY TECHNIQUES**

LEARNING OBJECTIVES:

The student should be able to:

- Define the term "simple serological techniques".
- Describe the benefit of the use of serological tests.
- Define the term "titer".
- Enumerate the environmental factors affecting the ag-ab interactions.
- Enumerate the different immunological names give to antibodies.
- Define the terms; prozone, equivalence zone, and post zone.
- Enumerate some examples of the major simple serological techniques, such as:
 - Agglutination reactions
 - Precipitation reactions
- Explain the principle of the agglutination reactions
- Enumerate some diagnostic test depend on the principle the of the agglutination reactions.
- Explain the principle of the precipitation reactions
- Enumerate some diagnostic test depend on the principle of the precipitation reaction.
- Explain the terms; lattice, cross-reacting antibodies. Latex, charcoal, and agar.
- Discuss the identity, partial identity, and non-identity
- Compare between agglutination and precipitation reactions.
- Explain the Immunodiffusion (single and double diffusion) methods (Ouchterlony technique).
- Explain the principle of the toxin-anti-toxin reaction.
- Explain the term "flocculation reaction".

LECTURE REFERENCE:

1. TEXTBOOK: ROITT, BROSTOFF, MALE IMMUNOLOGY. 6th edition. Chapter 27. pg. 417-434.
2. TEXTBOOK: MARY LOUISE TURGEON. IMMUNOLOGY & SEROLOGY. IN LABORATORY MEDICINE. 2ND EDITION. Chapter 6. pg 111-131.
3. TEXTBOOK: ABUL K. ABBAS. ANDREW H. LICHTMAN. CELLULAR AND MOLECULAR IMMUNOLOGY. 5TH EDITION. pg 522-534.

SIMPLE LABORATORY METHODS

I. GENERAL CONSIDERATIONS

Serologic reactions that are in vitro Antigen-antibody reactions provide methods for the diagnosis of disease and for the identification and quantitation of antigens and antibodies. Simple serological techniques are called simple, because, these procedures involving direct demonstration and observation of reactions, they do not require the participation of accessory factors such as; indicator system, or specialized equipment. Some examples of these tests are the agglutination reactions, and precipitation reactions.

A. The antibody "TITER", or level of antibody in serum, can be measured by using known antigens.

1. Antibody titers can be of diagnostic and prognostic importance.
2. For example, a rise in the antibody titer between serum taken during the acute phase of an illness and during the convalescent phase (acute and convalescent serums) can be diagnostic for that illness.

B. Environmental factors can profoundly affect the forces involved in antigen-antibody interactions for example:

1. **Physiological pH and salt concentration** promote optimal union. Forces of attraction tend to be weaker in conditions that are acid (below pH 4.0) or alkaline (above pH 10.0).
2. **Temperature**, the higher the temperature (up to a maximum of 50C), the greater the kinetic motion of the reactions and therefore the more rapid the rate of reaction.

C. the serologic identification of antibody types and types of antigen-antibody reactions are based on the physical state of antigen:

1. Agglutinins are antibodies that aggregate cellular antigens.
2. Lysins are antibodies that cause dissolution of cell membranes.
3. Precipitins are antibodies that form precipitates with soluble antigens.
4. Antitoxins are antibodies that neutralize toxins.

D. The relative sensitivity of the various tests for antigens and antibodies are presented in Table-1

Test	Approximate Detectable Amount (µg/ml)	
Precipitation	20.0	1.0
Immunelectrophoresis	20.0
Double diffusion in agar gel	1.0
Complement fixation	0.5
Radial immunodiffusion	0.05	0.5
Bacterial agglutination	0.01
Hemolysis	0.01
Passive hemagglutination	0.01
Hemagglutination inhibition	0.001
Antitoxin neutralization	0.01
Radioimmunoassay (RIA)	0.0005	0.000005
Enzyme-linked immunosorbent assay (ELISA)	0.0005	0.000005
Virus neutralization	0.000005

