**TABLE OF CONTENTS**

**INTRODUCTION**

- The growth of viruses in eggs ................................................................. 2
- Advantages of eggs over animal host systems ................................... 3
- Viruses that can grow in embryonated chicken eggs ........................ 3

**MATERIALS AND METHODS**

- Amniotic inoculation ............................................................................. 6
- Allantoic inoculation ............................................................................ 17
- Harvesting and identification of infecting agents .............................. 19
- Chorio-allantoic membrane inoculation .............................................. 19
- Intravenous inoculation ..................................................................... 23
- Yolk sac inoculation .......................................................................... 24

**FREQUENTLY ASKED QUESTIONS** ......................................................... 26

**References** .......................................................................................... 26

**Websites** ............................................................................................. 26
INTRODUCTION

Viruses can only grow in living cells but they are particular about the type of cell they infect and grow in – there is no universal cell that will support all viruses. Viruses tend to be host-specific; therefore human viruses grow best in cells of human origin, bovine viruses in bovine cells, canine viruses in canine cells, while some viruses will not grow in vitro at all. Therefore in the laboratory the suspected virus must be grown in a culture method known to support its growth.

- **animals** are used for studying viruses which do not grow in cell cultures or eggs, and for testing vaccines,
- **eggs** support a fairly wide range of animal and human viruses – hence their importance in the diagnostic service,
- **cell cultures**: different types of cell lines will support different types of viruses.

The growth of viruses in eggs

Embryonated hen’s eggs will support the growth of some viruses. Not all viruses will grow in the tissues of embryonated eggs initially but many can be adapted to growth in eggs without much difficulty. Eggs provide a suitable means for the primary isolation and identification of viruses, the maintenance of stock cultures and the production of vaccines.

The viruses grow in the cells of the embryo and membranes and can be detected in several ways. These include mortality, deformity or haemorrhages in the embryo, lesions on the membrane in the form of pocks, oedema of the developing membranes, inclusion bodies in sections prepared from embryo tissues or the presence of viral antigens in the egg fluids.

An embryonated hen’s egg contains cells (the embryo and its membranes) that will support the growth of some viruses. They grow either in the cells of the embryo or its membranes, or in both and when these cells die they are liberated into the egg fluids. Collection of the virus-infected egg fluid is relatively simple, if somewhat messy.

Eggs are inexpensive and easy to maintain. Eggs come in a usually sterile package surrounded by a porous shell. As they should arrive clean in the laboratory, they should not be washed or immersed in water as this may allow bacteria to enter the egg. Use a quickly evaporating agent, such as alcohol, tincture of iodine or ether, to sterilize the eggshell at the site re inoculation.

Eggs are freely available, especially hens’ eggs but ducks’ eggs have also been used. The immune system of the embryo has not matured; therefore antibodies are not produced against the inoculated virus. However, maternal antibodies are transferred from the hen to the
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

egg which implies that eggs should be obtained from non-vaccinated (especially against Newcastle disease virus), mycoplasma-free flocks.

**Advantages of eggs over animal host systems**

- Eggs are readily available, cheap and easy to maintain
- Preliminary incubation of the eggs is carried out at 38 – 39°C and 60 – 70% humidity. The eggs need to be turned at least twice a day or rolled continually in a specially designed egg incubator
- Once inoculated, the eggs are incubated at temperatures suitable for the growth of the virus, but still maintaining a high degree of humidity
- Eggs are easily manipulated under sterile conditions
- Eggs come in a sterile package surrounded by a porous shell. They should arrive clean in the laboratory. *Do not wash the eggs* or immerse them in water as this may allow bacteria to enter the porous shell. To sterilize the site of inoculation, use a quickly evaporating agent, such as alcohol, tincture of iodine or ether
- Eggs are sheltered from the natural diseases often observed in laboratory animals, and are relatively free from bacterial and many latent viral infections. However maternal antibodies are transferred from the mother hen, therefore the eggs should be obtained from non-vaccinated (especially Newcastle disease virus), mycoplasma-free flocks
- Eggs are generally free from natural factors of defence, specific or non-specific, that sometimes intervenes and prevents passage in adult animals. The immune system of the embryo has not matured therefore antibodies are not made against the inoculated virus. Also the embryo is sensitive of some viruses that are harmless to the adult bird
- Eggs are easily identified and labelled with details of date, virus inoculated and experimental procedure
- Different routes of inoculation for different viruses are available i.e.
  - the amniotic and / or allantoic cavities,
  - the yolk sac,
  - the chorio-allantoic membrane,
  - intravenous.

