Chapter 7

Isolation and Propagation of Coronaviruses in Embryonated Eggs

James S. Guy

Abstract

The embryonated egg is a complex structure comprised of an embryo and its supporting membranes (chorioallantoic, amniotic, yolk). The developing embryo and its membranes provide the diversity of cell types that are needed for successful replication of a wide variety of different viruses. Within the family *Coronaviridae* the embryonated egg has been used as a host system primarily for two avian coronaviruses within the genus *Gammacoronavirus*, infectious bronchitis virus (IBV) and turkey coronavirus (TCoV). The embryonated egg also has been shown to be suitable for isolation and propagation of pheasant coronavirus, a proposed member of the *Gammacoronavirus* genus. IBV and pheasant coronavirus replicate well in the embryonated chicken egg, regardless of inoculation route; however, the allantoic route is favored as these viruses replicate well in epithelium lining the chorioallantoic membrane, with high virus titers found in these membranes and associated allantoic fluids. TCoV replicates only in epithelium lining the embryo intestines and bursa of Fabricius, thus amniotic inoculation is required for isolation and propagation of this virus. Embryonated eggs also provide a potential host system for detection and characterization of other, novel coronaviruses.

Key words Embryonated egg, Allantoic, Amniotic, Chicken, Turkey

1 Introduction

Embryonated eggs are utilized as a laboratory host system for primary isolation and propagation of a variety of different viruses, including the avian coronaviruses, infectious bronchitis virus (IBV), turkey coronavirus (TCoV), and pheasant coronavirus [1–4]. They have been extensively utilized for propagation of these viruses for research purposes and, in the case of IBV, for commercial production of vaccines. In addition, embryonated eggs provide a potential host system for studies aimed at identifying other, novel coronavirus species.

The embryonated egg is comprised of the developing embryo and several supporting membranes which enclose cavities or "sacs" within the egg [5]. The shell membrane lies immediately beneath the shell; this is a tough fibrinous membrane that forms the air sac in the region of the blunt end of the egg (Fig. 1). In contrast to the

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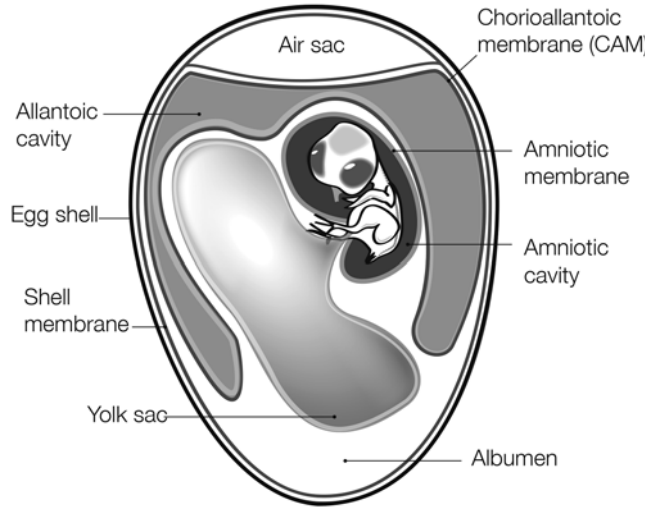

Fig. 1 Anatomical features of an embryonated chicken egg at approximately 11 days of incubation

shell membrane, chorioallantoic, amniotic, and yolk membranes are comprised largely of epithelium, and represent potential sites of coronaviral replication. The chorioallantoic membrane (CAM) lies directly beneath the shell membrane; this is a highly vascular membrane that serves as the respiratory organ of the embryo. The CAM is the largest of the embryo membranes, and it encloses the largest cavity within the egg, the allantoic cavity; in the embryonated chicken egg, this cavity contains approximately 5–10 ml of fluid, depending upon the stage of embryonation. The amniotic membrane encloses the embryo and forms the amniotic cavity; in the embryonated chicken egg, this cavity contains approximately 1 ml fluid. The yolk sac is attached to the embryo and contains the nutrients the embryo utilizes during embryonic development and the immediate post-hatch period.

