LABORATORY MANUAL

PRATICAL VETERINARY MICROBIOLOGY

VMC- Unit- 4 & 5

Course title – Veterinary Immunology and Serology & General and Systematic Veterinary Virology

Compiled By:
Prof. G.C. Chaturvedi

Dr .Priyanka Singh
(Associate Professor)

Dr. Neha Singh
(Assistant Professor)

Name ……………………………..……………………………………………..

Roll No / Enroll No -…………………...…………/……………….……….…..……

Batch -……………………………….Session –…………………………………..

Department of Veterinary Microbiology
Mahatma Jyotiba Fule College of Veterinary & Animal Science
Chomu, Jaipur

FOREWORD

I am glad to see the Laboratory Manual of ***Veterinary mycology, Microbial Biotechnology and Veterinary Immunology and Serology*** *prepared by* ***Dr. G. C. Chaturvedi, Dr. Priyanka Singh and Dr. Neha Singh, Dept. of Veterinary Microbiology****. It is appreciable to note that the manual covers the practical syllabus of B.V.Sc.&A.H. course as per the standards laid down by Veterinary Council of India.*

Dr. G. C. Chaturvedi, Dr. Priyanka Singh and Dr. Neha Singh have devoted keenly to prepare this manual with their excellent knowledge and expertise in the field of microbiology. They have covered all the aspects like objectives, outline and description, material and methods and observation to be taken care off.

Definitely this manual will be helpful for smooth and effective conduction of practicals and ensure a handbook for students for entire life in the profession.

I congratulate Dr. G. C. Chaturvedi, Dr. Priyanka Singh and Dr. Neha Singh for their strenuous efforts and excellent presentation of this manual.

Dean
Mahatma Jyotiba Fule College of Veterinary & Animal Science, Chomu, Jaipur

PREFACE

This Laboratory Manual has been prepared for the undergraduate students of B.V.Sc. & A.H. in accordance with the syllabus designed by the Veterinary Council of India. The efforts have been made to make the manuscript worthy, realistic and easily understandable for the students, teachers and Veterinary Practitioners for diagnosis of different microbial diseases of animals. We hope this manual will serve very useful tool to the undergraduate and graduate students of Veterinary Science who are undergoing courses in veterinary microbiology.

It's our pleasure to thank Dean Sir, M.J.F College of veterinary and Animal Sciences, Chomu, Jaipur for providing necessary facilities and rendering all helps in preparing this course manual.

Computer operator and typist Mr. Ashutosh Sharma worked hard for very existence of this manual so I acknowledge his efforts.

Course Incharge

Deptt. of Veterinary Microbiology

UNIT-4

Sl. No.	Practical	Page No.	Date	Signature
1.	Preparation of antigen			
2.	Raising of antiserum			
3.	Concentration of immunoglobulin's			
4.	Agglutination: a)Plate agglutination test b)Tube agglutination test			
5.	Haemagglutination: a) Passive(Indirect) haemagglutination test(PHA) b) Reverse Passive haemagglutination test(RPHA) c) Latex agglutination test			
6.	Precipitation test: Agar gel precipitation test(AGPT)			
7.	Immunoelectrophoresis: a) Counter immunoelectrophoresis(CIE) b) Rocket immunoelectrophoresis(RIE)			
8.	Complement fixation test(CFT)			
9.	Fluorescent antibody technique(FAT)			
10.	Immunoperoxidase test(IPT)			
11.	Enzyme linked immunosorbent assay(ELISA)			
12.	Assay of cell mediated immune(CMI) response			
13.	Veterinary Biological(visit and appraisal)			

UNIT-4

PRACTICAL NO. 1

PREPARATION OF ANTIGEN

The methodology of preparation of antigens varies depending upon type and source of the antigens. The following exercises describe the preparation of few commonly used antigens.

A. Preparation of Gamma Globulins:

Gamma globulins can be gradually precipitated from serum at 35 to 50% saturation with ammonium sulfate. Usually three cycles of precipitation are necessary for fractionation of gamma globulins.

Materials required:

1. Saturated $(NH_4)_2\ SO_4$ solution, pH 7.2
2. Serum.
3. Normal Saline Solution (NSS)
4. Dilute ammonium solution
5. Dialysis sac.
6. Magnetic stirrer
7. Beaker, pipettes, centrifuge tubes.
8. Centrifuge machine
9. Refrigerator

Procedure:

1. Adjust pH of a 20 ml aliquot of saturated $(NH_4)_2\ SO_4$ solution to 7.2 with dilute ammonia solution just before use.
2. Take 20 ml of serum in a beaker (100 ml capacity) and add drop wise 20 ml saturated $(NH_4)_2\ SO_4$ solution with mild, constant stirring over a magnetic stirrer.
3. Allow the suspension to stand for 1 hour at room temperature with mild stirring.
4. Centrifuge the suspension at 3000 rpm for 30 min.
5. Discard the supernatant and dissolve the precipitate in 10 ml NSS (i.e., half the original serum sample taken for precipitation) and re-precipitate again by gradual addition of saturated ammonium sulfate solution.

6. Repeat the precipitation cycle twice more as described above.
7. Collect the final precipitate and dissolve it in 10 ml NSS.
8. Remove the sulfate ions from the crude immunoglobulin suspension by extensive dialysis against NSS.

B. Preparation of Erythrocyte Antigen:

The following procedure has been worked out for the preparation of erythrocyte antigens from sheep.

Materials required:

1. Sheep blood.
2. Elsevier's solution
3. NSS
4. Erlenmeyer flask 50 ml capacity
5. Graduated cylinder graduated pipette.
6. Graduated pipette.
7. Graduated centrifuge tubes.
8. Centrifuge machine.

Procedure:

1. Collect 20 ml of sheep blood from jugular vein in equal volume of Elsevier's solution in an Erlenmeyer flask.
2. Transfer the blood suspension into centrifuge tube and centrifuge the suspension at 2500 rpm for 20 min.
3. Carefully remove the supernatant along with Buffy coat layer with a graduated pipette.
4. Re suspend the cell pellet in 10 ml NSS and centrifuge again as in step 2.
5. Remove the supernatant and re suspend the cells in NSS and centrifuge again as in step 2.
6. Following the third centrifugation, note the volume of the packed cell and then carefully remove the supernatant as described above.

7. To make a suspension of a given concentration, pour an equal volume of NSS into a fresh Erlenmeyer flask and suspend the packed cells in it by carefully adding the cells in it from the centrifuge tubes with a graduated pipette (to make 1% suspension, use a volume of NSS 99 times that of the packed cells).

C. Preparation of Freund's Adjuvant Modified Antigen:

Materials required:

1. Antigen – bovine serum albumin, (5% solution in NSS) or any other antigen.
2. Freund's complete adjuvant (FCA)
3. Serological pipettes.
4. Syringes
5. Needles, 13 gauge and 20 gauge.
6. Beaker.

Procedure:

1. Take 10 ml of FCA in a beaker.
2. Draw 10 ml of 5% solution of BSA into a syringe and add it in drops or aliquot's of 1 to 1.5 ml to FCA.
3. Emulsify the mixture thoroughly after each addition of BSA by ejecting the emulsion repeatedly drawn into a 20 ml syringe with a 13 gauge needle until the emulsion becomes milky in appearance.
4. Check the emulsion by placing a drop of emulsion into a beaker containing ice chilled water. If the emulsion is stable the droplet will not disperse.

Note: The emulsion should be made just prior to use. Re-emulsification is necessary if it is stored before use.

PRACTICAL NO. 2

RAISING OF ANTISERUM

Antibodies to an antigen or immunogen can be raised easily under laboratory conditions suing certain species of animals. The antiserum or immune serum which is raised by giving repeated injection of antigen (Hyper immunization) is referred to as hyper immune serum.

The production of a good hyper immune serum depends on various factors like:

i. Choice of animal
ii. Nature of antigen
iii. Dose of antigen
iv. Use of adjuvant with antigen
v. Route of immunization.

The various routes used for immunization of animals are:

a. Intra-dermal – Used for viscous and slowly dispersing forms of antigen such as Freund's adjuvant emulsified antigen.
b. Subcutaneous – Suitable for emulsions, precipitates and viscous materials.
c. Intramuscular – Suitable for adjuvant modified antigens.
d. Intravenous – **S**uitable for particulate, however not advisable for viscous and non-aqueous immiscible antigens.
e. Intra-peritoneal – Suitable for complex and particulate antigens.
f. Foot pad – Used for inoculating particulate or cellular antigens into mouse and guinea pigs.

Table 1.Site of injection for different routes in animals and birds.

Species	Intradermal	Subcutaneous	Intramuscular	Intraperitoneal	Intravenous
Rabbit	Flank of back	Abdomen	Thigh muscle	Abdomen	Marginal vein
Guinea pig	Flank of back	Groin, back	Thigh muscle	Abdomen	Dorsal and inner aspect of hind leg
Mice	Flank of back	Abdomen	Thigh muscle	Abdomen	Tail vein
Sheep/ goat	Neck	Neck	Thigh muscle	Abdomen	Jugular vein
Pig	Ear	Behind ear	Thigh muscle	Abdomen	Ear vein
Chicken	Wattle, Thorax	Wing, Thorax	Breast, thigh	Abdomen	Wing vein

Immunization schedules: The following schedules can be used for raising hyper immune serum against soluble antigen in animals and birds.

Rabbit:

1. For primary immunization, inject 50 to 500 µg protein in FCA intra-dermally into 6 to 8 sites on the back (o.1 to 0.2 ml/ site).
2. Boost the animal at 14th day of primary immunization with 0 to 200 µg of protein in PBS or FIA by i\m or s\c. route.
3. If a good precipitating antibody titter is not obtained 2 weeks after the second injection, continue boosting with 300µg of alum – absorbed protein through marginal ear vein route.

Guinea pigs and rats:

1. For primary immunization, inject 10 to 100 up protein in FCA by i.d., or s.c., route on the animal's back (0.1 ml/site) at several sites.
2. Boost at 8th day of primary immunization with 10 to 50 µg protein in FIA or PBS by i\m or s\c and i\p. route.

Mice:

As for rats but use 5 to 50 µg protein for both primary and secondary immunization.

Chickens:

1. For primary immunization, inject 50 to 200 µg protein in FCA by i\m. route into 2 to 4 sites of the breast muscle (0.25 to 0.3 ml).
2. Boost after 10 days with 50 to 200 µg protein in PBS.

Sheep and Goats:

1. For primary immunization, inject 0.5 to 10 mg protein in FCA by s\c., i\m. or i\d. route at several sites.
2. Boost after 14 to 28 days with 0.5 to 10 mg protein in PBS or FIA at several sites by i\m. or s\c. rote.

PRACTICAL NO. 3

CONCENTRATION OF IMMUNOGLOBULINS

Various techniques are used for concentration of immunoglobulin’s or other proteins. These include: Osmotic pressure technique, Lyophilization, Negative pressure technique, Ultra-filtration, Chromatography technique and salt precipitation technique.

In the following exercise osmotic pressure technique is outlined for concentration of immunoglobulin’s.

Materials required:

1. Crude immunoglobulin suspension
2. Polyethylene glycol (M. Wt. 6000)
3. Dialysis sac.
4. Petri dish, large size

Procedure:

1. Fill the crude immunoglobulin suspension in a dialysis sac of required size.
2. Place the dialysis sac in a large petridish in slanting position at 4 C and cover it with powdered polyethylene glycol.
3. At every 15-30 min. intervals, strip off the wet gummy PEG by pulling it with fingers from the outer surface of the sac.
4. Sprinkle additional PEG over the sac to achieve the desired reduction of volume of fluid within the sac.
5. Finally wash the outer surface of the dialysis sac with distilled water and collect the concentrated sample aseptically.

PRACTICAL NO. 4

AGGLUTINATION TESTS

The term agglutination refers to clumping of particulate antigens like bacteria, erythrocyte etc by specific antibodies. Agglutination can be observed microscopically or macroscopically depending upon the technical methods employed. For a visible agglutination reaction to occur, the size of the antigen should be more than 200-250 nm. An IgM antibody is much more (750 times) efficient than an IgG molecule in agglutination reaction. The important pre-requisites for an agglutination reaction are-

i. Presence of electrolytes.
ii. Hydrogen ion concentration.
iii. Temperature.

Types of Agglutination Test:

A. Tube agglutination test
B. Plate agglutination test.
C. Slide agglutination test.
D. Viral haemagglutination test.
E. Viral haemagglutination inhibition test.
F. Direct haemagglutination test.
G. Indirect or passive haemagglutination test.
H. Reverse passive haemagglutination test.
I. Latex agglutination test.
J. Co-agglutination test.

A. **Tube Agglutination Test:**

This is simple serological test used for the diagnosis of bacterial diseases and also for the detection and quantitative estimation of specific antibodies against a bacterium in suspected serum.

Principle:

Antibodies to bacterial antigen contain two binding sites that specifically combine with the determinant groups of antigen which are present on the surface of bacteria. Under suitable conditions, an antibody molecule combines with the determinant groups on the surfaces of two bacteria and in this way the bacteria become joined or agglutinated.

Application:

1. For the diagnosis of infectious bacterial diseases like Brucellosis, Salmonellosis etc.
2. To determine the presence or concentration of antibodies in serum samples to a particular bacterial antigen.

In the following exercise tube agglutination test for diagnosis of Brucellosis is outlined.

Materials required:

1. Antigen – *Brucella abortus* plain antigen.
2. Antiserum – *B. abortus* hyper immune serum (known positive serum).
3. Suspected serum (test serum).
4. Phenol saline (Diluent)
5. Serological tubes rack.
6. Graduated serological pipettes.
7. Serological water bath.

Procedure:

Test protocol of tube agglutination test

Test Tube	1	2	3	4	5	6	7	8	9	10
Phenol saline (ml)	0.9	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.75	0.5
Test serum (ml)	0.1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	Discard 0.5 ml from tube No.8	
Antigen (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.25	0.5
Serum dilution (Final)	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	50% opacity tube	Antigen control tube

1. Put 10 serological tubes in a serological rack.
2. Add 0.9 ml of phenol saline in the 1st tube and 0.5 ml in tube No. 2,3,4,5,6,7,8, and 10. Add 0.75 ml phenol saline in tube No. 9.
3. Put 0.1 ml of test ser4um into the 1st tube with a graduated pipette, mix and transfer 0.5 ml of the diluted serum to the second tube.
4. Mix thoroughly and transfer 0.5 ml to the 3rd tube.
5. Continue to transfer up to the tube No. 8 and finally discard 0.5 ml from this tube (No.8).
6. Add 0.5 ml of antigen suspension each to all the tube except tube No. 9 to which add 0.25 ml of antigen.
7. Shake the rack.
8. Incubate overnight at 37^0C in a serological water bath and record the results after allowing the tubes to equilibrate at room temperature for few minutes.

Interpretation:

If the test serum contains specific antibodies to the antigen, clumping occurs in the form of floccular sediment or carpet at the bottom of the tube leaving a clear supernatant. In negative reaction there may be simple setting of antigen or homogenous turbidity in the tubes.