**Viruses that can grow in embryonated chicken eggs**
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

Embryonated chicken eggs are not routinely used for the isolation of viruses. Viruses of veterinary importance which grow in eggs are tabulated under disease caused, age and route of infection, incubation temperature and outcome of infection.

**Table 1: Viruses of veterinary importance that can be grown in embryonated chicken eggs**

<table>
<thead>
<tr>
<th>ANIMAL</th>
<th>VIRUS GENUS</th>
<th>DISEASE</th>
<th>ROUTE OF INOCULATION</th>
<th>OUTCOME OF INFECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td><em>Rhabdovirus</em></td>
<td>Vesicular Stomatitis</td>
<td>Inoculate onto allantoic Sac 8 days old embryo 3 – 4 days incubation</td>
<td>Embryo death</td>
</tr>
<tr>
<td></td>
<td><em>Capripoxvirus</em></td>
<td>Lumpy skin disease</td>
<td>Inoculate onto CAM 7 – 9 days incubation 33,5 – 35°C Staining of membrane</td>
<td>Pocks / lesions on membrane</td>
</tr>
<tr>
<td></td>
<td><em>Orthopoxvirus</em></td>
<td>Cowpox</td>
<td>Inoculate onto CAM Can be distinguished from pseudocowpox (parapox) which does not grow on the chorio–allantoic membrane.</td>
<td>Pocks / lesions on membrane</td>
</tr>
<tr>
<td></td>
<td><em>Orthomyxovirus</em></td>
<td>Swine influenza</td>
<td>Amniotic/allantoic inoculation into 9-10 day old embryonating eggs Incubate 48 hrs at 33.5°C</td>
<td>Haemagglutinates chick RBC</td>
</tr>
<tr>
<td></td>
<td><em>Iridovirus</em></td>
<td>African swine fever</td>
<td>Yolk sac inoculation Causes death in 6 – 7 days</td>
<td></td>
</tr>
<tr>
<td>Swine</td>
<td><em>Orbivirus</em></td>
<td>Bluetongue</td>
<td>Yolk sac / IV 10 – 11 days old embryonating eggs Incubate 3-5 days at 33.5°C Causes death of embryo. Further processed for presence of virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Flavivirus</em></td>
<td>Wesselsbron disease</td>
<td>Yolk sac</td>
<td></td>
</tr>
<tr>
<td>Sheep and goats</td>
<td><em>Orthomyxovirus</em></td>
<td>Equine influenza</td>
<td>Amniotic/Allantoic inoculation into 10 day old embryonating eggs Incubate 48 hrs at 33°C Haemagglutinates chick and pig RBC.</td>
<td></td>
</tr>
<tr>
<td>Horses</td>
<td><em>Reoviridae Orbivirus</em></td>
<td>African horse sickness</td>
<td>Yolk sac /IV 10 – 12 days old embryonating eggs Incubate at 33°C for 3 – 7 days Causes death of embryo.</td>
<td></td>
</tr>
</tbody>
</table>
**Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs**