The developing embryo and its membranes (CAM, amniotic, yolk) provide the diversity of cell types that are needed for successful replication of a wide variety of different viruses. Embryonated eggs may be inoculated by depositing virus directly onto the CAM, or by depositing virus within allantoic, amniotic, and yolk sacs [6]. For avian coronaviruses, inoculation of eggs by allantoic or amniotic routes has been shown to provide these viruses with access to specific cell types that support their replication [2–4]. IBV is an epitheliotropic virus that replicates in a variety of epithelial tissues in the post-hatch chicken including respiratory tract, gastrointestinal tract, kidney, bursa of Fabricius, and oviduct [7]. In the embryonated chicken egg, IBV replicates well regardless of inoculation route; however, the allantoic route is favored as the virus replicates extensively in epithelium of the CAM and high titers are shed into allantoic fluid [8]. A pheasant coronavirus has been isolated and

propagated in embryonated chicken eggs using procedures similar to those utilized for IBV (allantoic route inoculation) [3]. TCoV also is epitheliotropic in post-hatch chickens and turkeys, but replicates only in epithelium lining the intestinal tract and bursa of Fabricius [1, 4, 9]. These cellular tropisms of TCoV also are observed in the embryonated egg; the virus replicates only in embryonic intestines and bursa of Fabricius, sites that are reached only via amniotic inoculation.

2 Materials

2.1 Preparation and Collection of Samples for Egg Inoculation

1. Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 % fetal bovine serum (FBS) and antibiotics: penicillin 1,000 U/ml, gentamicin 0.05 mg/ml, amphotericin B 5 μg/ml. Adjust pH to 7.0–7.4 using either 1 N NaOH or 1 N HCl. Tryptose phosphate broth and other cell culture basal media (minimal essential medium, RPMI 1640, etc.) may be substituted for DMEM.
2. Sterile cotton-tipped swabs are used for collection of antemortem samples (e.g., respiratory secretions, feces, etc.). Type 4 Calgiswab (Puritan Medical Products) is useful for collection of respiratory secretions from small birds.
3. Sterile Whirl-Pak® bags (Fisher Scientific) are used for collection of tissues.
4. Tissue homogenizer. Mortar and pestle, Ten Broeck homogenizer, or Stomacher® (Fisher).

2.2 Egg Inoculation and Incubation

1. Fertile eggs are obtained, preferably, from specific-pathogen-free (SPF) flocks (e.g., Charles River/SPAFAS). Alternatively, fertile eggs may be used that are from healthy flocks free of antibody to the virus of interest (*see* **Note 1**).
2. Disinfectant: 70 % ethanol, 3.5 % iodine, 1.5 % sodium iodide.
3. A vibrating engraver (Fisher Scientific) or drill (Dremel) is used to prepare holes in egg shells. Prior to use, disinfect the tip of the engraving tool/drill to prevent contamination of the egg.
4. Plastic cement, glue, tape, or nail varnish are used to seal holes in egg shells after inoculation.
5. Egg flats.
6. Egg candlers are available from a variety of commercial sources.
7. A suitable egg incubator is needed; these are available from a variety of commercial sources. Commercially available egg incubators generally are equipped with heat source, humidifier, and a timer-based mechanical turning system.

2.3 Collection of Specimens from Inoculated Eggs

1. Sterile scissors and forceps.
2. Sterile pipettes or 5 ml syringes with 1 in., 18 gauge needles.
3. Sterile plastic tubes, e.g., 12 × 75 mm snap-cap tubes or microcentrifuge tubes.

3 Methods

Embryonated chicken and turkey eggs are extensively utilized for isolation and propagation of IBV and TCoV, respectively [2, 4]. These same eggs and techniques may be useful for amplification of other coronaviruses, and this has been demonstrated with isolation and propagation of pheasant coronavirus in embryonated chicken eggs [3]. However, many viruses exhibit host specificity and this should be considered when attempting to isolate and propagate novel coronaviruses.

