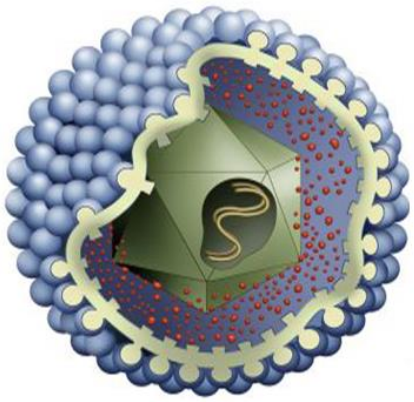
Laboratory Manual

VETERINARY MICROBIOLOGY

VMC Unit-V



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

LABORATORY MANUAL
FOR
VETERINARY MICROBIOLOGY

VMC

(New Syllabus As Per MSVE 2016)

Unit – V

Veterinary Microbiology

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LABORATORY MANUAL
FOR
VETERINARY MICROBIOLOGY

CERTIFICATE

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

Name of the student:_____

Registration No.: _____

Exam seat No.:_____

Course Teacher

ANNUAL EXAMINATION

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

Course Teacher **Sectional Head**

Examiner

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Practical 1

COLLECTION, PRESERVATION, TRANSPORT OF CLINICAL SAMPLES & PROCESSING OF MATERIAL FOR VIRUS ISOLATION

Collection of clinical samples for diagnosis of viral diseases

1.PPR: Eye (ocular swab), mouth and rectal swabs in PBS on ice, about 10ml or more blood at the height of body temperature in anticoagulant, Prescapular lymph nodes, spleen, tonsil etc. in HBSS and on ice. Tissue materials from 5 to 6 or more animals be collected and dispatched for better picture of disease / outbreak. Cotton buds can be used as swabs.

2.Foot and Mouth Disease: Vesicular fluid from unruptured oral vesicles and curetted epithelium from fresh lesions oeso-pharyngeal fluid in 50% PBS preferably on ice. About 10 ml blood at the height of body temperature in EDTA/heparin.

3.Bovine Spongiform Encephalopathy: Tissues from brain stem involving medulla oblongata, posterior cerebellar peduncle, rostral quadrigemina body and spinal cord in 10% formal saline for histopathological examination and for prion detection very thin pieces of above sites of brain in frozen state.

4.Bovine Virus Diarrhea / Mucosal Disease: Blood in EDTA, paired serum samples, semen, intestinal swabs, lymph nodes and spleen on ice.

5.Bovine Malignant catarrhal Fever (BMCF): Blood in EDTA, Paired serum samples, all internal organs including cornea, skin, muzzle on ice.

6.Rabies: Half portion of brain, salivary gland in 50% phosphate buffered glycerin in leak proof hard box. Alternative and preferable small pieces from hippocampus and brain (cerebellum, medulla, cerebrum spinal cord) in 50% buffered glycerin and on ice separately duly sealed and packed in thick polybags and hard box labeled **"SUSPECTED FOR RABIES"**. If available, fresh smears from brain may be stained with Seller's stain.

7.Pox Disease: Scabs in sterile containers on ice, scabs in 50%buffered glycerin.

8.Bovine Herpes Virus1,2, 3,/IBR/IPV/Bovine Mammilitis /

Parainfluenza 3 / Adenovirus :

Paired serum sample on ice, swabs from vagina and nasal lesions and pieces of trachea, liver, turbinate bone, lung on ice. From bulls semen and preputial washing in transport medium and paired serum on ice.

9.Enzootic bovine leucosis: Blood in EDTA, tumor tissues, lymph nodes, abomasum, right auricle of heart, spleen, intestine, liver, kidney, lung and uterus.

10.African Swine Fever: Blood in heparin or EDTA, spleen, tonsil, kidney lymph nodes, and bones on ice for virus isolation, paired serum samples.

11.Classical swine fever: Heparinized 20 ml blood at the height of temperature in sterile vials or test tube on ice from live animal. Heart blood, pieces of spleen, lymph nodes, pancreas (10 to 15 g each) in 50% GPB.. Tissue materials from 5 to 6 or more animals may be collected in order to give confirmatory diagnosis/true picture of disease. Materials for isolation and serological tests may be collected in sterile vials on ice without adding glycerin.

12.Transmissible gastroenteritis (TGE): Faeces, small intestine, lung, udder on ice for virus isolation and serological tests

13.Porcine reproductive and respiratory (PRRS):

Lung, liver, lymph nodes, tonsils, spleen, heart, brain, ascitic fluid and paired sera on ice

14.Aujeszky's disease (Pseudorabies): One half of brain, skin and subcutaneous tissue in sterile container on wet ice or in buffer glycerin for virus isolation. Paired serum sample.

15.Bluetongue disease: Blood at the height of body temperature in heparin (6-10 units/ml), paired sera in sterile containers on ice. Spleen, Lymph nodes on ice.

16.Rift valley fever: Blood, liver, spleen, brain, aborted fetus on ice for virus isolation.

17.Caprine arthritis encephalitis/ Maedi/Visna disease: Paired serum, joint capsule, lung, brain etc. on ice.

18.Canine Distemper: Pieces of lung, brain etc. on ice. Impression smears

from liver, pieces of liver and spleen on ice.

19.Infectious canine hepatitis: Impression smears from liver fixed in methanol. Spleen and liver in sterile containers on ice.

20.Canine parvovirus infection: Rectal swabs and faeces in PBS, pieces of intestine, heart on ice.

21.Equine influenza: Nasal swabs in PBS or Hank's solution on ice paired serum.

22.Equine Infectious anemia: Paired serum sample,spleen on ice/blood on ice.

23.African horse sickness: 20 ml unclotted whole blood in EDTA, paired serum samples, spleen, brain, lung in 50% buffered glycerin.

24.Equine rhinopneumonitis: Nasal swabs, liver, lung, spleen, thymus from aborted fetus, paired serum samples, blood from acute clinical cases.

25.Ranikhet disease: Freshly dead / moribund bird on ice, portion of liver, spleen, trachea, bronchi, lung in 50% buffered glycerin saline on ice.

26.Marek's disease: Live birds in acute stage of disease, feather follicles from chest and neck region in transport medium, paired serum samples .

27.Avian influenza: Intestinal contents, faeces, cloacal swabs, oro-nasal swabs, samples from trachea, lungs, liver, air sacs, intestine, spleen, kidney, brain, heart, separately as a pool , paired serum samples.

28.Infectious bursal disease (Gumboro disease): Live affected chick/bird, bursa of Fabricious in transport medium, paired serum sample .

29.Infectious bronchitis: Swab from exudates, lung, paired serum sample.

30.Infectious stunted chick syndrome: Liver, duodenal portion of intestine with pancrease, spleen on ice.

31.Leechi heart disease (Hydro pericardium syndrome):
Liver, spleen, thymus, bursa, kidney, heart on ice.

Exercise

Q1. Explain the importance of 'cold chain'.

Q2. Write the preservatives and transport media used for collection of material for the isolation of virus.