<table>
<thead>
<tr>
<th>Virus</th>
<th>CAM</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bornavirus</td>
<td>Canine distemper</td>
<td>CAM – needs adaptation, Produces lesions</td>
</tr>
<tr>
<td><strong>Dogs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paramyxovirus</td>
<td>Newcastle disease</td>
<td>Amniotic/allantoic inoculation into 9-10 day old embryonating eggs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Incubate 2 – 5 days at 37°C</td>
</tr>
<tr>
<td>Rhabdovirus:</td>
<td>Rabies</td>
<td>CAM – plaque formation and death of embryo, Difficult, but can be</td>
</tr>
<tr>
<td>Lyssavirus</td>
<td></td>
<td>adapted</td>
</tr>
<tr>
<td><strong>Poultry</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paramyxovirus</td>
<td>Infectious laryngotracheitis</td>
<td>CAM – plaque formation and death of embryo, Amniotic/allantoic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>inoculation. Incubate 5-7 days</td>
</tr>
<tr>
<td>Herpesvirus</td>
<td>Infectious laryngotracheitis</td>
<td>Amniotic/allantoic inoculation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Incubate 30 hrs at 37°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Effects on embryo include death, dwarving, and curling, plus uratic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>deposits in the mesonephrons.</td>
</tr>
<tr>
<td>Coronavirus</td>
<td>Infectious bronchitis</td>
<td>Amniotic/allantoic inoculation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Incubate 30 hrs at 37°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Effects on embryo include death, dwarving, and curling, plus uratic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>deposits in the mesonephrons.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Some strains require 10 days incubate for maximum virus. Further</td>
</tr>
<tr>
<td></td>
<td></td>
<td>processed for presence of virus</td>
</tr>
<tr>
<td>Coronavirus</td>
<td>Turkey enteritis (Bluecomb disease)</td>
<td>Amniotic inoculation – grows in embryo intestines or yolk sac, 3 – 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>days incubation</td>
</tr>
<tr>
<td>Orthomyxovirus</td>
<td>Avian influenza, Myna, turkey, chicken,</td>
<td>Amniotic/allantoic inoculation</td>
</tr>
<tr>
<td></td>
<td>duck, gull</td>
<td>Incubate 2 days at 35°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Death of embryo within 48 hours</td>
</tr>
<tr>
<td>Adenovirus type 1</td>
<td>Avian adenovirus infection</td>
<td>Allantoic inoculation</td>
</tr>
<tr>
<td>Chick embryo lethal orphan (CELO)</td>
<td></td>
<td>Incubate 3-4 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>May need up to five blind passages</td>
</tr>
<tr>
<td>Pox: Avipox</td>
<td>Fowlpox</td>
<td>CAM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Produces focal or diffuse pocks</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>Avian encephalomyelitis</td>
<td>Yolk sac inoculation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Incubate 9 days at 37°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Muscular dystrophy and paralysis, Signs of encephalomyelitis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>observed after hatching</td>
</tr>
</tbody>
</table>
**Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs**

<table>
<thead>
<tr>
<th>VIRUS AND DISEASE</th>
<th>EFFECT ON: CHORIO-ALLANTOIC</th>
<th>EFFECT ON: CHICKEN EMBRYO</th>
<th>HAEMAGGLUTINATION OF CHICK EMBRYO FLUIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newcastle disease virus (NDV), a paramyxovirus of chickens</td>
<td>No pocks</td>
<td>Lethal within 48-72 hours</td>
<td>HA positive</td>
</tr>
<tr>
<td>Laryngotracheitis, a herpesvirus of chickens and pheasants</td>
<td>Pocks</td>
<td>No effect</td>
<td>HA negative</td>
</tr>
<tr>
<td>Infectious bronchitis, a coronavirus of chickens</td>
<td>No pocks</td>
<td>Dwarfing</td>
<td>HA negative</td>
</tr>
</tbody>
</table>

**Table 2: The use of eggs to distinguish between clinically similar diseases**

**MATERIALS AND METHODS**

**Amniotic inoculation**

Although the volume of fluid in the infected amniotic sac is small [1 – 2 ml], the virus is introduced directly into the amniotic fluid that bathes the developing lung buds of the embryo whose cells are highly susceptible to infection with myxoviruses. The amniotic route is recommended for the primary isolation of mumps virus and influenza A, B and C viruses from humans but has little application in veterinary virology. Newly isolated influenza viruses may require several passages before they adapt to growth by other routes, such as allantoic.