Record the antibody titer as the highest final dilution of serum that gives a distinct agglutination of the antigen and decrease in the opacity of the supernatant to match the opacity of the tube No. 9 (opacity tube).

B.Plate Agglutination Test :

This is a rapid qualitative agglutination test. A colored antigen is used in this test which facilitates the reading of the reading of the test. It is commonly used as a screening test.

Application:

i) For rapid diagnosis of Salmonellosis, Brucellosis by detecting specific antibodies against the antigens used.

ii) To determine the immune status of a flock/herd.

Materials required:

i. Porcelain agglutination plate.
ii. Colored antigen (e.g. coloured *S. pulloram* or *B. abortus* antigen).
iii. Test serum.
iv. Pasteur pipette.
v. Applicator stick.

Procedure

1. Place a drop of colored antigen on a porcelain agglutination plate with a Pasteur pipette.
2. Add a drop of either test serum or whole blood onto the antigen drop with a separate Pasteur pipette and mix it with an applicator stick.
3. Tilt the plate gently for 2 minutes and look for agglutination.

Interpretation

If the test serum or whole blood contains specific antibodies to the antigen, the antigens will be rapidly clumped.

PRACTICAL NO.5

HAEMAGGLUTINATION

When erythrocytes are used as agglutinate, then it is called as haemagglutination. Certain viruses such as those causing mumps, measles, influenza and in poultry Newcastle disease (Paramyxoviruses) have ability to agglutinate red blood cells from certain species of animals especially chicken, guinea pig, human type 'O'red blood cells.

A. Passive(Indirect) Haemagglutination Test:

In passive haemagglutination soluble antigen is adsorbed on particulate matters like red blood cells and can participate in the agglutination reaction. Passive haemagglutination is a very sensitive test. Sheep red blood cells acquire the property of adsorbing soluble antigens (like blood sera, viruses etc) after they have been treated with a dilute solution of tannic acid.

Materials required:

1. Rabbit antiserum
2. Phosphate Buffer Saline (PBS) pH 7.4
3. Antigen
4. Sheep blood
5. 4% sodium citrate as anticoagulant
6. Tannic acid
7. Microtitre plate with U-shaped wells
8. Micropipette
9. Normal saline solution (NSS)

Tanning of sheep red blood cells:

Using 0.5 ml sod.citrate solution, collect 5 ml sheep blood. Centrifuge the blood to sediment the cells, discard blood plasma and wash the cells thrice and prepare 2% cell suspension in normal saline solution. Add equal volume of 2% sheep red blood cells in 1:20,000 freshly prepared tannic acid solution. Incubate the mixture at 37^0C for 10 min and then wash the cells with normal saline solution.Resuspend the cells in saline and prepare 2% cell suspension.

Sensitization of sheep red blood cells with antigen:

Sensitize tanned sheep red blood cells by mixing equal volume of antigen. Allow the coating at room temperature for 10 min and wash off excess of antigen at 2000 rpm for 3 min. Wash the cells once again in normal saline solution and prepare 2% cell suspension.

Procedure:

1. Take 0.9 ml of PBS in 1st well and 0.5 ml in the remaining wells of first row of microtitre plate.
2. Prepare 2-fold dilution by adding 0.1 ml of rabbit antiserum into the first well of first row. Mix well and then transfer 0.5 ml from the 1st well to the 2ndto 3rd well. Continue to transfer this way up to the last well and finally discard 0.5 ml from the last well.
3. Add 0.5 ml of antigen coated RBCs into all the wells.
4. Shake the plate gently and incubate at room temperature for 30 mins and take the reading.
5. Always kept the negative control for compare the results.

Interpretation:

Wells showing a layer of uniformly agglutinated cells covering the bottom are considered positive. The negative results are shown as compact, sharply demarked discs of sediment cells in the of the bottom of the wells.

B. ReversePassiveHaemagglutination (RPHA) Test:

The technique utilizes stabilized red blood cells coated with specific viral antibody. If the corresponding antigen is present, the red cells will agglutinate. This technique is useful for identification of non-haemaglutinating viruses.

C. Latex Agglutination test:

This is a simple one step method used for detecting some viral antigens in clinical specimens. Latex carrier particles coated with antiviral antibodies are commercially available. Drop of antibody coated particles are mixed with a drop of clinical specimen suspension on a slide. Presence of specific antigen in the clinical specimen causes binding of antibody coated latex particles and visible agglutination occurs.

PRACTICAL NO. 6

PRECIPITATION TEST

Precipitation tests are based on the interaction between soluble antigens (precipitates) with their specific antibodies (precipitins) that lead to the formation of visible precipitates.

Broadly precipitation tests are categorized as

1. Gel precipitation tests.
2. Tube precipitation test.

General procedures of gel precipitation techniques:

The procedure of preparation of gel slides, cutting wells/trough, loading of samples, staining etc are similar for most of the gel precipitation techniques. So a general procedure is described below to be followed by the details of the respective technique whenever necessary.

i. Precoating of slides

i) Prepare 0.2 to 0.5% agar solutions in distilled water.

ii) Dip the microslide in molten agar solution.

iii) Dry the slides in slanting position in air at room temperature overnight.

The precoated slide should be protected from moisture to avoid fungal contamination and should be stored at room temperature.

ii. Gel medium

Agarose or Noble agar gel is usually used in diffusion techniques. For gel electrophoresis, electrophoretic grade agarose showing high electro-endosmosis is used.

iii. Buffer

The choice of buffer may differ based on the type of precipitation technique (s) in gel. For agar gel precipitation test usually phosphate buffer saline (PBS, pH 7.2 to 7.4) or barbitone buffer (0.05 to 0.1 M, pH 8.0 to 8.6) is used.

If antisera of avian species are used in the test, 1 to 8% sodium chloride should be added to the buffer to improve precipitation.

To avoid the growth of contaminating microorganism in the buffer, 0.02% sodium azide or 0.01% merthiolate should be added.

iv. Gel solution

For plain gel to be used in agar gel precipitation test, prepare 1 to 1.5% agarose in appropriate buffer with constant stirring in a boiling water bath.

For preparation of antiserum impregnated gel, mix equal volume of 2% melted agarose solution (earlier prepared in appropriate buffer and cooled down to 56^0C) and suitable diluted pre-warmed antiserum.

v. Preparation of gel slides

1. Place precoated micro slide on a horizontal leveled surface.
2. Pour 3.5 to 4.0 ml of melted gel solution with a pre-warmed wide mouthed glass pipette onto the slide carefully.
3. Allow the gel to solidify at room temperature for 3-4 minutes and then transfer gel slide to plastic boxes or petridishes containing a piece of wet cotton gauge or sponge (to maintain humidity).
4. Store the gel slides at 4^0C for 2-3 hours of further solidification before use.

vi. Gel punching

i. Place the gel slide over the template and punch wells of 2 to 4 mm in diameter and 5 to 6 mm apart as per requirement of the technique with a gel puncher.

ii. To make a trough or trench as required in immune electrophoresis, place the gel slide over the template and cut the gel with a pair of parallel blade mounted on a 2 mm Perspex blade.

iii. Remove the cut gel from the wells or through by scooping with a sharp needle or by aspiration with a vaccum pump connected with the gel puncher.

vii. Loading of samples

i. Prepare the test samples in the same buffer as that used in the gel.

ii. Fill the wells or trough up to the brim with samples using fine capillaries or by micropipette fitted with a tip. Use separate capillaries or tips for each samples.

For a well 2 to 4 mm diameter, 10 to 20µl sample can be loaded. For a trough of 40 mm x 2 mm, 100 – 150µl samples can be loaded.

viii. Incubation

Incubate the loaded slide in a humid chamber at room temperature for at least 24 hours to allow diffusion of the immune-reactants.

ix Washing, drying and staining of gel slides

The intensification and visualization of thin and weaker precipitin lines can be achieved by staining the gel slide with either coomassie brilliant blue or amido black stain. Staining also helps photography of the immune-precipitins and stained slide can be preserved for permanent records. Because of its greater sensitivity and better staining reaction, coomassie brilliant blue stain is commonly used for staining immuno-precipitins.

Washing and drying:

i) Wash the gel slides in NSS with 3-4 changes of fresh NSS for 24 hours to remove the non-precipitated proteins.

ii) Immerse the slide in distilled water for 1 hour to remove the salts.

iii) Dry the gel overnight at 37^0C for 18 hours at room temperature by covering the gel slide with a wet filter paper strip.

iv) Remove the filter paper strip after slight wetting.

Staining of slide with coomassie brilliant blue stain:

i) Stain the dried gel slide with 0.1% coomassie brilliant blue staining (A) for 25 minutes.

ii) Destain with destaining solution (B) for 5 to 10 minutes until the back ground is clear. The immune-precipitins stain blue.

iii) Dry the stained slide in air at room temperature.

Coomassie brilliant blue staining solution (A):

Coomassie brilliant blue	-	0.1 gm
Acetic acid	-	10 ml
Ethanol	-	45 ml
Distilled water	-	45 ml

Destaining solution (B):

Acetic acid	-	10 ml
Ethanol	-	45 ml
Distilled water	-	45 ml

Agar Gel Precipitation Test (AGPT):

This test also known as ouchterlony technique is a simple effective qualitative test for determining the relationship between antigens and for knowing how many different antigen antibody system are present. One of the greatest advantages of this test is that several antigens can be tested simultaneously against a particular antiserum and several antigen-antibody systems can be demonstrated simultaneously.

Principle:

Antigen and antibody placed in the wells of the agar or agarose gel diffuse towards each other and form a visible opaque band of precipitation in the optimal proportions. (zone of equivalence).

Materials required:

1. Pre-coated slides.
2. 1% agarose in PBS (pH 7.2 – 7.4) containing 0.02% sodium azide.
3. Antigen – Bovine serum albumin, 1 mg/ml in PBS.
4. Antibody – Rabbit anti bovine serum albumin
5. Gel punch.
6. Template
7. Needle
8. Micro pipette

Procedure:

1. Prepare required number of gel slides as described earlier.
2. Place a gel slide over a template and punch wells of 2 to 3 mm diameter and 4 to 5 mm apart with a gel punch.
3. Remove the cut gel from the wells by scooping with a needle.

4. Fill the central well to the brim with 20ul neat serum and the peripheral wells with 20 ul antigen. Alternatively, fill one peripheral well with NSS or PBS as control.
5. Place the slide in a humid chamber and incubate overnight at room temperature.
6. Examine the slides for precipitin lines.

PRACTICAL NO. 7

IMMUNOELECTROPHORESIS

Immunoelectrophoresis is a very useful technique to study antigen-antibody reaction. It has the advantage over simple gel diffusion technique in that the different fractions of the electrophoresed proteins are separated in an electric field and give better precipitation reaction. Electrophoresis involves the migration of charged protein particles in an electric field. The rate of migration depends on the magnitude of electric charge on the particles and some other factors like voltage of the current, pH of the buffer etc.

The net charge on a protein depends mostly on the pH of the solution. At a buffer pH below the isoelectric point, the protein migrates positively or as a cation, with the mobility increasing with decreasing pH .Conversely above the isoelectric point, the protein migrates negatively, or as anion, with solubility increasing with increasing pH. Positively charged substance travel toward the cathode(-ve electrode)while negatively charged substance go towards the anode(+ve electrode)

There are five main groups under electrophoresis-

A. Classical immunoelectrophoresis
B. Counter Immunoelectrophoresis
C. Rocket Immunoelectrophoresis
D. Two- dimensional Immunoelectrophoresis
E. SDS- polyacrylamide gel electrophoresis(SDS-PAGE)

A. Counter lmmuno-electrophoresis (CIE):

This technique which is also known as crossover immuno-electrophoresis is like AGPT except that an electric force is applied to the antigen and antibody together in the agarose gel. The major advantage of this technique is that a result can be obtained in about 1 hour.

Principle:

Under certain electrophoretic conditions some antigens when placed on the cathodal end of the gel slide, move towards the anodal end while the antibodies placed on the anodal end move towards the cathodal end. During the migration process, when the antigen and antibody meet, a precipitin line is formed.

Application:

i) Generally used for rapid diagnosis of some viral disease like FMD, Rinderpest, duck plague etc.

ii) For identification of some bacteria and Mycoplasma.

Materials required:

1. 0.08 M Barbitone buffer (pH 8.2).
2. 1% Agarose in 0.08 M barbitone buffer (pH 8.2).
3. Electrophoresis apparatus with power pack.
4. Rest same for AGPT.

Procedure:

1. Prepare gel slide with 1% agarose in 0.08 M barbitone buffer.
2. Punch two wells 5 to 6 mm apart and remove the cut gel.
3. Place antiserum in the anodal well and antigen in the cathodal end.
4. Run the slide in an electrophoresis apparatus for 30 mm. to 1 hour using 7.5 Amp per slide.
5. Remove the slide from the electrophoresis apparatus and examine for the precipitin line.

Interpretation:

Precipitin line appears between the antigen and antibody charged wells in positive reaction. Sometimes due to the slow migration of the antigen, precipitin line may not develop under the conditions described above. This can be overcome by increasing the period used for electrophoresis.

B. Rocket Immunoelectrophoresis (RIE):

This is also known as one-dimensional single electro-immunodiffusion,the technique. Antiserum to a particular antigen or antigens is incorporated into an agarose supporting medium on a glass slide. The samples of unknown quantity of the antigen are placed in a small well. The wells are cut toward the cathode anode. Most protein antigens migrate towards the anode during electrophoresis. After allowing for immunodiffusion, the immunoprecipitation resembles a spike or rocket. The height of the rocket is directly proportional to the antigen concentration. Rocket heights and their corresponding standard antigen concentrations are plotted on linear graph paper. A calibration curve is obtained while can be used to determine the concentrations of unknown samples. The sensitivity of the test is 0.5µg/ml of protein

PRACTICAL NO. 8

COMPLEMENT FIXATION TEST (CFT)

This is a sensitive serological test which can be used to detect and quantitate antigens and antibodies.

Principle

When a specific antigen is added to a serum sample containing its specific antibodies, they form an antigen-antibody complex. Subsequently when complement is added to this complex, it binds to the complex and forms antigen + antibody + complement complex. Such a complex can be detected by using an indicator system comprising of sheep erythrocytes sensitized with anti sheep erythrocyte antibodies (haemolysin). Since the complement binds the antigen antibody complex, no more free complement is available to lyse the sheep erythrocyte of the indicator system. If a serum sample does not contain specific antibodies to the antigen, no antigen antibody complex is formed and hence when complement is added, it remains free. These free complement cause lysis of the sheep erythrocyte of the indicator system.

Application:

Used for identification of viral antigens like FMD virus, Rinderpest virus etc.

Complement fixation test can be carried out in microtitre plates (micro-CFT) or in tubes (Tube CFT).

Micro complement fixation test:

The test procedure described below is used for detection of FMD virus antigen is tissue samples.

Materials required:

Microtitre plates (U-bottom).

Antigen antiserum

Complement (Fresh guinea pig serum, pooled).

2% sheep erythrocyte antibody (haemolysin)

Rabbit anti-sheep erythrocyte antibody (haemolysin)

Diluents -5X veronal buffer (prepared by diluting 1 par stock buffer with 4 pars of distilled water)

PBS (pH7.4)

Dropping pipettes

Incubator /Hot water bath.