Table-1 Relative Sensitivity of Tests Measuring Antibody and Antigen

II. PROCEDURES INVOLVING DIRECT DEMONSTRATION AND OBSERVATION OF REACTIONS

A. Procedures involving cellular antigens

1. Agglutination reactions

- a. Agglutination reactions serve to detect and quantitate agglutinins and to identify **cellular antigens** such as bacterial cells, white blood cells, and red blood cells.
 - (1) When cells interact in vitro with the appropriate antibody, they clump together and eventually form masses that become large enough to be seen. (When antibody agglutinates bacteria in the body, opsonization occurs).
 - (a) Agglutination occurs because antibodies are at least bivalent (i.e., they have at least two combining sites).
 - (b) Two sites on the antibody and multiple sites on the antigen result in antigen antibody lattice formation that can build up into increasingly larger complexes (**Figure-1**).

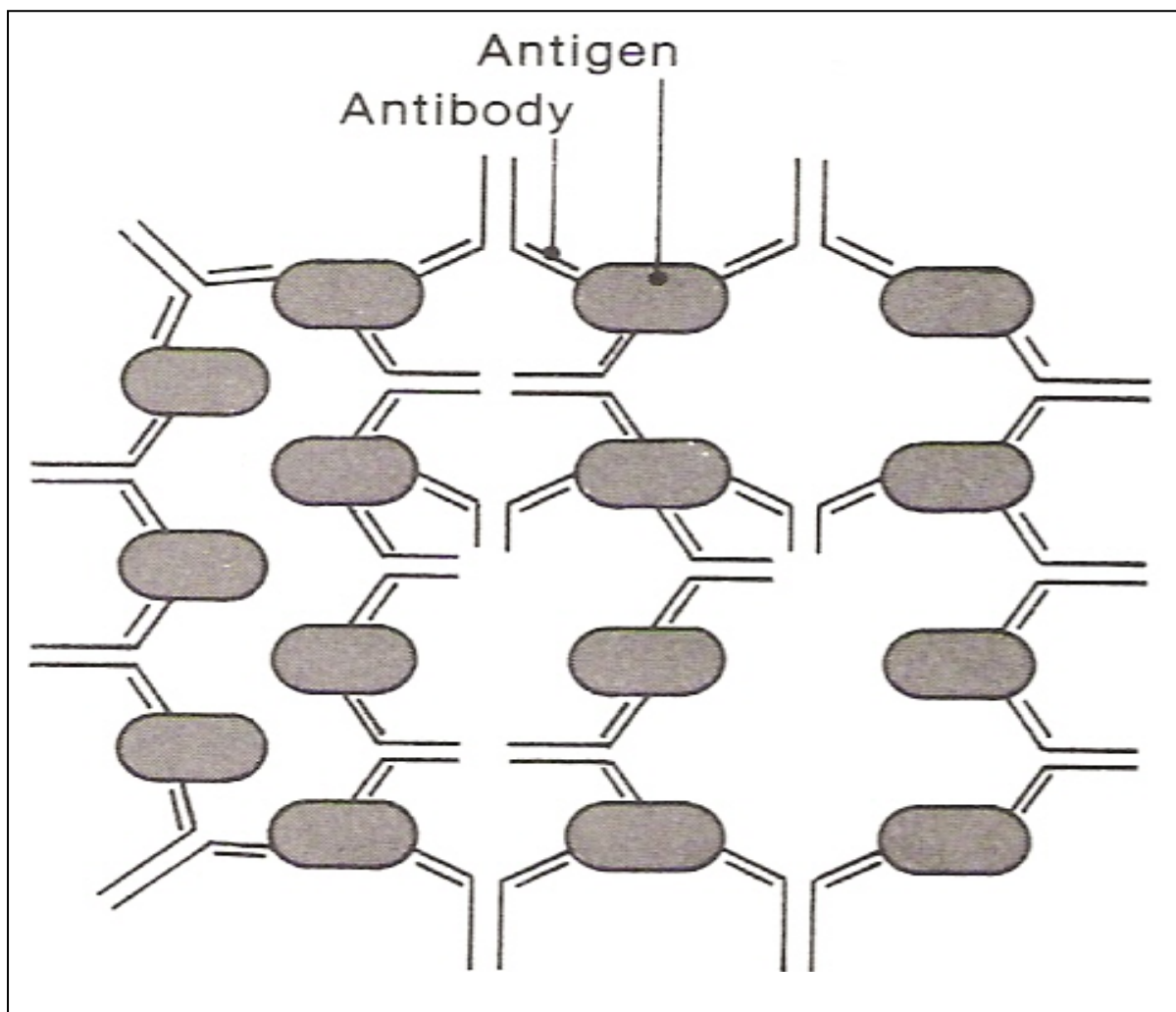


Figure-1 Lattice structure composed of antigen and antibody

- (2) The aggregates may be seen in the test tube or under the microscope.
 - (3) If red blood cells are employed as the agglutinin, the process is **hemagglutination**.
- b. The classic application of the agglutination reaction is the **Widal test** for the diagnosis of typhoid fever.**
- (1) In this test, the antibody content of the patient's serum is measured by adding a **constant amount of antigen** (e.g., *Salmonella typhi*) to **serially diluted serum**, that is, to progressively more dilute serum samples.
 - (2) After appropriate incubation, the serum samples are examined for visible agglutination. The highest dilution of serum that shows agglutination is referred to as the **titer**.

2. Lysis

a. Principle

- (1) In the presence of complement, an antigen-antibody reaction on a cell membrane may damage the membrane, leading to cell lysis (**cytolysis**).
- (2) The damage is presumed to be due to the enzymatic activity of activated complement components and the membrane-altering properties of complement components C8 and C9.