**Requirements**

- 9 – 10-day-old embryonated hen’s eggs and egg rack
- Egg candler
- Pencil for marking and labelling eggs
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

- Tincture of iodine or 70% alcohol and cotton wool swab for sterilizing egg shell
- Dental drill with flat-headed bit for drilling through egg shell
- Sterile needle
- Sterile Pasteur pipettes and teats
- Sterile liquid paraffin
- Sterile fine tipped forceps
- 1 ml syringe with fine needle (sterile)
- 2 cm wide sellotape to seal egg after inoculation
- Humidified egg incubator

![Image of candling an embryonated egg](image1.png)

Figure 2: Candling of a 10-day-old embryonated egg for amniotic inoculation

**Method**

- Candle eggs to ensure viability of the embryo
- Allow 6 eggs per specimen and 2–4 eggs for controls. Label eggs appropriately
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

- Mark the position of the air sac and embryo with a pencil, avoiding any major blood vessels
- Sterilize the top of the egg (blunt end) with a swab squeezed in tincture of iodine or 70% alcohol
- Drill, using a flat-headed bit, cut a circle (15 cm in diameter) on the top of the egg above the air sac
- While drilling, cut through the shell but leave the underlying shell membrane intact.

![Drilling the egg shell for amniotic inoculation](image)

**Figure 3: Drilling the egg shell for amniotic inoculation**

- Re-sterilize the drilled area and its surrounds with 70% alcohol
- Position the egg on a tray with the pencil mark denoting the position of the embryo in front
- Tip the egg forward (towards yourself) to ensure that the embryo falls forward
- Remove the drilled circle of shell and its tightly attached underlying membrane with a sterile needle
- The shell membrane lining the bottom of the air sac is now visible
Pipette a small drop of sterile liquid paraffin onto the exposed air sac membrane, above the embryo. The membrane will become transparent enabling the dark shape of the embryo to be seen.

NB: Use only a small drop of liquid paraffin, as any excess will run into the egg when the membranes are pierced and suffocate the embryo.

Flame a pair of fine pointed forceps and plunge them, when warm but not too hot, through the transparent shell membrane and pick up the amniotic membrane.

Important: Avoid the larger blood vessels, the heat of forceps should cauterize the smaller ones.

Pull the amniotic membrane through the shell membrane.

Inoculate 0.1 – 0.2 ml solution containing the virus into the amniotic cavity using a 1 ml syringe with a fine needle. Release the membrane and allow it to fall back into place.

Seal the eggshell with sellotape. The sticky surface of the sellotape is sterile if not touched.

Figure 4: Inoculating into the amniotic cavity
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

- Incubate at the required temperature in an upright position.
- Candle eggs daily. Discard any eggs containing dead embryos the day after the inoculation – these are usually traumatic deaths. Keep all the eggs that die subsequently at 4°C until harvested.

**Harvesting of amniotic fluid**

**Requirements**

- Eggcups – small beakers or damp cotton wool
- Sterile Petri-dishes
- Sterile scissors – small curved
- Sterile fine pointed forceps
- Wide necked bottles: x 2 containing 70% alcohol
- Wide necked bottles: x1 containing absolute alcohol
- Sterile Pasteur pipettes and teats
- Rack for holding pipettes
- Sterile McCartney bottles – 1 per egg
- Sterile bijou bottles – 1 per egg
- Discard bin/container for eggs
- Discard tray/bin for glassware and instruments
- Bottle slope
- Gloves, gown and mask
- Bunsen burner
- Bench cote - protective paper covering for surface of hood
- Safety hood or biohazard cabinet
Disinfectant to wipe up any spillage e.g. 3% tegador and 70% alcohol

Sterile swabs and paper towels for cleaning and wiping

Antibiotic cocktail

**Method**

- Candle the eggs and mark those containing dead embryos
- Chill the eggs at 4°C for 2 hours to constrict the blood vessels and kill the embryos
- Prepare harvesting area and label one McCartney bottle and one Bijou bottle per egg
- Set out several eggcups or small beakers or damp cotton wool on sterile Petri-dishes and prepare egg pipettes
- Harvest eggs individually in the following order:
  1\textsuperscript{st}: alive control eggs
  2\textsuperscript{nd}: alive infected eggs
  3\textsuperscript{rd}: dead control eggs
  4\textsuperscript{th}: dead infected eggs
- Remove the first egg to be harvested from the fridge and place in an eggcup. Keep the other eggs at 4°C until harvested. If the eggs warm up, the blood will start to flow and contaminate the harvested egg fluids
- Flame the curved scissors (warm, not hot) and cut eggshell below the pencilled line marking the air sac
- Pipette the allantoic fluid (5-10 ml) into the appropriately labeled McCartney bottle
- Flame the forceps (warm, not hot) and pierce the amniotic membrane
- Pipette, using a clean pipette, the amniotic fluid (1-2 ml) into the appropriately labeled Bijou bottle
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

