



Laboratory Manual

VETERINARY MICROBIOLOGY

VMC Unit-IV



Department of Veterinary Microbiology
College of Veterinary & Animal Sciences, Udgir
MAHARASHTRA ANIMAL & FISHERY SCIENCES UNIVERSITY, NAGPUR

**LABORATORY MANUAL
FOR
VETERINARY MICROBIOLOGY**

VMC

(New Syllabus As Per MSVE 2016)

Unit – IV

Veterinary Microbiology

Compiled
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FOR
VETERINARY MICROBIOLOGY**

CERTIFICATE

Certified that this is a bonafide record of practical work done in the laboratory for the course of **VETERINARY MICROBIOLOGY (VMC) Unit IV** during the year.

Name of the student: _____

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Evaluated the practical record submitted for the Annual Practical Examination held on _____.

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COLOUR PLATE

PLATE 1

1. RBPT: Plate Agglutination Positive Test
2. RBPT: Plate Agglutination Negative Test
3. Hemagglutination Inhibition test
4. Radial Immunodiffusion Test
5. Counter Immuno-electrophoresis

Practical 1

PREPARATION OF ANTIGEN AND RAISING ANTISERUM AGAINST WHOLE CELL BACTERIN

PREPARATION OF ANTIGEN

I. Heat killed / formalinized whole cell bacterin

1. Plate out the bacterial culture from appropriate stock culture onto a BHI agar plate, or other suitable medium. Incubate overnight at 37°C ($\pm 2^\circ\text{C}$).
2. Bacterial Growth is transferred to normal saline solution. Thoroughly mixed .
3. Expose to heat by heating the broth culture for 30 min at 100°C (Free Steam). OR Add Formalin (40% w/v) was added to the broth culture at a final concentration of 0.5% (V/V) and left 48 hrs at room temperature.
4. Centrifuge the killed bacterial suspension and resuspend the sediment in a small volume of normal saline. Repeat the step twice or thrice.
5. Adjust the opacity of suspension to the tube No.4 of Mac Farlands nephelometer by adding required amount of normal saline.
6. The antigen thus prepared can be used for immunization or testing antibodies against specific antigen by agglutination tests.

Preparation of somatic antigen *Salmonella typhi* antigen-Widal Test

- i) Plate out the Salmonella culture from the appropriate stock culture onto a BHI agar plate, or other suitable medium, for single colony growth. Incubate overnight at 37°C ($\pm 2^\circ\text{C}$).
- ii) Using a Pasteur pipette, wash off the culture, preferably inside a safety cabinet, with approximately 2 ml of absolute alcohol, and transfer into a sterile universal container.
- vi) Leave the antigen for 4–6 hours at room temperature to enable the alcohol to kill the bacteria and detach flagellae.

vii) Spin the universal container in a bench-top centrifuge for 5 minutes at 1000 g. Pour off the liquid and add enough phenol saline to make the antigen up to an opacity equivalent to suspension with approximately 10⁸ colony-forming units/ml.

viii) Carry out standard titration with known serum to ensure that the antigen is positive for the required factor.

ix) Store in a refrigerator at 4°C until required.

Preparation of Reagents and Standards:

McFarland's standard

McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing. An example of such testing is antibiotic susceptibility testing by measurement of minimum inhibitory concentration which is routinely used in medical microbiology and research. If a suspension used is too heavy or too dilute, an erroneous result (either falsely resistant or falsely susceptible) for any given anti microbial agent could occur.

Original McFarland standards were mixing specified amounts of barium chloride and sulfuric acid together. Mixing the two compounds forms a barium sulfate precipitate, which causes turbidity in the solution. A 0.5 McFarland standard is prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate (BaCl₂•2H₂O), with 9.95 mL of 1% sulfuric acid (H₂SO₄).

The standard can be compared visually to a suspension of bacteria in sterile saline or nutrient broth. If the bacterial suspension is too turbid, it can be diluted with more diluent. If the suspension is not turbid enough, more bacteria can be added.

McFarland Nephelometer Standards

McFarland Standard No.	0.5	1	2	3	4
1.0% Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1.0% Sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1X10 ⁸ CFU/mL)	1.5	3.0	6.0	9.0	12.0
% Transmittance*	74.3	55.6	35.6	26.4	21.5
Absorbance*	0.08 to 0.1	0.257	0.451	0.582	0.669

*at wavelength of 600 nm

Normal saline 0.9% NaCl

Add 9g NaCl to 1000 ml D/W. Autoclave at 15 lbs for 15 minutes.

Phosphate-buffered saline (PBS)

Reagent	Amount to add (for 1× solution)	Final concentration (1×)
NaCl	8 g	137 mM
KCl	0.2 g	2.7 mM
Na ₂ HPO ₄	1.44 g	10 mM
KH ₂ PO ₄	0.24 g	1.8 mM

To prepare 1 L of 1× PBS, dissolve the reagents listed above in 800 mL of H₂O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H₂O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle or by filter sterilization. Store PBS at room temperature.

ANTISERA RAISING

Aim:- Preparation of Antiserum Against Brucella abortus.

Principle:- An antiserum is the serum containing specific antibodies against particular antigen. In Microbiology Laboratory such antisera are used for various serological tests (antigen-antibody reactions) for diagnosis of infectious diseases e.g. diagnosis of brucellosis by agglutination test. The antisera raising is usually done in laboratory animals like rabbit.

Requirements:- Brucella abortus Bacterin (prepared as per the procedure described in practical no.1), 1ml and 5ml syringes with 24 G 1inch needles , scissors, spirit, absorbent cotton, rabbits etc.

Schedule of Immunization:-

Sr.no.	Day	Dose	Route
1	First	0.1ml	Intravenous
2	Third	0.25ml	Intravenous
3	Fifth	0.5ml	Intravenous
4	Seventh	1.0ml	Intravenous
5	Ninth	2.0ml	Intravenous
6	Twelfth	3.0ml	Intravenous

Procedure:- Secure the rabbit properly. Clip the hair of the area over marginal ear vein and sterilize with spirit. Inoculate the antigen at appropriate dose rate as per the above schedule. Bleed the rabbit by cardiac puncture after one week of last

injection, separate the serum, inactivate at 56 °C for 30 minutes, add merthiolate as a preservative and store in screw cap vial in refrigerator.

Exercise

1. Explain:
 - a. Antigen
 - b. Bacterin
2. Enlist bacterial antigens in veterinary use
3. Define Hyperimmune serum.

References

Joseph Mcfarland, M.D. Jama. (1907). The nephelometer: An instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines.; XLIX(14):1176-1178.
Alsever, J.B., and Ainslie, R. (1941). N.Y. State J. Med., 41, 126.