Titration of minimum haemolytic dose (MHD) of complement:

It is absolutely necessary to determine the titre of the complement prior to its use in the test.

Procedure:

1. Make 1:10 dilution of guinea pig serum.
2. Set up two fold complement dilutions as per the following protocol:

Tube No.	1	2	3	4	5	6	7
Veronal Buffer (ml)	0.1	0.2	0.3	0.4	0.5	0.6	0.7
Guinea pig serum	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final complement dilution	1:20	1:30	1:40	1:50	1:60	1:70	1:80

3. Mix 0.1 ml of each complement dilution with 0.2 ml veronal buffer and 0.1 ml of sensitized erythrocytes.
4. Incubate at 37^0 C for 30 min., and centrifuge at 3000 rpm for 15 min.

The highest dilution of complement which shows lysis of RBC is taken as 1 MHD. In the CFT proper 2 MHD are used.

Titration of haemolysin:

For sensitization of sheep erythrocytes, the titre of the haemolysin must be first determined. The titration of haemolysin is carried out as described below:

1. Make 1:100 dilution of haemolysin in veronal buffer
2. Set up various dilutions of haemolysin from 1:100 stock dilution using veronal buffer.
3. Take 2ml of every dilution of haemolysin in separate tubes (starting from 1:100).
4. Add equal volume of 2% sheep erythrocyte into each tube and incubate at 37^0 C for 30 min.
5. Finally add equal volume of 2 haemolytic dose of complement.
6. Incubate again at 37 C for 30 min and take the reading.

The highest dilution of haemolysin that shows complete lysis of erythrocytes is considered as the titer or one MHD. Usually 2 haemolytic dose is required for sensitization of sheep erythrocyte.

Sensitization of sheep erythrocytes:

1. Wash the sheep erythrocytes earlier collected in Elsevier's solution thrice in PBS (pH 7.4) by centrifuging at 2000 rpm for 20 min.
2. Prepare a 2% suspension of the washed erythrocytes in veronal buffer.
3. Mix equal volume of 2% sheep erythrocyte and 2 MHD of haemolysin.
4. Incubate at 37^0 C for 30 min. with occasional mild stirring for sensitization of erythrocytes.
5. Store the sensitized erythrocytes in ice.

Test procedure of micro CFT:

The procedure described below is routinely used for detection of FMD virus antigens in tissue sample.

Preparation of test antigen:

1. Triturate the tissue sample in pestle and mortar and prepare a 10% suspension in PBS (pH 7.4).
2. Centrifuge the tissue suspension at 3000 rpm for 15 min.

3. Mix ht supernatant with an equal volume of chloroform and again centrifuge at 3000 rpm for 15 min.
4. Collect the supernatant and use it as test antigen.

Test proper:

i) Take 25µl of antigen suspension to each of the 4 wells of the microtitre plate with a dropping pipette.

ii) Add 25µl of know virus – specific serum to all the four wells.

iii) Add 25µl of complement (2MHD) into each well.

iv) Gently shake the plate manually or by using a rotary shaker.

v) Incubate the plate at 37^0 C for 30 min.

vi) Add 50µl of sensitized erythrocytes to each well and further incubate at 37^0 C for 30 min.

Parallelly keep antigen and antibody control wells as follows:

Antigen Control: 25µl of diluents + 25µl of antigen + 25µl of each of the complement dilution + 50µl of sensitized erythrocytes.

Antibody Control: 25µl of diluents + 25µl of antiserum + 25µl of each of the complement dilution + 50µl of sensitized erythrocytes.

Interpretation:

Absence of haemolysis is the wells containing test antigen, antibody, complement and sensitized erythrocytes as well as presence of haemolysis in the antigen and antibody control wells indicates a positive result.

The degree of fixation of complement is read in terms of amount of settling of erythrocytes which is graded in the scale of 0 to 4 as given below:

Grade	**% of haemolysis**	**Interpretation**
0	100	Negative
1	75	Positive
2	50	Positive
3	25	Positive
4	0	Positive

A reading of 2 or above in a particular type specific serum is the indication of the type to which the virus antigen belongs. Ideally all the controls, i.e., antigen control, serum control, complement control would show a score of 0, i.e., 100% haemolysis.

PRACTICAL NO.9

FLOURESCENT ANTIBODY TECHNIQUE (FAT)

Flourescent antibody technique is another method o9f antigen reaction. Antigen in the form of a smear or a tissue section treated with homologous antibody which has been labeled with a fluorescent dye (e.g.fluorescein isothiocyanate) when examined under ultraviolet microscope, will fluoresce.

There are two methods of fluorescent antibody technique which are referred to as

1. Direct Fluorescent Antibody Technique:

In direct method specific antibacterial or antiviral serum is first conjugated with fluorescent dye and then treated with the antigen before it is examined under ultraviolet microscope.

Materials required:

1. Ultraviolet microscope
2. *Brucella abortus* culture/antigen
3. Fluorescent dye
4. Phosphate Buffer Saline (PBS) pH 7.0
5. Moist filter paper

Procedure:

1. Prepare smears of *B.abortus* culture on clean slides, allow to dry and fix over flame.
2. Place a drop of *B.abortus* fluorescent antibody on each fixed slide and spread evenly over the smear.
3. Place each slide in a petridish containing moist filter paper and kept at room temperature for 30 minutes.
4. Wash the slide with 1% phosphate buffer to remove excess labeled antibody. Place the slides in a jar containing phosphate buffer for about 10 minutes.
5. Remove the slides from the jar and blot dry.
6. Cover the stained area with a drop of glycerol and then with a cover slip.

7. Examine the slides under fluorescence microscope with an ultraviolet source.

Results:

If the antibody is specific for the antigen, antigen-antibody complexes form and the labeled dye fluoresce giving a visible colour (yellow-green) under the microscope.

2. Indirect Fluorescent Antibody Technique:

In the indirect method unlabelled specific antibacterial or antiviral serum is first treated with the corresponding antigen and then with a fluorescent conjugated antibody globulin specific for the species from which antibacterial or antiviral serum is obtained.

Materials required:

1. Ultraviolet microscope
2. *Brucella abortus* culture/antigen
3. *Brucella abortus* infected cow serum
4. Fluorescein conjugated anticow globulin rabbit serum
5. Phosphate Buffer Saline (PBS) pH 7.0

Procedure:

1. Prepare a thin smear of *B.abortus* culture on clean slides, allow to dry and fix over flame.
2. Pour appropriate dilution (1:10) of the suspected cow serum on the smear.
3. Incubate in humidity chamber for about 30 minutes at 37^0C.
4. Wash the smear 3 to 4 times with phosphate buffer saline (pH 7.0) to remove excess of serum.
5. Pour appropriate amount of fluorescein conjugated anticow globulin rabbit serum and incubate again for 30 minutes at 37^0C in a humidity incubator.
6. Wash the specimen 3 to 4 times with phosphate buffer saline.
7. Dry the smear, mount under coverslip and examine under fluorescent microscope.

Results:

Record the observation.

PRACTICAL NO.10

IMMUNOPEROXIDASE TEST (IPT)

In this technique the enzyme (horse radish peroxidase) is attached to the antibody either chemically or by immune reactions and the peroxidase labeled antibody thus obtained is reacted specifically with homologous intracellular or extracellular viral antigens or viruses. The reaction is detected by use of appropriate substrate which reacts with the peroxidase and produce colour. The preparations can be preserved and maintained for subsequent examination by light microscopy.

This test is simple and can be applied on any cells taken on the glass slides, either mucous membranes, impression smears, buffy coats which is the leucocyte contents, cell culture smears, histological sections, or any other material which can be formed as monolayer on the glass slide.

There are two methods of Immunoperoxidase techniques-

1. **Direct method:**

In direct method specific antibody conjugate (antibodies conjugated with horse radish peroxidase enzyme) is added to the monolayer of infected cells on the glass slide. After incubation, a substrate which is reactive to the enzyme is added and then examined under ordinary microscope. The cells with viral particles will take brown or blue colour depending upon the substrate used.

2. **Indirect method:**

In indirect method specific antibody is added on the monolayer (cells in single plane) of the cells on the glass slide and incubated. Then the anti-antibody conjugate (anti-antibodies conjugated with horse radish peroxidase enzyme) is added on to the slide and incubated. After adding substrate, the slides are washed dried and can be examined under light microscope for the colour reaction of either brown or blue.

The main advantages of the technique are its sensitivity, specificity, rapidity and the preparations can be observed under light microscope and the preparations are permanent.

PRACTICAL NO. 11

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Principal

When an unknown antigen is passively absorbed onto a solid phase, its presence can be detected by specific antibody covalently lined to an enzyme. If the unknown antigen is specific to the enzyme labeled antibody, an antigen-antibody-complex will be formed which will bind to the solid phase. To this bound complex, if a substrate which is specific to the labeled enzyme is added, the enzyme will cause breakdown of the substrate resulting the colour development. The colour development can be detected either visually or in an ELISA reader.

Application

i. To identify the causative agents of infectious bacterial and viral diseases as well as for the identification of fungi, protozoan and metazoan parasites.
ii. To detect the presence of specific antibodies, antigens and to quantify the antigens and antibodies.
iii. To measure the hormone level.

General requirement of ELISA:

a) Solid phase, preferable 96 wells ELISA microtitre plates.
b) Coating buffer.
c) Blocking buffer.
d) Washing buffer.
e) Substrate buffer.
f) Stopping reagent.
g) Single and multichannel pipettes.
h) Disposable tips.
i) Wash bottles.
j) Antigen.
k) Antibody
l) Enzyme-labelled antibody and anti-species antibody.
m) Refrigerator.

n) ELISA reader.
o) Water bath / incubator.

Buffer/solutions for ELISA:

i. Coating buffer

Carbonate-bicarbonate buffer (0.05M. pH9.6) is commonly used as coating buffer.

ii. Washing solution

PBS (pH7.4) containing 0.5% Tween-20 is used as washing solution.

iii. Blocking buffer

Any one of the following agent is incorporated at a concentration shown against them in PBS (pH7.4) to prepare the blocking buffer. The type of the blocking agent as well as its concentration to be used in preparing the blocking buffer must be first standardized prior to its use.

Blocking agent		Concentration
Tween-20	………..	0.05 – 0.1 %
Bovine serum albumin	………..	1.5 – 5 %
Foetal calf serum	………..	1.0 – 10 %
Skimmed milk powder	………..	3.0 – 5 %
Lactalbumin hydrolysate	………..	1.0 – 5 %
Hor4se serum	………..	1.0 – 50 %

Enzyme:

The enzymes which are used for labeling of specific antibodies in ELISA include:

i. Horseradish peroxidase (HRPO), RZ-3 and RZ-0.6
ii. Alkaline phosphatase
iii. B-D galactosidase

Substrate:

The substrates which are used for different enzymes are given below:

i. Ortho-phenylenediamine (OPD) and $H_2 O_2$ for HRPO

ii. P-nitrophenyl phosphate for alkaline phosphatase.

iii. O-nitrophenyl-D-galactoside (ONPG) for B-D galactosidase.

Substrate buffer:

i. For OPD – Citrate buffer (0.1M, pH 5.0)

ii. For P-nitrophenyl – Diethanolamine buffer

iii. For B galactosidase – P.B.S. (pH 7.4)

Substrate solution:

i. For HRPO

OPD – 40 mg

Citrate buffer – 100 ml

(0.1 M, pH 5.0)

Prepare freshly and protect from light. Finally add 10 of H_2O_2 (30%) to the above solution just before use.

ii. For alkaline phosphatase

P-nitrophenyl phosphate – 5 mg

Diethanolamine buffer – 5 ml

iii. For B-galactosidase

Stock Solution

ONPG - 90 mg

PBS (pH 7.4) - 10 ml

Heat to 50^0C to dissolve, Dilute the stock solution 1:10 on PBS (pH 7.4) containing 10m M Mg Cl_2 and 0.1 M2-ME.

Stopping reagent:

1 M $H_2 SO_4$ is used as stopping reagent.

Types of ELISA:

The various techniques of enzyme linked immunosorbant assay are as follows:-

A. Direct ELISA
B. Indirect ELISA
C. Sandwich /Trapping/Capture ELISA
D. Competition ELISA
E. Liquid phase blocking sandwich ELISA

In liquid following exercise, the methodologies of Indirect and Sandwich ELISA have been given.

A. Indirect ELISA:

The technique involves specific binding of the antigen with antibody. The antigen to be identified is presented to detecting antibody (1st antibody) on the solid surface absorbed by hydrophobic/covalent bonding. If the antigen and detecting antibody are homologous, antibody binds with the antigen. This antigen-antibody binding is detected by using an anti-species second antibody conjugated to an enzyme, e.g. horse radish peroxidase.

The antispecies antibodies are raised against serum globulins of that species in which antigen specific detecting antibodies have been raise. For example, if antiserum is raised in guinea pig, then antispecies antibody is raise in another species by immunizing with normal guinea pig serum globulins.

Advantages:

More specific for detection and quantification of unknown antibody in serum samples.

Disadvantages:

1. Direct coating of some antigen like insoluble antigen is sometime not possible.
2. Labile antigen like FMD antigen may loose specificity.
3. More time consuming.

Procedure:

The procedure described below is used for detection of Infectious Bursal Disease (IBD) antibodies in avian serum.

1. Make 1:100 dilution of IBD antigen (earlier prepared from supernatant of 50% suspension of IBD infected CAM and embryonic tissue) in coating buffer.
2. Coat each well of the microtitre plate with 50µl of antigen suspension.
3. Incubate overnight at 4^0 C.
4. Wash the wells three times with PBS (pH 7.4) containing 0.05% Tween-20.
5. Add 50µl of test sera diluted 1:100 to 1:800 in blocking buffer (PBS containing 8% NaCl and 0.05% tween-20) to each well.
6. Incubate the plate at 37^0 C for 1 hr.
7. Wash the wells with washing buffer.
8. Add 50µl of anti-chicken sheep globulin (1:8000 dil.) to each well.
9. Incubate at 37^0 C for 1 hr. and wash the wells with washing buffer three-times.
10. Dispense 50µl of anti-sheep peroxidase conjugate (1:7500 dilution in blocking buffer) to each well.
11. Incubate at 37 ^{0}C for 1 hr. and wash the wells with washing buffer as described earlier.
12. Ad 50µl of freshly prepared substrate solution to each well.
13. Incubate the plate at 37^0 C for 15 min.
14. Stop the enzyme reaction with 50µl of 1 M sulphuric acid.
15. Record the colour development visually and record the absorbance of each well in an ELISA reader after keeping the plates overnight at 4^0 C.
16. Keep appropriate antigen, serum, globulin and anti-globulin conjugate control in the test.

Interpretation:

Serum samples showing colour development similar to the positive control wells or samples showing an OD value of 0.2 or above after subtracting from blank or negative control wells are considered as positive.

B. Sandwich ELISA:

In this technique specific antibodies are first positively absorbed onto the solid surface to which antigen is then added. In the subsequent steps of the test, the detecting antibodies are added and the binding of the detecting antibodies is ascertained either directly or indirectly.

