Winter School on

'RECENT ADVANCES IN DIAGNOSIS AND MANAGEMENT OF DISEASES IN MARICULTURE'

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Course Manual

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IMMUNO - DIAGNOSTIC TECHNIQUES IN AQUATIC ANIMAL DISEASES

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Diagnosis plays an important role in any disease control programme. The realization that techniques applied for human medicine and farm animal medicine can be equally used for aquatic animal disease management led to the application of all diagnostic tests in this field also. The immuno-diagnostic tests utilize the body's reaction against foreign substances. These reactions are of two types, one that secrete the specialized globulins (immunoglobulins) and the other generating sensitized cells. Though both these mechanisms can be utilized for diagnosis, the first one is extensively used because of its easy interpretation, convenience and simplicity.

When an antigen comes in contact with the specific antibody they interact with each other forming a union. This union occurs between the Fab fragment of antibody and the particular grouping of antigen (antigenic determinant). The molecules are held together by non-covalent intermolecular forces. The methods used to demonstrate the antigen antibody reactions form the basis of immuno-diagnosis.

The antigen antibody reaction can be detected at two levels. The primary union of these reactants can be demonstrated by labeling one of the reactants with a suitable marker. Such markers are, fluorescent dyes, enzymes and radioisotopes. The union is detected by the reaction exhibited by these markers. Another method employs the physical changes that occur in the antigen-antibody complex after their union. These changes are termed secondary phenomena. The initiation and development of secondary effect involve a series of many events, which involve a number of variables. These secondary effects are broadly put under agglutination, precipitation, activation of complement cascade and release of histamine from mast cells.

Agglutination tests

In this reaction the antigen is part of the surface of some particulate material. Antibody added to a suspension of such particles combines with the surface antigens and link them together to form clearly visible aggregates. There are a number of tests using agglutination. These are slide agglutination, tube and micro plate agglutination, latex bead agglutination, haemagglutination and haemagglutination inhibition.

Slide agglutination test

Two drops of saline containing 0.5% phenol are placed on a slide. Suspension of the test bacterium is prepared in this saline. One drop of antiserum is added to one drop of bacterial suspension. The slide is rocked to mix the antibody with the bacterial suspension. Visible agglutination develops within 5 minutes. A set of positive controls and negative control are to be used for checking the results.

Tube or micro titre agglutination

Serial dilutions of antibody are prepared in 0.5 ml volumes in test tubes and the same volume of antigen is added to the tubes and mixed. Instead of test tubes micro-plates having 96 wells are also used; 50 µl saline is taken in each well of 96 well of micro-titre plates. Serial doubling dilutions of the serum are prepared by adding 50 µl

serum to first well, mixing and transferring 50µl to the second well and continuing to the third and so on. A saline control is also set up. 50µl bacterial suspension is added to each well; mixed and plates are incubated overnight at ambient temperature in moist chamber. Agglutination results in irregular settling of antigen. The reciprocal of the antiserum dilution at the end point is known as titre of serum. If agglutination occurs at a dilution of 1/3000, the titre is 3000. To make agglutination clear the antigens can be stained with dyes.

Haemagglutination

In this test the agglutination is made more visible using erythrocytes. The soluble antigens are adsorbed on the cell membranes of mammalian erythrocytes. Commonly used cells are sheep erythrocytes, which are treated with tannic acid and subsequently with the antigen. The antibodies cross link the erythrocytes and produce agglutination. Blood cells after saline washing are suspended in PBS (0.6 ml packed cell are suspended in 10 ml PBS). 10 ml of 1/20000 tannic acid + 10 ml erythrocyte suspension are incubated for 15 minutes at 37°C. The cells are washed in PBS. A portion of cells is kept as negative control. To 1 ml of treated RBC suspension, 1 ml buffer containing antigen to be coated to the cells is added. The mixture is incubated at 37° C for 30 minutes. The cells are centrifuged, washed 3 times in buffer containing 1% rabbit serum. The coated and uncoated cells are suspended in PBS containing 1% rabbit serum to give 1% cell suspension. Agglutination test is performed using coated RBC suspension in place of antigen suspension. Plates are left for 2 hours at ambient temperature and left at 4° C overnight. The settling of RBC in compact button form indicates negative test. Positive reaction indicated by formation of irregular clumps. Uncoated RBC suspension forms negative control.

Haemagglutination inhibition test

Certain viruses agglutinate RBCs of several animals. Mixing the virus with the appropriate red cell in the presence of antiserum inhibits haemagglutination. This test can be used for testing suspected patient's serum for specific antibody and for proving identity of virus using known antiserum

Latex agglutination

This is used to detect toxins released by microbes. Polystyrene latex particles are sensitized with antiserum. The cultures are freed of cells by centrifugation and supernatant diluents are prepared in wells of micro titre plates by serial doubling dilutions. To the wells add an equal quantity of sensitized latex suspension. Mix the contents of plates by rotating the plate. The plate is kept in a humid chamber at room temperature for 20-24 hours. Irregular settling of latex particles indicates the positive reaction.

