Serology is the study and use of immunological tests to diagnose and treat disease or identify antibodies or antigens. Direct testing involves using antibodies to find an antigen in a specimen. Indirect testing involves using an antigen and antibodies in serum or blood. There are two basic categories of immune testing using antibody-antigen interactions. For direct testing, the investigator is looking for the presence of an antigen, often a pathogen, in a specimen, which is typically taken from a site of infection. Indirect testing involves checking the blood or serum for antibodies that have formed against a particular antigen. Thus, in each category the investigator uses either antibody or antigen to test for the presence of its complement—antibody locates antigen in direct testing, whereas antigen locates antibody in indirect testing. The latter process can be used either to monitor the spread of an infection through a population or to establish a diagnosis in an individual. For example, the presence of antibodies against HIV in an adult’s serum is strong evidence that this individual has been infected with HIV, and a patient’s immune response to tuberculosis antigen indicates exposure to Mycobacterium tuberculosis.

Researchers have developed a wide variety of serologic tests to visualize antibody-antigen interactions, ranging from simple, automated processes to complex tests requiring skilled technicians. Physicians and medical laboratory technologists choose a particular test based on the suspected diagnosis, the cost to perform the test, and the speed with which a result can be obtained. In the following subsections we will consider several types of serologic tests: precipitation tests, agglutination tests, neutralization tests, and several tagged antibody tests. Some of these procedures are presented for historical reasons—more accurate and faster modern tests have replaced them.

**Precipitation Tests**

One of the simplest of serologic tests relies on the fact that when antigens and antibody are mixed in proper proportions, they form huge, insoluble, lattice-like complexes called precipitates. When, for example, a solution of a soluble antigen, such as that of the fungus Coccidioides immitis is mixed with an antiserum containing antibodies against the antigen, the mixture quickly becomes cloudy due to formation of a precipitate consisting of antigen-antibody complexes (also called immune complexes). When a given amount of antibody is added to each of a series of test tubes containing increasing amounts of antigen, the amount of precipitate increases gradually until it reaches a maximum. In test tubes containing still more antigen molecules, the amount of precipitate declines; in fact, in test tubes containing antigen in great excess over antibody, no precipitate at all develops. Thus, a graph of the amount of precipitate versus the amount of antigen has a maximum in the middle and lower values on either side. The reasons behind this pattern of precipitation reactions are simple. Complex antigens are generally multivalent—each possesses many epitopes—and antibodies have pairs of active sites and therefore can simultaneously cross-link the same epitope on two antigen molecules. When there is excess antibody, each antigen molecule is covered with many antibody molecules, preventing extensive cross-linkage and thus precipitation. Such soluble immune complexes can activate complement in the kidneys, leading to inflammatory damage to blood vessels in the kidneys. When the reactants are in optimal proportions, the ratio of antigen to antibody is such that cross-linking and lattice formation are extensive. As this lattice grows, it precipitates. In mixtures in
which antigen is in excess, each antigen molecule is bound to only two antibody molecules. There is no cross-linkage; because these complexes are small and soluble, no precipitation occurs. In the body, phagocytic cells do not easily remove these small immune complexes. As a result, immune complexes may be deposited in the joints and in the tiny blood vessels of the kidneys, where they trigger allergic reactions. Because precipitation requires the mixing of antigen and antibody in optimal proportions, it is not possible to perform a precipitation test by combining just any two solutions containing these reagents. To ensure that the optimal concentrations of antibody and antigen come together, scientists can use a technique involving movement of the molecules through an agar gel: immunodiffusion.

**Agglutination Tests**

Not all antigens are soluble proteins that can be precipitated by antibody. Because of their multiple antigen-binding sites, antibodies can also cross-link particles, such as whole bacteria or antigen-coated latex beads, causing agglutination. The difference between agglutination and precipitation is that agglutination involves the clumping of insoluble particles, whereas precipitation involves the aggregation of soluble molecules. Agglutination reactions are easy to see and interpret with the unaided eye. IgM antibodies, which have 10 active sites, are more efficient than IgG antibodies (with only 2 active sites) at causing agglutination. When the particles agglutinated are red blood cells, the reaction is called hemagglutination. One use of hemagglutination is to determine blood type in humans. Blood is considered type A if the red blood cells possess surface antigens called A antigen, type B if they possess B antigens, type AB if they possess both antigens, and type O if they have neither antigen. In a hemagglutination reaction to determine blood type, two portions of a given blood sample are placed on a slide. Anti-A antibodies are added to one portion, and anti-B antibodies to the other; the antibodies agglutinate those blood cells that possess complementary antigens. Another use of agglutination is in a type of test that determines the concentration of antibodies in a clinical sample. Although the simple detection of antibodies is sufficient for many purposes, it is often more desirable to measure the amount of antibodies in serum. By doing so, clinicians can determine whether a patient’s antibody levels are rising, as occurs in response to the presence of active infectious disease, or falling, as occurs during the successful conclusion of a fight against an infection. One way of measuring antibody levels in blood sera is by titration. In titration, the serum being tested undergoes a regular series of dilutions, and each dilution is then tested for agglutinating activity. Eventually, the antibodies in the serum become so dilute that they can no longer cause agglutination. The highest dilution of serum giving a positive reaction is the titer. Thus, a serum that must be greatly diluted before agglutination ceases (for example, has a titer of 1000) contains more antibodies than a serum that no longer agglutinates after minimal dilution.