- (3) The phenomenon is probably of importance in the host's defense against microbial infections, cancer, and so forth.
- (4) Cytolysis in vivo can be a major cause of tissue damage.

b. Types of cytolysis

- (1) In **hemolysis**, hemoglobin is released from the red blood cell; this is a requisite phenomenon for the complement fixation test.
- (2) In **bacteriolysis**, cells of gram-negative bacteria undergo immune lysis.
- (3) Cytolysis can involve the destruction of other cell types (e.g., tumor cells) under appropriate conditions in the presence of specific antibody and complement.

B. Procedures involving soluble antigen

1. Precipitation

- a. When soluble antigens come in contact with specific antibody, they aggregate (e.g., precipitate).**
- (1) Because the antigen is soluble instead of cellular, a large number of molecules are required for lattice formation.
 - (2) Moreover, a large lattice must be formed in order for the aggregate to be visible.
- b. Changing the amount of antigen affects precipitation (Figure-2).**
- (1) When the antigen concentration is very low and the antibody relatively superabundant (**zone of antibody excess**), formation of small complexes occurs.
 - (a) However, residual antibody will remain in the supernatant after the mixture has been centrifuged.
 - (b) This area containing excess antibody is known as the **prozone**.
 - (2) As more antigens are added, large aggregates form. In the **zone of equivalence**, there is neither antigen nor antibody in the supernatant.
 - (3) With increasing amounts of antigen (**zone of antigen excess**), the lattice size becomes too small to precipitate. Hence, instead of reaching a plateau, the curve comes back down to zero (**Figure-2**).

- (a) The area containing excess antigen is known as the **postzone**.
- (b) In extreme antigen excess, the complex will be **trimolecular** (e.g., one antibody molecules for every two antigen molecules).