*****

Practical 2

ISOLATION & CULTIVATION OF VIRUSES IN LABORATORY ANIMALS

GUINEA PIG FOOTPAD INOCULATION METHOD FOR ISOLATION OF FMD VIRUS

Material Required /Clinical specimen:

1 ml of lymph from an unruptured or recently ruptured cattle tongue vesicle / epithelium samples. Oesophageal-pharyngeal fluid collected by means of a probang cup. Two month old Guinea pig 2 Nos., 25 gauge needle, syringe etc.

Preparation of inoculum:

Take out the frozen specimen from deep freeze and thaw at room temperature.Transfer the specimen (ruptured mucosal /tongue epithelium/tissue collected) to the mortar.Cut into small pieces .Add small quantity of sterile sand & PBS (to make it 10-20% specimen) and grind into a fine paste.Centrifuge and collect supernatent.Add the antibiotics to avoid contamination i.e.,Penicillin @ 10,000 units and streptomycin @ 10 mg per ml and incubate for 30 minutes.

Inoculation Procedure :

1. Prepare one hind foot pad of each guinea pig. Clean the foot pad with 70% alcohol and dry.
2. Mark one longitudinal line on the sterilized foot pad of each guinea pig.
3. Intradermally inoculate 0.2 ml of above prepared inoculum with 25 gauge needle at five equidistant spots on the marked line on foot pads.
4. The Guinea pigs are maintained on good bedding and observed daily for the lesions on the foot pad and clinical signs.
5. Lesions in positive cases will be evident on 4^{th} or 5^{th} day of inoculation. The skin from fully developed lesions is removed and stored in deep freezer till further processing.

Exercise

Q1. Describe the clinical signs and lesions of FMD in cattle.

*****

Practical 3

ISOLATION OF VIRUSES IN EMBRYONATED EGGS (CHICK EMBRYO INOCULATION & HARVESTING OF VIRUS)

CULTIVATION OF RD VIRUS BY ALLANTOIC ROUTE

Material required: Spleen, lung, kidney tissue from RD suspected bird, 10-11 day old embryonated chicken eggs 5 nos. , drill machine, egg candler, egg incubator, syringes, needles, forceps, scissors, petridish, tincture of iodine, melted paraffin, phosphate buffer saline, penicillin & streptomycin.

Procedure:

1. Candle the egg and mark an area of CAM away from the from the embryo and amniotic cavity .The area should be free from large blood vessels and about 3 mm below the base of the air-cell. Make a pencil mark in the area at the point of inoculation.
2. Make another mark on the upper end of the air sac of the eggs.
3. Drill a small hole through the shell at each mark but do not pierce the shell membrane.
4. Apply tincture of iodine to the holes and allow drying.
5. With sterile precautions, make a small puncture in the shell membrane in the hole over the air sac. The hole over the air-cell is necessary for air vent to allow the equalization of pressure produced by the inoculum within the egg and to prevent the inoculum and embryo fluid from escaping through the hole on the side of the egg.
6. Inoculate 0.2 ml inoculum through the hole in the side of the egg to a depth of about ¼ inch. Use 1ml tuberculin syringe with 27-gauge ½ inch needle.
7. Seal the two holes with melted paraffin or suitable liquid adhesive.
8. Incubate the eggs for 4-5 days.

9. For collection of allantoic fluid(harvesting), apply disinfectant to the shell over air sac. Break the shell over air sac with forceps and remove the shell to a distance of about 8-10 mm from the top of the air sac.

10. With the help of a 10 ml syringe and 22-gauge 1-inch needle, collect about 5ml of allantoic fluid from the cavity through the air sac opening and expel the fluid in the container.

CULTIVATION OF FOWL POX VIRUS BY CHORIO ALLANTOIC MEMBRANE ROUTE OF INOCULATION

Fowl poxvirus can be isolated by the inoculation of suspected material into embryonated chicken eggs. Approximately 0.1 ml of tissue suspension of skin or diphtheritic lesions, with the appropriate concentration of antibiotics, is inoculated on to the chorioallantoic membranes (CAMs) of 9–12 days old developing chicken embryos. These are incubated at 37°C for 5–7 days, and then examined for focal white pock lesions or generalized thickening of the CAMs.

Material required: drill machine, egg candler, egg incubator, syringes, needles, forceps, scissors, petridish, tincture of iodine, melted paraffin, phosphate buffer saline, penicillin & streptomycin.

Procedure:

1. Candle the eggs and mark the position of the embryo.
2. Keep the long axis of the egg in horizontal position with embryo uppermost; mark an equilateral triangle with each side about 1 cm, equidistant between the ends of the egg.
3. With the help of small carborundum disc, cut the eggshell at the marks but do not pierce the shell membrane.
4. With needle, pierce the shell membrane over the air sac.
5. Apply tincture iodine to the grooves cut by the carborundum disc and allow

drying.

6. Gently remove the triangle of shell to expose shell membrane without rupturing the chorioallantoic membrane (CAM).
7. Using a teasing needle, pierce the exposed shell membrane on the side of the egg but do not pierce the CAM.
8. Place 0.1 ml of inoculum (0.1 ml of tissue suspension of skin or diphtheritic lesions) over the pierced point on the side of the egg and create a slight vacuum with a small rubber at the hole over the air sac by sucking the air through the air bulb. Inoculum over the shell membrane will pass through the opening in the shell membrane on the side of the side of the egg allowing the CAM to drop from the shell membrane underneath the triangular area and the inoculum will be taken in due to negative pressure created at the opening i.e., triangular area on the side of the egg.
9. Close the triangular opening in the shell on the side as well as over the air sac with the help of cut shell flap removed and melted paraffin wax or suitable adhesive tape.
10. Incubate the egg for 5-7 days at 37^{O} C maintaing the humidity.
11. Candle the eggs daily & discard the mortality of embryo within 24 hours of inoculation.
12. After 5-7 days remove all the eggs from the incubator, kill the embryos by chilling and collect CAM. Wash the CAM in a petridish with normal saline and

    examine.

CULTIVATION OF AVIAN ENCEPHALITIS VIRUS BY YOLK SAC ROUTE

Material required:

Brain tissue from the birds infected with AE virus- processed inoculum (10% emulsion in PBS-Practical No.3), incubated 7-8 day old embryonated chicken eggs, egg candler, egg incubator, syringes, needles, forceps, scissors, petridish, tincture of iodine, melted paraffin, phosphate buffer saline, penicillin, streptomycin & centrifuge machine.

Procedure:

1. Candle the egg with long axis in the horizontal plane and locate the yolk sac. Make a mark on the shell over the yolk sac about half way from the small end of the egg to the apex of the curvature of the shell.
2. Drill a small hole through the shell at the mark avoiding piercing of the shell membrane.
3. Using a 1 ml tuberculin syringe fitted with a 27 gauge needle, 1.25 needle insert the full length needle ion the long axis through the hole and inject the inoculum (0.2 ml of 10% emulsion of brain in PBS from suspected bird).
4. Seal the hole with melted paraffin/ suitable adhesive tape.
5. Incubate the egg for 5-7 days at 37^{O} C maintaing the humidity.
6. Candle the eggs daily & discard the mortality of embryo within 24 hours of inoculation.
7. Remove the dead embryos, if any and all the remaining embryos after 3 days of incubation, chill them and collect yolk, yolk sac and brain of the embryos.
8. Preserve the material in sterilized screw capped tubes in the deep freeze till further use.