Precipitation tests

When antigen molecules are in soluble form, they combine with antibodies to form insoluble complexes. These complexes will precipitate and produce cloudiness in solution. This aggregate formation will reach maximum, when the antigen and antibodies are in optimal proportion. If one component is in high proportion the precipitation will be inhibited. The precipitation can be performed in test tubes. This test is used for detecting and identifying antigens, for typing of microbes. The technique is also used in forensic studies for detecting adulteration of foodstuffs. The modifications of this test are the immuno-diffusion techniques. The antigen and antibodies are allowed to diffuse and a

concentration gradient forms in gel. The precipitation bands form in the gel in the position where the antigen and antibody reach optimal proportion after diffusion.

Simple test tube precipitation

Antiserum is taken in a test tube. Layer an extract of antigen/ microbe without mixing. Incubate the tubes at room temperature. Haziness will start appearing in the junction where the two suspensions meet.

Agar gel percipitation (Ouchterlony's immuno diffusion)

1% Agrose gel is prepared in phosphate buffer and poured in to a petri dish. Once the gel cools, wells are cut in gel layer. Antiserum antigen solutions are placed opposite to each other in the wells. The gels are incubated at 37° C in a damp chamber for a few days. Precipitin bands will form, where antigen and antibody meet.

Single radial immuno-diffusion

Antiserum is incorporated in agrose gel prepared in PBS (antiserum: gel = 1: 9). The gel is melted in water bath. When it is cooled to 50° C add the antiserum and mix it with agrose. The gel is poured in petri dish. Wells are cut. Place antigen solution in the well and incubate at 37° C. The formation of a ring of precipitation around the well indicates positive reaction. The precipitation ring forms at a distance from the rim of the well, where the optimum concentration of antigen is attained. Hence the diameter of ring of precipitation is directly proportional to antigen concentration. Using different known concentrations of antigen in single radial immuno diffusion test, the diameters of precipitation rings can be plotted in a graph against concentrations of antigen. In this way the concentration of antigen in a test sample can be directly found from the plotted graph.

Immuno-electrophoresis

A number of immuno diffuson tests use the help of movement of colloids in an electric field. The electrophoresis separates the antigen mixtures. These separated antigens are used to perform precipitation reactions with antibody. This is commonly used for studying serum proteins. A well is made in electrophoretic agrose gel 1/3 distance away from one end of the gel in the midline and a narrow trough is made parallel to the well extending 2/3 length of the gel. The well is filled with the antigen mixture with indicator dye, and the electrophoresis performed till the indicator dye reaches 2/3 distance of gel.(the gel is oriented with the antigen well near the cathode). After termination of electrophoresis the trough is filled with the antiserum and the gel incubated in a moist chamber as in immuno-diffusion. Several precipitation arcs will form in the gel, each representing one antigen.

Rocket electrophoresis

Antiserum is incorporated in the agrose gel (prepared in electrophoresis buffer) as in single radial immuno-diffusion. Then wells are cut in the gel, filled with antigen and electrophoresis is performed. Rising arc will be formed in the gel. The height of arc is proportional to antigen concentration. Like single radial immuno-diffusion a standard curve can be plotted for estimation of antigen.

Two dimensional immuno-electrophoresis

A glass plate area is divided in two halves by holding another glass plate vertically. In one half, ordinary electrophoretic gel is poured and allowed to solidify. The glass plate is removed after solidification of gel. The other half is filled with antiserum-incorporated gel. In the ordinary gel, a well is cut and antigen is filled. Electrophoresis is

performed connecting the ends of ordinary gel to buffer tanks after this electrophoresis, the plate turned 90° The ordinary gel portion is connected to buffer in cathode tank, while the antibody incorporated gel end is connected to anode tank. The electrophoresis is again performed, which result in development of arcs corresponding to each component of antigen mixture.

Counter current electrophoresis

This is a test, which demonstrate even minute quantity of antigen, hence a powerful diagnostic technique. Two wells are made in the gel in longitudinal direction the well near the cathode is filled with suspected antigen and the anode end well is filled with antiserum. Electrophoresis is run for one hour. In positive cases precipitation arc develops between two wells.

Activation of complement

Complement fixation test detects the presence of antibody in an animal or group of animals. Antibody combining with antigen exposes its FC portion, which makes the receptor to combine with the complements. Activation of complement system results in the lysis of cells bearing antigen. The test system consists of antigen (virus), dilutions of antiserum (serum is heated at 55 °C for 15 minutes) and a complement source (guinea pig serum). This system is incubated at 37 °C for 45 minutes. Indicator system consisting of SRBC and anti sheep erythrocyte serum (haemolysin) is added, absence of haemolysis after incubation indicate positive reaction. Positive and negative controls have to be run along with the test. This is highly effective for virus detection. The test has to be performed with different dilutions of antiserum and haemolysin.

