GENERAL CONSIDERATIONS

Immunological and serological tests are used in the diagnosis of animal diseases since very long. By these methods, one can detect the antibodies specific to a particular infection in serum or antigen in different body tissues or excretions or secretions. The antibodies are produced in animal body as a result of body defense responses against the causative agents.

AGGLUTINATION

Agglutination is the clumping of particulate antigens such as bacterial cells in presence of an immune serum. For agglutination, the antigen should be in particulate form of suspension and the antigen-antibody reacting sites must be present on surface of the particles. The antibodies present in serum binds with particulate antigen forming antigen-antibody complexes which settles down leaving a clear supernatant in case of tube agglutination test.

Applications

1. Diagnosis of bacterial disease such as brucellosis, salmonellosis, yersiniosis.

2. Blood typing prior to transfusion.

3. Diagnosis of rickettsial diseases like Q-fever.

4. Diagnosis of viral diseases like influenza.

Various types of agglutination tests commonly used in the diagnosis of animal diseases are mentioned below.

1- Slide Agglutination Test

This method is quick, simple and requires smaller quantity of reagents. In this test, take a clean, dry glass slide and place a drop of antigen suspension over the middle area of the slide. Add one drop of test serum and mix properly with the help of clean glass rod or tooth pick. If the homologous antibodies are present, the clumps of bacterial cells will occur through agitation. The clumping can be seen by naked eyes or by using a light microscope.

2- Tube Agglutination Test

The tube agglutination test is used for the diagnosis and screening of herds for brucellosis in animals. To perform this test the two-fold serum dilutions are made in carbol saline as 1:5, 1:10, 1:20, 1:40..... and so on. An equal amount of brucella antigen is added and mixed properly with agitation of the rack holding tubes. These tubes are then kept at 37°C in incubator for 24 hours. If agglutination occurs, the clumps of antigen and antibody complexes will settle down leaving the clean supernatant. In case of no agglutination, the turbid suspension remains same. The standard tube agglutination is useful in diagnosis of those diseases in which immune carriers are present. The titer should be expressed as the highest dilution of antiserum which gives visible agglutinations.

3- Plate Agglutination Test

Plate agglutination test is used for rapid agglutination reaction. In this test, a concentrated antigen is used, e.g. in brucella, rose bengal plate agglutination test. The dye is also added in antigen to stain the bacterial cells which facilitates the visual reading. The most often used dyes are methyl violet, crystal violet, rose bengal or malachite green. In this test, 0.03 ml of serum (2-3 drops) is placed in wells of hemagglutination perpex plate and equal amount of coloured antigen is added and this is mixed with a bacteriological loop or needle. For thorough mixing the plate should be rotated by keeping on a flat surface in clockwise and anticlockwise direction and is incubated for 3 minutes at 37°C. In case of a positive test, large clumps of cells will form, which can be seen by naked eyes.

Hemagglutination Test (HA test)

Hemagglutination test does not depend on formation of antigen-antibody complexes and is used for the diagnosis of certain viral diseases and identification of viruses possessing hemagglutinin. To perform hemagglutination test, take a microtitre plate ‘U’ shaped bottom or test tubes in a rack. These should be properly washed, and dried. Place 50 µl phosphate buffer saline in all the columns of the microtitre plate. Take 50 µl virus antigen and place in wells of first column, mix it and transfer from it 50 µl to next and this make the two fold dilution of virus antigen like 1:2, 1:4, 1:8, 1:16, 1:32 and so on. From 11th well discard 50 µl ml mixture and leave the 12 column. Place 0.1 ml of the 1% RBC suspension in each column from 1-12, mix it by rotating the plate clockwise and anti-

clockwise and leave it in incubator at 37°C for one hour and examine it at 15 minutes interval to note the results.

1. Positive (HA) is indicated by agglutination of erythrocytes covering by a thin layer on bottom of wells (+).

2. A negative HA is determined by the button formation at the bottom of the plates (–).

Hemagglutination Inhibition Test (HI)

The hemagglutination inhibition (HI) test is performed in which the specific serum is added with a known virus dilution which neutralizes the hemagglutinins in virus and does not permit the agglutination of erythrocytes. In other words the agglutination of erythrocytes is inhibited by premixed antibodies. This is known as hemagglutination inhibition test. The HI test is sensitive and specific because it measures antibodies directed against surface proteins which can change antigenically. The test is simple, inexpensive and rapid and is, therefore, recommended for identification of hemagglutinating viruses, e.g. Newcastle disease virus. To perform the HI test place 50 µl PBS (pH 7.2) in well 1 to 12 of the first row of microtiter plate. Add 50 µl antiserum of having Newcastle virus antibodies to first well and make two-fold dilution till the 11^{th} well, leave 12th well as such using a separate pipette, place 50 µl of New castle virus antigen (4 HA units) to each well and keep the plate for 15 minutes in incubator at 37°C. Add 50 µl 1% chicken RBC in each well and incubate the plate for 30 minutes. Read the plate for agglutination of RBC's. In HI positive, there will not be any agglutination of RBC and the highest dilution of serum, inhibiting agglutination of erythrocytes will be the HI titer.

