Chapter 2

Influenza Virus Isolation

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Abstract

The isolation of influenza viruses is important for the diagnosis of respiratory diseases in lower animals and humans, for the detection of the infecting agent in surveillance programs, and is an essential element in the development and production of vaccine. Since influenza is caused by a zoonotic virus it is necessary to do surveillance in the reservoir species (aquatic waterfowls), intermediate hosts (quails, pigs), and in affected mammals including humans. Two of the hemagglutinin (HA) subtypes of influenza A viruses (H5 and H7) can evolve into highly pathogenic (HP) strains for gallinaceous poultry; some HP H5 and H7 strains cause lethal infection of humans. In waterfowls, low pathogenic avian influenza (LPAI) isolates are obtained primarily from the cloaca (or feces); in domestic poultry, the virus is more often recovered from the respiratory tract than from cloacal samples; in mammals, the virus is most often isolated from the respiratory tract, and in cases of high pathogenic avian influenza (HPAI) from the blood and internal organs of infected birds. Virus isolation procedures are performed by inoculation of clinical specimens into embryonated eggs (primarily chicken eggs) or onto a variety of primary or continuous tissue culture systems. Successful isolation of influenza virus depends on the quality of the sample and matching the appropriate culture method to the sample type.

Key words: Influenza virus, Virus isolation, Virus detection, Sample collection, Embryonated eggs, Tissue culture

1. Introduction

Influenza is caused by a zoonotic RNA virus that is perpetuated in the wild aquatic bird reservoir (1) and is occasionally transmitted via intermediate hosts (pigs, quails) to humans and other mammals; on rare occasions these viruses can evolve into highly pathogenic (HP) or pandemic strains (2, 3). To understand the evolution of influenza viruses it is necessary to do surveillance in the reservoir, intermediate, and mammalian hosts. The emergence of the pandemic H1N1 2009 influenza virus can be traced from humans to pigs and ultimately to wild aquatic birds (4). The emergence of the Asian

HP avian H5N1 influenza virus evolved along similar pathways but is different in acquiring a highly cleavable hemagglutinin (HA) molecule that is associated with high pathogenicity in gallinaceous poultry (5). The HP H5N1 influenza virus has to date not acquired the characteristics of consistent human-to-human transmission. The purpose of this chapter is to consider surveillance strategies for influenza virus from the perspective of the isolation of influenza viruses to elucidate the natural history, evolutionary strategies, pathogenesis, and the molecular basis of pathogenicity and transmissibility. The isolation of influenza viruses plays an important role in pandemic planning and vaccine preparation.

2. Materials

2.1. Collection of Specimens

Details of materials for collection of samples for influenza are given in the World Health Organization Manual on Animal Influenza Diagnosis and Surveillance (http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025rev.pdf).

1. Cryovials, 2–3 mL.

2. Phosphate buffered saline (PBS), pH 7.2: For 10 L mix 80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4 or 21.7 g $Na_2HPO_4 \cdot 7H_2O$, and 2 g KH_2PO_4: if necessary adjust to pH 7.2 with 1 N HCl.

3. Transport media for egg inoculation: PBS, pH 7.2, autoclaved, 500 mL with 500 mL sterile glycerol with penicillin G $(2 \times 10^6 \, U/L)$ (Sigma, St. Louis, MO), streptomycin (200 mg/L) (Sigma), polymixin B $(2 \times 10^6 \, U/L)$ (X-Gen Pharmaceuticals, Inc., Long Island, NY), gentamicin (250 mg/L) (Hospira, Inc., Lake Forest, IL), nystatin $(0.5 \times 10^6 \, U/L)$ (Sigma), ofloxacin HCl (60 mg/L) (Sigma), sulfamethoxazole (200 mg/L) (Sigma) (see Note 1).

4. Transport media for tissue culture: Medium 199 with 0.5% BSA with penicillin G $(2 \times 10^6 \, U/L))$ (Sigma), streptomycin (200 mg/L) (Sigma), polymixin B $(2 \times 10^6 \, U/L)$ (X-Gen Pharmaceuticals), gentamicin (250 mg/L) (Hospira, Inc.), nystatin $(0.5 \times 10^6 \, U/L)$ (Sigma), ofloxacin HCl (60 mg/L) (Sigma), sulfamethoxazole (200 mg/L) (Sigma) (see Note 1).