Practical 2

SEPARATION AND PRESERVATION OF SERUM: CONCENTRATION OF IMMUNOGLOBULINS

Materials and reagents

1. Luria-Bertani broth (LB) medium: Bacto-tryptone, yeast extract
2. RNAase
3. Isopropanol
4. Ethanol

Equipment

1. Table-top centrifuge

Procedure

Objective: To separate the immunoglobulins from serum

Principle: The blood serum contains a number of different proteins. Among these largest amounts are made up by albumin and globulin. The globulins play an important role in defense mechanism of body since they are endowed with antibody activity and globulin processing antibody activity is referred as immunoglobulins.

The globulin can be separated from albumin on the basis of certain physical, chemical properties, which include their solubility in salts and solvents, electrophoretic mobility, molecular size and sedimentation in ultracentrifuge.

The globulin may be precipitated with saturated ammonium sulphate solution. Globulins are of two types' euglobulin and pseudo globulins. Euglobulins are gamma globulins (antibodies) and are insoluble in distilled water while pseudo globulins are alpha & beta globulin & are soluble in distilled water.

Requirements:-Pipette 5ml, immune serum 5ml, saturated ammonium sulphate solution 5ml, dialysis tube, distilled water, normal saline, centrifuge, etc.

Procedure:-

1. Add 5ml of saturated ammonium sulphate solution slowly to 5ml serum in a test tube. Mix the contents by continuous swirling of tube without agitating excessively.
2. Allow the mixture to stand for 5 min. so that globulin precipitates.
3. Centrifuge the material for 10 min. at high speed. This will make the precipitate to settle down at the bottom of test tube.

4. Pour off the supernatant liquid (containing albumin) and dissolve the precipitate in 3ml distilled water.
5. Put the dissolved precipitate in a semi permeable cellophane tube and tie each end of the tube and place it in the distilled water beaker. The distilled water should be frequently changed to dialyze out $(\text{NH}_4)_2\text{SO}_4$ ions completely.
6. Dissolve the contents of cellophane tube in 5ml normal saline and preserve the material for further use.

Exercise

1. What are immunoglobulins? state various classes of immunoglobulins.
2. Why antibodies are called as immunoglobulins.
3. Write down the important characteristics of immunoglobulin classes in a tabular form.

Practical 3

AGGLUTINATION REACTIONS: PLATE AND STANDARD TUBE AGGLUTINATION TEST

Agglutination Test

Principle:

When a particulate antigen is mixed with the specific antibody in the presence of electrolytes at suitable temperature and pH, clumps are formed at the bottom of the tube i.e., agglutination reaction is seen. STAT and Rose Bengal Plate tests are based on the principle of agglutination reaction.

1. Rose Bengal Plate Test

The test is used for preliminary screening and is of qualitative test.

Material required

Brucella colored antigen, test serum, positive serum, Negative serum, and slide or gooch plate.

Procedure

1. place one drop of suspected serum on the slide/plate.
2. Place a drop of colored antigen next to the serum sample.
3. Mix the reagents with the toothpick and rotate the slide/plate gently.
4. After few minutes, in case of positive reaction, clumping takes place and confirm the reaction with suitable controls.

2. Standard Tube Agglutination Test (STAT)

Material Required

Brucella plain antigen, test serum positive serum, Negative serum, Carbol saline (Normal saline with 0.5% carbolic acid), Agglutination tubes 8 Nos., Rack for holding test tubes.

Procedure

1. Place 8 agglutination tubes in a rack, number serially 1 to 8.
2. Add 0.9 ml of diluent to the first test tube and 0.5 ml to rest of the tubes.
3. Add 0.1 ml of serum to the first test tube mix it well and transfer 0.5 ml to the 2nd tube.
4. Mix thoroughly and transfer 0.5 ml to the third tube and continue this process up to 7th tube. Keep 8th tube as antigen control.
5. Discard 0.5 ml i.e., diluted serum from 7th tube.
6. Add 0.5 ml of Brucella plain antigen to all the tubes. Mix and incubate at 37 °C for 24 hours.
7. Keep positive control.

Interpretation

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 the same.

Exercise

1. What is particulate antigen?

Practical 4

HAEMAGGLUTINATION AND HAEMAGGLUTINATION-INHIBITION TESTS, INDIRECT / PASSIVE HAEMAGGLUTINATION TEST

HAEMAGGLUTINATION TEST

Some of the viruses belonging to Paramyxovirus, Orthomyxovirus, Reovirus, Enterovirus & Pox viruses agglutinate suspensions of washed RBC's of various species of animals. This haemagglutination property (ability to agglutinate erythrocytes) has been successfully used for identifying the viruses.

Principle of Haemagglutination Test:

Some of the viruses contain in their outer coat virus coded glycoproteins (Haemagglutinin) capable of binding to the mucoprotein receptors (Sialic acid) over the surface of the erythrocytes. Such viruses bridge the gap between the erythrocytes leads to the formation of lattice. This reaction is seen in micro plates/Perspex plates in the form of mat formation.

Material Required: Perspex plate, 1ml pipettes, rubber bulb, beaker, conical flask, Pasteur pipette, centrifuge, measuring cylinder.

Reagents: 1% Chicken RBCs, Phosphate buffer saline (pH 7.2), Allantoic fluid (collected from ECE on inoculation of suspected material for RD virus isolation)

Procedure:

1. Add 0.5 ml each of PBS in all the wells in a row of Perspex plate with the help of 1ml pipette and rubber bulb.
2. Add 0.5 ml of undiluted allantoic fluid in the first well (only).
3. With the help of pipette mix PBS and allantoic fluid (containing RD virus) from the first well properly and transfer 0.5ml from 1st well to 2nd well (Two fold dilution). Again in 2nd well mix the contents with the help of pipette and transfer 0.5ml from 2nd well to 3rd well. Repeat till 9th well and discard 0.5ml

(diluted allantoic fluid containing RD virus) from 9th well.

4. Add 0.5 ml of 1% chicken RBC suspension to all the wells including 10th well (RBC control).
5. Shake the plate well to mix the contents and incubate the plate at room temperature.
6. Read the test at intervals of 15,30,45 and 60 minutes.

Interpretation:

1. The presence of haemagglutinating virus in the allantoic fluid will result in mat formation (layer of uniformly agglutinated cells covering the bottom). The mat formation depends on the concentration of virus present in the suspension.
2. A negative test consists of a compact, sharply demarcated disc of sedimented red blood cells at the bottom of the wells i.e., button formation.
3. The end point of HA activity of the RD virus is the highest dilution of the virus showing mat formation i.e., haemagglutination. The virus titre is expressed as HA unit contained in undiluted allantoic fluid. HA titre is the reciprocal of the highest dilution showing mat formation i.e., haemagglutination.