Figure 5: Opening an egg for harvesting

- Tip the embryo out onto a sterile Petri-dish
- Using clean scissors and forceps, decapitate the embryo and open its chest cavity
- Remove the lungs and bronchi and pool with the amniotic fluid. Titurate the lungs using a Pasteur pipette to release the virus
- Discard the rest of the egg material and the dirty glassware
- Use a clean Petri-dish for each embryo and change egg cups frequently
- Rinse forceps and scissors in 70% and absolute alcohol, flame and allow cooling before harvesting the next egg
- Add antibiotics to the egg fluids: approximately 100 units/ml of penicillin and streptomycin, and 50 µg/ml of gentamycin.
- Test egg fluids individually for the presence of virus by haemagglutination using the appropriate red blood cells, such as guinea pig or fowl red blood cells
- Store egg fluids at 4°C – do not freeze during passages
- Passage egg fluids at a 10-1 or 10-3 dilution. Do not inoculate undiluted egg fluids as inhibitors, such as interferon, will inhibit viral growth
Preparation of a series of dilutions

The example below illustrates the following:

- A series of 2-fold dilutions of the virus sample
- Add 1 ml of diluent to each tube
- Add 1 ml of virus to first tube, mix well, carry 1 ml over, mix well, and repeat to end
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

**Haemagglutination and haemagglutination inhibition assays of fluid harvested from embryonated eggs**

**Requirements**

- Red blood cells – guinea pig or fowl cells
- Alsevers’ solution – anticoagulant solution
- Phosphate buffered saline (PBS) containing Mg2+ and Ca2+
- Bench top centrifuge
- Centrifuge tubes - large for washing and conical graduated for calculating packed cell volume
- Sterile tin foil squares for covering centrifuge tubes while spinning
- 250 ml sterile glass bottles
- 1 ml graduated pipettes
- Plastic Wassermann tubes and rack

**Method**

**Red blood cells**

- Collect in Alsevers’ solution, 3-5 ml guinea pig from heart puncture and / or 5 ml fowl blood from wing vein
- Suspend cells in x 10 volume of PBS containing Mg++ & Ca++ and spin to wash cells at 1000 rpm for 10 min
- Discard supernatant fluid (SNF) and resuspend cells in clean PBS
- Repeat washing process x2 and for the final wash, spin in a conical graduated centrifuge tube
- Make a 10% stock suspension of red blood cells and dilute for use to 0,6% suspension

**Haemagglutination assay**

- Set out and label two (2) Wassermann tubes per egg fluid sample to be assayed
- Add 0,8 ml cold PBS containing Mg2+ and Ca2+ to the 1st tube - to make 1/5 dilution
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

- Add 0.5 ml cold PBS containing Mg\(^{2+}\) and Ca\(^{2+}\) to the 2nd tube - to make 1/10 dilution
- Add 0.2 ml harvested egg fluid and mix well = 1/5 dilution
- Pipette 0.5 ml of the mixture to the 2nd tube = 1/10 dilution
- Add 0.5 ml of the 0.6% red blood cell suspension to the 1st tube ONLY
- Incubate at 4°C for 30-60 min
- Read haemagglutination patterns in 1st tube and titrate further if necessary (from the 2nd tube)
- Pool egg fluids from the same specimen showing a high positive titre

**Figure 8: Haemagglutination assay**

**Haemaglutination-inhibition assay**

This assay may be used to identify the isolated virus by testing it against known reference antisera. The antibodies in the reference sera bind to the viral haemagglutinins resulting in inhibition of viral haemagglutination. The haemagglutination-inhibition (HAI) assay is sensitive, specific, simple, inexpensive and rapid. The reference sera are usually obtained commercially and have been treated to remove any non-specific agglutinins as well as any non-specific inhibitors, both of which could interfere with the test results.