5. Swabs: cotton, polyester or Dacron tipped with plastic or wire shaft (see Notes 2 and 3).

6. Field Data Sheet.

7. Personal protective equipment.

8. Concentrated antibiotics: 1 mL contains 200,000 U penicillin G potassium salt (Sigma), 40 mg Streptomycin sulfate salt (Sigma), 20,000 U polymixin B for injection, USP (X-Gen

Pharmaceuticals), 4 mg gentamicin (Hospira, Inc.); store in 0.5 mL aliquots at –20°C.

9. 10% suspension of formaldehyde-fixed chicken red blood cells: one volume of fresh red blood cells is combined with nine volumes of sterile PBS and 37% formaldehyde solution is added dropwise with constant stirring to a formalin concentration of 1.5% and mixed at 4°C for 18–20 h with constant stirring. The mixture, which should be dark brown from reacting with the formaldehyde, is then washed five times with PBS to remove formalin. The fixed erythrocytes are diluted in PBS to a concentration of 10%.

2.2. Egg Inoculation

1. Egg sealant (wax, nail polish, household cement).
2. 1-cm³ syringe.
3. 27-gauge 1-1/2″ syringe needle.
4. 3-cm³ syringe with 21-gauge 1″ needle.
5. 10- to 11-day-old embryonated chicken eggs (7- to 8-day-old embryonated chicken eggs for influenza C).
6. 70% Ethanol.
7. PBS, pH 7.2: For 10 L mix 80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4 or 21.7 g $Na_2HPO_4 \cdot 7H_2O$, and 2 g KH_2PO_4: if necessary adjust to pH 7.2 with 1 N HCl.
8. Concentrated antibiotics: 1 mL contains 200,000 U penicillin G potassium salt (Sigma), 40 mg Streptomycin sulfate salt (Sigma), 20,000 U polymixin B for injection, USP (X-Gen Pharmaceuticals), 4 mg gentamicin (Hospira, Inc.); store in 0.5 mL aliquots at –20°C.

2.3. Tissue Culture Maintenance

1. Madin–Darby Canine Kidney Cells (MDCK), ATCC CCL-34.
2. Growth medium: Eagle's minimum essential medium (EMEM) (Invitrogen, Carlsbad, CA) with Earle's salts, L-glutamine and sodium bicarbonate augmented with fetal bovine serum (Thermo Scientific, Waltham, MA) added to a final concentration of 10% and Antibiotic Antimycotic Solution (100×), Stabilized (Sigma), 10 mL/L.
3. 0.5% Trypsin–EDTA (10×) (Invitrogen).
4. Trypsin Neutralizer (Invitrogen).
5. Sterile PBS.

2.4. Tissue Culture Infection

1. Madin–Darby Canine Kidney Cells (MDCK), ATCC CCL-34.
2. Infection medium: Eagle's minimum essential medium (EMEM) with Earle's salts, L-glutamine and sodium bicarbonate augmented with bovine serum albumin (Sigma) added to a final concentration of 4% and Antibiotic Antimycotic Solution (100×), Stabilized (Sigma), 10 mL/L.

3. Trypsin, TPCK-treated (Worthington Biochemical, Lakewood, NJ), diluted in ddH$_2$O to a concentration of 1 µg/mL, and sterile-filtered. Aliquot and store at –20°C.

4. PBS-ABC: 160 mL PBS-A (5×), 10 mL PBS-B (10×), 10 mL PBS-C (10×), 10 mL Antibiotic Antimycotic Solution (100×), Stabilized (Sigma), 1 mL gentamicin sulfate injection USP 80 mg/2 mL (Hospira, Inc.) in 810 mL sterile ddH$_2$O. (PBS-A (5×):100 g NaCl, 2.5 g KCl, 14.4 g Na$_2$HPO$_4$, 2.5 g KH$_2$PO$_4$, 0.2 g phenol red, bring to 2 L with ddH$_2$O and autoclave; PBS-B (10×): 13.2 g CaCl$_2$·H$_2$O, bring to 1 L with ddH$_2$O and autoclave; PBS-C (10×): 10 g MgCl$_2$·6H$_2$O, bring to 1 L with ddH$_2$O and autoclave).