Exercise

1. What is SPF eggs?
2. Write the source of SPF eggs.
3. Write the name of some important viruses and routes of egg inoculation for virus isolation.

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Practical 4

MEDIA & REAGENT PREPARATION FOR CELL CULTURE

Basic Components of Culture Media

Culture media contain a mixture of amino acids, glucose, salts, vitamins, and other nutrients, and available either as a powder or as a liquid form from commercial suppliers. The requirements for these components vary among cell lines, and these differences are partly responsible for the extensive number of medium formulations. Each component performs a specific function, as described below:

Buffering systems

Regulating pH is critical for optimum culture conditions and is generally achieved by one of the two buffering systems:

Natural buffering system

In a natural buffering system, gaseous CO_2 balances with the CO_3/HCO_3 content of the culture medium. Cultures with a natural buffering system need to be maintained in an air atmosphere with 5-10% CO_2, usually maintained by an CO_2 incubator. Natural buffering system is low cost and non-toxic.

HEPES

Chemical buffering using a zwitterion, HEPES, has a superior buffering capacity in the pH range 7.2-7.4 and does not require a controlled gaseous atmosphere]. HEPES is relatively expensive and toxic at a higher concentration for some cell types. HEPES has also been shown to greatly increase the sensitivity of media to phototoxic effects induced by exposure to fluorescent light.

Phenol red

Most of the commercially available culture media include phenol red as a pH indicator, which allows constant monitoring of pH. During the cell growth, the medium changes color as pH is changed due to the metabolites released by the cells. At low pH levels, phenol red turns the medium yellow, while at higher pH levels it turns the medium purple. Medium is bright red for pH 7.4, the optimum pH value for cell culture.

Inorganic salt

Inorganic salt in the media help to retain the osmotic balance and help in regulating membrane potential by providing sodium, potassium, and calcium ions.

Amino Acids

Amino acids are the building blocks of proteins, and thus are obligatory ingredients of all known cell culture media. Essential amino acids must be included in the culture media as cells can not synthesize these by themselves. They ae required for the proliferation of cells and their concentration determines the maximum achievable cell density. L-glutamine, an essential amino acid, is particularly important. L-glutamine provides nitrogen for NAD, NADPH

and nucleotides and serves as a secondary energy source for metabolism. L-glutamine is an unstable amino acid, that, with time, converts to a form that can not be used by cells, and should thus be added to media just before use.

Carbohydrates

Carbohydrates in the form of sugars are the major source of energy. Most of the media contain glucose and galactose, however, some contain maltose and fructose.

Proteins and Peptides

The most commonly used proteins and peptides are albumin, transferrin, and fibronectin. They are particularly important in serum-free media. Serum is a rich source of proteins and includes albumin, transferrin, aprotinin, fetuin, and fibronectin. Albumin is the main protein in blood acting to bind water, salts, free fatty acids, hormones, and vitamins, and transport them between tissues and cells.

Fatty Acids and Lipids

They are particularly important in serum-free media as they are generally present in serum.

Vitamins

Many vitamins are essential for growth and proliferation of cells. Vitamins cannot be synthesized in sufficient quantities by cells and are therefore important supplements required in tissue culture. Again serum is the major source of vitamins in cell culture, however, media are also enriched with different vitamins making them suitable for a particular cell line. The B group vitamins are most commonly added for growth stimulation.

Trace Elements

Trace elements are often supplemented to serum-free media to replace those normally found in serum. Trace elements like copper, zinc, selenium and tricarboxylic acid intermediates are chemical elements that are needed in minute amounts for proper cell growth. These micronutrients are essential for many biological processes, e.g. the maintenance of the functionality of enzymes.

Media Supplements

The complete growth media recommended for certain cell lines requires additional components which are not present in the basal media and serum. These components, supplements, help sustain proliferation and maintain normal cell metabolism.

Antibiotics

Although not required for cell growth, antibiotics are often used to control the growth of bacterial and fungal contaminants. Routine use of antibiotics for cell culture is not recommended since antibiotics can mask contamination by mycoplasma and resistant bacteria .

Serum in Media

Serum is a complex mix of albumins, growth factors and growth inhibitors . Serum is one of the most important components of cell culture media and serves as a source for amino acids,

proteins, vitamins (particularly fat-soluble vitamins such as A, D, E, and K), carbohydrates, lipids, hormones, growth factors, minerals, and trace elements. Serum from fetal and calf bovine sources are commonly used to support the growth of cells in culture . Fetal serum is a rich source of growth factors and is appropriate for cell cloning and for the growth of fastidious cells. Calf serum is used in contact-inhibition studies because of its lower growth-promoting properties. Normal growth media often contain 2-10% of serum. Supplementation of media with serum serves the following functions :

- Serum provides the basic nutrients (both in the solution as well as bound to the proteins) for cells.
- Serum provides several growth factors and hormones involved in growth promotion and specialized cell function.

EMEM contains Earle's balanced salt solution, nonessential amino acids, and sodium pyruvate. It is formulated with a reduced sodium bicarbonate concentration (1,500 mg/l) for use with 5% CO_2 (see Sodium Bicarbonate and Buffering, page 14). Because EMEM is a simple medium, it is often fortified with additional supplements or higher levels of serum.

Exercise

1. Write the difference between maintenance medium and growth medium.

*****

Practical 5

SUBCULTURE & MAINTENANCE OF CONTINUOUS CELL LINES

Subculturing, also referred to as passaging, is the removal of the medium and transfer of cells from a previous culture into fresh growth medium, a procedure that enables the further propagation of the cell line or cell strain.

Procedure for subculturing of cell lines:

1. Remove and discard the spent cell culture media from the culture vessel.

2. Wash cells using a balanced salt solution without calcium and magnesium .

3. Remove and discard the wash solution from the culture vessel

4. Add the pre-warmed dissociation reagent such as trypsin to the side of the flask; use enough reagent to cover the cell layer (approximately 0.5 mL per 10 cm2). Gently rock the container to get complete coverage of the cell layer.

5. Incubate the culture vessel at room temperature for approximately 2 minutes.

6. Observe the cells under the microscope for detachment. If cells are less than 90% detached, increase the incubation time a few more minutes.

7. When ≥ 90% of the cells have detached, tilt the vessel for a minimal length of time to allow the cells to drain. Add the equivalent of 2 volumes (twice the volume used for the dissociation reagent) of pre-warmed complete growth medium. Disperse the medium by pipetting over the cell layer surface several times.

8. Transfer the cells to a 15-mL conical tube and centrifuge then at 200 × g for 5 to 10 minutes. Note that the centrifuge speed and time vary based on the cell type.

9. Resuspend the cell pellet in a minimal volume of pre-warmed complete growth medium and remove a sample for counting.

10. Determine the total number of cells and percent viability using a hemacytometer, cell counter and. If necessary, add growth media to the cells to achieve the desired cell concentration and recount the cells.