HAEMAGGLUTINATION INHIBITION TEST

The haemagglutination inhibition test is used to assess the titres of haemagglutinating-inhibiting antibodies against RD virus in the serum of suspected/healthy bird used for diagnosis and monitoring the vaccine titres.

Principle:

HI-Test is based on the haemagglutination property of RD virus. Haemagglutinin binds to the mucoprotein receptors (Sialic acid) over the surface of the erythrocytes & bridges the gap between the erythrocytes leads to the formation of lattice. This reaction is seen in microtitre / Perspex plates in the form of mat

formation. But In the presence of antibodies (in the serum), abs interacts with the virus and thus makes the virus unavailable for agglutination of red blood cells. Red blood cells settles at the bottom of the wells thereby giving i.e., button formation in the presence of haemagglutinating-inhibiting antibodies against RD virus.

Material Required: Perspex plate, 1ml pipettes, rubber bulb, beaker, conical flask, Pasteur pipette, centrifuge, measuring cylinder.

Reagents: 1% Chicken RBCs, Phosphate buffer saline (pH7.2), 4HA Unit RD virus, and Test serum)

Procedure:

1. Add 50ul each of PBS in all the wells in a row of Perspex plate with the help of 1ml pipette and rubber bulb.
2. Add 50ul of undiluted Test serum in the first well (only) .
3. With the help of pipette mix PBS and Test serum in the first well properly and transfer 50ul from 1st well to 2nd well (Two fold dilution). Again in 2nd well mix the contents with the help of pipette and transfer 50ul from 2nd well to 3rd well. Repeat till 8th well and discard 50ul (diluted test serum) from 8th well.
4. Add 50ul each of 4 HA unit virus upto 9th well (Virus control).
5. Allow it to stand for 10 minutes at room temperature.
6. Add 50ul each of 1% chicken RBC suspension to all the wells including 10th well (RBC control).
7. Shake the plate well to mix the contents and incubate the plate at room temperature.
8. Read the test at intervals of 15,30,45 and 60 minutes.

Interpretation:

1. The presence of haemagglutinating-inhibiting antibodies against RD virus in the test serum binds to the RD virus and makes RD virus

unavailable for haemagglutination of Red blood cells. Resulting into sedimented red blood cells at the bottom of the wells i.e., button formation.

2. A negative test consists of absence of haemagglutinating-inhibiting antibodies

against RD virus in the test serum results in the availability of RD virus for haemagglutination of Red blood cells. Resulting into mat formation (layer of uniformly agglutinated cells covering the bottom).

3. The end point of haemagglutination inhibition test. The titre is expressed as HI titre of haemagglutinating-inhibiting antibodies against RD virus in the test serum .HI titre is the reciprocal of the highest dilution showing button formation i.e., haemagglutination inhibition.

Protocol for Haemagglutination Inhibition test:0.5ml

Well No.	1	2	3	4	5	6	7	8	9	10
Reagent	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	
PBS	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
Test serum	0.5ml							Discard 0.5ml		
4HA unit										
RD virus Dilution	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
of Test serum	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
1%Chicke										
n RBC Control	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
									Virus control	RBC control

Passive/ Indirect Haemagglutination Test (HI)

Principle:

In the passive haemagglutination, a soluble antigen is adsorbed on particulate matters like Red Blood Cells (RBCs) and therefore can participate indirectly in the agglutination reaction. Passive haemagglutination (PHA) is very sensitive test. Sheep red blood cells acquire the property of adsorbing soluble antigen (like blood sera, virus) after they have been treated with dilute solution of tannic acid. Antigen coated red blood cells are agglutinated by specific antibody to adsorbed protein antigen.

Materials:

1. Test tubes for diluting antiserum (10 nos.) and for test (12 nos.)
2. PBS & NSS
3. Rabbit antiserum (1 ml.)
4. Sheep blood
5. Sodium citrate solution as anticoagulant (4 %)
6. Tannic acid
7. Pipettes (10 ml., 1 ml.)

Procedure:

1) Tanning of sheep red blood cells: Using 0.5 ml. sodium citrate solution collect 5 ml sheep blood, centrifuge the blood to sediment obtain PCV, discard blood plasma and wash cells thrice and prepare 2% cell suspension in N.S.S. Add equal volume of 2%

R.B.C. in 1: 20,000 freshly prepared tannic acid solution, incubate the mixture at 37°C for 10 minutes and then wash the cells with N.S.S. Resuspend the cells in saline and prepare 2% cells suspension.

2) Sensitization of sheep red blood cells: The tanned sheep red blood cells are sensitized by mixing equal volume of antigen. Allow the coating at room temperature for 10 minutes and wash off excess antigen by centrifugation at 2000 rpm for 3 minutes, wash cells once again in N.S.S. and prepare 2% cell suspension.

3) Prepare 2 fold serial dilutions of rabbit antiserum as shown in table below: Use separate pipette for each dilution and mix the contents thoroughly.

Tube No.	Rabbit Antiserum	Diluent	Final dilution
1.	0.1 ml. of antiserum	9.9 ml.	1 : 100
2.	1 ml. from tube 1	1 ml.	1 : 200
3.	1 ml. from tube 2	1 ml.	1 : 400
4.	1 ml. from tube 3	1 ml.	1 : 800
5.	1 ml. from tube 4	1 ml.	1 : 1600
6.	1 ml. from tube 5	1 ml.	1 : 3200
7.	1 ml. from tube 6	1 ml.	1 : 6400
8.	1 ml. from tube 7	1 ml.	1 : 12800
9.	1 ml. from tube 8	1 ml.	1 : 25600
10.	1 ml. from tube 9	1 ml.	1 : 51200

4) Place the test tubes in a rack and number the tube from 1 - 12. Add the reagents in following quantity.

Reagent (ml)	Tubes											
	1	2	3	4	5	6	7	8	9	10 (Ab)	11 (Ag)	12 (RBC)
Diluent/ PBS	—	—	—	—	—	—	—	—	—	—	0.5	0.5
Diluted rabbi	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—	—
Antigen coated RBCs	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—	0.5	—
Non-coated RBCs	—	—	—	—	—	—	—	—	—	0.5	—	0.5

Tube no. 10, 11 & 12 are kept as Antibody, Antigen & RBC control respectively

- o Mix the content of tubes by gentle shaking and incubate the tubes at room temperature for 2 - 3 hours. Examine the tubes for agglutination and record the result.

Interpretation: Tube showing layer of uniformly agglutinated cells covering the bottom are considered positive. Negative result is seen as a compact sharply demarcated disc of sedimented RBCs at the bottom of the test tubes. All controls (tube 10, 11 and 12) should be negative.