Labeling techniques

Radioimmunoassay.
Fluorescent antibody technique.
Enzyme-Linked Immunosorbent Assay. (ELISA)
Immuno-Peroxidase

Fluorescent antibody technique

Fluorescein isothiocynate, and rhodamine isothiocynate produce fluorescence under UV light. Antibodies are conjugated with these dyes. Infected tissue smears are prepared, dried and fixed. A drop of conjugated antibody solution is placed over the smear. Incubate at room temperature for 15 minutes. Positive and negative controls are set up in parallel. The slide is washed thrice in PBS allowed to dry in dark. The slide is observed in a fluorescence microscope using appropriate UV light. 'Coombs' test is an indirect method and is more specific. Label an antiglobulin serum with the fluorescent dye. The slide/ smear is treated with the specific antisera, followed by labeled serum. The antigen in smear will combine with the antibody of first sera, which will be the antigen for the antibodies in the second sera.

Radioimmunoassay

Mostly used in the estimation of hormones. It is also used in the estimation of immunoglobulins like IgE. The radioiodine labeled antigen of competes with non-labeled antigen of a sample under test, for the antibodies with which the labeled antigen and test sample are mixed. The more of antigen (hormone) in the test sample the less chance the labeled hormone has of combining with the limited number of antibody molecules that are available in the anti-hormone serum. Measuring quantity of labeled hormone

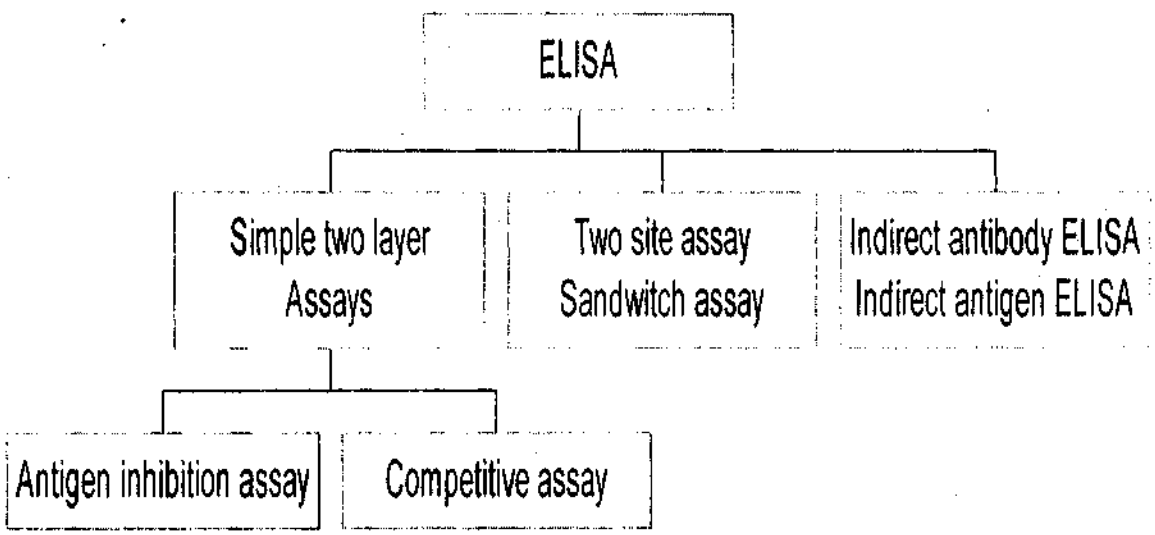
combined with antibody a measure of the hormone in the test sample can be obtained. Hormone antibody complexes are separated by electrophoresis or the antibody linked to an insoluble support (cellulose).

Enzyme labeling

- Enzyme Linked Immunosorbent Analysis (ELISA)
- Immuno-Peroxidase

Enzyme Linked Immunosorbent Analysis (ELISA)

Most antigens bind spontaneously to plastic surfaces like polystrene. Antibodies also attach while retaining their antigen binding activity. In subsequent steps, one or more layers of the solid phase captured immune complexes are formed. A variety of enzymes can be chemically coupled to either antibody or antigen without any change in their biological activity. An enzyme conjugate in antigen antibody complex leaves enzyme component available for substrate action. Addition of the substrate with the chromogen results in colour change. The reaction can be stopped at an appropriate stage and the colour signal determined by visual comparison with an appropriate standard or by optical density measurement. When the test requires the solid phase itself, the substrate should form an insoluble coloured precipitate. ----Dot. Enzymes commonly used are horseradish peroxidase, alkaline phosphatase and streptavidin (Avidin biotin complex).