Exercise

1. Name the continuous cell lines of animal origin.

Reference

https://www.vanderbilt.edu/viibre/CellCultureBasicsEU.pdf

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Practical 6

QUANTITATION OF CELLS BY VIABLE CELL COUNTS IN A HAEMOCYTOMETER

Material Required: Hemacytometers, Cell line trypsinized

Procedure :

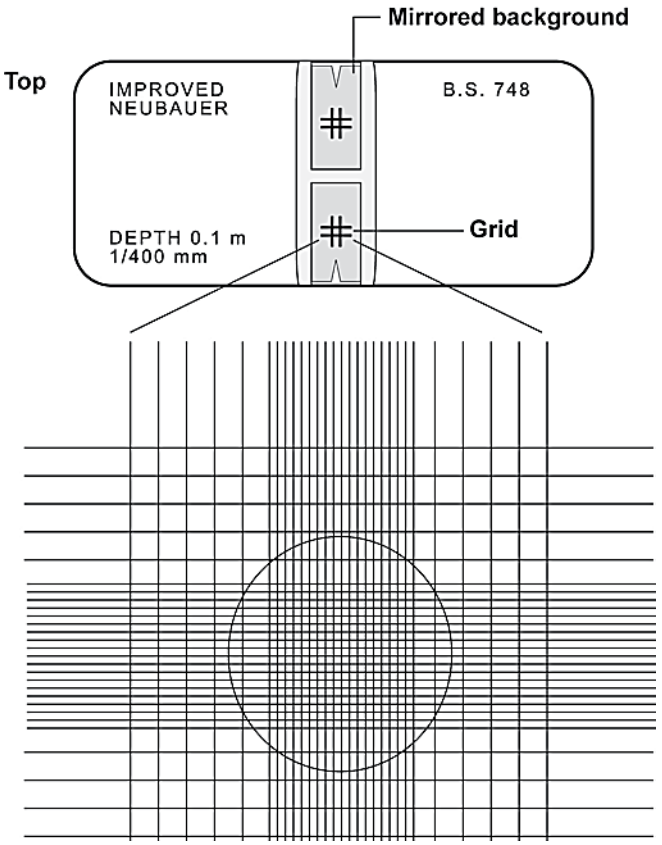
1. Clean the chamber of haemocytometer and cover slip with alcohol. Dry and fix the coverslip in position.

2. Harvest the cells. Add 10 µL of the cells to the hemacytometer. Do not overfill.

3. Place the chamber in the inverted microscope under a 10X objective. Use phase contrast to distinguish the cells.

4. Count the cells in the large, central gridded square (1 mm2). The gridded square is circled in the graphic below. Multiply by 104 to estimate the number of cells per mL.

5. Prepare duplicate samples and average the count.



Trypan Blue Exclusion method for viable cell count

The following procedure will enable you to accurately determine the cell viability. Cell viability is calculated as the number of viable cells divided by the total number of cells within the grids on the hemacytometer. If cells take up trypan blue, they are considered non-viable.

1. Determine the cell density of your cell line suspension using a hemacytometer.

2. Prepare a 0.4% solution of trypan blue in buffered isotonic salt solution, pH 7.2 to 7.3 (i.e., phosphate-buffered saline).

3. Add 0.1 mL of trypan blue stock solution to 1 mL of cells.

4. Load a hemocytometer and examine immediately under a microscope at low magnification.

5. Count the number of blue staining cells and the number of total cells. Cell viability should be at least 95% for healthy log-phase cultures.

% viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100

To calculate the number of viable cells per mL of culture, use the formula below. Remember to correct for the dilution factor.

Number of viable cells × 104 × 1.1 = cells/mL culture

Exercise

1. Explain total count and viable count.

******

Practical 7

CRYOPRESERVATION & RECOVERY OF CELL CULTURE

Cryopreservation

1. Prepare freezing medium and store at 2° to 8°C until use. Note that the appropriate freezing medium depends on the cell line.
2. For adherent cells, gently detach cells from the tissue culture vessel following the procedure used during the subculture. Resuspend the cells in complete medium required for that cell type.
3. Determine the total number of cells and percent viability using a hemocytometer, cell counter and Trypan Blue exclusion, or the Countess® Automated Cell Counter. According to the desired viable cell density, calculate the required volume of freezing medium.
4. Centrifuge the cell suspension at approximately 100–200 × *g* for 5 to 10 minutes Aseptically decant supernatant without disturbing the cell pellet.
5. Resuspend the cell pellet in cold freezing medium at the recommended viable cell density for the specific cell type.
6. Dispense aliquots of the cell suspension into cryogenic storage vials. As you aliquot them, frequently and gently mix the cells to maintain a homogeneous cell suspension.
7. Freeze the cells in a controlled rate freezing apparatus, decreasing the temperature approximately 1°C per minute. Alternatively, place the cryovials containing the cells in an isopropanol chamber and store them at –80°C overnight.
8. Transfer frozen cells to liquid nitrogen, and store them in the gas phase above the liquid nitrogen.

Thawing Frozen Cells:

The following protocol describes a **general procedure** for thawing cryopreserved cells.

1. Remove the cryovial containing the frozen cells from liquid nitrogen storage and **immediately** place it into a 37°C water bath.
2. Quickly thaw the cells (< 1 minute) by gently swirling the vial in the 37°C water bath until there is just a small bit of ice left in the vial.
3. Transfer the vial it into a laminar flow hood. Before opening, wipe the outside of the vial with 70% ethanol.
4. Transfer the thawed cells **dropwise** into the centrifuge tube containing the desired amount of pre-warmed complete growth medium appropriate for your cell line.
5. Centrifuge the cell suspension at approximately 200 × *g* for 5–10 minutes. The actual centrifugation speed and duration varies depending on the cell type.
6. After the centrifugation, check the clarity of supernatant and visibility of a complete pellet. Aseptically decant the supernatant without disturbing the cell pellet.
7. Gently resuspend the cells in complete growth medium, and transfer them into the appropriate culture vessel and into the recommended culture environment.

Exercise

1. What is cryopreservation?
2. What is the temperature of liquid nitrogen.

******

Practical 8

PRIMARY CELL CULTURE

Material Collected: Scabs in sterile containers on ice, scabs in 50%buffered glycerin.

Preparation of Lamb Kidney (LK) Cells:

1. Collect lamb kidney from a (young) wool sheep breed aseptically in a beaker containing Hank's Balanced Salt Solution.
2. Transfer the kidneys to sterile petri dish, peel off the capsule and cut the kidneys half longitudinally. Cut the cortex portion with sterilized curved scissors and mince finely.
3. Transfer the minced tissue in a 500ml-trypsinizing flask and wash thrice with HBSS.
4. Add trypsin (1:250 dilution) solution to the minced tissue and allow to stir about 30-40 minutes at room temperature.
5. After trypsinization, filter cell suspension through 3 layers of cheesecloth and pour cell suspension in a conical graduated centrifuge tube of 50ml capacity.
6. Centrifuge the suspension in a refrigerated centrifuge for 10 minutes at 1500 RPM. Decant the supernatant fluid, add equal amount of fresh BSS to the tube, mix the contents and repeat washing twice.
7. Collect 2 ml cell suspension and add to the 25 cm^2 tissue culture flask with suitable growth media.
8. Incubate at 37^OC (CO_2 incubator) for 3-5 days.
9. Examine the monolayer cell sheet under inverted microscope and preserve for further processing by replacing the growth medium with maintenance medium.