3. Methods

The collection of samples for influenza virus surveillance depends on the species to be tested. Thus, influenza in the original avian reservoir species is primarily an intestinal tract infection (6); however, initial infection occurs in the respiratory tract making it necessary to collect both respiratory and fresh fecal samples. From domestic poultry the same samples can be collected as for wild aquatic birds. For influenza surveillance in mammals including humans, pigs, horses, cats, dogs, etc., respiratory tract samples are the primary source of virus but occasionally replication does occur systemically (7) and additional samples should be collected (see below).

Prospective surveillance for influenza is often done on apparently healthy birds and mammals (8). Protective equipment and clothing should be worn and biological safety and good hygiene practices followed. All samples to be collected must be identified with a tracking number, and information on species, date, sample type, field data, and health status recorded. Ideally, two samples of each specimen are recommended, but practical considerations frequently limit the sampling to one vial. The type of swab to be used depends on the animal and in principal should be large, but keeping in mind that the safety of the animal is paramount. The swab is fully saturated with the sample and immediately placed in plastic vials of transport media appropriate for the system used for isolation, i.e., for egg inoculation, glycerol saline transport medium is recommended and for tissue culture, bovine serum albumin (BSA) enriched tissue culture medium is recommended. The shaft of the swab is broken off and the vial closed and placed in ice (water based). The aim is to transport the sample to the laboratory on wet ice as rapidly as possible or in the field to move the sample vials to a liquid nitrogen dry shipper before the wet ice melts. If the samples cannot be processed immediately in the laboratory, they

are stored at –70°C until processed. On receipt in the laboratory, the samples are either stored at –70°C until processed or are thawed ready for inoculation. The samples must be thawed rapidly in a 37°C incubator (or water bath) until melted, and then held at 0°C on wet ice and injected into eggs or tissue culture.

One of the most frequently made mistakes in influenza surveillance is to microfilter (0.2 µm) the sample to remove bacteria—especially from fecal samples. It is important to rely on antibiotics to eliminate bacteria because filters frequently remove viruses.

Details of collection methods are given in the World Health Organization Manual on Animal Influenza Diagnosis and Surveillance (http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025rev.pdf).

3.1. Samples to Collect for Virus Isolation

3.1.1. Avian Species (e.g., Chickens, Ducks)

1. Cloacal swab.
2. Tracheal swab.
3. Oropharyngeal swab—small birds where tracheal sampling is not feasible.
4. Fresh fecal sample (environmental).
5. Water trough sample—caged birds (e.g., song birds, live market poultry).
6. Tissue samples—from sacrificed or freshly dead birds (blood, brain, trachea, lungs, intestinal tract, pancreas, kidney, liver, spleen).
7. Blood/serum sample—for serological diagnosis; two samples 21 days apart from the same animal are necessary.

3.1.2. Mammalian Species (e.g., Pigs, Horses)

1. Nasal swabs.
2. Throat swab—oropharyngeal.
3. Tracheal swab (usually after slaughter).
4. Drinking water.
5. Rectal swab.
6. Fecal swab (environmental).
7. Tissue samples—from slaughtered or freshly dead animals (respiratory tract and lung samples, brain, intestinal tract, pancreas, kidney, liver).
8. Blood/serum sample—for serological diagnosis; two samples 21 days apart from the same animal are necessary.

3.1.3. Humans

1. Nasal swab.
2. Nasopharyngeal swab.
3. Throat swab.
4. Gargle (oropharyngeal wash).

5. Nasal wash.

6. Transtracheal aspirate.

7. Bronchioalveolar lavage.

8. Fecal sample.

9. Tracheal and lung tissue—postmortem.

10. Internal organs—postmortem.

11. Blood/serum sample—for serological diagnosis; two samples 21 days apart from the same person are necessary.

3.1.4. Isolation of Influenza from Large Volumes of Water

Low levels of influenza virus deposited by infected waterfowl can be concentrated from water (lake water, river water, etc.) using formalin treated erythrocytes (9).