Positive: no agglutination of RBC.

Negative: clumping and agglutination of RBC.

Agar Gel Precipitation Test

The agar gel precipitation test is based on the ability of antibodies that can form precipitin lines with antigen in gel system. In this test, the antigen and antibodies are allowed to migrate towards each other in gel and a line of precipitation is formed at the zone the equivalence, where the antigen antibody complexes are formed, which is visible with naked eyes. The test is used for the detection of antibodies in serum or antigen in tissues and excretions or secretions. Microscopic glass slides (25 X 75 mm) or even small

petridishes may be used to carryout the test. These slides are precoated with either 0.3% agarose or impregnated in the agar. Agarose is poured on clean slide; 4-5 ml on microscopic slide to have a 2 mm thickness of agar on slides. The slides should be left for 15-20 minutes to solidify the gel and should be transferred to the humid chamber and placed at 4°C for 15-30 minutes. Thereafter, wells are cut using a gel cutter; there should be a central well and 4-6 outer well. Before cutting wells, prepare a diagram on a piece of graph paper for equal distance of wells. The optimum size of wells is 5 mm diameter and distance from central well to outer well should be not more than 5 mm. The gels of wells are removed using suction or needles. If antibodies are to be detected then place the antigen in central well and serum or test samples in peripheral wells using separate pipettes for each sample. If antigen is to be detected then place the known serum or antibodies in central well and test sample in peripheral wells. It is always desirable to have a positive control invariably along with test samples. Place the slides in humid chamber; large petridishes with wet filter paper or cotton inside and keep it at 37°C in incubator. After an incubation of 24 hours, examine the slides for precipitin lines in between the central and peripheral wells. In some cases, it may take long incubation period even upto 7 days or 24 hours at 37°C and 24 hours at 4°C in refrigerator. However, the slides should be examined in morning or evening daily for the development of precipitin lines because over incubation may also leads to the loss of sharpness of bands.

COMPLEMENT FIXATION TEST

The complement is a normal component of serum of animals; the highest concentration being present in guinea pig serum. The complement combines with antigen antibody complex to cause the lysis of bacterial cells. The complement fixation test is used for the detection of foot and mouth disease virus antigen in vesicular fluid for diagnosis and typing of the virus.

Material required:

1. Wasserman's tubes.

2. Known standard antigen suspension.

3. Patient's serum (heated at 55˚c for 30 minutes to destroy the complement).

4. Complement (fresh serum of guinea pigs).

Procedure:

1. Mix the materials, and incubate in water bath at 37˚c for 30 minutes.

2. Sensitized sheep erythrocytes 5% suspension (as indicator for the fixation of the complement).

3. Mix, and incubate in the water bath for 30-60 minutes, and read the result:

a. Positive: no hemolysis.

b. Negative: hemolysis.

Enzyme Linked Immunosorbent assay (ELISA)

ELISA used for the immunodiagnosis of infectious diseases to measure either antigen (direct, capture or sandwich ELISA), or antibody (indirect or competitive ELISA) .

(١)Direct ELISA :

1- The antibody specific for the antigen to be detected is adsorbed to the surface of the wells of the microtiter plate .

2- A sample containing unidentified antigen is then added to each well

3- A second antibody specific for the antigen is then added if both the antibody adsorbed to the wall of the well and the antibody known to be specific for the antigen have reacted with antigen a "sandwich" will be formed ،with the antigen between two antibody molecules. This reaction is visible only because the second added antibody is linked to an enzyme, such as horseradish peroxidase or alkaline phosphatase unbound enzyme linked antibody is washed from the well .

4- The enzyme's substrate is added to it . Enzymatic activity is indicated by a color change that can be visually detected .

5- The test will be positive if the antigen has reacted with adsorbed antibodies in the first step. If the test antigen was not specific for the antibody adsorbed to the wall of the well the test will be negative because the unbound antigen will have been washed away.

(٢)Indirect ELISA :

1- A known antigen from the laboratory, rather than the antibody, is added to the shallow wells on the plate .

2- The antiserum is added to the well. If the serum contains antibody specific to the antigen, the antibody will bind to the adsorbed antigen. All unreacted antiserum is washed from the well .

3- The conjugate (Anti-HISG) is then allowed to react with the antigen-antibody complex .

4- The anti-HISG, which has been linked with an enzyme, reacts with the antibodies that are bound to the antigens in the well .

5- Finally, all unbound anti-HISG is rinsed away and the correct substrate for the enzyme is added. A colored enzymatic reaction occurs in the wells in which bound antigen has combined with antibody in the serum sample