Result: Highest dilution of rabbit antiserum showing agglutination is taken as PHA titre.

Preparation of 1% Chicken RBC suspension

1. Collect 2 ml of blood using Alsever's solution (Equal proportion) in a centrifuge tube.
2. Centrifuge at 2000rpm for 6 minutes.
3. Discard the supernatant plasma and buffy coat without disturbing the RBC.
4. Add 5ml of Phosphate buffer saline solution to the RBC pack and centrifuge for 2 minutes at 1000rpm.
5. Repeat step 4 for 2-3 times.
6. Finally collect the packed cell volume of RBC and prepare the RBC suspensions as required 1%.
(For preparing 1% RBC suspension: 1 ml of Packed volume of RBC should be added to 99 ml of Normal saline)

Preparation of Alsever's Solution

Alsever's solution is an isotonic, balanced salt solution. It is routinely used as an anticoagulant/ blood preservative, which permits the storage of whole blood at refrigerator temperatures for approximately 10 weeks.

For usage, an equal volume of blood is collected into a container of this product and gently, but thoroughly, mixed. The solution is placed in a refrigerator at 2-8 °C for 2 weeks. The container can then be removed and the plasma layer removed for use.

Reagents

- | | |
|---|--------|
| · Citric acid $C(OH)(COOH)(CH_2.COOH)_2.H_2O$ | 0.055g |
| · Sodium Citrate $Na_3C_6H_5O_7.2H_2O$ | 0.8g |
| · D-Glucose $C_6H_{12}O_6$ | 2.05g |
| · Sodium chloride NaCl | 0.42g |
| · Distilled water to make up to 100 mL | |

Method

1. Weigh out reagents into a conical flask.
2. Dissolve in distilled water and make up to 100 mL.
3. Dispense into sterile 10 mL bottles.
4. Sterilize by autoclaving at 116°C for 10 minutes. Use slow exhaust.

5. Allow to cool, then tighten the lids and label the bottles.
6. Store in the refrigerator.

Exercise

1. Plot the protocol for Hemagglutination test in tabular form.
2. Calculate 4HA unit of RD Virus. If the given titre of HA virus is 256 HA unit.
3. Differentiate between Direct and Indirect / Passive HA.

Reference: Passive HA Test-VMC IV Lab Manual Dr.(Mrs.) R. S. Gadge, BVC, Mumbai

Practical 5

PRECIPITATION REACTIONS

AGAR GEL IMMUNODIFFUSION TEST

Principle: When soluble antigen combines with specific antibodies in the presence of proper electrolytes at, optimum temperature, optimum pH, the antigen-antibody complex forms an insoluble precipitate.

The precipitation test can be carried out as qualitative or as quantitative Test.

Precipitation Test can be performed using liquid media or using gel such as agarose as in Agar Gel Precipitation Test.

Agar Gel Precipitation Test:

Double agar gel immunodiffusion test (Ouchterlony method):

The double agar gel immunodiffusion test can be used to diagnose viral infections. Viral (or other microbial) antigen is placed in the center well, and serum samples are placed in the wells surrounding the center well. The reactants will diffuse through the agar at a rate inversely related to their molecular weights. If antibodies to the antigen are present, a precipitate will be seen between the center well and sample well; the antibodies in the serum diffuse out and bind antigen that is diffusing from the center well to form large insoluble complexes.

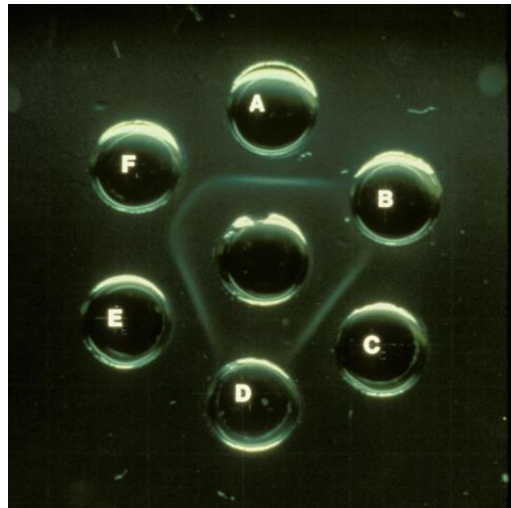
Material Required: 1.2% Agarose, Soluble antigen, Known Positive Serum and Negative serum sample, Gel Punch, Pasteur Pippette, Clean glass slide and needle.

Procedure:

1. Select a clean flat bottom petri dish and pour 12 ml of 0.9% agarose prepared in normal saline/PBS 7.2 solution. To avoid contamination add merthiolate in concentration of 1: 10,000 in molten agarose.
2. After solidification of agarose, Punch one central well and 6 peripheral wells of about 5.3 in diameter and 2.4 mm apart. The thickness of agarose should be 2.8 to 3 mm.
3. The agarose from the cut wells should be removed with the help of needle.
4. Seal the bottom of each well, with the help of drops of molten agarose.

5. Add Known standard test serum in central well and test antigens in peripheral wells. 50-60 UI.
6. Cover the plate and keep in the incubator at room temperature for 24 hours in humid atmosphere.

Result: Formation of precipitation line in between central and peripheral well is indicative of reaction of known antibody with specific antigen.



In the figure shown, all samples are positive except b and d.

Interpretation:

Line of Identity: When two similar antigens are placed in adjacent peripheral wells, reacts with homologous antiserum in the central well, the lines produced by them joins producing a continuous line in the form of an angle i.e., line of identity.

Line of Non-Identity: When two different antigenic preparations are allowed to react with a serum containing specificities for both, precipitation bands formed will cross each other. This is called as lines of non-identity.

Line of Partial Identity: If only one of the two antigenic preparations possesses specificity with the serum and other two possess two antigens (one specific and other non-specific), precipitation will be formed in the form of spur. This is called as line of partial identity.