Exercise:

Q1. Define Primary Cell Culture.

Q2. Write the procedure for CEF Primary cell culture.

*****

Practical 9

INFECTION OF CELL CULTURES WITH VIRUS & STUDY OF CYTOPATHIC EFFECTS- DEMONSTRATION

Preparation of inoculum

Take out the frozen specimen from deep freeze and thaw at room temperature. Transfer the specimen (tissue collected) to the mortar. Cut into small pieces .Add small quantity of sterile sand & PBS (to make it 10-20% specimen) and grind into a fine paste. Centrifuge and collect supernatent. Add the antibiotics to avoid contamination i.e., Penicillin @ 10,000 units and streptomycin @ 10 mg per ml and incubate for 30 minutes. Use for inoculation.

Preparation of blood for isolation of virus

Blood collected using suitable anticoagulant is stored at $4^{O}C$ till further processing. (some viruses loose the infectivity when stored at 4 ^{O}C). At the time of inoculation take out the blood from refrigerator bring it to the room temperature, either centrifuge it to collect the buffy coat used in many virus isolation procedures or subject it to **Ultra–sonicator**, centrifuge , collect the supernatant . Add the antibiotics to avoid contamination i.e., Penicillin @ 10,000 units and streptomycin @ 10 mg per ml and incubate for 30 minutes. Use for inoculation.

Pass on the inoculum through Sintered glass filter,cellulose acetate membrane filters with average pore diameter of 1.2u,0.8u and 0.2 u to remove the debris,bacterial cell etc, to prevent the contamination i.e., bacterial and fungal.

Inoculation:

1. One ml of clarified biopsy preparation supernatant is inoculated on to a 25 cm^2tissue culture flask of 90% confluent LT or LK cells.
2. Allow to absorb for 1 hour at 37°C.
3. The culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum.
4. The flasks should be examined daily for **14 days for evidence of cytopathic effect (CPE)**, and the medium is replaced if it appears to be cloudy. Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from

surrounding cells, and eventually r**ounding of cells and margination of the nuclear chromatin, characterized** by grape-like clusters of rounded cells gathered around a hole in the monolayer; sometimes giant cells with several nuclei may be observed. Experience is needed to recognize this characteristic appearance.

5. If no CPE is apparent by day 14,blind passage must be made. The culture should be freeze/thawed three times, and clarified supernatant inoculated on to fresh LT or LK culture.
6. The cell cultures are observed daily for CPE, which usually appears within 3 days after inoculation.
7. After third passage, if there is no CPE, declare the sample negative for presumptive isolation of virus using cell culture.

Exercise

Q1. Enlist the Cytopathic Effects (CPE) for various animal viruses**.**

******

Practical 10

TITRATION OF VIRUS BY $TCID_{50}$ & PLAQUE ASSAY IN CELL CULTURE

VIRUS TITRATION REED-MÜENCH METHOD

Titration of Rabies virus in vivo **by intracerebral inoculation**

After a virus is propagated in either cell culture or in a suitable animal, we need to know the infectivity titre of the virus material obtained. This can be determined in vivo by inoculating increasing dilutions of the virus material to a susceptible host animal such as laboratory mice and based on mortality seen in different dilutions, the infectivity titre which is the reciprocal of highest dilution showing 50% mortality in the inoculated mice and expressed as LD_{50}/ml and can calculated by suing either Reed-Müench or Kärber formula. As an example, titration of rabies virus is illustrated in this section.

Materials:

1. Microbiological safety cabinet.
2. Sterile Pipettes, 10ml and 1ml
3. A tray with ice flasks. Rabies virus suspension (cell culture supernatant of infected brain emulsion)
4. Mice (Swiss albino or Laka) 4-6 weeks old
5. One ml syringe and needle for I/c inoculation
6. Test tubes.
7. Sterile PBS pH 7.4
8. Sterile bovine or equine serum
9. Cages for housing mice.

Procedure:

1. Working in microbiological safety cabinet, prepare the diluting fluid which is PBS containing 2% serum and dispense 9ml in test tubes labelled 10^{-1} to 10^{-7} and keep the test tubes in rack immersed in plenty of ice.
2. To make 10 fold (log) dilutions of the virus material, dilute 1ml of virus in 9ml of dilutent to get the initial dilution i.e. $10^{-1.}$ Subsequently transfer 1ml of previous virus dilution to next dilution by using at each step a fresh pipette, to achieve serial tenfold dilutions.
3. Inoculate 0.03 ml of each virus dilution intracerebrally into mice, starting from the highest dilution (in this case $10^{-7).}$ Use at least 6 mice per dilution and transfer these into cages appropriately labelled.
4. Observe the mice for 14 days. Any death occurring within first 5 days should be considered non-specific. Observe for specific signs and symptoms of rabies i.e., ruffling of hair, incoordination, tremors and paralysis of hind and fore limbs and finally death. Note down total number of specific deaths in each dilution and calculate the virus titre by using Reed-Muench or Karber formula.

Microtitration of Poliovirus using Vero cell line

Most of the commonly encountered human viruses produce characteristic cytopathic effect in one or the other cell lines routinely used in virology laboratories. The infectivity titres of these viruses can conveniently be determined by infecting a particular cell line with increasing dilutions of the virus material and determining the highest dilution producing cytopathic effect in 50% of the inoculated cells. The 50% end point dilution which in this case is expressed as TCID 50/ml can be calculated by using either Reed-Muench or Karber formulae. As an example, titration of polio virus I is illustrated in this section.

Materials:

1) Microbiological safety cabinet
2) Sterile test tubes or Penicillin vials
3) Micro pipettes, 200 & 1000 microns
4) Co2 incubator.
5) 96 well flat bottom tissue culture plates(Nunc)
6) Minimum Essential Medium(MEM,Sigma,HI) supplemented with antibiotics
7) Fetal calf serum(FCS)
8) Trypsin versene glucose coln.(TVG)
9) VERO cells.
10) Fluid containing PI.
11) Inverted microscope

Procedure:

1. Make 10 fold serial dilutions, say from 10^{-3} to 10^{-7} of the virus material in MEM containing 2% FCS, dispensed in either test tubes or penicillin vials, changing pipettes or tips with each dilution. Keep the tubes in rack immersed in ice.
2. Transfer 100 ul of each dilution to 4 wells of the micro-titre plate, starting from highest dilution to the lowest.
3. Trypsinise one MD bottle containing a confluent monolayer of Vero cells and count the cells and dilute to $4x\ 10^5$ cells per ml. In MEM containing 10% FCS. Dispense 100 ul of cell suspension in to each of the well containing virus dilution and also include 4 wells as cell control in which 100Ul of cell suspension is mixed with 100 UL of MEM with 2% FCS. While dispensing the virus dilutions and cell suspension it is necessary to keep the plate on ice tray.
4. Cover the plate and keep it in CO2 incubator and adjust the temperature to 37 C.
5. Read the plate under an inverted microscope after 3 to 4 days when a confluent monolayer of Vero cells can be seen in control wells. Look for the cytopathic effect in the wells inoculated with virus dilutions. This consists of rounding of cells, i.e. if 2 of 4 wells inoculated with 10^{-8} dilution shows cytopathic effect then the titre is 10^{-8} per 0.1 ml. The titre can also be calculated by the method of Reed and Muench.