Embryonated eggs from avian species other than chickens and turkeys may be utilized; these are inoculated essentially as described for chicken and turkey eggs, primarily by making adjustments in the length of time embryos are incubated before inoculation. Embryonated chicken eggs are inoculated by the allantoic route at approximately the middle of the 21-day embryonation period, at 8–10 days of embryonation; they are inoculated by the amniotic route late in the incubation period, at 14–16 days of embryonation. Turkey and duck eggs have a 28-day embryonation period and generally are inoculated by the allantoic route at 11–14 days of embryonation, and by the amniotic route at 18–22 days of embryonation.

Embryonated chicken and turkey eggs are incubated at a temperature of 38–39 °C with a relative humidity of 83–87 %. They should be turned several times per day to ensure proper embryo development and to prevent development of adhesions between the embryo and its membranes. Fertile eggs may be stored for brief periods with minimal loss of viability [10]. Ideally, fertile eggs are stored at a temperature of 19 °C with a relative humidity of approximately 70 %. Alternatively, eggs may be stored at room temperature; these should be tilted at 45°, and daily alternated from side to side to minimize loss of embryo viability.

Indirect evidence of coronavirus replication in inoculated embryonated eggs may consist of embryo mortality or lesions in the embryos such as hemorrhage, edema or stunting; however, virus replication may occur in the absence of readily discernible effects on the embryo. Methods for specific detection of coronaviruses in inoculated embryonated eggs include electron microscopy, immunohistochemistry, and reverse transcriptase-polymerase chain reaction (RT-PCR) procedures [2, 4, 11, 12]. Electron microscopy is a particularly useful tool as this method depends solely on morphologic identification of the virus and does not require specific

reagents [13]. The characteristic electron microscopic morphology of coronaviruses allows their presumptive identification in embryonic fluids (e.g., allantoic fluid) or embryo intestinal contents. A variety of immunohistochemical and RT-PCR procedures have been developed for detection of coronaviruses, and these same procedures may be useful for detection of novel coronaviruses due to antigenic and genomic similarities among coronaviruses, particularly those within the same genus [2, 4, 9, 11, 12, 14–16].

3.1 Collection of Samples for Egg Inoculation

1. Swabs used to collect clinical samples such as respiratory secretions and feces are placed in 2–3 ml of DMEM supplemented with FBS and antibiotics.
2. Tissues are collected using aseptic technique and placed in clean, tightly sealed bags (Whirl-Pak bags).
3. Clinical samples should be chilled immediately after collection and transported to the laboratory with minimal delay. Samples may be shipped on ice, dry ice or with commercially available cold packs (*see* **Note 2**).

3.2 Preparation of Samples for Egg Inoculation

1. Use a vortex mixer to expel material from swabs, then remove and discard swab. Clarify by centrifugation (1,000–2,000 × *g* for 10 min) in a refrigerated centrifuge. Filter, if needed, through a 0.45 μm filter, and store at −70 °C (*see* **Note 3**).
2. Tissues and feces are prepared as 10–20 % suspensions in DMEM supplemented with FBS and antibiotics. Tissues are homogenized using a mortar and pestle, Ten Broeck homogenizer, or Stomacher^{R} (Fisher). Tissue and fecal suspensions are clarified by centrifugation (1,000–2,000 × *g* for 10 min) in a refrigerated centrifuge; this removes cellular debris and most bacteria. Filter, if needed, through a 0.45 μm filter, and store at −70 °C (*see* **Note 3**).

3.3 Allantoic Sac Inoculation

1. Chicken eggs (21 day embryonation period) are generally inoculated at 8–10 days of embryonation; eggs from other avian species may be used by making adjustments in the ages at which embryos are inoculated. Turkey and duck eggs (28 day embryonation period) generally are inoculated by this route at 11–14 days of embryonation.
2. Place eggs in an egg flat with the air-cell up. Candle eggs to ensure viability and mark the edge of the air-cell.
3. Disinfect the area marked on the shell and drill a small hole just above the mark so that the hole penetrates the air-cell, but not the portion of the egg below the air-cell.
4. A 1-ml syringe with a 25-gauge, 0.5 in. (12 mm) needle is used to inoculate eggs. The needle is inserted to the hub while holding the syringe vertically and 0.1–0.3 ml of inoculum is injected into the allantoic cavity.