As the antigen is finally trapped on sandwich between two antibodies, this technique is called sandwich ELISA.

Advantage:

Very specific for detection of unknown antigen present in very low level or in crude mixture.

Disadvantage:

Antibodies raised preferably in two species are required. For example, if the first antibody is raised in bovine, the enzyme labeled antibody should be preferably raised in rabbit.

The procedure described below is used for detection of foot and mouth disease (FMD) virus antigens.

Procedure:

1. Dilute the rabbit anti-FMD IgG raised against virus types O, A, C and asia-1 to a concentration of 10µg/ml in coating buffer.
2. Dispense 50µl of the diluted rabbit anti-FMD IgG into 4 rows of wells.
3. Cover the plate and incubate at 37^0C for 1 hr. following by overnight at 4^0C.
4. Empty the plate by inversion over a sink and wash the wells thrice with water.
5. Add 50µl of test antigen per well to all the wells.
6. Cover the plate and incubate at 37^0 C for 1 hr.
7. Dilute the pretitrated guinea pig anti-FMD serum in dilution buffer.

8. Add guinea pig anti-FMD serum at the rate of 50µl per well to each set of wells earlier coated wit rabbit anti-FMD IgG and also to known positive homologous control wells. Add negative serum control of normal, healthy guineapig serum diluted in dilution buffer to one set of antigen control wells. For sub-typing, add each dilution of serially diluted guineapig serum at the rate of 50µl to a set of (atleast 2 wells) antigen wells.
9. Wash the plate as in step 4.
10. Add anti-guineapig HRPO conjugate buffer at the rate of 50µl per well to all the wells.
11. Incubate the plate at 37^0C for 1hr.
12. Wash the plate as in step 4.
13. Add 100µl of substrate solution to each well.
14. Incubate the plate at 37^0C for 15 min or until colour develops in dark.
15. Stop the reaction by adding 50µl of $H_2 SO_4$ to each well.
16. Read the results visually or in an ELISA reader.

Interpretation

In typing test, the maximum O.D. obtained against the different sera after taking into consideration the controls is compared and is assigned to the type where maximum O.D. is obtained with a minimum of at least 0.2 O.D. differences with other sera.

PRACTICAL NO. 12

ASSAY OF CELL – MEDIATED IMMUNE RESPONSES

Cell-mediated immunity (CMI) plays an important role in host defence against intracellular pathogens and clearance of virus infected cells, tumor cells and foreign graft. CMI responses can be divided into two categories. One group involves direct cytotoxic activity while the second group involves a subpopulation of T helper (T_H) cells, called T_{DTH} cells that mediate delayed type hypersensitivity (DTH) reactions.

Some of the technique which are used to test the integrity of the CMI response are as follows:

A. DTH skin test.
B. Migration inhibition assay
   i. Leucocyte migration inhibition test
   ii. Macrophage migration inhibition test
C. Skin sensitization test.
D. Passive transfer fest.
E. Lymphocyte stimulation test
F. Skin graft rejection test.
G. Host rejection (graft V host reaction).

In the following exercise, methodology of DTH skin test has been outlined.

DTH skin test:

Intradermal test provides a classic model for study of delayed type hypersensitivity which reflects the CMI responses of the host.

Principle:

On intradermal injection of the antigen to which the animal has an earlier contact, an indurated, erythromatous inflammatory reaction develops at the site of injection usually after 24 hrs and reaching the peak at 48-72 hrs. The reaction occurs due to the interaction of T-lymphocytes with subsequent release of lymphokines by these cells. These lymphokines in association with the lysosomal enzymes released by the inflammatory cells like macrophages and neutrophils cause inflammatory reactions and tissue damage at the test site.

In the following exercise the procedure of tuberculin test is outlined.

Materials required:

1. Tuberculin
2. Tuberculin syringes with needles.
3. 70% alcohol
4. Razor
5. Measuring scale.

Procedure:

1. Shave the hair from an area of the neck region of a cow and measure the skin thickness.
2. Inject 0.1 ml of mammalian tuberculin with a tuberculin syringe into the shaved area intradermally.
3. Take the skin thickness reading of the test site after 48 hrs.

Observation and interpretation:

If the animal is suffering from tuberculosis an inflammatory reaction will occur at the site of inoculation. A skin thickness of 4 mm or more than the pre inoculation skin thickness is considered as positive.

PRACTICAL NO.13

VETERINARY BIOLOGICALS (visit and appraisal)

APPENDIX

1. Alsever's solution

Glucose ………………….. 20.5 gm

Sodium chloride …………. 4.2 gm

Sodium citrate …………… 8.0 gm

Citric acid..………………. 0.55 gm

Sterilize the solution at 101b pressure for 15 minutes and store at 4 C.

2. Barbitone buffer (0.08 M, pH 8.2)

Barbital sodium (5, 5 – diethyl barbitureic acid sodium salt) - 12.00 gm

Barbital (5, 5 – diethyl barbituric acid - 4.4 gm

i. Dissolve sodium barbital (12.00 gm) in 800 ml distilled water.

ii. Dissolve barbital (4.40 gm) in 150 ml of distilled water at 95^{0} C.

iii. Mix solution 1 and 2 adjust pH to 8.2 with 5 M sodium hydroxide and make upto 1 litre with distilled water.

3. 50% buffered glycerine

PBS (pH 7.4)	-	50 ml
Glycerine	-	50 ml

Sterilize at 15 lb pressure for 30 min. and store at 4^{0} C.

4. Hanks balanced slat solution (HBSS, Ca, Mg free)

Sodium chloride	-	8.0 gm
Potassium chloride	-	0.4 gm
Di-sodium hydrogen phosphate	-	0.06 gm
Potassium dihydrogen phosphate	-	0.06 gm
Dextrose	-	2.0 gm
Sodium red	-	0.32 gm
Phenol red	-	0.02 gm
Distilled water	-	1000 ml

Sterilize the solution at 15 lb pressure for 10 min.

5. Phenol saline (0.5%)

Sodium chloride	-	0.85 gm
Phenol	-	0.5 gm
Distilled water	-	100 ml

6.Phosphate buffered saline (PBS) pH 7.4

Sodium chloride	-	8.0 gm
Potassium di-hydrogen phosphate	-	0.2 gm
Di-sodium hydrogen ortho phosphate	-	1.16 gm
Potassium chloride	-	0.2 gm
Distilled water	-	1000 ml

6. Saturated ammonium sulphate solution

Ammonium sulphate	-	100 gm
Distilled water	-	100 ml

Dissolve ammonium sulphate in hot distilled water by stirring at 50 C. allow it to cool overnight at room temperature. Filter the solution and store at 4 C. adjust pH of the supernatant at room temperature to 7.2 just before use using dilute ammonia solution.

7. Veronal buffer (stock)

Sodium chloride	-	85.0
Barbital sodium	-	3.75 gm
(5, 5 diethyl barbituric acid, sodium salt)		
Barbital (5, 5 diethyl barbituric acid)	-	5.57 gm
Magnesium chloride	-	1.68 gm
Calcium chloride	-	0.28 gm

First dissolve 5.75 gm of barbituric acid in 500 ml hot distilled water. Then add other ingredients at room temperature and make exactly upto 2 litres with distilled water. Autoclave for 30 minutes and store at 4^0 C.

BUFFER FOR ELISA

1. **Coating buffer** (carbonate-bicarbonate buffer, 0.05M, pH 9.6)

Sodium carbonate	-	1.59 gm
Sodium bicarbonate	-	2.93 gm
Distilld water	-	1000 ml

Store at 4^0 C for maximum upto 14 days.

2. **Washing buffer** (PBS /Tween-20)

PBS(pH 7.4)	-	1000 ml
Tween-20	-	50 µl

3. **Blocking buffer**

Washing buffer	-	100 ml
BSA fraction V	-	1.0 gm

Or

Any other blocking agent at the concentration mentioned in the test.

Prepare just before use or store at 4^0 C.

4. Substrate buffer

i. Citrate buffer (0.1M, pH 5.0)

Citric acid	-	5.11 gm
Di-sodium hydrogen ortho phosphate	-	9.15 gm
Distilled water	-	1000 ml

ii. Diethanolamine buffer

Diethanolamine	-	97 ml
Sodium azide	-	0.2 gm
Magnesium chloride	-	100 gm
Distilled water upto	-	1000 ml

INDEX

Sl. No.	Practical	Page No.	Signature
1.	Preparation of Equipments and Glassware For Virological Work		
2.	Collection of Material For Diagnosis of Viral Diseases		
3.	Filtration and Filtration Technique		
4.	Cultivation of Viruses- Chick Embryo Inoculation		
5.	Cultivation of Viruses: Chick Embryo Inoculation-II (Methods of Inoculation)		
6.	Cultivation of Viruses In Tissue Culture		
7.	Cultivation of Viruses: Animal Inoculation		
8.	Laboratory Diagnosis of Important Viral Diseases of Animals		
9.	Laboratory Diagnosis of Poultry Viral Disease		

PRACTICAL No. 1

PREPARATION OF EQUIPMENTS AND GLASSWARE FOR VIROLOGICAL WORK

Various types of glassware and instruments commonly used in virology laboratory

Glassware:	Other materials:	Instruments
Conical flak	Scissors	Laminar flow
Beakers	Plastic vessels	Electric sterilizer
Pipettes – 1, 2, 10 ml	Rubber corks.	Deep freezer
Petri dishes	Scalpels	Egg Candler
Milk dilution bottles	Rubber tubing	Various grade filters
Prescription bottles	Pestle and mortar	Glass distillation unit
Trypsinization flask	etc	De-ionizer
Test tubes		Incubators (including B.O.D.)
vacuum filtration flask		Egg incubator
Tuberculin syringes		Centrifuges (Refrigerated & ultracentrifuges.)
Etc		Hot air oven
		Refrigerator.
		Water bath.
		Freeze dryer.
		Vacuum & pressure pump
		Electric monopan balance
		etc

Preparation of glassware –

A. **Washing:** Contamination of cultures with microbial agents is the problem in the tissue culture work. Also glassware contains toxic produces derived from manufacturing procedures, packing and hence they should be prepared with utmost care.

   a) **Washing of new glassware:**

      1) Immerse the glassware in dilute 4% HCI overnight.
      2) Rinse thoroughly in tap water.
      3) Boil in 1% Teepol or any detergent solution for one hour.

4) Rinse in tap water to remove detergent solution.
5) Immerse the glassware in double glass distilled-water and boil for one hr
6) Rinse with double glass distilled water.
7) Allow to drain the water and keep for drying in a hot air oven at 63-70°C

b) **Washing of used glassware:** If the used glassware contains infectious material, they should be autoclaved. Alter immersing in detergent solution overnight wash the glass ware in tap water and scrub out to remove dirt. Immerse the glassware in 1% detergent solution and boil for one hour (follow from step 4)

Pipettes are washed similar to glassware. Care should be taken to see that enough water is passed through it during rinsing. Dry overnight at 60°C.

Immerse bungs in dil l% $NaHCO_3$ solution, boil for l0 min. Wash under tap water for 10-15 min. Rinse and dry in oven at 60°C.

Scissors, forceps, scalpels etc may be boiled in 10% soap solution and then washed as for glassware. They can be wiped with a swab soaked in carbon tetrachloride before being dried and sterilized.

B. Sterilization: All the glassware should be dried immediately after washing. Tubes, flasks, beakers, pipettes etc, must be kept inverted so that water comes out quickly. After thorough drying, the mouth of the tubes, bottles and flasks should be plugged with non absorbent cotton wool and wrapped with muslin cloth followed by aluminum foil and packed in paper. In case of pipette, broad end must be plugged with non absorbent cotton and then packed in groups of 4-5 covering their mouth and tip with foil and place them in pipette box for sterilization.

All scissors, scalpels, forceps etc should he dried in oven and wrapped in aluminum foil individually and placed in stainless steel box for sterilization.

The glassware is sterilized in hot air oven. The instruments and glassware mounted with metal plates (filters etc) or fitted with rubber stoppers or rubber tubing's should be autoclaved. Plastic or polystyrene wares can be sterilized by immersing in absolute alcohol or by ultra-violet radiation for l hour.

PRACTICAL NO.2

COLLECTION OF MATERIAL FOR DIAGNOSIS OF VIRAL DISEASES

On the face of an outbreak or a disease, collection of clinical specimens in proper preservative, their transportation to the laboratory under ideal conditions and quick processing of the specimens in the laboratory to identify the causative agent are crucial in successful treatment and control of the disease outbreak. This attains additional importance when a highly contagious or infectious disease appears.

Purpose of collection of specimens:

1. For virus isolation
2. For serological investigation.
3. For direct examination (rapid diagnosis).

The specimens for virus isolation are usually taken from the affected regions e.g. throat swab or nasal swabs are taken when the infection involves the upper respiratory tract, and sputum is taken for the lower respiratory tract infections. Scrapings are taken from the lesions of the skin and mucous membranes, and if vesicles are present at these sites, the vesicular fluid is also removed. Blood is withdrawn in the cases of rash. Signs related to central nervous system (CNS) involvement indicate the collection of Cerebrospinal fluid. If involvement is due to large number of viruses whose normal habitat is the alimentary tract, stools or rectal swab are also obtained.

In case of serum, two sera samples (paired) one at the beginning and another about l-2weeks later, are taken to show the rise or fall of the antibody level. The following points should be remembered while collecting specimens.

1. The concentration of virus is usually the highest at the affected sites and during the early stages of the disease.
2. In diseases where viremia is noticed, the maximum liter of the virus often coincides with peak pyrexia and may precede the onset of other clinical signs, therefore serum collection is a must.
3. Secondary bacterial infections are a common sequel of viral disease. As such, samples taken from the later stages of the disease or at post—mortem are less likely to contain viable virus in detectable concentration.

Selection of specimens in relation to the disease has been shown below:

Table: A general outline for the samples to be collected in different diseases.

Samples to be collected		
Disease	**Biopsy material**	**Post-mortem material**
Respiratory and ocular disease		Tissues from selected areas of the affected system and the associated lymph nodes.
Skin diseases and lesions of mucous membranes of mouth, genital tract, gastroenteritis	Deep skin scrapings, swabs from affected area, blood, faecal sample, rectal and genital swabs etc.	Tissues from selected areas of the affected system and the associated lymph nodes.
Systematic disease	Blood, nasal and uriogenital swabs and faeces	Tissues from various organs, including lymph nodes, bladder, liver, spleen, kidney, bursa, ovaries, lungs, heart
Disease of CNS	Blood, CSF, deep nasal swabs	Brain, CSF, spinal cord & associated nerves, heart blood.
Diseases of urinary tract	Uriogenital swab, urine and blood	Kidneys, urinary bladder, urine, ovaries, testes etc.
Abortion	Blood from dam, vaginal mucus, biopsy of lymph node of that area, semen, blood from bull etc.	Tissues from placenta and other organs of fetus, blood from fetal heat, blood from aborted animals lymph nodes, testes, uterus. Ovaries etc.