1. To 1 L of the water sample add 5 mL of a 10% suspension of formaldehyde-fixed erythrocytes in PBS.

2. Set on ice and shake thoroughly at 10 min intervals for 1 h.

3. Aliquot into 250-mL centrifuge bottles.

4. Centrifuge in an RC-5B Plus Beckman centrifuge (or similar) for 5 min at $4054 \times g$ in a 4°C prechilled rotor chamber.

5. Discard all but 5 mL of the supernatant which is retained in the bottle.

6. Place bottle on ice and resuspend the pellet with the residual supernatant.

7. Transfer the resuspended pellet to a 50-mL plastic centrifuge tube and spin for 10 min at $4054 \times g$. Discard the supernatant.

8. Resuspend the pellet in a mixture of 0.3 mL antibacterial media and 0.3 mL PBS with antibiotics.

9. Transfer the mixture into a microcentrifuge tube and incubate for 1 h at 37°C. Mix by inverting the tube three or four times during the incubation period.

10. Inoculate three 10-day-old embryonated chicken eggs by the allantoic route (see Subheading 3.2.1) with equal volumes of the mixture.

11. Incubate eggs for 72 h at 35°C.

12. Chill eggs overnight at 4°C and harvest (see Subheading 3.2.2).

General Comments on Handling and Preparing Specimens for Culture

1. For samples where highly pathogenic (HP) avian influenza viruses of H5 or H7 subtype are endemic or suspected, the laboratory manipulation and virus isolation must be done in an approved biosafety level three enhanced (BL3+) laboratory wearing protective equipment (10). For other samples BL2 facilities are appropriate.

2. After the field samples are received in the laboratory they are handled in a class II biosafety cabinet.

3. Before the inoculation into fertile eggs and tissue culture, additional antimicrobial inhibitors are added. The sample is not centrifuged or filtered but injected directly into cultures.

4. Embryonated chicken eggs are tolerant of injection with fecal samples but they can be toxic to tissue cultures.

3.2. Virus Isolation in Egg Cultures

The embryonated egg most frequently used for influenza studies is from the chicken, but duck or other avian embryos also support the replication of influenza viruses (11). Fertile eggs are incubated at 38°C in a humidified atmosphere for 10 days. The eggs are candled with a bright candling lamp for fertility and are marked on the shell at the air sac-main vein connecting point. A major difference between avian and mammalian influenza viruses is that avian influenza viruses possess receptors on their HA molecule that are specific for α 2-3 sialic acid, while mammalian viruses possess predominantly α 2-6 sialic acid receptors (12). In the chicken embryo, the cells lining the allantoic cavity bear predominantly α 2-3 sialic acid while the amniotic cavity has predominantly α 2-6 bearing sialic acid (13, 14). Thus, avian influenza virus will replicate well in the cells lining the allantoic cavity, while mammalian influenza viruses will replicate preferentially in the amniotic cavity (Fig. 1), especially on initial isolation. However, since many mammalian influenza viruses will on initial isolation replicate in the allantoic cavity, both routes of inoculation are recommended for initial virus isolation. All influenza viruses that will replicate in the amniotic cavity of the chicken embryo can be adapted to replicate in the allantoic cavity—a necessary property for vaccine production in chicken embryos. It is noteworthy that this adaptation can result in the selection of antigenic variants that can have influence on the selection of vaccine strains (15). Since inoculation of the amniotic cavity is not as simple as inoculation of the allantoic cavity, it is recommended that those using this technique practice inoculation of the amniotic cavity using Coomassie brilliant blue stain (0.5%) followed by opening the egg to determine that the virus was injected into the amniotic cavity. The detailed methods and safety considerations are given on the WHO website in the document entitled "Laboratory-based surveillance of influenza virus infections—Part B Procedures" (http://whqlibdoc.who.int/publications/1982/a86910_partB.pdf).