**The Complement Fixation Test**

The classical complement system by antibody leads to the generation of membrane attack complexes (MACs) that disrupt cytoplasmic membranes. This phenomenon is the basis for the complement fixation test, which is a complex assay used to detect the presence of specific antibodies in an individual’s serum. The test can detect the presence of small amounts of antibody—amounts too small to detect by agglutination—though complement fixation tests have been replaced by other serological methods such as ELISA (discussed shortly) or genetic analysis using polymerase chain reaction (PCR).
Complement fixation test (CFT) belong to indirect two-system reactions. Like other serological reactions it is used for serological diagnosis (determination specific complement-fixing antibodies in the blood with a help known antigen or determination of the antigens by the known antibodies). As in other serological reactions in CFT antibody and antigen interact, but complement takes part in it too, that is fixing with antibodies (IgM, IgG), which interact with the antigen. Let as discuss the case when CFT is made for serological diagnosis of infectious diseases. Add to the patients blood serum known antigen and complement. If this serum has specific antibodies to the antigen, the antigen + antibody complex will form, which binds the complement. But this process is invisible. It was the first system of CFT. For determination of the complement state (fixed or not fixed) one should add the second system, called hemolytic. Hemolytic system is prepared to consider the reaction and consists of hemolytic serum that contains sheep erythrocytes antibodies (hemolysin) and sheep erythrocytes (SE). Immune reaction of hemolysis in this system occurs in the presence of unfixed component in the first system. Before production reaction investigated serum of the patient is warmed at 56 ºC for 30 minutes to inactivate their own complement, which is located in it. A serum of Guinea pigs is used as a complement to CFT. All CFT components, except examined serum, must be preliminary titrated and used in the working dose. In case there are specific antibodies in the sample of serum, complex antigen + antibody is formed. This complex fixes complement through Fc-fragment of the antibodies, but it is not visually noticeable. We add the hemolytic system to the mixture and if complement is fixed in the first system, there is no hemolysis in the second system and reaction is positive – sheep erythrocytes precipitate. In case there are no antibodies in the studied serum, complement remains unfixed in the first system. When we add second (hemolytic) system we see hemolysis of erythrocytes, which indicates that the CFT is negative. CFT is used for diagnosis of syphilis, tuberculosis, brucellosis, candidiasis, influenza, and other diseases.

**Labeled Antibody Tests**

A different form of serologic testing involves labeled (or tagged) antibody tests, so named because these tests use antibody molecules that are linked to some molecular “label” that enables them to be detected easily. Labeled antibody tests using radioactive or fluorescent labels can be used to detect either antigens or antibodies. In the following sections we will consider fluorescent antibody tests, ELISA, and the western blot test.

**Fluorescent Antibody Tests**

Fluorescent dyes are used as labels in several important serologic tests. One of these dyes is fluorescein, which can be chemically linked to an antibody without affecting the antibody’s ability to bind antigen. When exposed to ultraviolet light, fluorescein glows bright green. Fluorescein-labeled antibodies are used in direct and indirect fluorescent antibody tests. Direct fluorescent antibody tests identify the presence of antigen in a tissue. The test is straightforward: A scientist loads a tissue sample suspected of containing the antigen with labeled antibody, waits a short time to allow the antibody to bind to the antigen, washes the preparation to remove any unbound antibody, and examines it with a fluorescent microscope. If the suspected antigen is present, labeled antibody will adhere to it, and the scientist will see fluorescence. This is not a quantitative test—the amount of fluorescence observed is not directly related to the amount of antigen present. Scientists use direct fluorescent antibody tests to identify small numbers of bacteria in patient tissues. This technique has been used to detect Mycobacterium tuberculosis in sputum and rabies viruses infecting a brain. In one use, medical laboratory technologists employ
a direct fluorescent antibody test to detect the presence of yeast in a sample. Indirect fluorescent antibody tests are used to detect the presence of specific antibodies in an individual’s serum via a two-step process:

1. After an antigen of interest is fixed to a microscope slide, the individual’s serum is added for long enough to allow serum antibodies, if present, to bind to the antigen. The serum is then washed off, leaving the antibodies bound to the antigen (but not yet visible).

2. Antibodies against human antibodies (anti–human antibody antibodies; in this example, anti-IgG) labeled with a fluorescent dye are added to the slide and bind to the antibodies already bound to the antigen. After the slide is washed to remove unbound anti-antibodies, it is examined with a fluorescent microscope. The presence of fluorescence indicates the presence of the labeled anti-antibodies, which are bound to serum antibodies bound to the fixed antigen; thus fluorescence indicates that the individual has serum antibodies against the antigen of interest.