All materials collected for viral diseases, should be in 50% glycerin saline for virus isolation or detection

Mention the materials to be collected for the diagnosis of diseases mentioned below

BOVINE:

Sr. No.	Diseases	Material to be collected
1	Ephemeral fever	
2	Malignant catarrhal fever	
3	Rinderpest	
4	Mucosal disease/viral diarrhoea.	
5	Foot and mouth disease	
6	Infectious bovine Rhinotracheits/ infectious vulvovaginitis	
7	Bovine Enterovirus	
8	Cowpox, buffalo pox	
9	Influenza and Para influenza	
10	Rabies	
11	Adenovirus	

EQUINE:

1	African horse sickness	
2	Equine herpes virus I and II	
3	Influenza	
4	Equine encephalomyelitis	
5	Equine infectious anemia	
6	Rabies	

OVINE AND CAPRINE:

1	Tongue	
2		
3	Louping ill (encephalomyelitis)	
4	Visna and maedi	
5	Scrapie	

PORCINE:

1	Seine pox	
2	Viral pneumonia	
3	Swine fever	
4	Swine influenza	
5	Vesicular exanthema and vesicular Stomatitis	
6	Pseudo rabies	
7	Foot and mouth disease, swine vesicular disease	

CANINE:

1	Rabies	
2	Infectious canine hepatitis	
3	Canine distemper	
4	Canine parvovirus	

AVIAN:

1	Ranikhet disease.	
2	Fowl pox	
3	infectious laryngeotracheitis	
4	Infectious bronchitis	
5	Merek’s disease.	
6	Avian encephalomyelitis	
7	Duck virus hepatitis.	
8	Infectious bursal disease	
9	HPS/Leechi disease	

PROCESSING OF MATERIAL FOR VIRUS ISOLATION

As in the collection of the specimen, it is essential that aseptic techniques should be followed in the processing of all specimens. Specimen should be maintained throughout the processing in ice water baths. Prechilled diluents should be used. Mortars when used should also be prechilled. The chilling temperature varies from 1°C to 3°C.

1. **Blood:** A viremic phase is a part of the pathogenesis of certain viruses eg. Togaviruses, Poxviruses, Enteroviruses etc. These viruses can be isolated from the blood collected during viremicphase. After collection the blood should be allowed to clot for about 20 minutes at room temperature and it is then transferred to a refrigerator for 1-2 hours at 4°C. Serum is separated from the clot and stored in sterile containers. In the case of heparinised blood samples, the plasma is separated from the cells after centrifugation at about 1000 r p m.in those cases in which a suspension of the blood clot has to be tested for virus, the clot is around in a mortar with the aid of allundum and suitable diluents, such as phosphate buffer saline (PBS) at pH 7.2. The suspension is centrifuged at 1000 G and supernatant is collected for further processing.
2. **Faeces and rectal swabs:** Enteroviruses, parvoviruses and rotaviruses are commonly encountered in the faeces.
   - **(i)** **Faeces:** A 10% or 20% suspension of faeces is made in prechilled PBS. Large particles from this suspension are allowed to settle by keeping the tube at 4°C for half an hour. The supernatant fluid is then decanted and centrifuged at 1000 G (l500rpm) for 10 min. After centrifugation the supernatant fluid is aspirated with a pipette and antibioses are added to it to give final concentration of 1000 units of sod. Penicillin G and 2 mg of streptomycin per ml. A second centrifugation may be necessary if the supernatant fluid cannot be properly separated from the sediment.
   - **(ii)** **Rectal swabs**: The cotton swab is removed from the application stick, with the aid of forceps and is introduced into the barrel of a 5 ml syringe with about 2 ml NSS The plunger is forcibly driven home, the expressed fluid being collected in a tube or vial. This process is repeated three times. The fluid is then centrifuged at 1500 G (2000 rpm) for one hour or at 9000 G(1000 rpm) for 20 min at a temperature of4°C and the fluid aspirated is stored for further processing.

(iii) Saliva, nasal and pharyngeal washings: The following viruses can be isolated from saliva, nasal and pharyngeal washings.

a. Saliva: Rabies, FMD, Mumps and Herpes viruses

b. Nasal washing: Influenza and Rhinoviruses

c. Pharyngeal washing: Rabies Adeno, Entero, Rabies Influenza, Para influenza. Reo. Rhino, herpes, mumps and Pox viruses.

For ticks, the quantity of diluents used series with the stage (larva, nymph or adult) of the ticks, the size of pool and on the state of the ticks whether engorged or un engorged. Between 2 to5 ml of diluents is used.

After processing the samples for virus isolation, all the sterile sample are eventually either inoculated in laboratory animals embryonated eggs of in tissue culture tribes depending on the type and nature of the virus.

PRACTICAL No. 3

FILTRATION AND FILTRATION TECHNIQUE

Filter is a material which is capable of stopping and filtering out the smaller dirt panicles and the larger bacteria and fungi. It cannot inhibit the virus particles. Filtration efficiency depends on the material of which the filter is made, the temperature of the liquid to be filtered, density of` particulate matter in the solution and the viscous nature of the material to be filtered. Warm material filter faster than colder ones. Liquid media filter faster than serum. Filtration is used for sterilization in virus work.

The technique of filtration is especially useful in making preparation of the soluble produce of bacterial growth such as toxins and in sterilizing liquids that would be damaged by heat such as serum and antibiotic solution. These filters have very small pores which arrest the passage of bacteria and fungi. The British pharmaceutical codex test for bacterial filters requires that efficient filter should be able to retain ***Serratiamarcesens***. This indicates an average pore diameter of 0.25 micron of less. The various types of filter used are as follows

1. Earthenware candles e.g. Berk field (Made up of diatomaceous earth and graded in 3 porosities) or Chamber lands filters (Made up of porcelain molded into candle form and graded).
2. Asbestos and asbestos paper discs e.g. Seitz filter. They are Consists of discs of compacted asbestos composition through which coarser particles can be passed.

   Procedure:

   The filter is assemble with asbestos disk in position and central tube of filter holder is passed through a rubber bung which is placed and fixed on mouth of filtration flask. The whole apparatus is wrapped and autoclaved. This disc is first conditioned with sterile distilled water (about 1 liter) by passing in though asbestos filter disc which removes the loose asbestos particles from the disc before pouring media or solution to be filtered. Then pour medium in metal container connect the side arm of vacuum conical flask to the vacuum pump. The creation of negative pressure in the flask will allow the fluid to come down.
3. Sintered glass filter. Its use uncommon
4. Cellulose and colloidal membrane filters — Millipore filters e.g. **Gradocol membrane:** The Gradacol membranes are micro cellulose membranes of graded porosity and are of two types.

The microgel— It has coarse structure visible microscopically.

Ultragel — The standard elements are not visible under microscope, but it is made of particulate matter. These membranes are not sufficiently permeable to filtration work. They are sterilized by steaming not by autoclaving.

Modern membrane filters:

Developed by Millipore filter steaming co-operation., USA and therefore referred as Millipore fillers. The filler consists of cellulose acetate and are therefore composed of two layers Basal layer with pores of 3 to 5 micron and upper layer with the pores of` 0.5 to l micron India meter. This structure gives the remarkable degree of porosity and yet ensures that bacteria are trapped on the surface. The filters are effective in retaining ***Serratiamarcesens***. They also withstand sterilization by autoclaving and can be stored indefinitely in dry condition. Their size range from 7 cm to 14 cm and can be filled into metal holder.

Cellulose membrane filter has several advantages over the widely used Seitz asbestos filter since they are much less absorptive and rate of filtration is much greater. Secondly the filter free from any debris from filter membranes.

Assembling of filter:

The filler consists of limb, body and funnel in which the metal disc with. Pore sis placed. The disc whether asbestos or cellulose acetate is placed with correct side up. In case of asbestos disc smooth surface should lace upward. This is placed on metal disc. The funnel is attached to mouth of filtration flask with help of rubber bung in cellulose acetate filters shining surface should face upward.

The body of filter is then placed gently over membrane without displacing the membrane and tightened.

The nozzle of funnel should be facing away from side nozzle of filtration flask so that during suction the filtrate do not suck in pump. The side nozzle of flask should be plugged with non absorbent cotton to prevent entry of moisture or contaminant. The whole assembly is then secured and autoclaved.

The rate of filtration through any filter depends on

(1) Size of particle

(2) Amount of fluid

(3) Electrical charge of particle

(4) Electrical charge of filter membrane

(5) Diameter of pores of filter membrane.

(6) Pressure employed- positive or negative pressure

(7) Viscosity and pH of medium

(8) Duration of Filtration

PRACTICAL No. 4

CULTIVATION OF VIRUSES- CHICK EMBRYO INOCULATION

Viruses grow intracellularly and hence living cells are required for cultivation. Like animals, embryonated chicken eggs also possess highly specialization tissues and organs and are frequently used to grow various viruses particularly those infecting chickens and other birds.

Purpose of chick embryo inoculation:

(i) Primary isolation of pathogenic viruses.
(ii) Blind pass aging for isolation of unknown viruses.
(iii) Titration of` viruses
(iv) Preparation of stock strains of viruses in laboratory
(v) Yielding large quantities of virus for vaccine production
(vi) Attenuation of viruses
(vii) Study of mode of multiplication and growth curve of viruses
(viii) Performing neutralization test for detection of antibodies
(ix) Study of effect of chemotherapeutic agents

Advantage of chick embryo inoculation

(i) Cheap and economical as compared with animals and tissue culture.
(ii) They are free from antibodies if obtained from parents of disease free flock. (SPF-Specific pathogen free)
(iii) Methods of inoculation and harvesting embryonic tissues and fluid are also simple.
(iv) Large sample size can be used for each virus i.e. 6 to 12 eggs for each sample.
(v) Required minimum space for incubation, no manpower for cleaning, feeding and watering is needed.

Selection and incubation of eggs

(i) The eggs should be from healthy disease free and unvaccinated flock.
(ii) The white shelled egg are easy for candling, hence preferred
(iii) Clean and porous eggs should be selected whereas, those having rough, bloody spots are rejected.

(iv) Eggs should be of normal size and shape. Too tapering or too flat eggs are generally infertile.

(v) Eggs should be from poultry maintained on antibiotic free diet.

Eggs are to be incubated at 37°C with 60-80% humidity. The temperature below 33C and above 44°C is lethal to embryos. They are kept in incubator with their broad end upwards and turned twice daily.

Candling:

Candling is the transillumination of the content of eggs with the help of electric light in a dark background. It is done in box with 40 W bulb attached inside & a black paper with hole in center is placed in front of the bulb to direct beam of light. The egg is held with broader end into beam of light so that inner structures are visible, these are air space, blood vessels, a moving embryo, yolk shadow and eye spot.

A dead embryo will show air space, yolk shadow and no intact blood vessels (Static or non moving embryo).

An in fertile egg will show yolk shadow and air space.

Some factors which effect the multiplication of the viruses in chicken egg are:

(i) Age of embryo

(ii) Route of inoculation.

(iii) Concentration and volume of inoculum

(iv) Temperature of incubation

(v) Time of incubation after inoculation

Harvesting of chick embryo:

After inoculation by any of the methods described above, the eggs are incubated at 37Cand candled daily to check whether embryos are dead or alive. Once the embryo dies the dead eggs are transferred to freeze between 4C-10C for chilling of the contents. After 3-4 hours the eggs are removed and placed on an egg rack, with air sac facing upward. The chilling process is done to contract blood vessel, so that the RBCs do not leak out into surrounding fluid during harvesting procedure. The shell over air sac is broken gently with forceps to expose inner shell membrane.

Shell membrane is pealed off gently and CAM is picked up with forceps and Pasteur pipette is introduced and 5-10 ml of allantoic fluid is collected from 10 day old and placed in sterile glassware which is then placed in deep refrigerator till further use in serological test or haemagglutination test.

The chorioallantoic is then cut open and other contents of egg i.e. yolk sac and embryo are emptied into sterile petridish. The embryo and yolk sac are observed for any lesions of virus which was inoculated. The chorioallantoic membrane is removed off from shell and observed for lesions (pock lesion) after placing it in petridish with N.S.S. if any lesions are observed, the yolksac, embryo and CAM are preserved in deep freezer for further use.

Certain pathogenomic lesions and changes seen in embryo /CAM/ Yolk sac:

1. Haemorrhage of subcutaneous tissues seen in NDV.
2. Congestion of epidermis
3. Curling and drawing of embryo eg. Infection Bronchitis
4. Thickening and fibrosis of amnion
5. Reduction in amniotic fluid
6. Thickening and edema of CAM
7. Pock on CAM
8. Inclusion bodies in embryo tissues.

PRACTICAL No. 5

CULTIVATION OF VIRUSES: CHICK EMBRY0 INOCULATION-II

(Methods of inoculation)

(a) Chorioallantoic membrane method (CAM):

Candle is 9 to 11 day old embryo. Wash the egg Mark two spots on egg, one spot on side of egg and other over the center of air space Apply spirit and drill the hole using 16 gauge needles at both spots scrape off shell membrane gently without puncturing it at spot on the side. Keep the egg horizontal and with the help of rubber bulb placed over hole on air sac suck out the air. This causes artificial air sac below hole on side of egg. By holding artificial air sac upwards inoculate 0.l-0.2 ml of virus using half inch 27 gauge needles and tuberculin syringe into artificial air sac. Seal holes with molten paraffin wax and place the egg in incubator horizontally with artificial air sac facing upward to prevent virus suspension from touching shell membrane. Candle eggs daily to check whether embryo is dead or alive

Advantages:

Can be used or a pock counting assay of those viruses which show pocks. This method can also be used for quantification of these viruses.

(b) Allantoic Method:

Candle a 6 to 9 day old visible embryo and wash the eggs embryo with soap and water. Mark the air space. Mark a spot l cm below the air space in an area free of blood vessels and away from the eye spot. Drill the hole with the help of I6 gauge needle without damaging underlying shell membrane. Apply spirit to sterilize the area and inoculate 0.1 to 0.2 ml of virus suspension using 27 gauge half inch needle and tuberculin syringe. Insert only the below of the needle and press the piston of the syringe to release the inoculum. After inserting the needle's bevel aspirate a little fluid. Allantoic fluid is straw colored translucent thin fluid. After inoculation seal the hole with molten paraffin or wax. Place the egg in incubator at 37°C and candle daily to check whether embryo is dead or alive.

Advantage:

A l0 days old embryo contains about 8 to l0 ml of` allantoic fluid so large amount of viruses can be obtained after inoculation. Therefore this method is used for vaccine production e.g. Lasota vaccine used against New castle disease of poultry.

(c) Amniotic method:

Candle a 6 to 9 day old embryo and make spot near the eye spot of embryo, approx1 cm below the air space. Wash the egg and drill a hole in shell at the spot without puncturing shell membrane. Using 27 gauge l inch needle inoculate 0.05-0.1ml. of virus suspension into amniotic cavity with tuberculin syringe. To check whether needle is in amniotic cavity, aspirate fluid which is very thick and then inoculate. Seal the hole with molten paraffin wax and place the egg in incubator at 37°C. Candle daily to check whether embryo is dead or alive.

Advantage:

Due to swallowing movement of embryo the virus come into contact with mouth and respiratory system embryo produces lesions. Other viruses can be directly absorbed through skin.

