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Special Issue: Manual guidance of veterinary clinical practice and laboratory

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Research Article

Serological and immunological test

Immunological and serological methods can detect the antibodies specific to a particular infection in serum or antigen in different body tissues or excretions or secretions. The animal diseases have been diagnosed by immunological and serological test since very long. The antibodies are produced in animal body as a result of body defense responses against the causative agents. However, detection of antibodies in serum is not sufficient to conclude diagnosis of animal's disease. The presence of antigen in body tissues or fluids directly correlates with the presence of incrimination agent in the system. It is, therefore, advisable that paired sera should be tested before arriving at any diagnosis. The detection of antigen is considered more specific for diagnosis. Here, many serological and imrnunological tests are described and the readers may use any of these according to their suitability and availability of materials. Serology is the science which study about serum and other body fluids. In laboratory term, serology is termed to the diagnostic identification of antibodies in the serum [1].

Serologic testing for infectious agents or for an antibody response demonstrating exposure to an infectious agent is one of the most common ways immunologically based tests are used in clinical medicine. Immunologic testing is also used to evaluate the immune system itself in cases of suspected hypersensitivity or autoimmunity, to estimate resistance to a particular disease, or to document an immunodeficiency state. Immunologic principles are used in diagnostic tests not intended to evaluate either infection or immunity. Serologic tests can be classified as tests of primary antigen-antibody interaction, secondary tests of interaction, or tertiary tests of interaction [2].

Agglutination test

Agglutination is the clumping of particulate antigens, such as bacterial cells, in presence of an immune serum or antibody or complements. For agglutination, the antigen should be in particulate form of suspension and antigen-antibody reacting sites must be present on the surface of the particles. The interaction between antibody and a particulate antigen results in visible clumping called agglutination. Agglutination reactions are more sensitive than immune precipitation [3].

The application of agglutination is to:

- ✓ In diagnosis of bacterial diseases such as brucellosis, salmonellosis, yersiniosis.
- ✓ Blood typing prior to transfusion.
- ✓ Diagnosis of rickettsia1 diseases like Q-fever.
- ✓ Diagnosis of viral diseases like influenza.

Various types of agglutination tests commonly used in the diagnosis of animal diseases are mentioned below Agglutination test commonly used in the diagnosis of domestic animals at veterinary laboratory can be classified into slide agglutination test, Tube agglutination test, capillary tube agglutination test and others [3-5].

Slide methods of agglutination

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 a clean glass rod or toothpick. If the homologous antibodies are present, clumps of bacterial cells will occur through agitation. The clumping can be seen by naked eyes or by using a light microscope. A saline-antigen control should also be used for comparison of the results. The test is mostly used for identification of salmonella organisms or pollorum disease in poultry [5].

Tube agglutination test

The tube agglutination test is used for the diagnosis and screening of herds for brucellosis in animals [6,7]. For diagnosis of brucellosis, a 0.5% carbol saline is used in cattle and buffaloes and 5.0% carbol saline for sheep and goats. To perform

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this test 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 into tubes and kept at 37 °c in incubator for 24 hrs. 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. There are some sets up to which the test is considered to be negative, e.g. brucellosis positive reactions at 1:20 and 1:40 dilutions are interpreted as negative and doubtful respectively [6,7].

The titer should be expressed as the highest dilution of antiserum which gives visible agglutination and expressed in IU (International Units). In the brucellosis it is considered 40 IU positive in sheep and goats and 80 IU in man and cattle. To avoid discrepancies in the results, the paired sera should be tested and an increase in fourfold titer of agglutination should be considered as a result of recent infection [8].

ELISA Test in veterinary laboratory practice

ELISA (Enzyme linked immune sorbent assay) is an antigen antibody reaction. In 1971, ELISA was introduced by Peter Perlmann and Eva Engvall at Stockholm University in Sweden. It is a common laboratory technique which is usually used to measure the concentration of antibodies or antigens in blood. A number of enzymes have been used for ELISA such as alkaline phosphatase, horse radish peroxidase and beta galactosidase. Specific substrate such as ortho-phenyldiaminedihydrochloride (for peroxidase), paranitrophenyl phosphate (for alkaline phosphatase) are used which are hydrolyzed by above enzymes to give colored end product. Antibodies or antigens present in serum are captured by corresponding antigen or antibody coated on to the solid surface[9].