**Titration of haemagglutinating virus**
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

The virus is titrated to determine the haemagglutinating dose to be used, i.e. 4HAD’s. The test must be done on the same day as the HAI test using the same batch of RBCs.

**Method**

- On a round-bottomed HA plate mark a row of wells from 1/10 – 1/1280 (or higher)
- Make doubling dilutions of the virus antigen in 0.25 ml amounts
- Add 0.25 ml PBS (or diluent used) per well – (in lieu of the immune serum)
- Add 0.5 ml of the 0.6% red blood cell suspension
- Incubate at the appropriate temperature for the required time
- Read the results. The last well with complete haemagglutination is the end point of the titration. This dilution contains one haemagglutinating dose or unit/0.25 ml (HAD)
- Determine 4 HADs/0.25ml. This is the dilution of virus that will be used in the HAI assay
- Dilute the virus to 4 HADs

**Assay**

- On a round-bottomed HA plate mark a row of wells from 1/10 – 1/1280
- Allocate one row for each reference sera used
- Allocate two wells for the 1/10 dilution – one of which will act as a serum control
- Make doubling dilutions of the reference sera in 0.25 ml amounts
- Add 0.25 ml of virus antigen at 4 HADs to each serum well - except the serum control wells
- Add 0.25 ml diluent to the serum control wells

Antigen control
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

- Mark a row of wells – 4HAD; 2HAD; 1HAD; ½ HAD; ¼ HAD
- Make doubling dilutions of the virus antigen in 0.25ml amounts
- Add 0.25 ml diluent (in lieu of antiserum)
- Red cell control
- Mark one or two wells
- Add 0.5 ml diluent
- Incubate to allow the virus / antibody to interact
- Temperature and time of incubation varies according to the virus (NDV = 30 – 60 min at RT)
- Add 0.5 ml of the 0.6% red blood cell suspension to all wells
- Incubate – again the time and temperature will depend upon the virus tested (NDV = 30 – 60 min at 4°C). NDV contains neuraminidase, which will elute the virus from the RBC if the temperature rises

Read the results

- The red blood cell and serum controls should show no haemagglutination
- The antigen control should show positive haemagglutination in the 4, 2, and 1HAD wells, a 50% haemagglutination in the ½ HAD well and no haemagglutination in the ¼ HAD well
- In the sample wells no haemagglutination signifies inhibition, and therefore a positive reaction

Allantoic inoculation

Many viruses such as fowl plague and Newcastle disease virus, grow readily in the endoderm of developing chicken eggs. Other viruses such as influenza, may require repeated amniotic passages before becoming adapted to the egg and grown in the allantoic cavity. Allantoic inoculation is a quick and easy method that yields large amounts [8 – 15 ml] of virus-infected egg fluids.
Requirements

- 9 – 11 day old embryonated hen’s eggs and egg trays
- Egg candler
- Egg incubator – at correct temperature and humidity
- Pencil for marking and labelling eggs
- Tincture of iodine or 70% alcohol and cotton swab for sterilising egg shell
- Metal egg prick
- 1 ml syringe with a 3 cm long 24 or 26g needle (sterile)
- Sellotape or wax to seal egg after inoculation
- Virus to be inoculated, diluted appropriately

Method

- Candle eggs to ensure viability of the embryo
- Allow 6 eggs per specimen plus 2 – 4 eggs for controls
- Label eggs with the appropriate specimen number
- Candle the eggs and mark the position of the air sac, and a point on the side of the egg, which is not adjacent to any major blood vessels
- Sterilise the egg shell over the air space with tincture of iodine or 70% alcohol
- Flame the metal egg prick
- Jab a small hole in the shell over the air space, just above the pencilled mark
- Resterilise the area with tincture of iodine or 70% alcohol
- Draw up inoculum in a 1 ml syringe
- Plunge the needle through the hole in the shell directly into the allanotic cavity
- Inoculate 0.1 ml virus dilution/egg
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