Calculation of 50% endpoints

In any biological quantization, the most desirable endpoint is one representing a situation in which half of the inoculated animals or cells show the reaction (death in the case of animals and in CPE case of cells) and the other half do not. In other words, the endpoint is taken as the highest dilution of the biological material , which produces desired reaction in 50% of the animals or cells. The 50% endpoint can be based on several types of reactions. The most widely used endpoint, based on mortality, is the LD_{50} (50% lethal dose). This terminology can also be applied to other host systems-for example, tissue cultures - in which the $TCID_{50}$ represents the dose that gives rise to cytopathic effect in 50% of inoculated cultures. When computing, if closely-placed dilutions are used and in each dilution large number of animals or cells are used, it may be possible to interpolate a correct 50% end point dilution, but it is neither practical nor economical. Reed and Muench devised a simple method for estimation of 50% endpoints based on the large total number of animals, which gives the effect of using at the 2 critical dilutions between which the endpoint lies, larger groups of animals than were actually included in these dilutions.

Calculation of the LD 50 titre by the Reed-Muench method

Let us presume that we have titrated a virus suspension by inoculating mice and death is the reaction. The number of deaths and survivals in each dilution is tabulated as given in table I below:

T A B L E I

Arrangement of data used in computation of LD_{50} titre by the method of Reed-Muench

VIRUS DILUTION	DIED	SURVIED	ACCUMULATED VALUES			
				MORTALITY		
			DIED	SURVIVED	RATIO	RATIO (%)
10^{-3}	6	0	17	0	17/17	100
10^{-4}	6	0	11	0	11/11	100
10^{-5}	4	2	5	2	5/7	71
10^{-6}	1	5	1	7	1/8	13
10^{-7}	0	6	0	13	0/13	0

Accumulated values for the total number of animals that dies or survival are obtained by adding in the direction of lowest to the highest values. The accumulated mortality ratios and the percentage mortality for each dilution is calculated.

In the example depicted in the table it can be seen that mortality in the 10^{-5}, is higher than 50% and in the next higher dilution, 10^{-6} it is only 13%. So, we need to find the 50% endpoint dilution, which obviously lies between these dilutions. First, we have to calculate the proportionate distance (PD) of the 50% endpoint from these dilutions by using a simple formula.

PROPORTIONATE

DISTANCE = (% mortality at dilution next above 50%) - 50%

(% mortality at next Dilution above 50%) _ (% mortality at next dilution below 50%)

EX.: $\frac{(71 - 50)}{(71 - 13)} = \frac{21}{58} = 0.36$ or 0.4

The proportionate distance obtained thus has to be corrected by the dilution factor, which is the logarithm of the dilution step employed , which in this case is 1 (log of 10). Then the 50% endpoint dilution can be calculated thus:

Negative logarithm of LD_{50} titre = (negative logarithm of the next dilution above 50% mortality + PD) x dilution factor i.e. In this example, (-5 + 0.4) x 1 =-5.4 or log LD_{50} titre = $10^{-5.4}$ per 0.03 ml which is the amount of material inoculated I/C. The same methodology can also be applied to compute $TCID_{50}$ when cell culture system is used for virus titration. Each dilution is inoculated into a minimum of 4 to 6Wells in a 96 well tissue culture plate and observed for CPE , which in this case replaces mortality.

Exercise

1. Explain Plaque assay.

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Practical 11

VIRUS NEUTRALIZATION TEST IN CELL CULTURES FOR DIAGNOSIS OF CAPRIPOX

A test serum can either be titrated against a constant titre of capripoxvirus (100 $TCID_{50}$ [50% tissue culture infective dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the use of 100 $TCID_{50}$, the neutralisation index is the preferred method, although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes. The use of Vero cells in the virus neutralisation test has been reported to give more consistent results (Kitching & Taylor, 1985).

Procedure:

Test sera including a negative and a positive control are diluted 1/5 in Eagle's/HEPES (N- 2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) and inactivated at 56°C for 30 minutes.

Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and 10, and 50 µl of Eagle's/HEPES without serum is placed in columns 11 and 12 and to all wells of row H.

A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over $\log_{10}$ 6 $TCID_{50}$ per ml is diluted in Eagle's/HEPES in bijoux bottles to give a log dilution series of $\log_{10}$ 5.0; 4.0; 3.5; 3.0; 2.5; 2.0; 1.5 $TCID_{50}$ per ml (equivalent to $\log_{10}$ 3.7; 2.7; 2.2; 1.7; 1.2; 0.7; 0.2 $TCID_{50}$ per 50 µl).

Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row A.

The plates are covered and incubated for 1 hour at 37°C.

LT cells are prepared from pregrown monolayers as a suspension of 10^5 cells/ml in Eagle's medium containing antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining wells of row H are cell and serum toxicity controls.

The microtitre plates are covered and incubated at 37°C for 9 days.

Using an inverted microscope, the monolayers are examined daily starting at day 4 for evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated according to the Kärber method. If left longer, there is invariably a 'breakthrough' of virus in which virus that was at first neutralised appears to disassociate from the antibody.

Interpretation of the results: The neutralisation index is the log titre difference between the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be made more sensitive if serum from the same animal is examined before and after infection. Because immunity to capripox is predominantly cell mediated, a negative result, particularly following vaccination in which the response is necessarily mild, does not imply that the animal from which the serum was taken is not protected.

A constant-virus/varying-serum method has been described using serum dilutions in the range 1/5 to 1/500 and fetal calf muscle cells. Because these cells have a lower sensitivity to capripoxvirus than LT cells, the problem of virus 'breakthrough' is overcome.

Exercise

1. Explain the difference between Virus neutralization and Serum Neutralization test.

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Practical 12

DIAGNOSIS OF INFECTIOUS BURSAL DISEASE BY AGID

For detection of antigen in the bursa of Fabricius, the bursae should be removed aseptically from about ten chickens at the acute stage of infection.The bursae are minced using two scalpels in scissor movement, then small pieces are placed in the wells of the AGID plate against known positive serum.

Preparation of agar

Dissolve sodium chloride (80 g) and phenol (5 g) in distilled water (1 litre). Add agar (12.5 g) and steam until the agar has dissolved. While the mixture is still very hot, filter it through a pad of cellulose wadding covered with a few layers of muslin. Dispense the medium in 20 ml volumes into glass bottles and store at 4°C until required for use.

The linear pattern of wells is preferred although a hexagonal pattern may be used. Each test serum or bursa should be placed adjacent to a positive control antibody (AB) or antigen (AG), respectively. Wells, 3 mm deep, 6 mm in diameter, and 3 mm apart, are used.

Test procedure

1. Prepare plates from 24 hours to 7 days before use. Dissolve the agar by placing in a steamer or boiling water bath. Take care to prevent water entering the bottles.
2. Pour the contents of one bottle into each of the required number of 9 cm plastic Petri dishes laid on a level surface.
3. Cover the plates and allow the agar to set.
4. Cut three vertical rows of wells 5 mm in diameter and 3 mm apart, using a protocol and tubular cutter
5. Remove the agar from the wells using a pen and nib, taking care not to damage the walls of the wells.
6. Using a pipette, dispense 50 µl of the test sera into the wells .
7. Dispense small pieces of finely minced test bursa suspension by means of curved fine pointed forceps/pipette into the wells, to just fill the wells.
8. Dispense 50 µl of the positive and negative control reagents into the relevant wells.
9. Incubate the plates at between 22°C and 37°C for up to 48 hours in a humid chamber to avoid drying the agar.
10. Examine the plates against a dark background with an oblique light source after 24 hours.