Enzyme linked immune sorbent assay methods can be classified in to three based on the binding structure between antibody and antigens such as: indirect, direct and sandwich method [9]. ELISAs are typically performed in 96-well (or 384-well) polystyrene plates, which will passively bind antibodies and proteins.A positive control serum and a negative control serum would be included among the 96 samples being tested in 96 well as it described (Figure 1) [10]. ELISA is the abbreviation of enzyme-linked immunosorbent assay. It is a useful and powerful method in estimating mg/ml to µg/ml ordered materials in the solution, such as serum, urine, sperm and culture supernatant [11].

Direct ELISA

For direct detection, an antigen coated to a multi-well plate is detected by an antibody that has been directly conjugated to an enzyme. Direct ELISAs involve attachment of the antigen to the solid phase, followed by an enzyme-labeled antibody [9,10]. Direct ELISA test is considered to be the simplest types of ELISA the antigen is adsorbed to a plastic plate, then an excess of another protein is added to block all the other binding sites. After excess enzyme- antibody complex is washed off, enzyme antibody bound to antigen left [12].

Procedure (Figure 2):

- A buffered solution of the antigen to be tested for is added to each well of a microtiterplate; where it is given time to adhere to the plastic through charge interactions.
- A solution of nonreacting protein, such as bovine serum albumin or casein, is added to well (usually 96-well plates) in order to cover any plastic surface in the well which remains uncoated by the antigen.
- The primary antibody with an attached (conjugated) enzyme is added, which binds specifically to the test antigen coating the well.
- A substrate for this enzyme is then added. Often, this substrate changes color upon reaction with the enzyme. The higher the concentration of the primary antibody presents in the serum, the stronger the color change. Often, a spectrometer is used to give quantitative values for color strength.

Indirect ELISA

Procedure (Figure 3):

* Antigen is coated in microtiter well
* Coat the micro titer plate wells with antigen.
* Block all unbound sites to prevent false positive results.
* Add sample containing antibody (e.g. rabbit monoclonal antibody) to the wells and incubate the plate at 37°c.

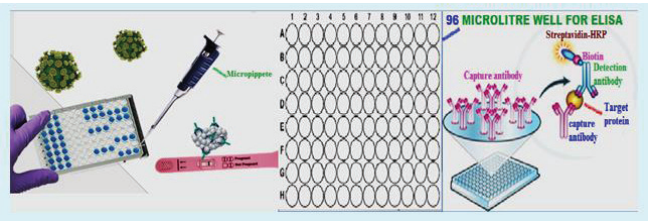

Figure 1: The microliters well for ELISA.

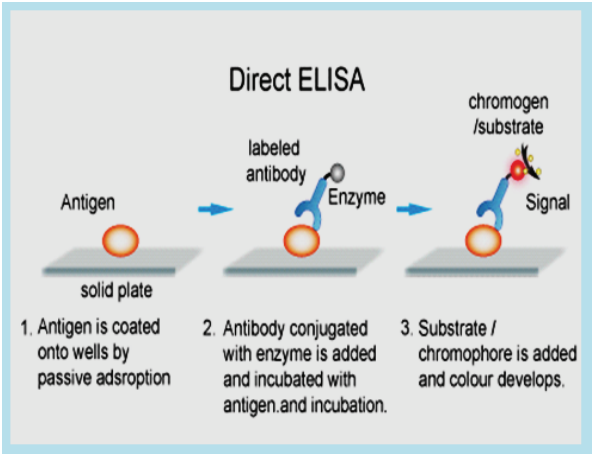

Figure 2: Direct ELISA and its procedure.

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* Wash the plate, so that unbound antibody is removed.
* Add secondary antibody conjugated to an enzyme (e.g. anti- mouse IgG).
* Wash the plate, so that unbound enzyme-linked antibodies are removed.
* Add substrate which is converted by the enzyme to produce a colored product.
* Reaction of a substrate with the enzyme to produce a colored product

Sandwich Method

In methods of sandwich, antibody is coated in microtiter.