(d) Yolk sac method:

Candle a 5 to 7 day old embryo wash the egg. Mark the eye spot of embryo and cm diametrically opposite side marl spot over yolk shadow in area devoid of all blood vessels. Apply spirit and drill hole in shell over yolk shadow without puncturing the shell membrane. Using 27 gauge needle and tuberculin syringe inoculate 0.05-0.08 ml into yolk sac. To check whether needle is in yolk sac; aspirate little of content if yellow yolk is seen, inoculate the virus into yolksac Seal hole with molten paraffin wax. Place egg in incubator at 37°C and candle eggs daily to check whether embryo is dead or alive.

Advantages:

After inoculation viruses multiply in yolk sac and produce inclusion bodies which can be observed by staining impressions smears of egg yolk with Giemsa stain. Passing of blue tongue virus for 100 passages produces attenuated vaccine for sheep.

(e) Intravenous method:

Candle l2-13 day old embryo, wash the egg with soap and water. Mark a spot where there is large blood-vessel and make a square window with help of carborandom disc, without puncturing shell membrane. Put drop of saline to make

shell membrane transparent. Invert the egg to fix b pod vessel against the window using a gauge needle inoculate 0.01-0.02 ml of virus suspension downward in direction of blood flow. Insert shell back in position and seal with molten paraffin wax and incubate egg at 37°C. Candle egg daily to check whether embryo is dead or alive.

Advantages:

Used for testing mutagenic and other toxic drugs which are used for treatment of cancer and other diseases and to check their effects on blood vascular system.

(f) Intracerebral method:

Candle is l2-13 day old embryo and wash. Mark a spot over eye spot of embryo. Drill a small window Remove shell without puncturing shell membrane and add drop of saline to make the shell membrane transparent. Invert me egg to fix the head of embryo against the windows and inoculate 0.01-0.02 ml intracerebrally into embryo using 27 gauge needle. Replace shell in position after inoculation. Seal and place the egg in incubator at 37°C. Candle egg daily to check whether embryo is dead or alive.

PRACTICAL No. 6

CULTIVATION OF VIRUSES IN TISSUE CULTURE

Cell Culture:

The term is used to denote cultivation of cells in vitro including growth of single cells in artificial or natural media. In cell culture cells are no longer organized into tissues. There are 2 types of cultures, organ and cell cultures.

(i) **Organ culture:** The culture of fragments of organs, intended to keep the cells growing in an organized and differentiated manner. This technique has been used by virologist to detect viruses by their effect on ciliated epithelium. Normal tissue culture media are used eg. Tracheal explants.

Preparation of tracheal explant:

i. Trachea is removed from a 8 to 20 day old chick embryo or newly hatched chick and transferred to petridish containing medium.
ii. Remove surrounding connective tissue gently
iii. Cut single tracheal transversely and place in petridish with medium
iv. Incubate at 37C for few hours, beating cilia may be seen by using an inverted microscope.

It is used for viruses like Infectious bronchitis, Influenza, infectious laryngeotrachitis of poultry wherein loss of ciliary activity is seen on inoculation of these viruses due to excessive mucous secretion the goblet cell.

Types of Cell Culture - Three types of cell culture as defined below

Primary Cell cultures:

When cultures are established initially from tissues taken directly from animals like foetal organs. They contain several cell types most of which are capable of limited growth *in vitro* (At the most 6-8 divisions on subculture)

Due to presence of diverse ramie of differentiated cell type tend to be very sensitive to many animal viruses. Therefore it is common practice to inoculate samples suspected to contain virus into primary cell cultures derived from same species as that from which sample is received.

Disadvantages:

As require sub culturing primary cell cultures cannot be used for vaccine production because of high cost and inconvenience to obtain fresh tissues each time as well as lack of consistency from batch to batch of same cell culture further more donor animals often harbor latent viruses which can confuse diagnosis or contaminate vaccines.

(i) **Diploid cell cultures:** These are the cells that are capable of undergoing number of divisions in culture during passaging or sub culturing that is roughly related to life span of species of animal. About 50passages can be carried out for foetal human cells and l0 subcultures for foetal cells from horses and cows. They retain their original diploid chromosome number throughout. Diploid strains of fibroblast established from human foetal embryo are used for human diagnostic virology and vaccine production.

(ii) **Heteroploid/Continuous cell lines:** These are the cells of single type which are capable of immortal propagation in virus. Such immortal cell lines originate from cancers or by spontaneous transformation of diploid cell culture. Often they no longer bear close resemblance to their cell of the origin because they undergo many mutations during their prolonged culture. The usual vindication of these cultures is the calls have lost specialized morphology and biochemical abilities that they possess as a differentiated cells *in vivo*.

For example, it is not possible to distinguish microscopically between epithelial cell lines arising from various cells of ectodermal or endodermal origin or between fibroblastic cell lines arising from cells of mesodemial origin. Cell of continuous cell lines are often an uploid in chromosome number especially if malignant in origin

Advantages:

1. They can be propagated indefinitely by sub culturing cells at regular interval (every 6-10 days
2. They retain viability for many years when frozen in medium containing dimethyl sulphoxide (DSO) and stored at -l96° to -70° c

Cell Cultures can be grown in two ways

(a) Monolayer cultures

(b) Suspension cultures

(a) Monolayer cultures: When cultures are grown on supporting surface they are called monolayer cultures eg. Cells grown in glass or plastic bottles, test tubes, cover slips or microtitre plates etc. After dispensing cells in these vessels, incubated in stationery conditions so that the cells form a confluent monolayer.

(b) Suspension cultures: When cells are grown in suspension and are not allowed attaching to any support surface like glass. Such cultures are known as suspension cultures. For this purpose cells are agitated continuously in roller bottles or fermenters so that the cells remain in suspension. This method is useful in chemical studies of viral replication because large numbers of identical cells are continuously available for regular sampling and processing. Various methods have been derived to maximize surface area to which cells attach, while keeping overall size of vessel and volume of medium within reasonable limits.

Roller bottle can be continuously rolled or filled with beads or a spiral plastic film. The most useful method for growing cells on large scale for vaccine production is on polystyrene orsephadexbeads known as micro carriers which are then maintained in suspension. In medium filled large fermenter tanks.

Applications of Cell Culture:

In immunological studies it is important to select cell line that will allow optimum growth of virus under study. Some viruses replicate in almost any cell line and some cell lines support replication of only a specific type or different types of viruses. Since some viruses require only specific type of cells to attach and multiply, on adaptation by serial passages, however mutants with somewhat greater growth potential for a given cell line can be selected.

Cell cultures serve three main purposes

1. Isolation of viruses from clinical specimen for this purpose a type of cell culture should be selected which is known for high sensitivity and in which cell abnormalityis readily recognized.

2. Production of vaccines and antigens for serological diagnosis. For this principle requirement is a cell line giving a high yield of virus and free from contamination.
3. Biochemical studies of viral replication for which continuous cell lines growing suspension culture are chosen.

Preparation of Primary Cell culture:

Chick embryo fibroblast culture:

1. Select a 10 day old fertile egg received from disease free flock and incubated properly. Select only well developed active embryos with good blood supply.
2. Apply tincture iodine on entire surface of egg and keep it on egg tray with marked air sac end upwards.
3. With help of sterile forceps break egg shell over air sac flaming forceps during process
4. Remove the shell by cutting and cut through CAM
5. Using a curved forceps take out embryo by catching its neck and place it in petridish containing HBSS. (Likewise remove several embryos using fresh sterile set of instruments every time)
6. Wash embryo several times with HBSS so that it becomes free from egg contents and debris
7. Using a sterile pair of forceps and scissors remove head and limb buds, then remove internal organs by cutting and expressing the abdominal wall
8. Transfer embryo to a beaker containing HBSS and wash it several times with HBSS so that blood and other debris are removed.
9. Mince the embryo with the help curved scissors.
10. Wash the minced tissue with HBSS, several times by pouring HBSS and shaking beaker well to allow tissue fragments to settle down and discarding supernatant.
11. Give a final washing to tissue with 0.25% trypsin solution warmed to 37°C.
12. Transfer the tissue pieces to trypsinisation flask containing Teflon coated magnet and pour sufficient amount of0 25% trypsin to just cover the tissue.
13. Place flask on a magnetic stirrer and spin it a moderate speed, i.e. about 200-300 rpm
14. After 30 minutes of continuous stirring, allow tissue fragments to settle and pour cell suspension in a beaker covered with gauze.

15. Transfer suspension to centrifuge tube and centrifuge at 10,000 rpm for l0 minutes and give 2 washing to cell with HBSS and finally with growth medium.
16. Suspend cells in growth medium. Count the cells in haemocytometer and adjust cell density to 104 cells/ml in growth medium Distribute cell suspension in milk dilution bottles or prescription bottles.
17. Incubate bottle horizontally at 37°C. Monolayers are seen in 2-4 days and if required medium is changed on 3 day by addition of maintenance medium.

Subculturing of continuous cell line:

1. Remove grown medium from bottle.
2. Add 2 ml of trypsin EDTA solution which contains 0.02% EDTA + 0.04% trypsin in calcium magnesium free balanced salt solution (CMFBSS).
3. Allow it to act for l-5 minutes in bottle at 37°C and observe under inverted microscope When cells of monolayer show roundness and tend to loosen from unless surface decant the EDTA (pour off slowly) trypsin solution.
4. Add 5-l0 ml of fresh growth mo.li urn and detach the cells by pipetting to make a cell suspension Acid an additional amount of growth medium so as to dispense into 8 bottles
5. Incubate bottles at 37°C incubator
6. Replace growth medium twice a week since monolayer formation takes place within a week.
7. Continue serial passage with l:3 split ratio that is from one bottle cells are transferred to another 3 fresh bottles every week up to l0 such passages.

Inoculation of viral sample in cell lines: Either primary or continuous cell lines known to be susceptible to suspected virus may be inoculated

1. Select a monolayer cell culture grown on cover slips in test tube or in bottles and pour growth medium
2. Wash the monolayer twice with HBSS
3. Inoculate 0.l ml of virus suspension and incubate test tube at 37°C for 60 minutes to allow the virus to adsorb into the cells
4. Add one ml maintenance medium or medium without serum and incubate tube at 37°C for3-7 days. Also keep control tube without any virus inoculums

Observation for virus action on cell is known as cytopathic effect (CPE). It is essential top passage each specimen or virus suspension in cell culture at least 3-5 times before declaring any specimen as negative.

Mytopathic effects (CPE)

Morphological changes securing in cell culture due to multiplication virus is known as CPE. It occurs due to certain biochemical changes such as shut down of cellular DNA, RNA and protein synthesis which follows synthesis of virus coded proteins. The viral proteins form a large crystalline aggregates or inclusion which visibly destroys the cell. Secondly as high concentration of virus protein or capsid protein has toxic effect on cell and produces CPE. Some viruses produce CPE by altering cellular microtubules. Alteration of mesosomal membrane may activate release hydrolytic enzymes which cause CPE. Cell fusion or syncytia formation occurs when newly synthesized budding virus from cell fuses with plasma membrane of an adjacent cell of there is fusion of virus specific areas of two adjacent cells.

PRACTICAL No. 7

CULTIVATION OF VIRUSES: ANIMAL INOCULATION

For the isolation of virus, processed clinical specimens can be inoculated into any of the following host systems depending on their availability and susceptibility.

1. Animal
2. Developing chick embryos
3. Cell cultures

Cultivation of viruses in cell cultures and developing chick embryos are the easier and more feasible methods

Purpose of animal inoculation:

Cultivation of viruses by inoculating animals is done only when the facilities for cell culture and chick embryo inoculation are no available. Sonic viruses can only be isolated in experimental or laboratory animals and hence are cultivated by animal inoculation.

Laboratory animals:

The laboratory animals commonly used in the laboratory are monkeys, rabbits, rats, mice, hamsters, guinea pigs, chicken, pigs, etc.

Natural hosts:

Natural hosts of the virus are used when laboratory animals are not available or for cultivation of those viruses which have a very narrow host range and cannot be cultivated in laboratory animals. When natural hosts are used young animals life preferred for one year old calves are used for cultivation of FMD virus.

Routes of animal inoculation:

The routes commonly used for inoculation of viruses into laboratory animals depends upon target organ and are -intravenous, intramuscular, subcutaneous, intradermal, intraperitoneal, Intracerebral, intranasal,intraplanter. intralingual, corneal scarification, checkpouch and per oral.

1. **Intravenous:** This route is used when the material is to be introduced directly into the circulation. Different sites are used in different laboratory animals. The site of injection is cleaned with alcohol and an appropriate size needle (usually a 26 gauge needle) is inserted into the vein. When the needle is fell inside the vein the syringe is slightly withdrawn till the blood enters the syringe this ensures that the needle is in the vein in the correct position. The appropriate inoculum is then injected slowly.
2. **Intramuscular:**This is a very simple route of inoculation. Any suitable muscle can be used. Usually the thigh muscle is selected. A 26 gauge needle is inserted 2-4 mm into the flesh pan and the inoculums are injected.
3. **Subcutaneous:**Any area on the animal where there is loose skin is suitable for subcutaneous inoculation. Usually the back of the neck or the flank region are used. The hairs at the site are clipped, area is sterilized, a fold of skin is pinched up and the injection made through the fold a subcutaneous injection shows formation of swelling.
4. **Intradermal:**Any suitable site may be chosen. The hairs are removed and the site is cleaned. The needle is inserted horizontally at an angle so that it does not penetrate below the skin.
5. **Intraperitoneal:**All the laboratory animals are suitable for this inoculation. The inoculation is to be made to one side of the midline of the lower abdomen. As the needle is withdrawn the skin around it should be gently pinched together and held for a few seconds to prevent the inoculum from leaking back. The animal is held with the ventral surface uppermost. The needle is inserted through the abdominal wall about 5 mm lateral to the midline. The needle is inserted l en deep to avoid puncturing of the viscera. A volume of 0.5 to 2 ml is injected.
6. **Intracerebral:** For this the animal is first anaesthetized with oilier (In suckling mice and very small laboratory animals no anesthetic is required.) The hairs over the head are removed and skin is disinfected with alcohol. The animal is held firmly with the dorsal side uppermost. A 26 gauge needle is inserted vertically in frontal foramen (seen as a pitted area). The site is a point mid way between outer can thus of eye and anterior margin of ear on the cranial midline. The needle is inserted to about 2 mm. and 0.1-0.3 ml is inoculated and the needle is withdrawn.