- Inoculate controls with 0.1 ml diluent (PBS containing Mg++ and Ca++)
- Seal hole with sellotape or hot wax
- Incubate for 48-72 hours, in upright position, at a temperature appropriate for the growth of the virus
- Candle daily. Chill eggs before harvesting

Harvesting and identification of infecting agents

See ‘Harvesting of amniotic fluid’ steps 1 – 7

See ‘Haemagglutination assay’

Chorio-allantoic membrane inoculation

This method has been widely used in veterinary virology because many viruses grow readily or can be adapted to grow on the CAM and produce visible foci or ‘pocks’, inclusion bodies, oedema or other abnormalities. Viruses include the herpesviruses (infectious laryngotracheitis and Aujeszky's disease virus) and poxviruses (fowlpox, ectromelia and vaccinia).

Requirements

- 10-13 day old embryonated hen’s eggs on egg rack
- Egg candler
- Pencil for marking and labelling eggs
- Egg incubator – at correct temperature and humidity
- Ether for sterilising egg shell at site of inoculation
- Dental drill with rounded burr head
- Sterile injection needle
- 2 ml rubber teat
- Sterile Pasteur pipettes
- PBS warmed at 56°C
- 1 ml syringe with fine short needle
Applied Veterinary Virology: The isolation and identification of viruses using embryonated chicken eggs

- Sellotape to seal holes
- Bunsen burner

**Method**

- Candle large white eggs and choose those with well-developed CAM membranes
- Mark the air sac and a point on the side of the egg not adjacent to any major blood vessels, which will be the site of inoculation
- Sterilise both areas with ether. Iodine is not used as it may cause non-specific pocks
- Drill a hole through the shell and shell membrane, above the middle of the air sac
- Drill a lengthways line through the shell at the site of inoculation leaving the underlying shell membrane intact. Drill slowly to avoid producing heat that can cause non-specific pocks
- Blow off shell dust with a Pasteur pipette and flame pipette lightly
- Pipette a drop of pre-warmed PBS onto the hole at the site of inoculation
- Stroke the fibres of the exposed shell membrane with the sterile needle
- The fibres of the shell membrane run transversely across the egg, while those of the CAM run lengthways along the egg. Therefore, by stroking the shell membrane along the drilled line, the fibres can be parted. This allows the PBS to run in-between the two membranes and aids in their separation
- Apply gentle suction, using the rubber teat, to the hole overlying the air sac. The suction will pull the air out of the air sac, and, as air is pulled into the hole at the site of inoculation, the CAM will drop. Too vigorous a suction may pull the membrane down too fast, causing haemorrhage and non-specific pocks
Figure 9: Schematic diagram of the inoculation of the chorio-allantoic membrane

- Candle the eggs to ensure that the CAM has dropped. The original air sac should have disappeared and been replaced by one at the site of inoculation
- Incubate the eggs for an hour to allow the membranes to settle
- Do not leave the eggs any longer as the membranes will become refractive and develop non-specific pocks. This will lower the membrane’s sensitivity to the inoculated virus
- Inoculate the virus directly onto the CAM. Do not attempt to touch the membrane, as it is fragile and easily ruptured
- Roll the egg gently to distribute the inoculum evenly
- Seal the two holes with sellotape
- Incubate the eggs, inoculation side uppermost, at the appropriate temperature

Harvesting chorio-allantoic membranes

Requirements

- Gloves and gown
- Cotton wool eggcups on Petri-dishes
- Curved scissors
- Sterile forceps, several pairs
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- Two Petri-dishes with sterile PBS per egg

**Method**

- Candle eggs and record any deaths
- Mark the position of the artificial air sac with a pencil
- Chill eggs in the fridge for at least 2 hours to constrict blood vessels. Do not freeze them
- Work under a hood, wear gloves and a gown
- Place egg in eggcup and cut eggshell with a sterile pair of curved scissors. Start incision at the hole above the true air sac and cut egg in half below line of artificial air sac
- Trim the shell around the artificial air sac while holding the eggshell with a sterile pair of forceps
- Using a 2nd pair of forceps, peel the CAM from the shell
- Wash the membrane in a Petri-dish containing PBS and then place in the 2nd dish with clean PBS
- Examine for pocks over a dark background
- Count number of pocks

![Figure 10: Lesions on the chorio-allantoic membrane](image)

Figure 10: Lesions on the chorio-allantoic membrane
Intravenous inoculation

This method is used in the identification of bluetongue virus. It causes haemorrhages in the embryo.

