EXPERIMENT NO. 2

Egg Inoculation

Embryonated chicken egg inoculations were first used with propagation of virus by Roux and Marfey in 1911. In 1931 Woodruff and Goodpasture cultivated fowl poxvirus on the chorioallantoic membrane. Burnet used chicken embryo for cultivation of viruses very extensively. Since that time and until mid 1950 almost all research and testing with viruses depended on animal or embryonated eggs for source of living tissue. Later on there were replaced gradually by tissue culture which is now most commonly used for propagation of viruses. The embryonating egg is nevertheless still a convinient and easily manipulated source of living tissue for propagation and testing of virus.

The use of fertile hen's egg in diagnostic virology has a number of advantages:-

1. Readily available, cheap and easily maintained.
2. Sheltered from the natural diseases often observed in laboratory animals, and are relatively free from bacterial and many latent virus infection.
3. Easily manipulated under sterile conditions.
4. Generally free from natural factors of defence, specific or non-specific, that some time intervene and prevent passage in adult animals.
5. Sensitive to some virus that are harmless to the adult birds.
6. Easily identified and labeled with the details of the date, nature of the virus and the experimental procedure.

Facilities and Equipments

Small commercial egg incubator which are equipped to supply balance of humidity, air circulation and heat is most ideal. Generally incubation of embryonating egg should occur under following condition.

Temperature - 37.5℃

Humidity - 62%

Forced air circulation to ensure a-balanced temperature and automatic turning trads to rotate the eggs during incubation.

Candling area and Equipments

A room, which can be darkened, is essential. This can be relatively small for it needs to accommodate person during candling. It should have 4 to 5 feet space on which to work and provide adequate ventilation. If this is not possible construct divides setting of a part of lab bench in corner or installation of black photographic curtain at height suspended from wall mounted red.

Equipment needed are suitable light source apparatus to make small holes in shell of eggs. Candling lamps are available. Best focus light source substituted, however can be made by a detachable dissecting microscope lamp with a variable rheostat present excellent choice present. Either an egg punch or drill commercially available can be used in making shell pores. If this is unavailable the sterile needle or rubber cork with an alpin can be used.

Inoculation Area Equipments

Ideal inoculation area is a small room, which need not exceed 5 feet length. It should have lab lamp space and an area for an equipment. It should preferably have a laminar flow benches fitted with ultraviolet lighting. To attain a clean room atmosphere, where facilities permit several inoculation rooms should be constructed. It is performed that each type of virus be handled in isolation. This avoid danger of contaminating the strain with each other. Under no circumstances inoculation or harvesting of one virus should be done in a room where other have been handled recently unless cleaning of surface of entire room has been done.

Egg Supply

Certain information about egg supplying flock should be a part of equipment data Following points should be recorded for all eggs used - Flock immunization history-

a) Type of vaccine used, date of manufacture, dose and route of administration.

b) Tests results on evaluation

c) *Production records* - It should essential to purchase egg from a single producer who can supply the necessary informations, preferably have your own hatchery supply the eggs. The present international standards as laid down by WHO, OIE and EDA of various countries emphasize that specific pathogen free (SPF) embryonating eggs be used for propagation of virus either for research or production.

Anatomy of Embryonating Egg

The term embryonating egg identifies any eggs in which an embryo is developing. It is common to refer to fertile egg which have been incubated as being 3,5,10 days etc. This does not refer to the time that has been exposed since the egg was laid but to the time that it has been incubated. Although certain early primitive developmental stages occur of fertilization and while the shell wall forms around egg contents. This development does not continues after bird lay eggs. Subsequently incubated artificially further development. This potential does not lost so long as eggs are not subject to extreme of temperature, it is this characteristic of suspended development which make it possible for avian to production their natural habitat. Clutch of eggs before setting down to incubate them, same principle of control storage which make it possible for common hatcheries to hatch thousand of chicks on special day.