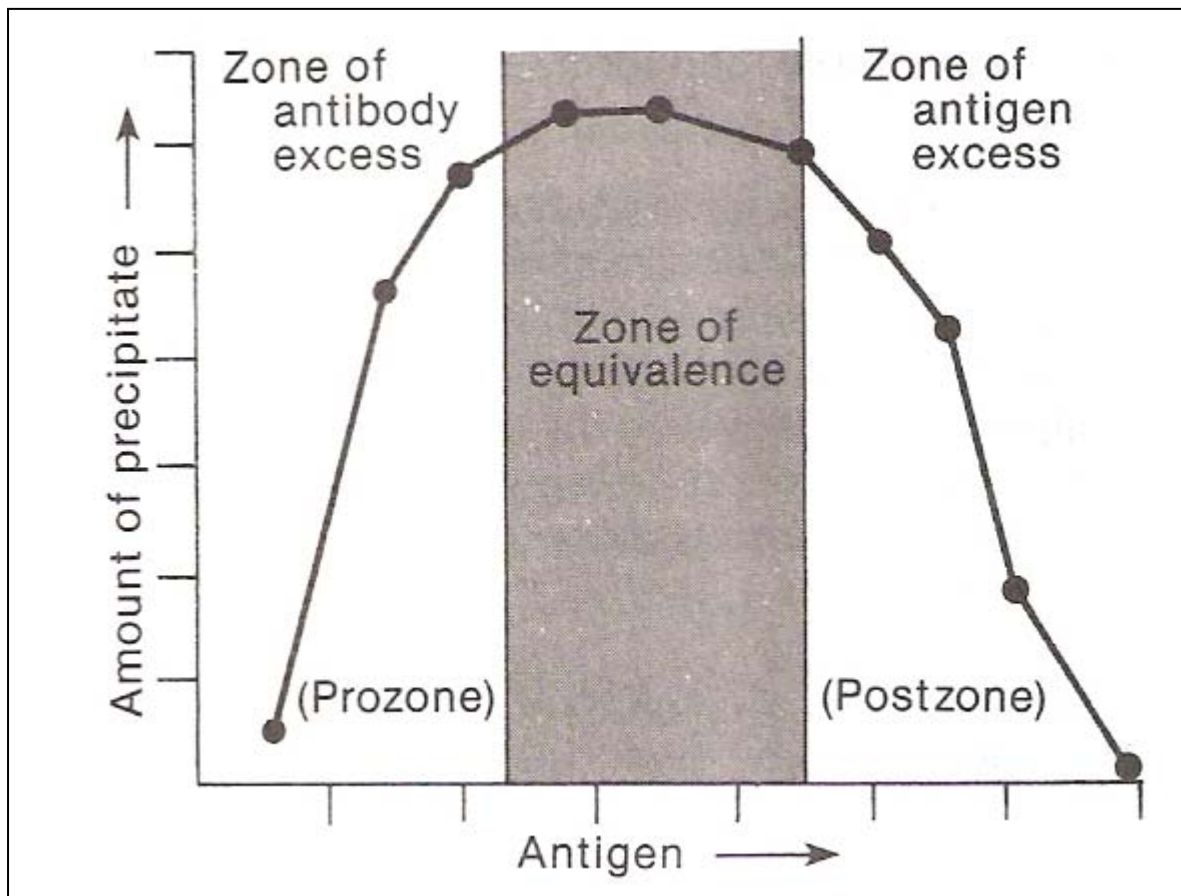


Figure-2 Effect of increasing amounts of antigen on the total immune precipitate obtained in a mixture of soluble antigen and its homologous antibody.

2. In vivo consequences of soluble complexes

a. When soluble complexes form in vivo, they may cause serum sickness.

- (1) An example of this is the administration of **diphtheria antitoxin** made from horse serum, which is a foreign protein to humans.
- (2) People given too much antitoxin (i.e., antigen) may develop serum sickness.

b. The soluble complexes are not handled well by the reticuloendothelial system (RES).

3. Immunodiffusion.

This laboratory technique is based on the phenomenon of precipitation.

a. If an antigen-antibody reaction takes place in a **semisolid medium** (e.g., agar), bands of precipitate will form.

b. The **Ouchterlony technique**, a **double immunodiffusion** method, is a useful example.

(1) The technique is called **double diffusion** because; the two components diffuse toward each other. (In **single diffusion**, one component is fixed in place).

(2) In the Ouchterlony procedure, antigen and antibody preparations are placed in separate wells that are cut into a thin layer of agar in a Petri dish.

(a) The reactants diffuse toward each other through the agar until they meet.

(b) At optimal antigen-antibody proportions, bands of precipitate form (**Figure-3**).