Indirect fluorescent antibody testing is used to detect antibodies against many viruses and some bacterial pathogens, including Neisseria gonorrhoeae, the causative agent of gonorrhea. Scientists routinely identify and separate B and T lymphocytes by using specific monoclonal antibodies produced against each cell type. The researchers can attach differently colored fluorescent dyes to the antibodies, allowing them to differentiate types of lymphocytes. Such identification tests can quantify the numbers and ratios of lymphocyte subsets, information critical in diagnosing and monitoring disease progression and effectiveness of treatment in patients with AIDS and other immunodeficiency diseases.

**ELISAs** is another type of labeled antibody test, called an enzyme-linked immunosorbent assay, the label is not a dye, but instead an enzyme that reacts with its substrate to produce a colored product that indicates a positive test. One form of ELISA is used to detect the presence and quantify the abundance of antibodies in serum. This test, which often takes place in commercially produced plates, has steps:

1. Each of the wells in the plate is coated with antigen molecules in solution.

2. Excess antigen molecules are washed off, and another protein (such as gelatin) is added to the well to completely coat any of the surface not coated with antigen.

3. A sample of each of the sera being tested is added to a separate well. Whenever a serum sample contains antibodies against the antigen, they bind to the antigen affixed to the plate.

4. Anti-antibodies labeled with an enzyme are added to each well.

5. The enzyme’s substrate is added to each well. The enzyme and substrate are chosen because their reaction results in products that cause a visible color change.

A positive reaction in a well, indicated by the development of color, can occur only if the labeled anti-antibody has bound to antibodies attached to the antigen of interest. The intensity of the color, which can be estimated visually or measured accurately using a spectrophotometer, is proportional to the amount of antibody present in the serum.
ELISA has become a test of choice for many diagnostic procedures, such as determination of HIV infection, because of its many advantages:

- Like other labeled antibody tests, ELISA can detect either antibody or antigen.
- ELISAs are sensitive, able to detect very small amounts of antibody (or antigen).
- Unlike some diffusion and fluorescent tests, ELISA can quantify amounts of antigen or antibody.
- ELISAs are easy to perform.
- ELISAs are relatively inexpensive.
- ELISAs can simultaneously test many samples quickly.
- ELISAs lend themselves to efficient automation and can be read easily, either by direct observation or by machine.
- Plates coated with antigen and gelatin can be stored for testing whenever they are needed.

A modification of the ELISA technique, called an antibody sandwich ELISA, is commonly used to detect antigen. In testing for the presence of HIV in blood serum, for example, the plates are first coated with antibody against HIV (instead of antigen). Then the sera from individuals being tested for HIV are added to the wells, and any HIV in the sera will bind to the antibody attached to the well. Finally, each well is flooded with enzyme-labeled antibodies specific to the antigen. The name “antibody sandwich ELISA” refers to the fact that the antigen being tested for is “sandwiched” between two antibody molecules. Such tests can also be used to quantify the amount of antigen in a given sample.

Western Blot Test

A technique for detecting antibodies against multiple antigens in a complex mixture is a western blot test. The name “western blot” is a play on words that refers to the similarity of this technique to a Southern blot test, named for the man who developed it. Western blots are also called immunoblots. Western blot tests are currently used to verify the presence of antibodies against HIV in the serum of individuals who are antibody-positive by ELISA. Compared to other tests, western blot tests can detect more types of antibodies and are less subject to misinterpretation. A western blot test has three steps:

1. Electrophoresis. Antigens in a solution (in this example, HIV proteins) are placed into wells and separated by gel electrophoresis. Each of the proteins in the solution is resolved into a single band, producing invisible protein bands.

2. Blotting. The protein bands are transferred to an overlying nitrocellulose membrane by absorbing the solution into absorbent paper—a process called blotting. The nitrocellulose membrane is then cut into strips.

3. ELISA. Each nitrocellulose strip is incubated with a test solution—in this example, those from each of six individuals who are being tested for antibodies against HIV. After the strips are washed, an enzyme-labeled anti-antibody solution is added for a time; then the strips are washed again and exposed to the enzyme’s substrate.
Color develops wherever antibodies against the HIV proteins in the test solutions have bound to their substrates, as shown in the positive control. In this example, the individual tested in strip 3 is positive for antibodies against HIV, whereas the other five individuals are negative for antibodies against HIV. Colored bands common to all patients are normal serum proteins.
Topic: Serological reactions: the purpose and a principle of reactions. Agglutination test. Precipitation test. CFT, IFT, ELISA, RIA.

QUESTIONS FOR DISCUSSION

1. The main principles and aims of the serological tests in medical practice.
2. Agglutination test and indirect (passive) hemagglutination test (PHAT): definition, mechanism, and practical use.
3. Precipitation reaction: identification, mechanism, types, practical use.
5. Complement fixation test (CFT): the aim of its carrying out, components, mechanisms.
6. Immunofluorescent test (IFT): the variety, aim of its carrying out, components, mechanism.
7. Enzyme linked immunosorbent assay (ELISA): the purpose of its carrying out, components, mechanism.
8. Radioimmunoassay (RIA): the purpose of its carrying out, components, mechanism.