Requirements for inoculation

- 11-day old eggs on egg tray
- Egg candler in darkened room
- 70% alcohol and swab to sterilize egg shell
- Pencil for marking
- Dental drill
- Sterile forceps
- Syringe and needle – 26g 3/8
- Sellotape
- Humidified egg incubator

Requirements for harvesting

- Egg cups
- Petri-dishes
- Sterile forceps and scissors
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- Pasteur pipettes and teats
- Bottles

**Method**
- Candle the eggs for viability. Locate the large vein of the CAM and ascertain the direction of blood flow. Mark this area
- Sterilize the eggshell with 70% alcohol
- Using a dental drill remove (slowly and gently!) a triangular piece of egg shell over the vein – do not pierce the vein
- Using the candler in a dim room to aid you, inoculate 50 µl of the virus in the direction of the blood circulation
- Seal the hole with sellotape and incubate at the appropriate temperature for the virus
- Candle daily and refrigerate dead embryos at 4°C

**Harvesting**
- Chill the eggs
- Cut open the eggshell and harvest the egg fluids into a labelled McCartney bottle
- Tip the embryo onto a sterile Petri-dish for examination

**Yolk sac inoculation**

The yolk sac route is particularly suitable for the cultivation of *Chlamydia, Rickettsia* and certain viruses, such as louping ill and other togaviruses.

**Requirements**
- Embryonated eggs, 6 – 8 days old, on tray
- Punch or drill
- Tincture of iodine and swab on stick
- Egg candler in darkened room
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- Pencil for marking eggs
- Sellotape for sealing hole
- Syringe with long (2”) fine-bore needle
- Petri-dishes
- Scissors and forceps
- Saline
- Glass slides
- Giemsa stain solutions
- Microscope with 100 x objective, microscope oil

**Method**

- Candle the eggs and mark the air sac
- Label the eggs (virus inoculum and controls)
- Disinfect the shell above the air sac with tincture of iodine or 70% alcohol
- Punch or drill a hole through the shell over the air sac, in the centre
- Using a syringe with a long needle, introduce the needle vertically through the hole for 1½"
- Inoculate 0.1 – 0.5 ml virus material directly into the yolk sac
- At this age the yolk sac is large while the embryo is small and unlikely to accidentally damaged
- Withdraw the needle and seal the hole with sellotape, wax or nailpolish
- Incubate at the temperature suitable for the virus
- Candle daily and discard embryos that die within 24 hours

**Harvesting**
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- Chill the eggs to kill the embryo
- Cut the egg-shell open and empty egg contents into a sterile Petri-dish
- Gently empty the yolk sac of yolk using forceps and scissors
- Remove the yolk sac to a clean Petri-dish and rinse in saline

FREQUENTLY ASKED QUESTIONS

1. **How old must the embryo be for isolation of a virus?**
   
   It depends on the specific virus and route of inoculation used

2. **Why is the haemagglutination test used after infection of ECE?**
   
   This is used to confirm the presence of some viruses

3. **When should egg material be harvested**
   
   It depends on the specific virus isolated and the clinical signs present e.g. death of the embryo. In general 3 – 5 days

4. **Should SPF eggs be used to isolate viruses and why?**
   
   Yes SPF eggs should be used. Although no immune system is present in the egg, antigens (vaccines) could be present from the hen.

5. **Why is the intravenous route of inoculation of ECE an important route to use?**
   
   This is a sensitive route of inoculation where the virus is directly inoculated into the vein and circulation of blood in the embryo increase the replication of the virus.

6. **Does one need specialized equipment and training to use ECE for virus isolation?**
   
   A dedicated laboratory area for aseptic work is needed. Not very expensive but basic laboratory equipment is needed. Skills training of staff are important.

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