Shell and Shell Membrane

If using sharp pointed forceps gently pick away shell of egg; a thin white membrane seen, so closely attached to shell itself that if broken piece of shell examined most would find it difficult to accept the fact that there is a membrane underneath the shell. Scrapping of inner shell of eggshell with forceps show this to be true. This is shell membrane. Outermost hard calcareous whitish covering of egg is egg shell. The shell itself is only are of the several system of avian eggs within function as an exchange system across its surface. Gaseous and liquid molecule passes in both directions. This is why the egg are incubated in presence of humidity and adequate circulation. If an egg is incubated in an atmosphere too low in humidity it will use its moisture. The content will be dehydrated and the death of the embryo will result. Same is true for circulation. If embryonating eggs are tightly packed for too long a period of time while under incubator the impairment of circulation will result in embryo death. The shell and shell membrane are not

just inert covering of a living content but themselves function to maintain life formed within it.

Air Space

All normal eggs have a rounded end and opposite to this is the end formed by more acute angle of shell that is pointed end under the shell and shell membrane of the blunt rounded end its an empty space. The egg contents are prevented from entering this area by layering of several membranes, which act as a barrier. The space is called air shell or air space. Its function both in respiration and pressure adjustment.

Chorio allantoic sac/ membrane (CAM)

Attached to developing embryo and originating hindgut, is a sac whose function is to remove wastes. As the embryo increases in size the sac also enlarges until it surrounds the embryo much like a double layered umbrella. Membrane which forms the sac is usually called chorio allantoic membrane together with the content of sac is designated as CAS or allantoic cavity. The fluid content in sac is known as allantoic fluid. It is necessary to differentiate between CAM inoculation when intend is to place the virus on/in membrane CAS or AF inoculation where intend is to place the virus in the allantoic fluid, allantoic cavity formed by CAM.

Yolk Sac and Yolk

Not only there is a CAS extending from developing embryo but structure called the yolk sac completely surround the yolk sac contents of the egg through its membrane and blood vessels, metabolites of yolk are transferred to embryos. As the embryo matures contents of yolk sac noticeably diminishes until the sac is approximately 1 cm. in diameter about three days before embryo hatch. At this state the embryo has attained such size that it nearly fills the egg and except for small opening in its abdomen from which yolk is produced the embryo is perfectly formed.

In the final days remaining to hatching time the opening of abdomen fills slowly and the contents will be absorbed in the digestive system of embryo.

Amniotic Sac

This vary with membrane system is most easily seen in embryo of 4 to 9 days. It appears as translucent clear liquid field stretched over developing embryo. Infact it is completely surrounding embryo except for areas where

chorio allantoic sac and yolk sac are attached. It's involvement with embryo and its function can just be seen by gently removing the entire contents of an egg into a petridish and remaining the intact structure. This membrane and its contents serve to protect the embryo against the physical damage and also function as an area of exchange. As the embryo enlarge the membrane stretches to accommodate the increase in size and serve less and less in the role of buffer against physical motion. By the time the embryo has hatched the membrane is thinly stretched and barely visible as its surrounds to fully developed form.

Selecting Eggs for Virus Propagation

1. **Determination of Viability/Egg-The** viability of embryo and the progress of embryo development is determined by candling. The egg at interval during incubation candling consist of giving the egg against the constructed light source preferably in dark room so that the shadow of embryo and associated structure specially blood vessels are visible for routine purpose, it is necessary to candle the egg only on the day of use to establish that the embryo has reached the normal size and is alive generally the healthy embryo shows the characteristics movement will impart an orange yellow color to its egg because of blood circulating in vessel. In embryo which is dead or dies will have low, no; or sluggish movement and will be easily detached because of the diminish of vessel or their complete absence. If the embryo has grown large enough nearly to fill the egg lack of motion may be only due to death: An egg obtaining a small embryo (dead) will usually have a cleared yellow color. Any coloration tending to green or black is an indication of extreme contamination and such egg should be carefully removed and placed in a discard container. In addition the content may have consistency of water rather than the viscous character of an healthy egg. Candling also enables in selecting point of entry through the shell. The point should not be situated directly over the large blood vessels adhering to the underlying shell membrane. As any puncture in them during inoculation may lead to hemorrhage and death of embryo.