SINGLE RADIAL IMMUNODIFFUSION (SRID)

The anti serum (antibody) is incorporated in agar gel on slide or Petri dish. The wells are cut on the surface of gel. Antigen is added to the well. The antigen diffuses radially and ring shaped bands of precipitation (halos) are formed around the well. The diameter of the ring is directly proportional to the concentration of antigen

Material required

Agarose, SRID slides, PBS (without KCL, pH 7.4), antiserum, antigen, moist chamber, oven, drier, water bath, brilliant blue stain, destain, std antigen, scale

Procedure

- 1) Wash the SRID glass slide, make it grease free and let it dry
- 2) Prepare 2% suspension of agarose (3ml) in PBS, melt it and spread on the slide
- 3) Keep this slide in a hot oven for drying.
- 4) Prepare 2% suspension of agarose (65ml) in PBS and melt it
- 5) Mix predetermined quantity of antibody in 6ml PBS and keep it at 560 OC in water bath
- 6) Let the temperature of antibody solution rise to 560 OC and also that of melted agar by keeping in water bath. Now mix agarose gel with antibody dilution without forming bubbles
- 7) Pour the mixture on dried gel in such a way that it shall form a agar gel layer of uniform thickness on slide. Let the gel solidify
- 8) Keep this gel at 40 OC for 10 mins
- 9) Punch the wells of 2mm diameter in agar gel using borer as show in the template leaving 10 ml space between two adjacent wells
- 10) Remove the gel from wells by using needles
- 11) Fill upper three wells with serial dilution of known standard soluble antigen using a pasture pipette and rubber bulb
- 12) Add a known positive and negative control antigen and test antigen in a remaining well in same fashion

- 13) Do never overflow the wells with antigen samples. Let the contents of wells diffuse in the agar gel
- 14) Cover the plate with the lead and incubate the plate 37°C temperature in humid atmosphere for 24- 48 hrs
- 15) Dry the gel in gel dry and stain with 0.1% brilliant blue for 12 hrs followed by destaining
- 16) Measure the diameter of stained precipitation rings produced
- 17) Plot a graph of standard (log diameter against concentration) and decide the antigen concentration in unknown samples. the SRID can also be employed for estimation of the serum immunoglobulin concentration

Applications:

- a) It has been widely employed for estimation of immunoglobulin classes that is IgG, IgM, IgA in sera
- b) It has also been used for screening in sera for antibodies to influenza viruses

COUNTER IMMUNOELECTROPHORESIS (CIE)

Principle: Electrophoretic migration of antigen and antibody towards each other in an agarose gel using a suitable buffer system results in a precipitation reaction at point intermediate between their origin. It is possible to enhance the speed of the reaction by driving antigen and antibody together by creating an electrical field.

Material required: Agarose, Barbitol buffer, antigen solution, antiserum, and glass plates, Micropipettes with tips, gel punch and Electrophoresis unit with power supply

Procedure:

- 1) Prepare 1% agar gel in barbitol buffer on counter-immuno-electrophoresis plate
- 2) Using a template cut two rows of wells in the gel
- 3) Take out the cut gel pieces with the needle without damaging the edges of the wells

- 4) Seal the bottom of the well with a little drop of molten agarose in each well
- 5) Load one row of wells with different antigen samples in a volume of 5 μ l/well
- 6) Load the other row of wells with the antiserum in a volume of 5 μ l / well
- 7) Place the gel plate carefully in the electrophoresis unit and make its connection with the power supply. Buffer tank through the filter paper wick
- 8) Connect the unit to the power supply and run electrophoresis at 5-10 V/cm.
For about 15-30 Min. for the development of precipitation lines/arcs
- 9) Dry the gel and stain with amido-black stain followed by destaining as done in Agar Gel.

Results:

Positive: Formation of precipitation arc/line between the opposing anodic and cathodic wells by the migration of antigen toward the anode and antibody towards the cathode in opposite directions under the influence of electric current.

ROCKET IMMUNOELECTROPHORESIS (RIE)

Rocket immune-electrophoresis (RIEP), also known as electro-immunoassay, is a simple, quick and reproducible method for quantitation of a specific protein in a mixture by using monospecific antibody in the agarose gel.

When a particular antigen in different concentration is electrophoresed in agarose having specific antibody in a suitable buffer system, antigen-antibody reaction at the zone of equivalence results in precipitation of the Ag-Ab complexes. The shape of the precipitating complex is like a rocket and the peak height and hence area of the rocket is proportional to the antigen concentration.

Material required: Antigen, Antiserum, Agarose, Glass plates, Glass pipette, Electrophoresis unit

Procedure:

- 1) Melt 1% agarose in barbitone buffer by keeping in a boiling water bath, cool it to 50°C and place it in water bath maintained at 50°C
- 2) Take about 10 ml of the agarose with a pre warmed pipette and transfer it to the beaker.
- 3) Quickly add between 75-100 µl of the prewarmed antiserum at 45-50°C and mix properly.
- 4) Pour onto the clean glass plate 10 ml of the antiserum mixed-molten agarose as soon as possible with the help of a 10 ml glass pipette, and wait for its gelling.
- 5) After the gel is set, cut 8-10 wells of 1-2 mm diameter in a row, toward the cathode end. The position of the row being dependent on the nature of the proteins.
- 6) Place the glass plate on the cooling plate of the electrophoresis unit and make its firm contact with filter paper wicks soaked in the electrode buffer.
- 7) Fill the well with different dilutions of the samples in a volume of 2-5 µl/sample well as quickly as possible.
- 8) Connect the electrophoresis unit to the power supply and run the gel at 2-5/cm for 15 min and then at 25-30V/cm for next 4-5 hrs
- 9) Disconnect the power supply. Take out the gel, observe 'rockets' of the precipitate and record the results as peak heights from the centers of the wells
- 10) Stain the gel as in AGPT

Observation:

Record the reading as peak heights from the center of the wells

Results:

Draw a calibration curve of peak heights of rockets on y- axis against antigen concentration on x- axis and record Ag concentration in the unknown samples using its peak heights obtained in gel.

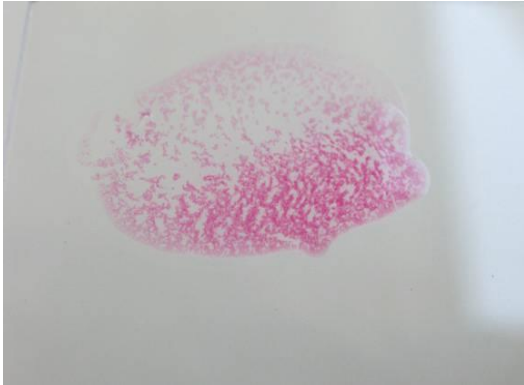
Exercise

1. Give diagrammatic representation illustrating simultaneous comparison of different antigenic preparation against an antiserum
2. What are advantages of electrophoresis over simple immunodiffusion test?
3. Give few examples of disease where AGID can be used as a diagnostic tool?
4. Perform the test and plot a standard graph and calculate antigen concentration in given samples by RID.