7. **Intranasal:** This route is commonly employed in mice and ferrets. The animal is anaesthetized, is held keeping the head up, the needle is brought- to the nostril and the required inoculum (0.05-0.1ml) is dropped slowly.
8. **Intra planter:** This route is employed for diseases such as Mouse pox and FMD in which case the plantar pad or footpad is inoculated For FMD. Usually guinea pigs are employed. The assistant holding the animal keeps the toes pressed with the thumb of the left hand and the needle is inserted with the right hand and about 0.1 nil of inoculum is injected in each pad.
9. **Intradermolingual:** This route is specially used for inoculating ophthovirus (FMD virus) in cattle. No sterilization is required. The tongue of the animal is withdrawn and held lightly by inking the tip of the tongue in grip. A 26 gauges needle is then inserted intradermally into the tongue by keeping the needle horizontal to the tongue surface.
10. **Corneal scarification:** This route is employed in rabbits. No sterilization is required. The animal is placed on its side. One drop of inoculum is placed on the corneal surface and gently scarified with a scalpel blade.
11. **Cheek pouch:** Hamsters are inoculated by this route. No sterilization is required. The animal is restrained and its mouth is opened. Cheek pouch is held and the mucous membrane of the cheek pouch is exposed for inoculation A 26 gauge needle is introduced into the pouch tissue as superficially as possible and slowly O.1 ml of inoculum is inoculated. This route is employed for studying the oncogenic viruses for their ability to produce tumors in hamsters
12. **Per oral:** This route is employed in a few circumstances. The usual procedure is to hold the head high to open the mouth, and the inoculum is discharged into the posterior part al cavity. Keep the head high for some time and allow the animal to swallow the contents.

Bleeding techniques:

The experimental animal may be bled either by venipuncture (from the vein) or by cardiac puncture. The venipuncture is preferred when small volume of blood is to be recovered. Different sites are used in different animals.

Rabbit:

When small amounts of blood are required, marginal ear vein is distended with xylene or alcohol and blood is withdrawn. If large amount of blood is needed heart bleeding is done. The anime is held in a supine position and the heart is located by palpating with the fingers in the space between 3rd and 4th rib on the left thoracic side. The needle is inserted through the intercostal muscles towards the ventricle and as soon as the needle touches the heart, the heart bent is felt. The needle is pushed inside the heart in one stroke and the plunger is slightly withdrawn so that blood enters the syringe freely About 10 to 15 ml blood is withdrawn and the needle is withdrawn, iodine applied

Rats, Mice and Hamsters:

For small volumes, bleeding can be done from the retro-ocular plexus of veins of the eye. A capillary pipette is specially prepared for this purpose; the diameter of the tip is about 1 mm. The assistant holds the animal with the dorsal surface facing up. The eyelids are opened and the tip of the capillary pipette is placed in the orbital cavity, the pipette is pushed further so that its tip touches the retro-ocular plexus, and the capillary pipette is rotated and then slightly the pressure of the pipette is loosened to allow the blood to be sucked in by capillary action. The pipette is withdrawn and the blood is transferred to a small tube. When a large volume is required, it is collected from either from the heart or by sacrificing the animal.

Chicken:

About 3-5 ml blood is collected from the humoral (wing) vein with a 21 gauge needle, when large amount is required blood is collected from jugular vein.

Cattle, Sheep and Goat:

Blood is collected from the jugular vein, for volumes of blood, bleeding canula may beused. For smaller amount of blood the ear vein in case of cattle and buffaloes may be used.

PRACTICAL No. 8

LABORATORY DIAGNOSIS OF IMPORTANT VIRAL DISEASES OF ANIMALS

Rinderpest:

Rinderpest generally known as cattle plague is one of the most dreaded diseases of ruminants (mainly cattle and buffaloes) and of the swine is characterized by ulceration of mucous membranes of the mouth, erosion and necrosis of mucous membranes of the digestive tract with shooting diarrhea and lachrymal discharges

Diagnosis:

1. Based on clinical symptoms as above.
2. The examination of stained impression smears prepared from the epithelia of tonsils and other lymphoid tissues reveals the presence of syncytia and acidophilic inclusion bodies.
3. Isolation and identification of virus:

For isolation of virus from live animal collect whole blood in EDTA. lymphnodes etc. These clinical materials are obtained preferably during the prodromal or early mucoid phase of the disease. From dead animal collect pieces of lymph node, spleen in 50% glycerin saline.

The Rinderpest virus can be isolated in laboratory animals like rabbits, hamsters and mice or in cell culture.

Cattle: The material is preferably inoculated in susceptible and known immune cattle. The susceptible cattle will react in 2 to 10 days.

Chick embryo: The virus has been adopted to grow in chick embryo by CAM, intravenous and yolk sac route of inoculation

Tissue culture: The virus grows well in primary or continuous cell lines and produce CPE. The susceptible cells are from cattle, sheep, goats, chick embryos, pigs, hamsters, doe and man. If the virus is present characteristic CPE develops within 10-l2 days. The CPE is characterized by multi nucleated giant cells or syncytia with intranuclear inclusions. The serum neutralization test is carried out in cell culture or in chicken embryo.

Other serological test: Soluble antigen of Rinderpest can be detected by complement fixation and agar gel diffusion test. The tissue of choice from fresh carcass or visceral lymph nodes or spleen taken at third to sixth day of pyrexia.

The specific neutralizing, complement fixing antibodies develop in the sera of cattle which develop up to fourth day of the disease or later. Other tests which are more sensitive and rapid have also been developed like single radial haemolysis lest, reverse passive haemagglutmation test, fluorescent antibody technique, immunoperoxidase test, enzyme linked imunosorbent assay (ELISA) etc.

Agar gel immuno diffusion test (AGIDT):

The presence of virus (antigen) in the tissues of affected animal or presence of antibodies in serum of affected animals can be detected by agar gel immunodiffusion test

Principle:

The AGPT is based on the ability of antigen antibody complex to form a visible line of precipitate in an agar when antigen and antibody interact after diffusion form separate points. The number of precipitation lines formed will depend upon number of antigen & fractions. It can be used to identify either antigen or antibody by using any one of the reagents which is of the known standard reference.

Materials: l) Noble agar gel. 2) Slides.

3) Pasteur pipette 4) Punch

5) Antigen (known) 6) Antiserum

7) Suspected antigen or sera sample.

Method:

1. A molten l% solution of noble agar in PBS is poured in 3.5-4 ml quantity with the help of pipette on to a clean grease free slide to give a layer of2-3 mm thickness and allowed to solidify.
2. The slides are kept in petridish moistened with cotton and transferred to 10°C in fridge to allow complete solidification of gel
3. Circular wells 3 mm in diameter are punched into agar using punch. The distance between wells being about 4 mm.

4. If antibody is to be detected, then standard antigen is added in central well and known positive and known negative sera are added in any two peripheral wells and rest of the wells are to be filled with suspected sera samples.

AGID for detection of antigen

AS – standard antiserum A^+ known positive antigen A- known negative antigen B- T1 to T4- test antigens	A-STANDARD ANTIGEN AS+ KNOWN POSITIVE ANTISERUM AS - - KNOWN NEGATIVE SERUM T1 TO T4 TEST ANTIGENS

AGID for detection of antibody

If antigen is detected then central well should be filled with standard antiserum and the known positive and known negative antigen should be added in any two of the peripheral wells. The suspected antigen sample should be added in the other remaining wells. The slides are then incubated in humid chamber of petridish containing moist cotton at room temperature for l8-24hours.

Result:

A positive reaction between antigen-antibody is indicated by appearance of tine lines of precipitation between antigen and antibody wells.

Foot and Mouth Disease

Foot and mouth disease is a highly contagious disease of cattle, sheep, goats and pigs as well as of a number of wild animals like deer, wild angulates. Man is occasionally infected. The disease is characterized by formation of vesicles on the mucosa of buccal cavity alimentary tract and on the skin of the testis and udder. There is a sudden death in young animals due to heart failure.

Diagnosis:

1. Based on clinical signs and lesions.
2. Isolation and identification of the virus.

Material for collection:

The specimens for diagnosis should be collected from those animals that have developed clinical signs recently i.e. when the animals have temperature, nasal discharge and the vesicles appear in the mouth and / or the feet. Samples from at least few animals should be collected. The samples include blood in anticoagulant serum, pharyngeal and oesophageal fluid collected with cup probang. The quantity of epithelium available varies from animal to animal but a piece of2x2 cm is appropriate.

From dead animals sample may be collected from lymph nodes, thyroid and heart. The samples are frozen and dispatched to the laboratory in frozen state. Where it is not possible to send samples in frozen state, the samples are transported in glycerol buffer pH- 7.6.

Virus isolation:

Cattle, guinea pigs, suckling mice and cell culture are used for isolation of viruses when the concentration of the virus in the sample is low.

Cattle:

On inoculation the pyrexia, vesicles and other symptoms usually develop between 24 to 72 hours, the vesicular epithelium is collected for carrying out serological test.

Mice:

The unweaned mice (6-8 days old) are highly susceptible to experimental infections if any animal dies between 1 to 7 days its carcass is used as source of antigen. The mice after 3 to 4 days of inoculation show paralytic symptoms and die.

Guinea pigs:

Guinea pigs weighing about 400 to 500 gms body weight are inoculated by intradermalroute into planter pads, which becomes red and vesicles appear usually by 3^{rd}day. The tissues form the lesions are collected and used as source of antigen.

Cell culture:

Bovine thyroid, BHK -21.l B RS-2 cells are used for isolation of virus A marked degenerative type of cytopathic effect is observed within 24 to 48 hours. The cell culture fluid is used as antigen

Serological tests for diagnosis:

The serological tests are applied directly to field.

Neutralization test:

The test is used to detect specific antibodies in the sera of recovered animals offer identification of virus with known hyperimmune sera.

Gel diffusion test:

The test can also be used for identifying unknown FMD virus strains. The test can becarried out in slides. The test is not very sensitive.

Radial immunohaemolysis test:

The virus antigen is coupled with sheep erythrocytes in presence of chromium chloride and is used for detection of antibodies in serum.

Immune fluorescence test:

The immunofluorescence test is used for detection of antigen in tongue epithelium, oesophageal fluid and various organs of dead animals suspected to have died of FMD. It gives a quick diagnosis in the face of outbreak where the virus cannot be isolated from the samples or for screening of large number of carrier animals by examining the esophageal fluid. The test can also be used for typing of FMD virus strains.

Immunoperoxidase test:

The enzyme horseradish peroxidase is attached with antibody. The peroxidase labeled antibody reacts with viral antigen (intracellular or extracellular). The reaction is observed by use of substrate which reacts with peroxidase and produces a colour. The test can also be applied for typing of FM D strains.

Enzyme linked immuno sorbent assay (ELISA):

The test is highly sensitive. This test is used for sertoyping of` FMD virus. Sandwich ELISA using guinea pig anti 146 S sera and rabbit hyperimmume sera against O, A, C, Asia -l is also used for diagnosis of FMD virus.

Recently electrofocussing and finger printing of ribonuclease T l oligonucleotides have been used in FMD virus diagnosis and typing of strains.

Complement fixation fest:

Principle:

When a complement is added to a mixture of antigen and specific antibody, the complement is utilized and fixed. It no antigen-antibody reaction occurs, the complement remains free, it is then detected by an indicator system or haemoltytic system which is in the form of sheep red blood cells sensitized by the antibody against sheep RBC; which are lysed or not lysed when added and incubated at 37°C depending on the availability of the complement from the antigen—antibody reaction mixture.

Material required:

1. Complement: - It is a guinea pig serum obtained from male guinea pig weighing 600-700 gms by bleeding them through intracardiac puncture. This serum is used as complement
2. Haemolysin
3. Sheep erythrocytes
4. Virus antigen
5. Anti-serum
6. Veronal buffer saline
7. Micro titer plates, Micro pipettes.

Test procedure for typing of Apthovirus from field samples:

25ul of virus antigen is added in duplicate wells of 96-well "U" bottom plate and correspondingly l:10 and 1:40 dilutions of antisera against serotypes O.A.C, and Asia-l added to each of the wells in 25ul quantity. All dilutions are made in Veronal Buffer saline (VBS). Fifty micro liters of 3 units of pre titrated guinea pig complement is added

to all the wells except the cell control. The contents are shaken and the plate is incubated at

37°C for 30 min. Suitable antigen, antiserum, complement and sheep erythrocyte controls are kept as follows.

Protocol for complement fixation test:

	Antigen control	Anti control	Serum complement	Cell control
VBS	---	25 ul	50 ul	100 ul
Antigen	25 ul	---	---	---
1:10/1:40 Complement	25 ul	25 ul	---	---
Incubate at 37° C for 60 min				
Haemolytic system	25 ul	25 ul	25 ul	25 ul

Incubate at 37° C for 30 min.

The plates are read after keeping them overnight at 4°C. The wells showing no haemolytic similar to antigen control and cell control are taken as positive for presence of FMD virus. (CFT +ve)

RANIKHET DISEASE

HAEMAGGLUTINATION (HA) TEST

Object:

To detect the presence of Ranikhet disease virus in a given suspension and to find out haemagglutination titer of virus.

Principle:

Certain viruses contain in their envelope virus coded glycoproteins (peplomers) capable of binding to the complementary mucoprotein receptors over the surface of erythrocytes. Such viruses bridge the gap between the red cells leading to format of lattice. This reaction is seen in the polypropylene plates as mat formation. This property of virus may be employed for detection of concentration of virus in a given suspension.

Material required:

Laxbro plates, N.S.S., dropping pipettes (0.25/0.50 ml). Test suspension, 1% fowl RBC suspension.

Preparation of 1% Fowl RBCs:

Take 5 ml of citrated fowl blood in test tube and centrifuge it to separate plasma and cells. Discard the plasma and add equal volume of NSS. With the help of pipette agitate RBCs. Then centrifuge tube at 1500 rpm for 10 min. Discard the supernatant and add fresh NSS. Repeat this procedure 3 times. After last centrifugation discard the supernatant. Then take 1 ml of this packed cell volume and add to 99 ml of NSS to give 1% washed fowl RBCs or 0.1 ml + 9.9 ml of NSS to give l% FRBCs.

Text procedure:

1. Mark first row of wells with dilutions 1:2, 1:4. 1:8, 1:16... and mark any last two other wells as virus control (VC) and fowl cell control (FCC).
2. Add 0.25 ml of NSS in the wells of first row and in FRBC control (FCC) & virus control (VC).
3. Add 0.25 ml of test virus suspension to the first well. Mix and transfer 0.25 ml to next well and so on. From last well discard 0.25 ml.
4. Add 0.25 ml of test suspension to virus control
5. Add 0.25 ml of FRBC’s to all the well including virus and FRBC control.
6. Rotate the plate gently to mix the contents and incubate at room temperature for 30 min
7. The titer is read when the FRBC control shows a button formation appearance.

Result:

Reciprocal of the highest dilution of virus showing complete haemagglutination or lattice formation is taken as virus titer. It is expressed as H.A. units.

Advice to farmers:

Depending on the mortality pattern in the flock, lesions observed on post mortem, age of birds and the symptoms shown, the farmer should be advised as follows.

In case of positive haemagglutination fest:

The possibility of presence of Ranikhet disease infection. Therefore collection of serum from unaffected and affected birds is must for confirmation of heamagglutination inhibition test. Farmer should be advised to do vaccination of healthy birds with live or killed Ranikhet disease vaccines, culling of the affected birds, disinfecting the sheds, waterers. Feeders and other equipment.

PROTOCOL FOR HAEMAGGLUTINATION TEST (HAT):

Sr.No	1	2	3	4	5	6	7	8	VC	FCC
NSS	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Virus	0.25	Mix and transfer						Discard from last well		
Virus									0.25	
1%FRBC	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256		
	Incubate at room temperature for 30 min									
	M	M	M	M	M	B	B	B	M	B

M – Mat formation B – Button formation

VC – Virus control FCC – Fowl cell control

HEAMAGGLUTINATION INHIBITION (HI) TEST: BETA PROCEDURE (DECREASING SERUM CONSTANT VIRUS)

Object: To determine HI titer units in given serum.