   The point of inoculation and air space should be marked at the time of egg candling.

2. **Sterilization of egg** - Egg shell is sterilized with the swab tightly squeezed in tincture of iodine, taking care that not too much of the solution soak into the shell. Alternatively 70% ethyl alcohol may be used .
3. **Age of embryonating egg and suitability to various inoculation *route** - The age of embryo best suited to propagation of virus and route of inoculation as worked out should be used. Little success follow it attempts are made substitute the route of inoculation.
4. The age of embryo and route of inoculation commonly employed for propagation is shown in table

Table

Route of inoculation	***Age of embryo***	***Volume of inoculum***	***iruses for which used***
	10-12	0.1	Canine distemper, herpes zoster, vesicular stomatits, infectious laryngotracheitis, pseudorabies, herpes simplex, fowl pox, vaccinia, pigeon pox.
Allantoic cavity	9-12	0.1	Ranikhet disease virus, infectious bronchitis influenza virus , mumps, eastern, western, venezuelen equine encephalitis virus, para influenza.
Amniotic cavity	7-15	0.1	Influenza virus , mumps virus
Yolk sac	5-8	0.1	Mumps virus
Intravenous	10-15	0.01	Blue tongue virus
Intracerebral	8-14	0.01	Herpes simplex .rabies

Determination of embryo position

Embryo position was determined so that inoculation into specific membrane or cavity can be done. Choose position, which will place inoculation route. A little to one side or the other of larger vessel still keeping as close vesicle to desired route.

Efficiency in candling

It is generally more efficient to make after an hole in shell at time after candling the exception to may be an unusually large number of egg being found dead then remove first the died egg free candle and then make a desired hole.

Piercing the shell

The size of opening necessary for inoculation varies considerably depending on the route employed and for this purpose the dental drill is suitable. With this type of device simple holes may be cut with an ordinary drill attachment, while whole segment may be cut from shell with a rotating carborandum disc. The segment are then removed gently piercing of with a sterile scalpel what ever the size of opening; the actual drilling process should cut through the shell only leaving the underline shell membrane intact; the latter must never be pierced by drill.

Techniques of embryo inoculation -

The methods of inoculation are as follows

1) Allantoic sac inoculation
2) Amniotic sac inoculation
3) Yolk sac inoculation
4) Chorioallantoic membrane (CAM) inoculation
5) Intraembryonic
6) Intracerebral
7) Intravenous

The first four methods are in common use and will be described briefly.

The other three methods require considerable experience and skill to perform

and are limited to special work.

A) Allantoic sac inoculation-

Materials - Embryonated 9-10 days incubated eggs, inoculum or virus suspension, tuberculin syringe with 27 gauge $\frac{Z}{A}$ to 1 inch needie, rubber cork with a pin, egg candler, rubber bulb, tincture iodine, molten wax etc.

Procedure -

1. Candle the egg and mark the boundary of air sec with pencil.
2. Select an area of the chorioallantoic membrane free of large blood vessels about 3mm below the base of the air sac.
3. In this area make a pencil mark at the point for inoculation and other mark on air sac.
4. Apply tincture of iodine at the sites selected for making holes.
5. Rubber cork with a pin dipped in tincture iodine is used for making holes on the air sac as well as on other selected site.
6. Using a 1-ml tuberculin syringe fitted with a needle through a hole in the side of egg to a depth of about % inch and deposits the inoculum.
7. Withdraw the needle and inoculate the next egg or keep the syringe in sterile tube from where it was removed.

For use of 0.1ml or more of inoculum the hole over the air sac is a necessary air vent to accommodate the inoculum within the egg and to prevent the inoculum from escaping through the hole on the side of the egg.

1) Seal the holes in the eggs with molten wax.

