Isolation and Propagation of Coronaviruses in Embryonated Eggs

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Abstract

The embryonated egg is a complex structure comprised of an embryo and its supporting membranes (chorioallantoic, amniotic, and yolk). The developing embryo and its membranes provide a diversity of cell types that allow for the 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). IBV replicates well in the embryonated chicken egg, regardless of inoculation route; however, the allantoic route is favored as the virus replicates 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, propagation, and characterization of other, novel coronaviruses.

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

1 Introduction

Embryonated eggs are utilized as laboratory host systems for primary isolation and propagation of a wide variety of different viruses, including the well-characterized avian coronaviruses, infectious bronchitis virus (IBV), a cause of a highly contagious disease of chickens, and turkey coronavirus (TCoV), a cause of enteritis in turkeys [1–3]. This host system also has been utilized to isolate coronaviruses from a wide variety of other avian species, including pheasants, pigeons, peafowl, and teal, and they provide a potential host system for studies aimed at identifying other, novel coronaviruses [4–6]. Embryonated eggs are routinely utilized for propagation of IBV and TCoV for research purposes and, in the case of IBV, for commercial production of vaccines.

The embryonated egg is comprised of the developing embryo and several supporting membranes which enclose cavities or “sacs”
within the egg [7]. The shell membrane lies immediately beneath the shell (Fig. 1). This membrane is a tough, fibrinous membrane that forms the air sac in the region of the blunt end of the egg. In contrast to the shell membrane, chorioallantoic, amniotic, and yolk membranes are comprised largely of epithelium, and these represent potential sites for 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 [8]. 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 [1–6]. 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 [9]. In the embryo-
nated 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 [10]. Coronaviruses obtained from pheasants,
pigeons and peafowl have been isolated and propagated in embry-
nated chicken eggs using procedures similar to those utilized for
IBV (allantoic route inoculation) [4–6]. TCoV also is epitheliotro-
pic in post-hatch chickens and turkeys, but replicates only in epi-
thelium lining the intestinal tract and bursa of Fabricius [1, 3,
11]. These same cellular tropisms of TCoV occur in the embryo-
nated 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
   1000 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.
   Other cell culture basal media (minimal essential medium,
   RPMI 640, etc.) or tryptose phosphate broth may be substi-
tuted for DMEM.

2. Sterile cotton-tipped swabs are used for collection of antemor-
tem samples (respiratory secretions, feces, etc.). Type 4 Calgis-
wabs (Puritan Medical Products) are useful for collection of
respiratory secretions from small birds.

3. Sterile Whirl-pak® bags (Fisher Scientific) are used for collec-
tion of tissues.

4. Tissue homogenizer (e.g., 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), drill (e.g., Dremel) or
   similar is used to prepare holes in egg shells. Prior to use,
disinfect the tip of the engraving tool/drill to prevent contam-
nination of the egg.
4. Plastic cement, glue, tape or nail varnish are used to seal holes in eggshells 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, 3]. These same eggs and techniques may be useful for amplification of other coronaviruses [4–6]. However, many viruses exhibit host specificity and this should be a consideration 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 [12]. 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. Eggs should be tilted at 45 °C, with daily alternation 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, 3, 13, 14]. Electron microscopy is a particularly useful tool as this method depends solely on morphologic identification of the virus and does not require specific reagents [15]. 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, 3, 11, 13, 14, 16–18].

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 (e.g. 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 (1000–2000 × 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. Prepare tissues and feces as 10–20% suspensions in DMEM supplemented with FBS and antibiotics. Homogenize tissues using a mortar and pestle, Ten Broeck homogenizer, or Stomacher® (Fisher). Clarify tissue and fecal suspensions using centrifugation (1000–2000 × 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 by the allantoic route at 8–10 days of embryonation (Fig. 2). Turkey and duck eggs (28 day embryonation period) generally are inoculated by this route at 11–14 days of embryonation. Eggs from other avian species may be used by making adjustments to the ages at which embryos are inoculated.
2. Place eggs in an egg flat with the air sac up. Candle eggs to ensure viability and mark the edge of the air sac.

3. Disinfect the area marked on the shell and drill a small hole just above the mark so that the hole penetrates the air sac, but not the portion of the egg below the air sac.

4. A 1-ml syringe with a 25-gauge, 0.5 in. (12 mm) needle is used to inoculate eggs. Insert the needle to the hub while holding the syringe vertically and inject 0.1–0.3 ml of inoculum into the allantoic cavity (Fig. 2).

5. Seal holes and return eggs to incubator.

6. Incubate eggs for 3–7 days. Evaluate embryos and allantoic fluid for the 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 (Fig. 3).
2. Candle eggs to ensure embryo viability. Place eggs in an egg flat with the air sac up.

3. Disinfect the shell at the top of the egg, over the center of the air sac. Drill a small hole through the shell at center of air sac using a vibrating engraver or drill.

4. A 1-ml syringe with a 22-gauge, 1.5 in. (38 mm) needle is used to inoculate chicken, duck and turkey embryos. Insert the needle to the hub while holding the syringe vertically and inject 0.1–0.2 ml of inoculum into the amniotic cavity (see Note 4).

5. Seal holes and return eggs to incubator.

6. Inoculated embryos generally are examined for the presence of virus after incubation for 2–5 days. Evaluate inoculated embryos for the 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. Using an egg candler, visualize and mark the general location of the embryo (see Note 5).
2. Place eggs in an egg flat with the air sac up. Disinfect the shell at the top of the egg, over the center of the air sac. Drill a small hole through the shell at center of air sac.

3. A 1-ml syringe with a 22-gauge, 1.5 in. (38 mm) needle is used to inoculate chicken, duck and turkey embryos. Inoculate eggs 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 is 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 generally are examined for the 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 sac up. Disinfect the portion of the egg shell that covers the air sac, and use sterile forceps to crack and remove egg shell over air sac.

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 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 the 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. Euthanatize 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 euthanatized 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 sac up. Disinfect the portion of the egg shell that covers the air sac, and use sterile forceps to crack and remove the egg shell over air sac.

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).

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4 Notes

1. Fertile eggs from non-SPF flocks may be used; however, the presence of antibodies may interfere with isolation and propagation, and the presence of egg-transmitted infectious agents may result in contamination of any viruses obtained with these eggs.

2. If dry ice is used, place samples 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 is only done 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 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 that inoculum is 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 to the embryo or its supporting membranes.

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, and/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 the presence of coronavirus using electron microscopy or RT-PCR procedures. Alternatively, immunohistochemical detection may be accomplished by staining either sections of CAM, or the 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 euthanatized by cervical dislocation or exposed briefly to carbon dioxide gas.

12. Intestinal contents commonly are examined for the 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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