Principle:

In this test there is an antigen-antibody reaction in which the antibody attaches to the specific virus present and the reaction is indicated by scaling down of the RBCs in the well to give button formation (HI positive). If the serum contains antibodies specific to the virus, such reaction will be seen at the lower dilutions. As the dilution of antibodies increases, a stage arises when there is very little or no antibody to attach to virus and virus combines with receptors on RBCs it is tree in the solution, to give a lattice formation at higher dilution of the serum (iii negative).

Requirements:

Laxbro plates, N.S.S., Test unknown serum, 8 HA units Ranikhet disease virus suspension, dropping pipettes

Preparation of 8 HA units of virus:

The neat Lasota vaccine virus is titrated using a HA test and the number of HA is determined. For example, if the Lasota vaccine contains 1024 HA units, then 8 HA units can be calculated as 1028/8=128 which is a dilution factor to be used in order to obtain 8 HA units. Therefore 1 ml of neat virus added to 127 ml of N.S.S. gives 8 HA units of Ranikhet disease virus.

Test procedure:

1. Mark first row of wells with dilutions l:2,l:4,l:8,l:l6, and mark last three wells as virus control (VC) and fowl cell control (FCC) and serum control (SC).
2. Add 0.25 ml of NSS in the wells of first row and in all the controls.
3. Add 0.25 ml of test serum to the first well Mix and transfer 0.25 ml to next well and soon. From last well discard 0.25 ml.
4. Add 0. 25 ml of scrum to serum control.
5. Add 0.25 ml of8 HA units of RDV to all the wells and virus control. (Not to the serum control and FRBC control).
6. Incubate the plate at room temperature for 10 min.
7. Add 0.25 ml of l% FRBCs to all the wells including all the controls.
8. Incubate the plate at the room temperature for 30 min. and read the first results as soon as in fowl cell control button formation occurs. After another 15 min read the final result i.e. after 45 min.

Results:

The reciprocal of the highest dilution of serum which shows complete inhibition of haemagglutination or it shows button formation is taken as serum antibody titer and is expressed in HI units

Advice to farmers:

In case of serum titer between 4-8 HI units, it indicates that flock is having a normal antibody response. Titer above 16 or 32 indicates a recently vaccinated flock and in case

unvaccinated flock with titre 16 or 32 indicates an active infection of Ranikhet disease. In case of low titers the farmer should he advice l0 revaccinate the flock. In case of unvaccinated flock showing high titers the effected birds should be culled. Vaccination of healthy birds along with disinfection of shed and other preventive measures should be undertaken.

HAEM AGGLUTINA'T̀ION INHIBITION TEST: BETA PROCEDURE

(SERUM UNKNOWN-STANDARD VIRUS)

Sr.No	1	2	3	4	5	6	7	8	VC	FCC	SC
NSS	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Test serum	0.25	Mix and transfer					Discard from last well				
Test serum	---	---	---	---	---	---	---	---	---	----	0.25
8HA unit virus	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	---	---
Incubate at room temperature for 10 min											
1%FRBC	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256			
	Incubate at room temperature for 30 min										
	B	B	B	B	B	M	M	M	M	B	B

M – Mat formation B – Button formation

VC – Virus control FCC – Fowl cell control

SC – Serum control

HAEMAGGLUTINATION INHIBITION (HI) TEST: ALPHA PROCEDURE

(CONSTANT SERUM DECREASING VIRUS)

Object:

To find out whether birds is actively suffering from Ranikhet disease.

Principle:

By this test bird is confirmed for the presence of Ranikhet disease virus. The test can also be used for checking potency of Ranikhet disease vaccine. It can be used as indirect test to find out antibody level to Ranikhet disease virus. An unknown Ranikhet disease virus previously titrated by heamagglutnation test is diluted to serial dilutions of 1:4, 1:8. etc. in N.S.S. and constant quantity of 1:5 dilution of antiserum is added to it. After which FRBCS are added. In first few wells there is heamagglutination i.e. lattice formation, whereas in higher dilution of virus inhibition or button formation is observed.

This is because in first few wells concentration of virus is higher than dilution of antibody and therefore virus attaches to receptors on RBCs to give a lattice formation. As dilution of virus increases, a stage arises when the virus and antibody are in equilibrium and therefore antigen-antibody reaction is complete and inhibition is seen in the form of button formation.

Requirements:

Laxbro plates, N.S.S, Dropping pipettes, 1% FRBCs, Ranikhet disease virus of known

HA titer. Hypermnuine Ranikhet disease serum diluted 1 in 5.

Test procedure:

1. Mark first row of wells with dilutions 1:2 1:4 1:8. 1:16. and mark three other wells as scrum control, virus control (VC) and fowl cell control (FCC).
2. Add 0.25 ml of NSS in all the wells of first row and in all the controls.
3. Add 0.25 ml of virus to the first well, mix and transfer 0.25 ml to next well and so on. From last well discard 0.25 ml
4. Add 0.25 ml of virus to virus control
5. Add 0.25 ml l: 5 diluted antiserum to all the wells of first row and to the serum control
6. Incubate the plate at room temperature for 10 min.
7. Add 0.25 ml of 1% FRBCs to all wells in first row and to all the control
8. Rotate the plate to mix the contents and incubate the plate at room temperature 40 to 60 min. As soon as button formation is observed in FRBC control and serum control the test is read.

Result:

The reciprocal of the highest dilution of virus which shows complete inhibition of heamagglutination or it shows button formation is taken as serum end point i.e. reciprocal of dilution of first wells showing button formation alter lattice

Formation is taken as serum end point.

The serum antibody titre can be calculated as

$$\text{HI units} = \frac{\text{Virus end point}}{\text{Serum end point}} \text{ X Dilution factor of serum}$$

Advice to the farmer:

Titer between 5-10 HI units indicate normal birds on farm and above 40 HI units suspicious for Ranikhet infection in unvaccinated birds and above 80HI units indicates a positive response to infection therefore accordingly a farmer should be advised to cull infected birds incase presence of Ranikhet is confirmed by above titer.

PROTOCOL FOR HAEM AGGLUTINATION INHIBITION TEST

(ALPHA PROCEDURE)

(CONSTANT SERUM DECREASING VIRUS)

Sr.No	1	2	3	4	5	6	7	8	VC	FCC	SC
NSS	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Test virus	0.25	Mix and transfer					Discard from last well				
Test virus	---	---	---	---	---	---	---	---	0.25	----	---
Standards	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25			0.25
Incubate at room temperature for 10 min											
1%FRBC	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256			
	Incubate at room temperature for 45 min										
	M	M	M	M	B	B	B	B	M	B	B

M – Mat formation B – Button formation

VC – Virus control FCC – Fowl cell control

SC – Serum control

Rabies:

Rabies is highly fatal disease of warm blooded animals and is of zoonotic importance.

Collection of material for diagnosis of rabies

(a) Samples from dead animals brain

Large animals:

Break open the temporal bone below the horns, which is a triangular area in case of cattle and buffalo, use trochar and canula which is passed through the temporal bone and aspirate the cerebellum or the cerebrum about 3-5 ml in 8 ml of 50% glycerin sa1ine.In ease of dogs, cats and other small animals open the head- skull and expose the brain and collect following parts from brain

1) Frontal & lateral bones 2) Cerebellum

3) Hippocampus 4) Medulla oblongata

(b) Samples from live animals

1. Biopsy materials — cerebrospinal fluid, corneal impression smears.
2. Other sample - Saliva.

Precautions to be taken while sending specimens

The brain is to be collected in 50% buffered saline if it is to be diagnosed on same day of collection. If it is to be sent to longer distances, it should be sent on dry ice and labeled AS

MATERIAL SUSPECTED FOR RABIES

The name of owner, date and time of collection, address of laboratory where it is to be sent and designation and name of person or institute sending sample should be mentioned in dispatching notes. Collection should be done in clean bottles or vials. The ratio of sample to the buffered glycerin saline should be l: 3.

Once suspected material vial is received in laboratory following two tests are performed to confirm presence of rabies virus in the brain tissue.

1. Staining of impression smears of brain tissue for detection of Negri bodies which are the aggregates of rabies virus particle.
2. Mouse inoculation test for detection of rabies virus.

Staining of impression smear by Seller’s staining:

Preparation of seller’s stain is as follows

1. 1 % alcoholic solution of basic follows
2. 1 % alcoholic solution of methylene blue

The working solution of seller’s stain is prepared by mixing l part of alcoholic solution of basic fuschin and 3 parts of alcoholic solution of methylene blue. The stock solution and working solution are kept in refrigerator in airtight bottles to prevent evaporation of methanol.

Material required:

Slides, a howl containing 2% bleaching powder solution, masks, gloves, scissors, forceps, and enamel trays, sterile hood, filter paper, seller's stain and staining tray.

Procedure:

Put on the gloves, remove brain from bottle with the help of forceps and place it in enamel tray The brain is cut into small pieces of 2-3 mm size and 3 such pieces are taken. Make3 impression smears of each piece on fresh slide and while smear is wet flood slide with working solution of Seller’s stain for about l0-20 seconds. Wash with tap water. The water is allowed to drain and slide is air dried.

After staining procedure is complete the brain is preserved at -20°C. All the scissors, forceps, remaining brain tissues should be put in 2 % bleaching powder. Wash & immersegloves in l% sodium hypochloride solution and then wash with soap. Remove gloves. & wash hands with soap and water. The carbolic acid soap should be used i.e. Life-buoy soap.

Observe of the slide, under oil immersion lens. In case of positive diagnosis, intraeytoplasmic eosinophilic inclusion bodies which appear like black granules, arranged

in a cart wheel shape are seen. These are called Negri bodies and are characteristic only of rabies virus. The nucleus of the neuron stains deep purple in color whereas, the cytoplasm stains light purple.

Mouse inoculation test:

Materials required:

Suckling mice, brain sample, pestle and mortar, scissors, forceps, a bowl containing l% bleaching powder, sterile hood, gloves, test tubes, antibiotic solution (Penicillin- l000 I.U. and streptomycin- 100 micro gm/ml), PBS, tuberculin syringe and 26 gauge needle.

Procedure:

Wear gloves and put a small piece of brain in mortar and triturate brain with pestle by adding sterile sand. Add l ml of PBS containing antibiotic; make uniform suspension of brain sample. Pour suspension into test tube and label test tube with brain number and date. Discard all other material into bleaching powder solution. Centrifuge tube at 1000 r p m for l5 min. The supernatant is collected in another test tube and inoculated into mice as follows.

In suckling mice (10-l5 days old) 0.01 ml of` suspension is inoculated through frontal bone using a 26 gauge needle and tuberculin syringes. In adult mice, inoculation is done through temporal fossa. About 6 mice are inoculated per brain and observed for period of 10 days for development of symptoms of rabies.

Observation:

If death of mice within first 3 days, it may be due to trauma during inoculation or contamination of inoculum and other nonspecific factors. Death after 4 days onwards with circular movements, dullness, ruffled hair coat and paralysis of hind limbs, tail etc. indicates symptoms of rabies. The dead mice brain impression smears are made similarly and strained with seller's staining. Presence of negri bodies confirms the presence of rabies virus in brain sample. All brain samples from animal suspected for rabies whether positive or negative for negri bodies are subjected to mice inoculation test to confirm the presence of viable rabies virus. Therefore seller's staining is only a screening test, whereas mouse inoculation test is confirmatory test in diagnosis or rabies.

PRACTICAL No. 9

LABORATORY DIAGNOSIS OF POULTRY VIRAL DISEASE

(A) INFECTIOUS LARYNGEOTRACHEITIS (ILT)

ILT is highly contagious respiratory disease of poultry characterized by respiratory distress, gasping, expectoration of blood stained exudates and high mortality. All breeds and ages of birds are affected. The virus which is double stranded DNA belongs to family herpesviridae.

Collection of material -	Live bird-	tracheal exudates, lung biopsy
	Dead bird-	lung

Diagnosis:

1. Based on clinic of signs: The chickens of all ages are susceptible but common in birds of 4-18 months of age. After an incubation period of 2-8 days, there is a mild coughing and sneezing which is followed by nasal and ocular discharge, dyspnoea, gasping. There is hemorrhagic tracheitis and the birds extend its head and take a prolonged inspiration, expectoration bloody mucus and frank blood.
2. Demonstration of type a intra nuclear inclusions in tracheal or conjunctival tissue.
3. Virus isolation from clinical material (tracheal exudate, lung suspension) in chicken embryo. Typical pocks are produced on the CAM after 4-5 days of inoculation.
4. Serological test:
5. Serum Neutralization
6. Fluorescent antibody test
7. Agar gel diffusion test.

(B) BNFECTIOUS BURSAL DISEASE (IBD):

IBD is infectious disease of young chickens in which virus destroys immunological organ namely bursa and leads to breakdown of immunity. IBD virus belongs to family birnaviridae which is a double stranded RNA virus.

Collection of material - kidneys, spleen, bursa

Diagnosis:

1. Based on clinical signs, gross lesions and histopathological changes in bursa. Age, history of birds, clinical signs and mortality pattern also helps in diagnosis.
2. Confirmation based on isolation and identification.

(C) INFECTIOUS BRONCHITIS (IB) :

IB is acute highly contagious respiratory disease of chicken. Single stranded RNA virus belonging to the family coronaviridae is the causative agent of the disease.

Collection of material: Live animal- tracheal scrapings
Dead animal- trachea, bronchi and lungs

Diagnosis:

1. Clinical signs: Coughing, gasping, tracheal rales, watery eyes and nasal discharge
2. Isolation
   (a) Chick embryo 9-l2 day's embryo by allantoic cavity inoculation shows dwarfing and curling of embryos.
   (b) Tissue culture -chicken embryo kidney, lungs and liver cells. Vero cells and chicken tracheal organ cultures. Virus produces syncytia in affected cell cultures
3. Fat of tracheal scrapings
4. Agar gel immuno diffusion test.

(D) MAREK'S DESEASE:

It is a transmissible virus disease which mainly a affects domestic fowl and is characterized by mononuclear infiltration around peripheral nerves and to lesser extent in skin muscle, iris and internal organs. The disease affects mainly domestic poultry and is common in young birds of 2-5 months old. The virus belongs to family herpesviridae.

Collection of material: Spleen, tumor tissue or other lymphoid cells, skin and feather tips

Diagnosis:

1. Clinical symptoms and lesions - paralysis of legs or wings, curling of toes. Torticollis swelling of vagus and sciatic nerves
2. Isolation —
   (a) Chick embryo — Characteristic pocks are produced on the CAM b
   (b) day old chicks are inoculated intra peritoneally
   (c) Cell culture — duck embryo fibroblast and chicken kidney cell culture. Characteristic plaques are produced in 6-14 days.

3. Serological tests

   (a) Agar gel immuno diffusion test

   (b) Indirect fluorescent antibody test or passive haemagglutination lest