Amniotic sac inoculation Procedure-

1. Candle 12-13 days old egg and make a pencil mark on the shell below the base of the air sac to locate the embryo.
2. Draw a circle parallel to and above 5 mm, above the base of air sac.
3. Using a small corborundum disc cut through the shell at the circle but do not pierce the shell membrane.
4. Apply tincture iodine to the groove cut by the disc and allow to dry.
5. Using forceps, remove the cap of shell over the air sac to expose the shell membrane.
6. Apply a few drops of sterile saline solution to the shell membrane over the embryo to make the membrane transparent. To cover more than about % of the shell membrane will interfere with the respiration and may result in the death of the embryo.

7. Using a 1 ml tuberculin syringe fitted with a 27 gauge, 1 inch needle, insert the needle into the amniotic cavity and inject the inoculum.
8. Close the opening in the shell over the air sac by sealing a disc of sterile heavy paper to the shell with molten wax.

C. Yolk sac inoculation Procedure -

1. Candle 5-7 days old egg and mark the position of embryo and yolk sac.
2. Apply tincture iodine and disinfect the site of inoculation.
3. Drill a small hole through the shell at the upper extremity of the shell over the air sac.
4. Use a 20-22 gauge 1% to *YA* inch needle and insert it perpendicular through the hole to the depth of at least 1 inch and deposit the inoculum.
5. Seal the hole in the egg with molten wax.

D) Chorioallantoic membrane (CAM) inoculation

Procedure -

1. Candle 10-12 days old egg and make a pencil mark on the shell below the base of the air sac. Then select a site on the side of embryo about 3mm above & parallel to the base of the air sac for making the whole without puncturing the CAM.
2. Mark the second site for making hole over the air sac. Apply tincture iodine on both the sites.
3. Make the hole on both the sites taking care not to puncture the CAM with the help of rubber cork containing small pin.
4. For producing an artificial air sac over the CAM, air is sucked with the help of rubber bulb from the hole in air sac. Thus air will pass through the opening made over embryo side permitting the chorioallantoic membrane to drop from the shell membrane at this point. The embryo membranes and fluid will fill normal air sac, thus creating an artificial air sac on the side of egg
5. Using a 1 ml tuberculin syringe with a 27 gauge, ***Y₂*** inch needle, insert the needle through the shell membrane over the artificial air sac and deposit the inoculum on the CAM.
6. Seal the holes with molten wax

Post inoculation procedure - Sealing

The egg is sealed immediately after inoculation to prevent desiccation and maintain sterility. Simple drill holes are readily sealed with a piece of adhesive tape which is water proof and needs no sterilization before use. Large “windows” in the shell are best sealed with paraffin wax and a glass coverslip. A shoulder of wax is first built up round the neck, which is then covered with paraffin wax and a glass coverslip; a shoulder of wax is first built up round the hole which is then covered with a warm, sterile cover subsequent cooling of the glass serves to seal the hole.

Incubation

Temperature

The optimum temperature for the development of the embryo (38-39℃) is too high for proper replication of most viruses and incubation temperature is accordingly reduced during the post incubation period to about 37℃, the exact temperature depending upon the virus being studied. The lower temperature has no significant effect upon the embryo during the few days. It is usually under observation.

Humidity

The same strict precautions concerning humidity of 65% during the preinoculation period also observed after the egg has been inoculated.

Air circulation

Forced air circulation by fan should be available to insure balanced temperature distribution throughout incubation.

Position of egg

The position of the egg after inoculation depends upon the location in the shell of the largest aperture. The later must remain uppermost, so that there is the least risk of the egg content being lost. Thus after inoculation by the amniotic route the egg is incubated in a horizontal position. Contrary to the pre-inoculation procedure; the egg must not be turned at any time after inoculation.