Procedure (Figure 4):

- Prepare a surface to which a known quantity of antibody is bound.
- Add the antigen-containing sample to the plate and incubate the plate at 37°c.
- Wash the plate, so that unbound antigen is removed.
- Add the enzyme-linked antibodies which are also specific to the antigen and then incubate at 37°c.
- Wash the plate, so that unbound enzyme-linked antibodies are removed.
- Add substrate which is converted by the enzyme to produce a colored product.
- Reaction of a substrate with the enzyme to produce a colored product

Competitive methods

The antigen-antibody mixture is then added to the microtitre well which is coated with antigen.

Procedure (Figure 5):

- Antibody is incubated with sample containing antigen.

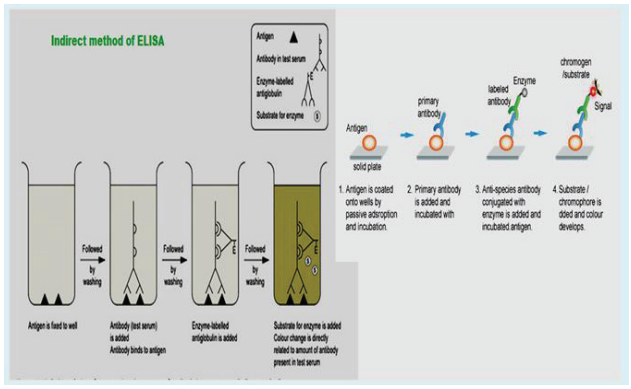

Figure 3: Indirect ELISA procedure [8].

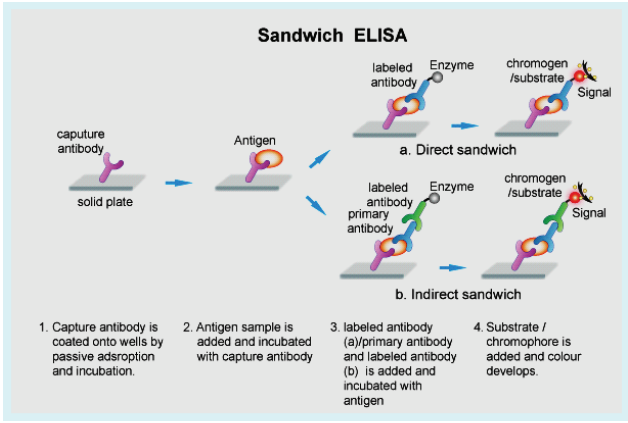

Figure 4: Procedure of sandwich ELISA techniques.

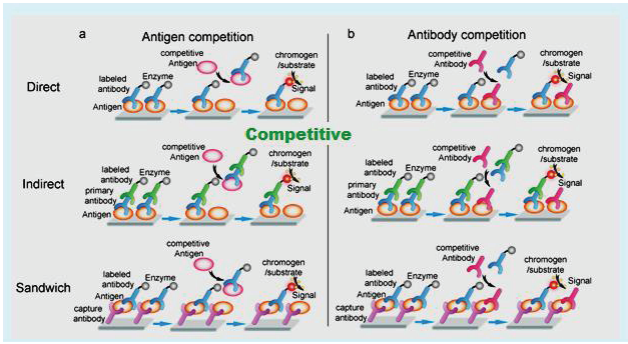

Figure 5: Competitive ELISA procedure.

- Antigen-antibody complex are added to the microtitre well which are pre-coated with the antigen.
- Wash the plate to remove unbound antibody.
- Enzyme linked secondary antibody which is specific to the primary antibody is added.
- Wash the plate, so that unbound enzyme-linked antibodies are removed.
- Add substrate which is converted by the enzyme into a fluorescent signal.

Allergic test: Histologically, the area which has been injected with allergen is heavily infiltrated with mononuclear cells comprising mainly of macrophages and lymphocytes. The cell involved in allergic reactions ate T-lymphocytes, microphage, regulatory B-cell and basophiles [13,14]. Allergic reaction test is being used to diagnosis chronic disease like tuberculosis, Para tuberculosis and glander. The allergens are prepared by killing the causative organisms of disease and filtering them from culture media. These allergens are named after their principal organisms like tuberculin for *Mycobacterium tuberculosis*. Special purified protein derivative (PPD) is used now a days for eliciting delayed type of hypersensitivity reactions and detection of reactors. Single intradermal (SID) and comparative intradermal (CID) tests are recommended for routine screening of animals to detect the reactor of tuberculosis and Johne's disease.