Reference:

1. www.microbelibrary.org/library/laboratory-test/3097-the-double-agar-gel-immunodiffusion-test-for-viral-infection

PLATE 1



1.RBPT- Plate Agglutination positive test



2.RBPT- Plate Agglutination negative test



3. Haemagglutination Inhibition Test



4.Radial Immunodiffusion Test

<https://www.fishersci.com/shop/products/dvotek-radial-immunodiffusion-kit-radial-immunodiffusion-kit/s90248nd>



5. Counter Immuno-electrophoresis

<https://en.wikipedia.org/wiki/Counterimmunoelectrophoresis>

Practical 6

CELL MEDIATED IMMUNE RESPONSE (DTH)

Delayed-type hypersensitivity (DTH) is an in vivo assay of cell-mediated immune function. DTH reactions are often divided into two phases: the sensitization phase, referring to the initial immunization with specific antigen, and the efferent or challenge phase of the DTH response, which usually follows 6 to 14 days after sensitization.

The diagnosis of bovine tuberculosis is based on tuberculin test, which involves the intradermal injection of bovine tuberculin purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72 hours later. This may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculins. The tuberculin test is usually performed on the mid-neck, 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. Turning red and swelling, pain, increase in temperature of local injection site are considered as signs of inflammation. Measure the diameter of the inflamed area using a ruler or, better yet, a caliper. The maximum swelling should occur between 24 and 72 hours.

Test procedure

- i) The injection sites must be clipped and cleaned. A fold of skin within each clipped area is measured with callipers and the site marked prior to injection. A short needle, with tuberculin attached, is inserted obliquely into the deeper layers of the skin. The dose of tuberculin is then injected. The dose of tuberculin injected must be no lower than 2000 International Units (IU) of bovine or avian tuberculin. A correct injection is confirmed by palpating a small pea-like swelling at each site of injection. The distance between the two injections should be approximately 12–15 cm. The skin-fold thickness of each injection site is re-measured 72 hours after injection. The same person should measure the skin before the injection and when the test is read.

- ii) In the interpretation of the intradermal comparative test, a reaction is usually considered to be positive if the increase in skin thickness at the bovine site of injection is more than 4 mm coupled with all signs of inflammation.

Exercise

1. Write the interpretation of test.

Practical 7

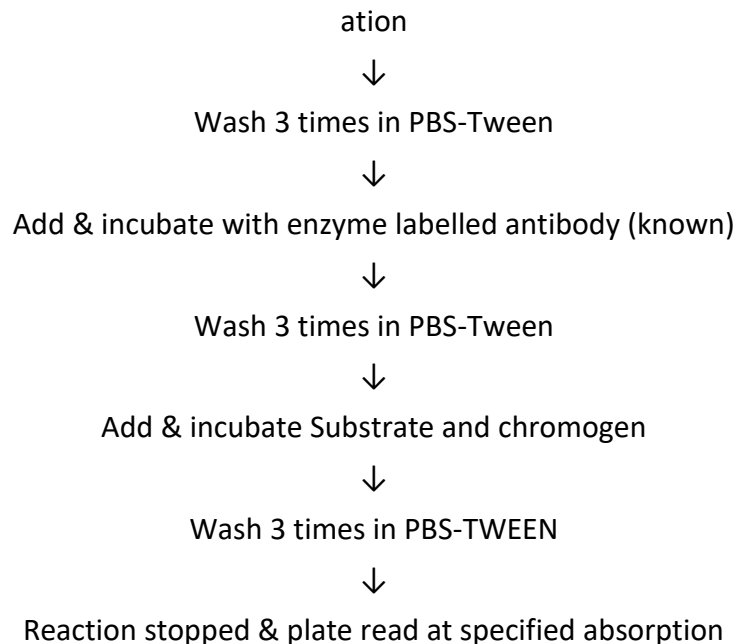
ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) DIRECT , INDIRECT, SANDWICH, DOT-ELISA

DIRECT ELISA

Principle: Antigen which is to be detected is coated on a solid surface and the known antibody coupled to the enzyme is then reacted upon and the presence of the enzyme activity is the indication of the positive reaction.

FLOW CHART :

Passive adsorption of test antigen (Unknown) in defined buffer to the microtitre plate by incubation

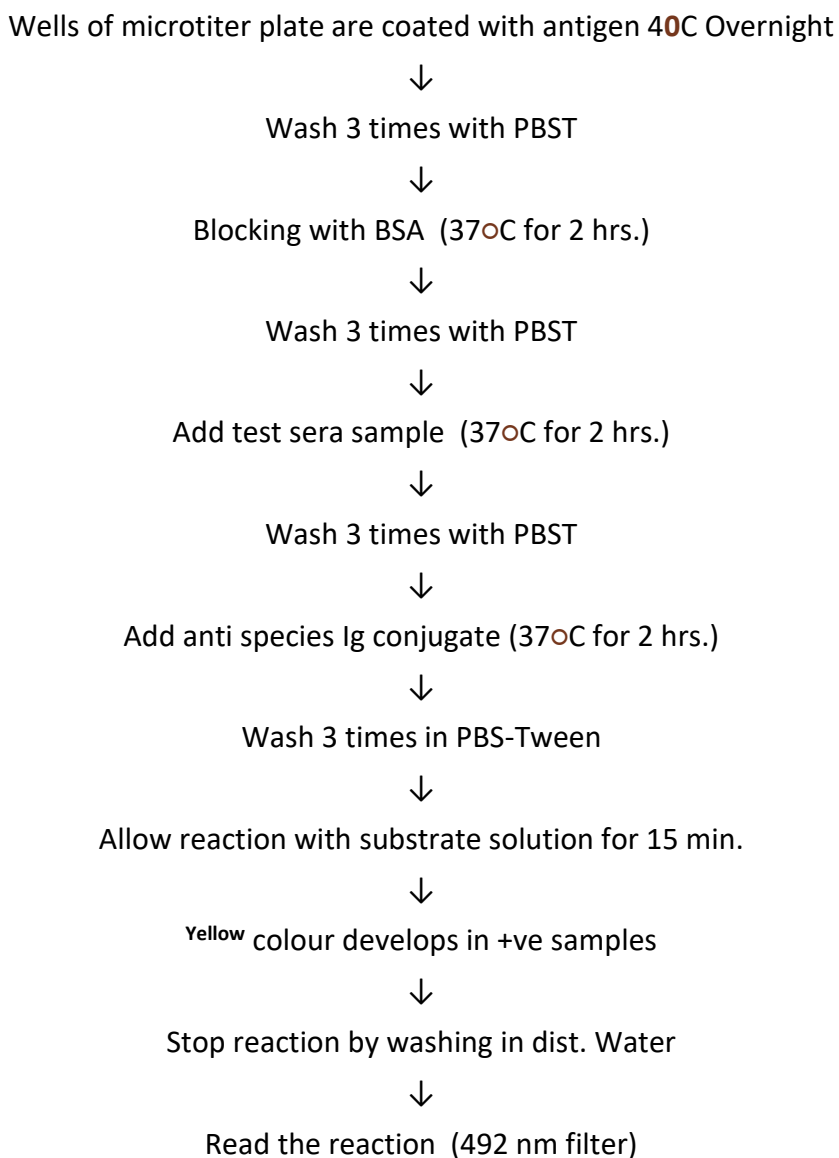


INDIRECT ELISA

Principle : For antibody detection, the wells microtiter plate are coated with antigen. Sera to be tested are added in these coated wells. If antibody is present in specimen, it binds to coated antigen. To detect this antigen antibody reaction, a goat anti-human immunoglobulin antibody conjugated with enzyme is added. Enzyme conjugated anti-human immunoglobulin binds to substrate to produce colour in a positive reaction. This procedure is named as INDIRECT ELISA.