Examination schedule

Inoculated egg should be candled with same frequency until determination made concerning length of time necessary to observe the length of the inoculation. The proformed schedule is make examination

approximately 18-20 hours but never late then 24 hours. The examination is to find which are dead or dying from trauma of inoculation. Some eggs may die within the time period due to virus inoculation. Its even balance of egg also inoculated with some virus suspension will provide sufficient material to harvest. Its surface generally not necessary to concerned about harvesting material from their early death. The so eggs are always best, discarded.

If for some reason an excessive concentration of virus suspension are inoculated which killed the egg s within this incidental death period. The best approach is to return another sample of original material and reinoculate the egg but with the higher duration of suspension make as well as subsequent examination there after 24 hour interval atleast where embryo dead or dying remove them and refrigerate immediately. Total duration of candling period can be determined by

1 Nature of virus

2. Response pattern of embryo

3. Tissues/ cell harvested

For example yolk is to be harvested the embryo should be removed before 12^{th} day of incubation. The surving embryo should still be examined for death or gross pathology. Changes initially candling for examination for eggs can be continued until hatching time but practically 15^{th} day of inoculation until can be seen. Because of the increased size of embryo and hence opacity.

Common embryo response to virus inoculation -

Embryo can respond to virus infection in many ways. A few responses which may be noticed following inoculation of the embryonated eggs. Some of the changes and lesions, which may be induced by the virus growth, are listed below.

1. Embryo death
2. Hemorrhages of subcutaneous tissues, feather follicles; occipital region.
3. Congestion of vessels of the wings and feet of the entire embryo.
4. Growth of the embryo is stunted.
5. Tucking of the embryo.
6. Decreased amount of amniotic fluid.
7. Increased amount of allantoic fluid.

8. Thickening andoedema of chrioallantoic membrane.
9. Pocks or areas of leucocytic infiltration, often with central necrosis.
10. Microscopic lesions.
11. Formation of inculsion bodies.

It is desirable to refrigerate the embryos for 4-5 hours before examination. This will reduce hemorrhage into the fluid if the embryo is still alive.

If material are to be saved for further passage or for vaccine production it is important to disinfect the shell with alcohol or tincture of iodine before opening the egg so as to avoid bacterial contamination.

Gross embryo abnormalities as stunning destroying of muscles and limb body disfunction

Histopathological changes can be seen only under microscope. Lesion of extra cellular membrane may be pock formation hemorrhagic oedema etc.

Harvesting material from inoculated eggs

Inoculated route v/s material harvested most important aspects of harvesting sequence in that inoculation. Does not naturally dictate the tissue to be harvested may partly be embryo associated (heart muscle etc) are tissue which is indirectly exposed to inoculation (yolk sac). The construction for harvesting are outlined all according to various tissues which are to be harvested.

Preparation of work area

Harvesting desired tissue should be done with as much care taken in preparation of work area. At the time of inoculation generally an air draft free close room supplied with air filtration system and work area which can be easily disinfected need more requirement. A covered pan containing disinfectant objects for egg material tissue fluid separate container advisable of such tissue can be enumerated heavy duty plastic bag packed in container should be used. All infected tissue should be autoclaved before disposal.

Material require for harvesting

Following are requirement for harvesting (regular less) material to be harvested all must be sterile pipette, sharp straight and curve scissors. Tincture of iodine, 70% of alcohol, discarding pan with lid, container for used

pipette, sterile flask or container with stopper or the fluid collecting harvested material sterilized syringe, 26-28 gauge needle.

General procedure

1) Selection of embryo to be harvested

Since the virus require viable tissue for multiplication most patent means all obtaining from embryo dying but not dead. That is why handling of egg frequently is recommended with some viruses. Some viruses collect tissue which dead or over a time period hold under a refrigerator and harvest all of them at one time at the end of the day where test is determined although all embryo are viable. Harvest all the tissue or fluid directly without refrigerator. Chilling of embryo is during 2-4 hours at 30℃ to 70℃ this immobilizes the embryo and reduces blood flow permitting better viability. When egg opened and blood free harvesting fluid.