5. Seal holes and return eggs to incubator.
6. Incubate eggs for 3–7 days. Evaluate embryos and allantoic fluid for presence of virus as described below.

3.4 Amniotic Sac Inoculation (Method A)

1. Fertile embryonated eggs are inoculated late in the incubation period. Chicken eggs are inoculated at 14–16 days of embryonation; turkey and duck eggs are inoculated at 18–22 days of embryonation.
2. Candle eggs to ensure embryo viability. Place eggs in an egg flat with the air-cell up.
3. Disinfect the shell at the top of the egg, over the center of the air-cell. Drill a small hole through the shell at center of air-cell using a vibrating engraver.
4. A 1-ml syringe with a 22-gauge, 1.5 in. (38 mm) needle is used to inoculate chicken, duck, and turkey embryos. The needle is inserted to the hub while holding the syringe vertically and 0.1–0.2 ml of inoculum is injected into the amniotic cavity (*see* **Note 4**).
5. Seal holes and return eggs to incubator.
6. Inoculated embryos are generally examined for presence of virus after incubation for 2–5 days. Evaluate inoculated embryos for presence of virus as described below.

3.5 Amniotic Sac Inoculation (Method B)

1. Fertile embryonated chicken eggs are inoculated, as above, at 14–16 days of embryonation; turkey and duck eggs at 18–22 days of embryonation. Candle eggs and mark the general location of the embryo (*see* **Note 5**).
2. Place eggs in an egg flat with the air-cell up. Disinfect the shell at the top of the egg, over the center of the air-cell. Drill a small hole through the shell at center of air-cell.
3. A 1-ml syringe with a 22-gauge, 1.5 in. (38 mm) needle is used to inoculate chicken, duck, and turkey embryos. Eggs are inoculated in a darkened room, as the embryo must be visualized for this method of amniotic inoculation. Hold the egg against an egg candler and insert the needle into the egg and toward the shadow of the embryo. As the tip of the needle approaches the embryo, a quick stab is used to penetrate the amniotic sac. Penetration of the amniotic sac may be verified by moving the needle sideways; the embryo should move as the needle moves (*see* **Note 4**).
4. Seal holes and return eggs to incubator. Inoculated embryos are generally examined for presence of virus after incubation for 2–5 days.

3.6 Collection of Allantoic Fluid from Eggs Inoculated by Allantoic Route

1. Candle eggs once daily after inoculation. Discard all eggs with embryos that die within the first 24 h after inoculation (*see* **Note 6**).
2. Collect allantoic fluid from all eggs with embryos that die >24 h after inoculation and from eggs with embryos that survive through the specified incubation period. Eggs with live embryos following the specified incubation period are refrigerated at 4 °C for at least 4 h, or overnight, prior to collection of allantoic fluid (*see* **Note 7**).
3. Place eggs in an egg flat with the air-cell up. Disinfect the portion of the egg shell that covers the air cell, and use sterile forceps to crack and remove egg shell over air cell.
4. Use forceps to gently dissect through the shell membrane and CAM to expose the allantoic fluid. Use forceps to depress membranes within the allantoic cavity so that allantoic fluid pools around the tip of the forceps. Use a pipette or syringe with needle to aspirate fluid. Place fluid in sterile, 12×75 mm snap-cap tubes, or other vials. Store at –70 °C (*see* **Note 8**).
5. Examine allantoic fluid for presence of coronavirus using electron microscopy, immunohistochemistry or RT-PCR (*see* **Note 9**).