Colour intensity can be read by spectrophotometer or ELISA reader. Colour change can also be seen by naked eyes. Positive and negative controls are always put up along with test serum. Incubation and washing is done at every step to wash off unbound enzyme. The enzyme (Horse radish peroxidase, alkaline phosphatase) gives rise to a colour change by adding specific substrate (O-phenyl-diamine dihydrochloride for peroxidase, p-nitrophenyl phosphate for alkaline phosphatase). Alkaline phosphatase with this substrate produces a ^{yellow} colour.

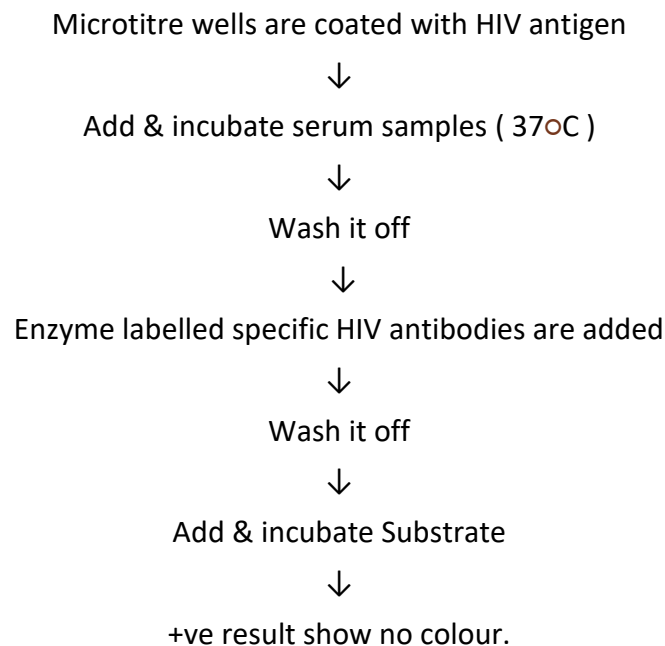
FLOW CHART :



COMPETITIVE ELISA

Principle: There are two specific antibodies, one conjugated with enzyme & other present in serum, competition occurs between two antibodies for same antigen. Positive result shows no colour whereas appearance of colour indicates a negative test.

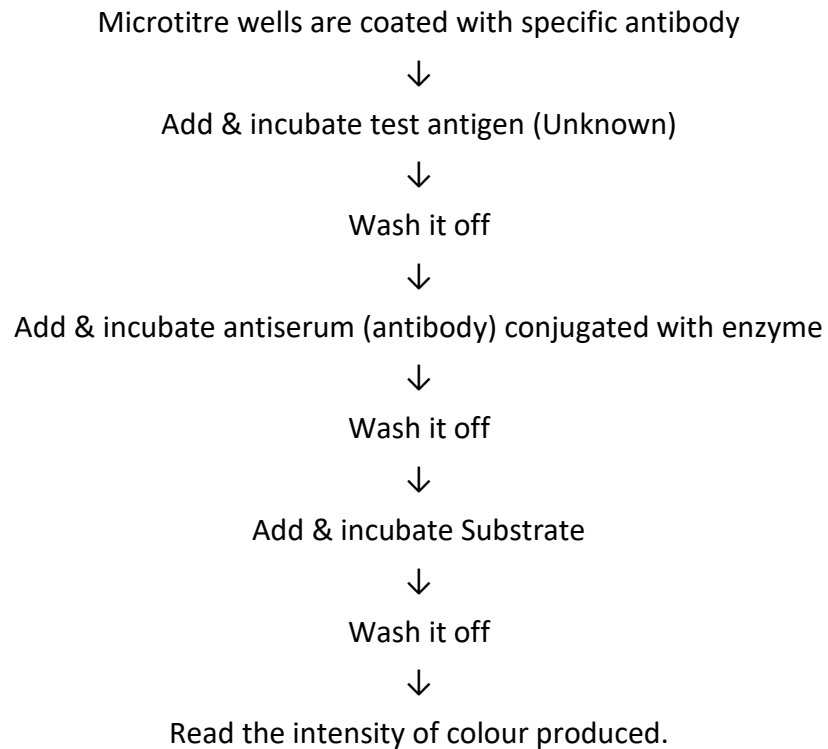
FLOW CHART :



SANDWICH ELISA

Principle: Enzyme labeled antibody can be produced in the same animal that produced passively adsorbed antibody, or from a different species immunized with the same antigen that is captured. Unbound conjugate is washed out and substrate is added. After another incubation, the degree of substrate hydrolysis is measured. The amount of substrate hydrolyzed is proportional to the amount of antigen in the test solution.