Antigen inhibition assay

Coat the wells with standard antigen. Antibody enzyme conjugate is diluted to get optimal binding. The conjugate so diluted can be quantitatively inhibited by traces of free antigen in test samples. The diluted conjugate is then re-incubated with different dilutions of positive test samples in coated wells (the reactions are carried out in antigen coated wells). After washing the substrate is added there is inhibition of colour development. This inhibition is inversely proportional to the concentration of antigen in test sample.

Competitive assay

Wells are coated with specific antibody. Enzyme labeled standard antigen is titrated on the antibody to determine optimal dilution, which is easily competed for by free antigen. Standard free antigen dilutions are then used in competition with labeled antigen to construct a standard competition curve. Antigen from test samples is estimated by the inhibition.

Two-site (sandwich) assay

Antibody is coated on wells. Test samples are added to the antibody coated wells. The complex is exposed to diluted reference antibody conjugate to the same antigen. The test is carried out under the assumption each antigen molecule has multiple epitopes for antibody attachment, hence free antibody combining sites will be available after combination with coated antibody.

Indirect antibody ELISA or indirect antigen

Valuable tool to detect specific antibody in epidemiological studies. Antigen is coated in wells, followed by application of antiserum. Specific antibody is detected by the use of anti-globulin conjugate directed against first immunoglobulin (first antibody). It can utilize a "Universal" ant species immunoglobulin.

ELISA-Avidin- biotin system

The low molecular weight coenzyme, biotin can be linked to the antibody using biotinyl-N-hydroxysuccinimide. It has less risk of steric interference with the binding site. The enzyme is brought into solid phase by using enzyme-coupled streptavidin, which has high affinity for biotin. One molecule of avidin bind with 4 molecules of biotin.

Immuno-histochemistry and immunoperoxidase

Permanent preparation can be made. Chemical and molecular components of cells can be studied. Enzymes such as horseradish peroxidase, alkaline phosphatase and glucose oxidase are used for conjugating antibodies. These reactions can be performed on histological section. Hence it is widely used for demonstration of macromolecules, microbial antigen in tissues/cells. The reaction products are insoluble coloured precipitates. These precipitates can be viewed under conventional microscope. The precipitate can also made electron dense by osmium fixation, hence electron microscopic studies are possible. Conjugating antibodies with gold and treating the section with conjugated antibodies enables electron microscopic detection of antigens. Direct and indirect methods are available.

Immuno-Dots or Dot blots

Modification of ELISA for practical field application. Antigen or antibody is spotted on to nitrocellulose paper using a fine pipette. The strip is then incubated in a blocking solution. Enzyme conjugated antibody or antigen is applied to the spot along with substrate.

Western blot analysis

This method combines enzyme-linked immunoassay with electrophoresis. Sample subjected to electrophoresis to separate components. The separated antigens are transferred to immobilising medium — nitrocellulose paper. Enzyme linked immunoassy is carried on the paper. This test is useful for characterization of antigens and antibodies. It can detect presence of antigens in tissue fluids, sera; blood etc. The test is also used for detection of antibodies in a population following exposure to pathogens. Poly acrylamide gel electrophoresis of suspected antigen source is done first. Blotting of separated proteins to nitrocellulose paper follows this. Either capillary blotting or electro-blotting achieves this.

Blotting of separated proteins to nitrocellulose paper

Capillary blotting

2-3 sheets of absorbent filter paper are saturated with transfer buffer and connected to buffer reservoirs. The gel is placed on top of the filter paper and the nitrocellulose paper is placed on the top of gel. 2-3 dry layers of dry blotting paper and several layers of paper towels are placed above the transfer paper and gel. A one-litre flask filled with water is placed over the paper towels. Blotting take 24- 48 hours.

Electro-blotting

It is done as per instructions of the manufacturers. Two or three layers of Whatman filter paper soaked in cathode buffer are placed over cathode plate of transfer system. The gel is placed over the filter papers and the nitrocellulose paper moistened with distilled water placed over the gel. Layers of the filter paper soaked in anode buffer follow this. Anode plate is placed in position and the system is connected to power supply. Electrophoretic transfer can be carried out at 100 volts for about 1.5 hours. After transfer the nitrocellulose paper is placed in an appropriate blocking buffer. Nitrocellulose paper is washed with tween tris buffered saline. Nitrocellulose paper is placed in diluted antibody for 1 hr at room temperature. Washing of nitrocellulose paper followed by placing the paper in conjugated antibody solution.

Though cell mediated reactions are of diagnostic value, they cannot be routinely used because of high technical skill required and the non-specific nature of many of these tests. However delayed hypersensitivity reactions are useful in diagnosing chronic bacterial diseases (tuberculin reaction in Mycobacterial infections) and systemic fungal infections.

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