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Single Intradermal (SID) Test: Single intradermal (SID) test: This test is applied by the intradermal injection of 0.1ml of bovine tuberculin PPD into a skin fold at the base of the tail or into the cervical fold and the subsequent detection of swelling as a result of delayed hypersensitivity. The reaction is read between 48 and 96 hours after injection with a preference for 48 – 72 hours for maximum sensitivity and at 96 hours for maximum specificity. The positive reaction constitutes a diffuse swelling at the site of injection [15].The main disadvantage of the SID test is its lack of specificity and the number of no visible lesion reactors (NVLs) which occur.

Mammalian tuberculin is not sufficiently specific to differentiate between reactions due to infection with *M. bovis* and infection with *M. avium*, *M. tuberculosis* and *M. paratuberculosis* including vaccination or *Nocardiafarcinicus*. The maximum permissible of NVL reactors is 10% and when this rate is exceeded, tests other than the SID test should be used [16]. The other disadvantages of SID test include failure to detect cases of minimal sensitivity, in old cows and in cows which have recently calved; as well as in early infection, in some cattle in an unresponsive state, referred to as energy which is developed due to antigen excess or immunosuppression which in-turn caused by nonspecific factors such as malnutrition and stress [17].

Tuberculin Test: Tuberculin was invented by koch in 1890, and was first used experimentally in treating tuberculosis in man. Numerous experiment showed this to be the case and since 1891 the use of tuberculosis as the diagnositic agents for tuberculosis of cattle. Tuberculin is the sterilized and filtered glycerin extract of cultures tubercle basilli. It is cantains the cooked products of the growth of these bacilli, but not the bacillli themselves [18]. Tuberculin testing is done for the diagnosis of tuberculosis in animals and birds. In bovines, the tuberculin is injected in the skin of neck; in pigs, in ear and in birds, in wattle.

Tuberculin skin test: The tuberculin test based on a delayed type hypersensitivity to *mycobacterial* tuberculo-protein, is the standard ante-mortem test in cattle [17]. It is convenient, cost effective method for assessing cell mediated responses to a variety of antigens and it is "gold standard" for diagnostic screening for detection of new or asymptomatic *M. tuberculosis* complex infection [19]. The reaction in cattle is usually detectable 30-50 days after infection [20]. The tuberculin is prepared from cultures of tuberculosis or *M. bovis* grown on synthetic media [21]. The tuberculin test is usually performed between the mid necks, but the test can also be performed in the caudal fold of the tail.

The skin of the neck is more sensitive to tuberculin than the skin of the caudal fold. To compensate for this difference, higher doses of tuberculin may be used in the caudal fold of the tail [22]. Bovine tuberculin is more potent and specific and the potency of tuberculins must be estimated by biological methods, based on comparison with standard tuberculins and potency is expressed in the international unit (IU) [23]. In several countries, bovine tuberculin is considered to be of acceptable potency if its estimated potency guarantees per bovine dose at least 2000 IU in cattle. In cattle with diminished allergic sensitivity, a higher dose of bovine tuberculin is needed and the volume of each injection dose must not exceed 0.2ml [24]. Cell mediated hypersensitivity, acquired through infection can be demonstrated systematically by fever or ophthalmically by conjunctivitis, or dermally by local swelling, when tuberculin test or its purified protein derivative (PPD) is given by the subcutaneous, conjunctival or intradermal route, respectively [25].

Procedure [26]:

- In single intradermal test, 0.1 ml of tuberculin or PPD is injected intradermally in the middle portion of neck of bovines, a site preferred due to maximum sensitivity.
- Prior to tuberculin injection, the skin should be cleaned, shaved and thickness of skin fold measured using a Vernier calliper.
- The reaction can be read after 72 hours of the injection; the positive reaction is characterized by a hot, red and painful swelling with an increase of 4 mm or more in skin fold. However, some diagnostic consider that skin thickness should be double to declare a positive.