Exercise

Q1. Explain: Preparation of antigen from infected bursa of Fabricius.

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Practical 13

DIAGNOSIS OF NEWCASTLE DISEASE

HAEMAGGLUTINATION AND HAEMAGGLUTINATION INHIBITION TEST

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

Principle of Haemagglutination Test:

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

Material Required: Perspex plate1ml pipettes, rubber bulb, beaker, conical flask, Pasteur pipette, centrifuge, measuring cylinder.

Reagents: 1% Chicken RBCs, Phosphate buffer saline (pH7.2), Allantoic fluid (collected from ECE on inoculation of suspected material for RD virus isolation)

Procedure:

1. Add 0.5 ml each of PBS in all the wells in a row of Perspex plate with the help of 1ml pipette and rubber bulb.
2. Add 0.5 ml of undiluted allantoic fluid in the first well (only).
3. With the help of pipette mix PBS and allantoic fluid (containing RD virus) from the first well properly and transfer 0.5ml from 1^{st} well to 2^{nd} well (Two fold dilution). Again in 2^{nd} well mix the contents with the help of pipette and transfer 0.5ml from 2^{nd} well to 3^{rd} well. Repeat till 9^{th} well and discard 0.5ml (dil.allantoic fluid containing RD virus)from 9th well.
4. Add 0.5 ml of 1% chicken RBC suspension to all the wells including 10^{th} well (RBC control).
5. Shake the plate well to mix the contents and incubate the plate at room temperature.
6. Read the test at intervals of 15,30,45 and 60 minutes.

Interpretation

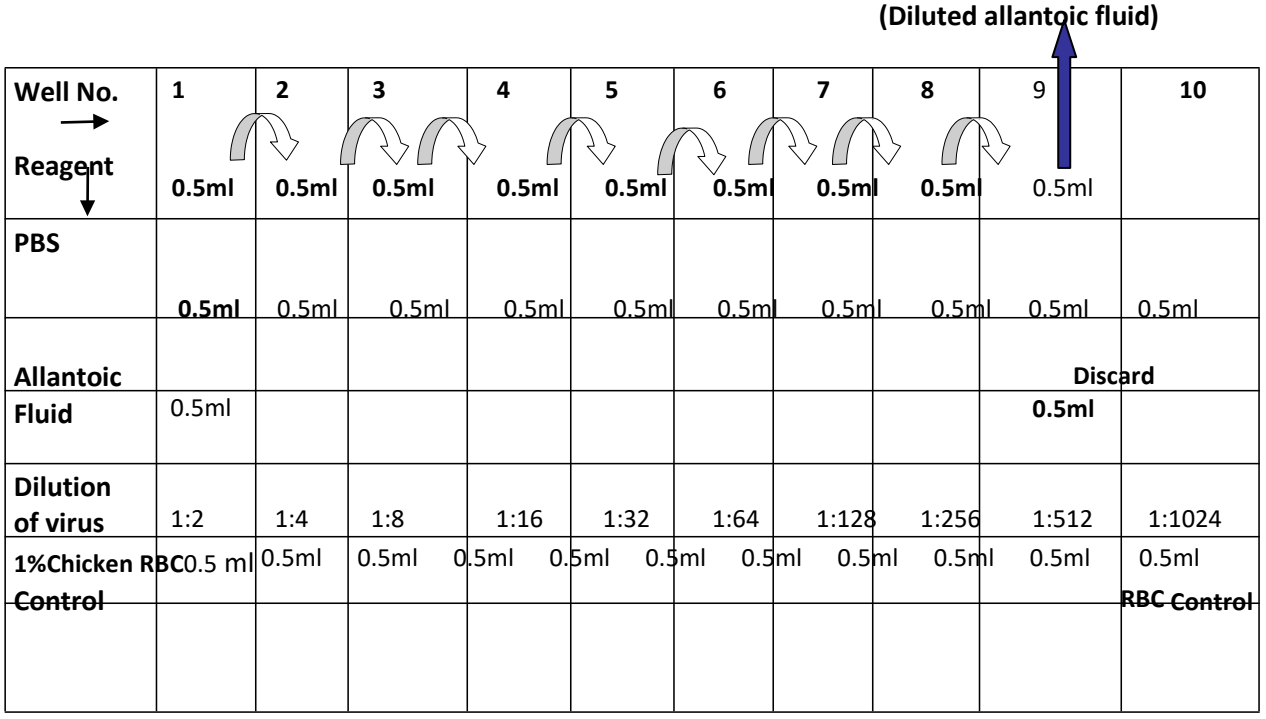
1. The presence of haemagglutinating virus in the allantoic fluid will result in mat formation (layer of uniformly agglutinated cells covering the bottom). The mat formation depends on the concentration of virus present in the suspension.
2. A negative test consists of a compact, sharply demarcated disc of sedimented red blood cells at the bottom of the wells i.e., button formation.
3. The end point of HA activity of the RD virus is the highest dilution of the virus showing mat formation i.e., haemagglutination. The virus titre i8s

expressed as HA unit contained in undiluted allantoic fluid. HA titre is the reciprocal of the highest dilution showing mat formation i.e., haemagglutination.

Protocol for Haemagglutination Test:

0.5ml

(Diluted allantoic fluid)



Well No. → / **Reagent** ↓	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	9	**10**
	0.5ml	**0.5ml**	**0.5ml**	**0.5ml**	**0.5ml**	**0.5ml**	**0.5ml**	**0.5ml**	0.5ml	
PBS	**0.5ml**	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
Allantoic										**Discard**
Fluid	0.5ml								**0.5ml**	
Dilution of virus	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
1%Chicken RBC	0.5 ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
Control										**RBC Control**

Haemagglutination Inhibition Test

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

Principle of Haemagglutination Inhibition Test

HI-Test is based on the haemagglutination property of RD virus. Haemagglutinin binds to the mucoprotein receptors (Sialic acid) over the surface of the erythrocytes& bridges the gap between the erythrocytes leads to the formation of lattice. This reaction is seen in microtitre / Perspex plates in the form of mat formation. But In the presence of antibodies (in the serum), abs interacts with the virus and thus makes the virus unavailable for agglutination of red blood cells. Red blood cells settles at the bottom of the wells thereby giving i.e., button formation in the presence of haemagglutinating-inhibiting antibodies against RD virus.

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

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

Procedure:

1. Add 0.5 ml each of PBS in all the wells in a row of Perspex plate with the help of 1ml pipette and rubber bulb.

2. Add 0.5 ml of undiluted Test serum in the first well (only) .

3. With the help of pipette mix PBS and Test serum in the first well properly and transfer 0.5ml from 1^{st} well to 2^{nd} well (Two fold dilution). Again in 2^{nd} well mix the contents with the help of pipette and transfer 0.5ml from 2^{nd} well to 3^{rd} well. Repeat till 8^{th} well and discard 0.5ml (diluted test serum) from 8^{th} well.