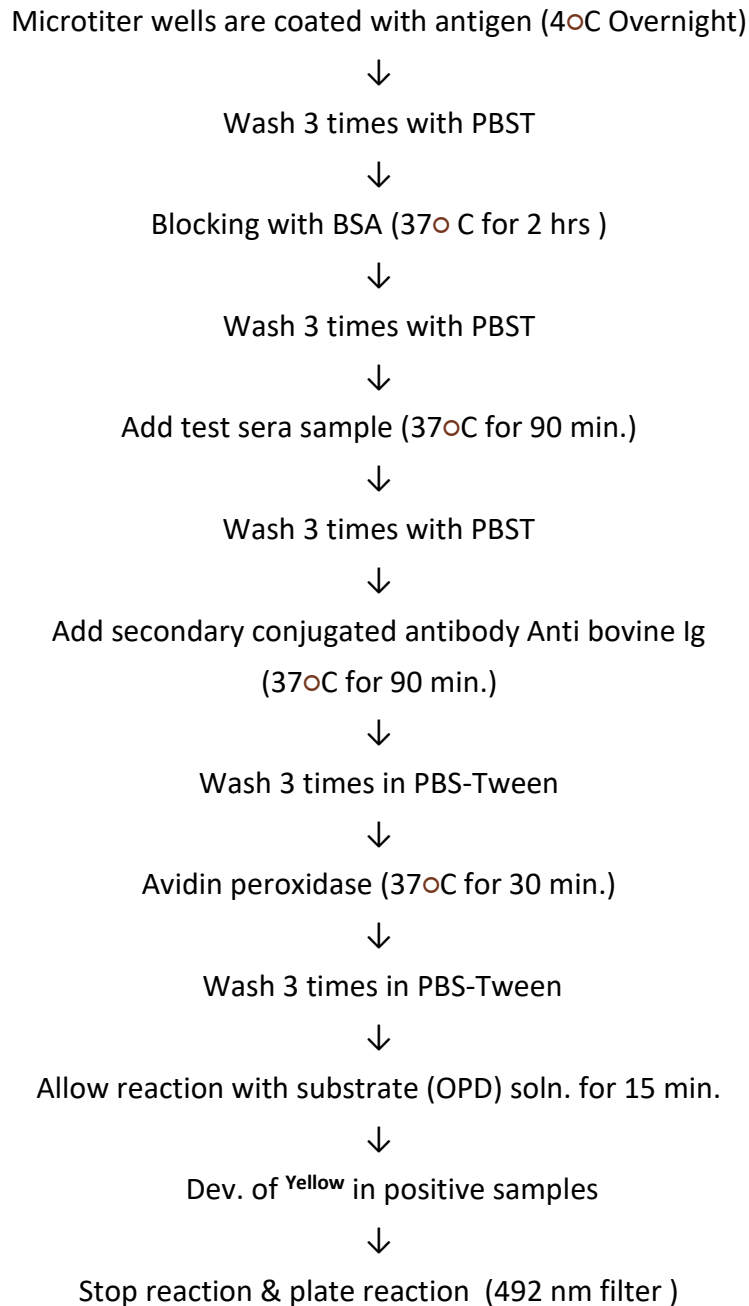
FLOW CHART :



AVIDIN - BIOTIN ELISA

Principle: The secondary antibody is labelled with biotin which is detected either avidin labelled with peroxidase or bridged avidin biotin method or avidin-biotin complex. The secondary antibody is conjugated with biotin and peroxidase is conjugated with avidin producing ^{Yellow} colour.

FLOW CHART :



Exercise

Q1. Write the applications of ELISA.

Q2. Write different enzymes (conjugate) and substance used in ELISA.

Practical 8

VISIT AND APPRAISAL OF VETERINARY BIOLOGICAL INSTITUTE

Institute of Veterinary Biological Products, Pune

"Pioneering National Institute" Imperial Bacteriological Laboratory was established in 1889 at Pune. The Institute was subsequently shifted to Mukteshwar and renamed as Indian Veterinary Research Institute. This was the only institute in India catering to the needs of veterinary biologicals. Thereafter the Serum Institute was established at Bombay in the year 1947 to meet the need of the then Bombay State. Initially only three products viz. Hemorrhagic Septicemia, Black Quarter, Goat blood Rinderpest disease vaccines were produced at Bombay. The Ranikhet Disease vaccine was produced in a specially established unit at Pune.

The institute from Bombay was shifted to Pune during the year 1959 and since then it is working in 25 hectares of land with necessary building and facilities. The Institute is manufacturing various types of vaccines, antigens and diluents. In all 8 types of bacterial vaccines 10 types of viral vaccines, 4 types of diagnostic antigens & reagents and 4 types of diluents are manufactured. On an average 600 lakh doses are manufactured annually.

The Institute is headed by the Joint Commissioner of Animal Husbandry and assisted by different technical and non-technical staff. The total strength of the staff is 153.

All the biologicals are produced as per the Drugs and Cosmetics Act 1940. There are 15 Production Laboratories and 4 main sections.

- Bacterial Vaccine Section
- Viral Vaccine Section
- Small Animal Breeding Center
- Test and Quality Control Section.

The biologicals produced are mainly supplied free of cost to state institutes. Some of the biologicals are sold on cash within the state and outside the state also.

The Institute is unique of its type functioning at the state level under Govt. sector, in the production of veterinary biologicals and is headed by Joint Commissioner Animal Husbandry.

The institute is presently undergoing overall up gradation as required under Schedule M of Drugs and Cosmetics Act, 1940. The bacterial vaccines HS, BQ and ET are also planned to be manufactured by using fermenter technology.

Institute of Animal Health and Veterinary Biologicals, Bangalore

The Institute of Animal Health and Veterinary Biologicals had a modest beginning as “Mysore Serum Institute” founded by 1926 by the erstwhile Maharaja of Mysore to produce hyper-immune serum for the control of Rinderpest Disease in the state. The name of the Institute was changed from “Mysore Serum Institute” to "Institute of Animal Health and Veterinary Biologicals" in view of the multifaceted activities undertaken by the Institute in the production of various veterinary biologicals and problem-oriented research on existing, exotic and emerging diseases of livestock.

The Institute was registered as Scientific Society under the societies registration act in 1978 to give a broader perspective and autonomous status to organize and manage its diversified activities of production of biologicals and diagnostic services through a network of units in the state.

The objectives includes large scale production of various biologicals for prophylaxis and diagnostic agents for speedy and prompt diagnosis of various livestock and poultry diseases. Produces 7 Bacterial Vaccines, 9 Viral vaccines and Diagnostic antigens. To adopt new technologies in the development of cost effective and efficient biologicals and diagnostic aids. And to provide prompt and effective diagnostic services for identification of disease problems with an aim to eradicate them.

Biological Product Division, IVRI, Izzatnagar, Bareilly

The Biological Products Division was originally founded as Imperial Veterinary Serum Institute in 1930 to produce antisera against rinderpest and hemorrhagic septicemia the most devastating diseases of cattle. In 1936 with the reorganization of the Institute, it was renamed as Division of Biological Products. The divisional activities are organized under three sections viz. viral vaccines, bacterial vaccines

and diagnostics. These are directed towards research and development on improvement of biological products, up-scaling of technologies to production level, introduction of newer vaccines, diagnostics and other biological products, production and supply of veterinary biologicals and transfer of technology on veterinary biologicals. In addition to it organization of training programmes for the state biological production units through national diploma and specialized short-term training courses, besides rendering advisory and consultancy services on veterinary biologicals are also important activities.

Exercise

1. Visit the veterinary biological institutes and record your observations.

Veterinary Microbiology

Practical Manual

Unit IV (As per MSVE 2016)

Department of Veterinary Microbiology

College of Veterinary & Animal Sciences, Udgir

MAHARASHTRA ANIMAL & FISHERY SCIENCES UNIVERSITY, NAGPUR



COVASU

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