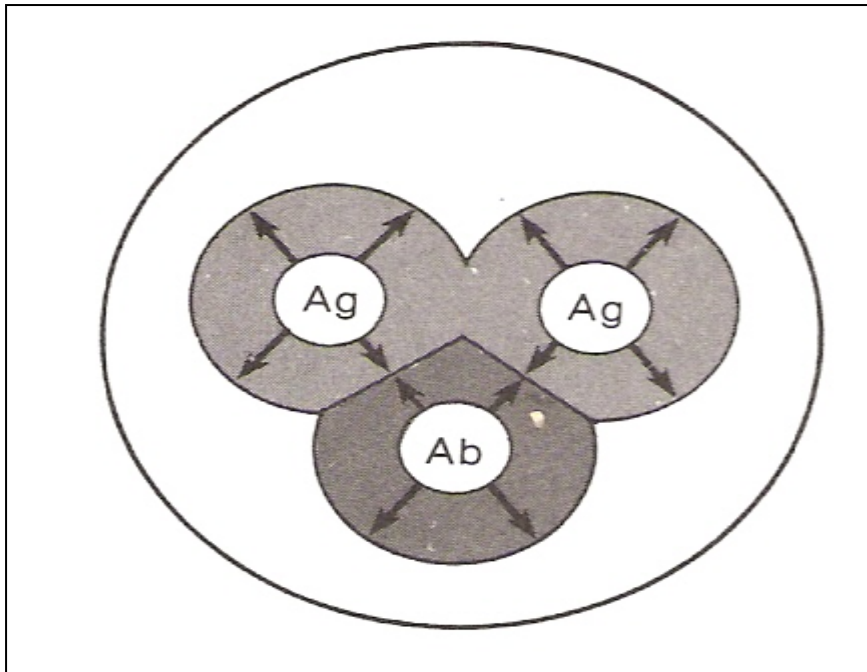


Figure-3 Diffusion of reactants in double immunodiffusion, Ag = antigen; ab = antibody.

c. One advantage of immunodiffusion procedures is that **antigenic relationships** can be detected from **precipitation patterns (Figure-4)**.

- (1) In **reactions of identity**, two identical antigens will diffuse at the same rate, and their two precipitin bands will merge into a solid **chevron**.
- (2) In **reactions of nonidentity**, the two antigens are completely different, and the lines of precipitate will cross.
- (3) In **reactions of partial identity**, a **spur** will form indication that the two antigens are cross-reactive but not identical.

- (a) The spur occurs because one of the antibodies does not react with the cross reacting antigen but migrates past that antigen until it reaches an antigen with the epitope for which it has specificity.
- (b) The spur in figure-4 contains antibody **b** only; antibody **a** reacted with epitope **a** on antigen **ac**.