Johnin test

Johnin purified protein derivative (PPD) procured from IVRI, Izatnagar was used for the test. One square inch area in mid neck of each goat was shaved and mopped with medispirit. Skin thickness prior to injection was measured using a sliding vernier calipers and 0.1 ml of Johnin PPD was injected intradermally. A pea sized bubble was formed at the site of injection. After 72 h skin thickness was measured and an increase in skin thicknessof 4 mm or above from initial reading was taken as positive [27]. An intradermal injection was given to each animal on the left side of the neck at the crossing point of a line between the ear and the shoulder joint and a line between the jaw and the shoulder crest [27].

Procedure of johninn test:

* First restraint animals and clean and shave the area of cervical region
* Inject 0.1 ml of johnin in intradermally route into cervical shaved area
* If tuberculin and johnin test done at the same time, just inject johnin in opposite side of neck
* An increase of 5 mm or more in thickness of skin fold after 72 hours is considered positive for Johne's disease.
* This test is also not able to detect cases of minimal sensitivity such as may occur in the early or late stages of disease, in old cows and in cow which have recently calved.

Double Intradermal Test (DID): The double intradermal test is performed in same manner as in single intradermal test, the only difference is that a second injection of tuberculin or

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Johnin is given 7 days after the first injection at the same site and the skin thickness is measured 24 hrs after the last injection. In positive reactor, there should be an increase of 5 mm or more in skin thickness. Double intradermal test is useful to detect those animals which are poorly sensitized for any reason.

Comparative Test: Comparative intra-dermal (CID) test: In this test, two sites on the mid neck, 10-12cm apart, are shaved and the thickness is measured in millimeters with caliper before the injection of tuberculin [28]. In the CID test, 0.1ml of avian PPD and 0.1ml of bovine PPD are injected intradermally into separate clipped sites on the side of the neck. Care must be taken in placing the injection as varied from place to place in the skin. After 72 hours the thickness of the skin at the sites is measured again [29].

When the change in skin thickness is greater at the avian PPD injection site, the result is considered negative for BTB. When the change in skin thickness increased at both sites, the difference between the two changes is considered. Thus, if the increased in the skin thickness at the injection site for the bovine (B) is greater than the increase in the 1+-skin thickness at the injection site at the avian (A) and (B – A), is less than 1mm, between 1 and 4 mm, or a 4 mm and above, the result is classified as negative, doubtful, or positive for bovine tuberculosis, respectively and the animal with the evidence of infection is termed as reactor [16].

The comparative test is used to differentiate between animals infected with *M. bovis* and those responding to bovine tuberculin as a result of exposure to other *mycobacteria*. This sensitization can be attributed to the antigenic cross reactivity among *mycobacterial* species and related genera [30].

Short thermal test: Intradermal tuberculin (4ml) is injected subcutaneously into the neck of cattle which have a rectal temperature of not more than 39°C (102°F) at the time of injection and for 2 hours later. If the temperature at 4, 6 and 8 hours after injection rises above 40°C (104°F), the animal is classed as a positive reactor. The temperature peak is usually at 6 - 8 hours and is generally over 41 °C (105.8°F) [21].

Stormont test: Stormont test is a more sensitive test than short thermal test of tuberculosis in cattle. This test relies on the increased sensitivity of the test site, which occurs after a single injection [31]. The test is performed similarly to the SID test in the neck with a further injection at the same site 7 days later. An increase in the thickness of 5mm or more, 24 hours after this second injection, is a positive result. The loss of sensitivity is probably due to the general immunological hyporeactivity that occurs associated with parturition [32].

Mallein Test: The mallein is a bacteria free filtrate from a fluid cultivated of *Pseudomonas mallei*, the cause of glanders inequine. The mallein test is routinely test to screen the equines for glanders and is considered quite specific. As the glanders is a communicable and notifiable disease, the test has its significance in detecting positive reactors in eradication programme of disease.

Procedure of mallein test:

- ✓ Inject 0.2 ml of mallein intradermally into skin of lower eyelid using tuberculin syringe
- ✓ After 48 hours the positive reaction like edema of eyelid within blepharospasim and severe purulent conjunctivitis will be marked.

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