Labelling

Write all labels and affix to vial'before harvest material are placed in them. It is nearly impossible to after label and chilled glass surface on which moisture from atmosphere is condensing unless labels are already placed and container before harvesting will be impossible to do as later. Transparent tape is an excellent material to one efficiency label. An alternative white surface adhesive tape can be used. It does not become unviewed from the vial during chilling.

Disinfection

Disinfecting surface of egg in harvesting of material given egg may be done with egg left in traps in which, may were incubated disinfect the surface of egg with cotton gauge or swap dip in tincture iodine or 70% alcohol. All the eggs to be opened may be disinfected one time. Rub the swab gently over the outer surface of egg particularly on area of anticipated opening.

Opening the egg

With ultra hot flaming sharp pointed forceps or scissors take the egg shell around the line marking the area to be opened. Top with sufficient force to break the shell without penetrating the egg any piece of become coarse in the process. Removal into small container few disposal disinfect amount should be in the container. Since the inner surface of shell will be

contaminated with virus of egg has been sufficiently infected break along with a natural egg shell or in a case of artificial egg shell.

Exposing tissue or fluid to be harvested

Pull away the shell membrane with sterile forceps and with the help of various harvesting equipment remove tissue and fluid to the sterile labelled container.

Treatment of harvesting material

1. **Cooling** - It is always better to use a container that is large enough to cool all tissue as fluid to be harvested in shadow of using small vial for each egg. When the harvest is completely mixed cool the material thoroughly and then allocate to individual labeled vials.
2. **Sterility check** - Do sterility check at the time of harvest to identify and bacterial examination which may have occurred during inoculation, incubation and harvesting. This is most conveniently done by inoculating the apparent bacterial media for aerobic and anaerobic sample may be obtained for the tip of pipette or forceps used in harvesting. Incubate the bacterial media at 37℃ an d examine bacterial growth after 24-48 hrs. If growth is observed either treat the harvest with any one of suitable antibiotic or discard specimen storage. After sterility check has been completed the harvested material should be dispanded in previously labeled vials and stored at -70℃ in deep freeze

Collection of specimen from embrvonating chicken eggs -

Extra embryonic fluids and yolk are collected with 5ml or 10ml syringe fitted with a 20 gauge, 1 inch needle. The membrane and embryo are collected with forceps.

Allantoic fuid -

1. Apply tincture iodine or another suitable disinfectant to the shell over the air sac. Break the shell over the air sac with forceps and remove the shell to within 5-10 mm of the base of the air sac.
2. Insert the needle into the allantoic cavity and aspirate the fluid. The amount collected per egg will vary, but on an average 5-10ml can be

collected from an egg. Collect the allantoic fluid in individual container or pooled them.

Amniotic fluid -

1. Remove the shell and shell membrane from air sac end of the egg.
2. Collect the amniotic fluid as described above. Apply few drop of saline solution to the shell membrane to render it partially transparent. Instead of applying saline solution the shell membrane and the chorioallantoic membrane may be removed from the base of air sac to permit a better view of the amnion.
3. Using another syringe and needle insert the needle into the amniotic cavity and aspirate the fluid. Collect the fluid individually or pooled into a proper container.

Yolk-

For harvesting yolk from embryos insert a 20 gauge *YA* inch needle, perpendicularly through the air sac as was done for inoculation by this method and aspirate the yolk in the syringe. Collect the yolk into a proper container.

Chorioallantoic membrane -

1. Apply tincture of iodine or 70% of alcohol to the shell surface over the air sac. Break the shell with forceps and remove the shell.
2. Using forceps, remove the shell membrane from the upper pole of the chorioallantoic membrane. Rupture the chorioallantoic membrane. Invert the egg and deposit the embryo, yolk sac and extra embryonic fluid and membrane in a petri dish.
3. If the chorioallantoic membrane adheres to the shell membrane in the shell, then strip it with another forceps. Put the membrane in a petri dish containing normal saline or phosphate buffer solution. Wash it thoroughly, drain. Collect in a container.