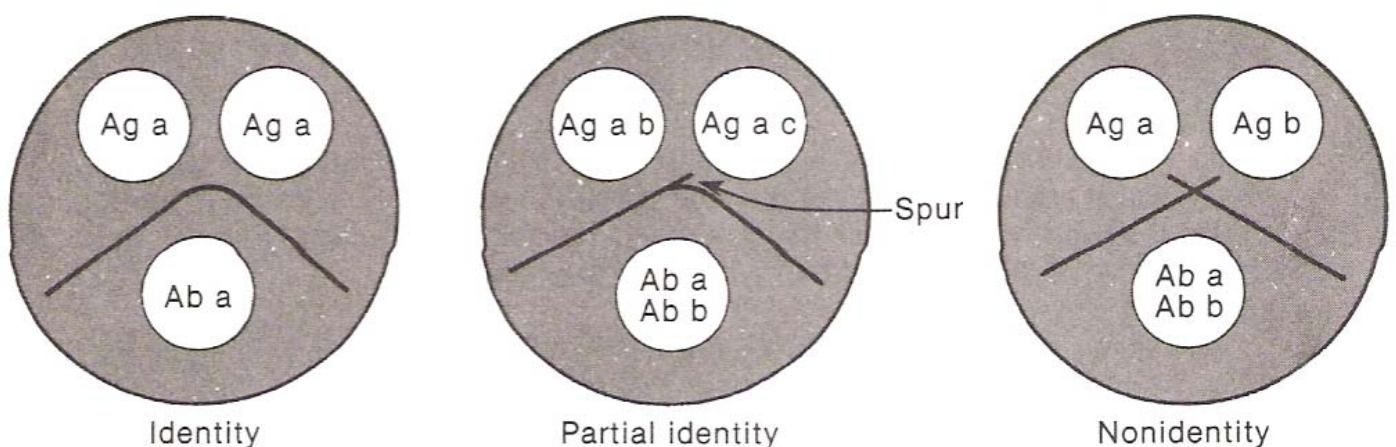


Figure-4 The types of patterns seen in immunodiffusion, Ag = antigen; ab = antibody.

4. **Quantitative radial immunodiffusion** is a variation that allows the quantitation of antigens. It is used routinely to determine human serum immunoglobulin levels; in this case, the **immunoglobulin** is the **antigen**.
 - a. For this purpose, an agar-coated slide is used, the agar being impregnated with antiserum) e.g., antibody to human IgG). Serum samples are then placed in wells in the agar.
 - b. As each sample diffuses through the agar and encounters the antibody, the IgG will form a concentric ring, or halo, of precipitate. The **diameter of the halo** directly correlates with the **concentration of IgG** in the sample.
 - c. The level of IgG in the sample can be determined by reference to a standard curve based on halo diameters of known concentrations of IgG.

5. **Immuno-electrophoresis**. The double-diffusion technique could not always resolve highly complex mixtures of antigens; therefore, this more sophisticated technique was developed.
 - a. In Immuno-electrophoresis, antigen is placed in wells in agar on a glass slide and is then subjected to electrophoresis via application of an electric current. Under these conditions, the individual antigenic components will migrate through the agar at variable rates.
 - b. If antibody is then placed in a well running the length of the slide and parallel to the path of migration, the reactants will diffuse toward one another and form separate arcs of precipitation for each antigenic component.

6. **Counter-immunoelectrophoresis**. This variant of double diffusion adds an electric current as the migratory force, which greatly speeds up the reaction (18 to 24 hours by double diffusion; 30 to 90 minutes with electric propulsion) and intensifies the precipitin bands.
 - a. Antigen and antibody are placed in wells and current is applied.
 - b. In suitable buffer, the negatively charged antigen migrates in the opposite (counter) direction as a result of endosmosis. Precipitation occurs where the reactants meet.

- C. **Toxin-antitoxin reactions**. If a serum contains an antitoxin (i.e., an antibody to a toxin) the antibody will neutralize the toxin. The **presence of antitoxin** can be demonstrated either directly or indirectly.
 1. **Direct testing**. The presence or absence of antitoxin in an individual's serum can be shown by intradermally injecting a small amount of the toxin. An example is the **Schick test**, which is used to test for immunity to diphtheria by testing for antibody to diphtheria toxin.
 - a. A **positive test** (inflammation that develops at the injection site within a few days) indicates the **absence of antibody**.
 - b. A **negative test** (no reaction) indicates that there was **sufficient antitoxin** to neutralize the toxin injected, and the individual is immune.
 2. **Indirect testing**. Antitoxin can also be detected via neutralization in vitro.
 - a. An individual's serum is mixed with toxin in vitro, and then, after a few minutes, a small amount of the mixture is injected into an experimental animal.
 - b. If the serum contains antitoxin, the animal will be protected against the deleterious effects of the toxin.

D. Procedures involving insoluble particulates. Flocculation is an antigen-antibody reaction that occurs if the antigen is neither cellular nor soluble, but is an **insoluble particulate**.

1. The **Venereal Disease Research Laboratory (VDRL) test** and the **rapid plasma reagin (RAR) test** are flocculation tests used for the diagnosis of syphilis.
2. These tests make use of **cardiolipin**, a hapten from normal beef heart that cross-reacts with a heterophile (Heterogeneous) antigen of the spirochete of syphilis.
3. Cholesterol particles with water-insoluble cardiolipin on their surface are used in the test. Visible aggregates form in the presence of an antibody (**reagin**) in the serum of patients with syphilis.

Dr. MUSTAFA HASAN LINJAWI