3.7 Collection of Embryo Tissues from Eggs Inoculated by Amniotic Route

1. Candle eggs once daily after inoculation. Discard all eggs with embryos that die within the first 24 h after inoculation (*see* **Note 6**).
2. Examine all eggs with embryos that die >24 h after inoculation and eggs with embryos that survive through the specified incubation period (*see* **Note 10**).
3. Euthanize live embryos by placing eggs in a plastic bag or plastic bucket filled with carbon dioxide gas, or refrigerate (4 °C) overnight. Alternatively, embryos may be euthanized by cervical dislocation upon removal from eggs using the handles of a pair of scissors (*see* **Note 11**).
4. Place eggs in an egg flat with the air-cell up. Disinfect the portion of the egg shell that covers the air cell, and use sterile forceps to crack and remove the egg shell over air cell.
5. Use forceps to dissect through the shell membrane and CAM.
6. Grasp the embryo with sterile forceps and gently remove from the egg.
7. Remove selected tissues and/or intestinal contents from embryo for coronavirus detection using electron microscopy, immunohistochemistry, or RT-PCR (*see* **Note 12**).

4 Notes

1. Fertile eggs from non-SPF flocks may be used; however, presence of antibodies may interfere with isolation and propagation, and presence of egg-transmitted infectious agents may result in contamination of any viruses obtained with these eggs.
2. If dry ice is used, samples must be placed in tightly sealed containers to prevent inactivation of viruses from released carbon dioxide.
3. The supernatant fluid should be filtered if the specimen is feces or other sample that likely is contaminated with high concentrations of bacteria. Filtration of samples will reduce virus titer, and should be used only when necessary.
4. The accuracy of delivering an inoculum into the amniotic sac using this method may be determined by sham-inoculation of embryos with a dye such as crystal violet (0.2 % crystal violet in 95 % ethanol), then opening eggs and determining site of dye deposition.
5. A distinct advantage of Method A is that visualization of the embryo is not required. Method B requires visualization of the embryo, and this is may not be possible for embryonated eggs having a dark shell color (e.g., turkey eggs, brown chicken eggs). Method A also requires less skill for delivery of inoculum into the amniotic cavity, but is more prone to error than Method B with the possibility of inoculum being deposited at sites other than the amniotic cavity. If the embryo can be visualized, a potential advantage of Method B is more precise delivery of inoculum into the amniotic cavity as compared with Method A.
6. Embryo deaths that occur <24 h after inoculation generally are due to bacterial contamination, toxicity of the inoculum, or injury.
7. Refrigeration kills the embryo and causes the blood to clot. This prevents contamination of allantoic fluid with blood.
8. Multiple passages in embryonated eggs may be necessary for initial isolation of coronaviruses; allantoic fluid is used as inoculum for additional passages in embryonated eggs. Embryos at each passage should be evaluated for gross lesions. For IBV, embryo-lethal strains generally result in embryos with cutaneous hemorrhage; non-embryo-lethal strains result in stunting, curling, clubbing of down, or urate deposits in the mesonephros of the kidney. In some cases, virus replication in embryonated eggs may not be associated with readily detectable embryo lesions.
9. Allantoic fluids commonly are examined for presence of coronavirus using electron microscopy or RT-PCR procedures. Alternatively, immunohistochemical detection may be accomplished by staining sections of allantoic membrane or

the allantoic epithelial cells that are present in allantoic fluid (these should be collected by centrifugation prior to freezer storage of allantoic fluid).

10. TCoV rarely results in embryo mortality. Typically, only those eggs with live embryos are examined following the specified incubation period; however, the possibility of embryo-lethal viruses should not be overlooked.
11. The method of euthanasia employed will depend upon the method used to detect virus in inoculated embryos. Fresh tissues are required if immunohistochemistry is to be employed; for this, embryos should be euthanized by cervical dislocation or exposed briefly to carbon dioxide gas.
12. Intestinal contents commonly are examined for presence of coronaviruses using electron microscopy or RT-PCR procedures. Alternatively, immunohistochemical detection may be accomplished by staining sections of intestines or bursa of Fabricius.

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