4. Add 0.5ml each of 4 HA unit virus upto 9^{th} well (Virus control).

5. Allow it to stand for 10 minutes at room temperature.

6. Add 0.5 ml each of 1% chicken RBC suspension to all the wells including 10^{th}well (RBC control).

7. Shake the plate well to mix the contents and incubate the plate at room temperature.

8. Read the test at intervals of 15,30,45 and 60 minutes.

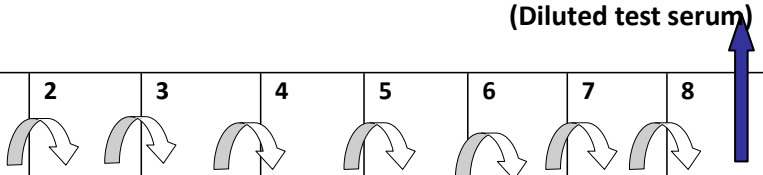
Interpretation:

1. The presence of haemagglutinating-inhibiting antibodies against RD virus in the test serum binds to the RD virus and makes RD virus unavailable for haemagglutination of Red blood cells. Resulting into sedimented red blood cells at the bottom of the wells i.e., button formation.
2. A negative test consists of absence of haemagglutinating-inhibiting antibodies against RD virus in the test serum results in the availability of RD virus for haemagglutination of Red blood cells. Resulting into mat formation (layer of uniformly agglutinated cells covering the bottom).

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

Protocol for Haemagglutination Inhibition test: **0.5ml**

(Diluted test serum)



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

Exercise

1. Write the use and composition of Alserver's solution.
2. Calculate 4HA unit of RD Virus.
3. Calculate the HI titres of given sera samples and interprete the results based on the history of vaccination in broilers.

******

Practical 14

ELISA FOR DIAGNOSIS OF INFECTIOUS BOVINE RHINOTRACHEITIS

(IBR dot-ELISA)

(Prescribed Test Protocol by OIE.int.org)

Collection and processing of samples

Nasal swabs are collected from several (from five to ten) affected cattle in the early phase of the infection. These cattle still have serous rather than mucopurulent nasal discharge. In cases of vulvovaginitis or balanoposthitis, swabs are taken from the genitals. The swabs should be vigorously rubbed against the mucosal surfaces. The prepuce can also be washed with saline; the washing fluid is then collected. The specimens are suspended in transport medium (cell culture medium containing antibiotics and 2–10% BoHV-1-free fetal bovine serum to protect the virus from inactivation), cooled at 4°C, and rapidly submitted to the laboratory.

During necropsy, mucous membranes of the respiratory tract, and samples of the tonsil, lung and bronchial lymph nodes are collected for virus detection. In cases of abortion, the fetal liver, lung, spleen, kidney and placental cotyledons are examined. Samples should be kept on ice and sent to the laboratory as quickly as possible.

After arrival at the laboratory, swabs are agitated at room temperature for 30 minutes in the transport medium to elute virus. Following removal of the swabs, the transport medium is clarified by centrifugation at 1500 g for 10 minutes. Tissues are homogenised to a 10–20% (w/v) suspension in cell culture medium before centrifugation at 1500 g for 10 minutes

Diagnostic Method: The ImmunoComb® test is based on solid phase “dot”-ELISA technology. Antigen is applied to test ‘spots’ on the solid phase, which is a comb-shaped plastic card (the Comb).

The serum specimen to be tested is diluted in a buffer in the first well of a multi-chamber developing plate. The test spots on the Comb are then incubated with the sera in the developing plate. Specific IgG antibodies from the specimen, if present, bind to the antigen at the test spot.

The Comb is then transferred to a well, where unbound antibodies are washed from the antigen spots. In the next step, the Comb is allowed to react with an anti-cow IgG Alkaline Phosphates conjugate, which will bind to antigen-antibody complexes at the

test spots. After 2 more washes, the Comb is moved to the last well, where a color result develops via an enzymatic
reaction. The intensity of the color result of test spots corresponds directly to the antibody level in the test specimen.

IBR dot-ELISA:

Specificity	86.2%
Sensitivity	94.6%

Preferred Method of Diagnosis: Serology is used to evaluate antibody responses to infection by the viruses associated with BRD. Serologic techniques are also helpful in evaluating the effectiveness of vaccination programs.

Interpretation: The level of antibodies (i.e., antibody titer) is Determined according to the intensity of the test color result.

Positive and negative control serum samples are included in the ImmunoComb® Bovine IBR-PI3-BRSV Antibody Test Kit.

The negative control consists of non-immune sera and should be read as zero. Specimens with colorless (white) or faint color result (i.e., less than S1) are considered negative.

The positive control spot on the Comb should develop a distinct grey color that is scored S3. The positive control has been calibrated to correspond approximately to 200 ELISA units (0.2 Optical Density). Specimens with identical or darker grey color results (S3 – S5) are considered positive.

Proper evaluation of the humoral immune response to infection is performed by comparing test results in paired serum samples, which are obtained at the acute and convalescent stages of illness. Negative serology would be expected to be found in unvaccinated and unexposed herds. Any seroconversion (S1 or greater) would indicate infection, while S3 or greater is
considered significant in previously vaccinated animals.

Applications:

1. To determine IBR infection in cattle by measuring IgG antibody titer.
2. To evaluate passive (maternal) antibody levels in calves to IBR Virus.
3. To evaluate humoral immunity status of herds for assistance in designing vaccination programs forIBR.

Exercise

1. Name other ELISA methods for diagnosis of animal disease.

References

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Tittle, D. (2000). A quantitative measure of the concentration of immunoglobulin G1, and antibodies to Infectious Bovine Rhinotracheitid, Parainfluenza 3 and Bovine Respiratory Syncytial Viruses in bovine colostrum and post-suck calf sera. Farm Animal Elective Project: Royal Veterinary College, University of London, England.

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Practical 15

MOLECULAR TECHNIQUES FOR VIRAL DISEASE DIAGNOSIS PCR, RT-PCR & QPCR

Nucleic Acid Recognition Methods

Amplification methods for detection of the viral DNA genome are specific to the genus and sensitive for detection throughout the course of disease including before and after the emergence of antibody responses. These methods include conventional PCR, real-time PCR, and most recently loop-mediated isothermal amplification. Nucleic acid recognition methods can be used to detect the viral genome in biopsy, swab or tissue culture samples.

Conventional PCR

Several conventional PCR methods have been reported with varying specificity for capripox, Herpes, Asfar viruses in general. Conventional PCR methods are particularly useful for obtaining sufficient genetic material necessary for species identification by subsequent sequence and phylogenetic analysis.

Real Time PCR

Several highly sensitive and specific fluorescent detection-based real-time PCR methods have been developed and validated. Each test detects a small conserved genetic locus within the viral genome are targeted.

Exercise

1.Write the applications of RT-PCR and Q-PCR.

2.Write one PCR protocol along with primers for diagnosis of DNA virus.

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Veterinary Microbiology

Practical Manual

Unit V (As per MSVE 2016)

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COVASU

Ashok V Bhosle