The following points need to be considered during influenza virus isolation:

1. The kind of red blood cells (erythrocytes) to be used in the HA test needs to be considered. For initial detection of mammalian influenza viruses especially from humans, guinea pig or human erythrocytes should be used keeping in mind that these are nonnucleated cells and take longer to settle (13). Turkey or goose erythrocytes give broader detection of all influenza viruses than chicken erythrocytes (16), and like chicken erythrocytes
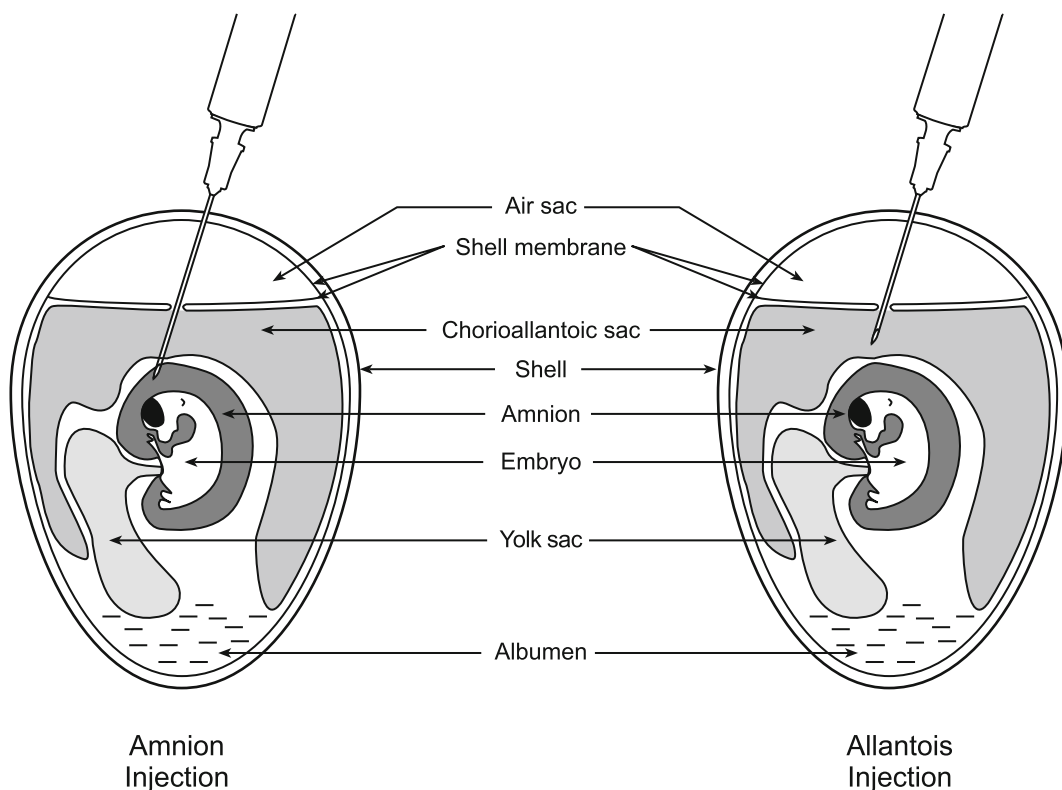
Fig. 1. Egg diagram illustrating the sites for injection of specimens into the amnion and allantois.

are nucleated and settle more rapidly than human or horse erythrocytes in hemagglutination assays. For some avian influenza viruses it is necessary to use horse erythrocytes, notably for HP H5N1 viruses from mammalian sources (17). Thus, for the initial detection of human and other mammalian influenza viruses, turkey, guinea pig, and horse erythrocytes can be used, and for avian influenza viruses, turkey and horse erythrocytes can be used.

2. Hemagglutination of erythrocytes can be caused by a number of agents including some parainfluenza viruses, bacteria, and by influenza viruses. It is important to note that some bacteria that do not kill chicken embryos can cause hemagglutination.

3. For rapid identification of the HA agent one of the rapid diagnostic kits (18) can be used. This will establish whether the HA agent is an influenza virus and will distinguish between influenza A and B. For identification of the subtype a hemagglutination inhibition (HI) test using specific immune sera is performed. If the available antisera do not identify the influenza subtype it is possible that the agent is a novel influenza virus, and sequencing of the HA and NA is recommended.

Additionally, the test results and the virus should be provided to the regional health or agricultural authority.

4. Two passages in embryonated eggs are recommended by WHO for mammalian influenza samples (particularly for humans). However, with the widespread use of molecular screening (real-time RT-PCR), experience has shown that a single passage in embryonated eggs detects almost all influenza viruses in field samples from apparently healthy avian species. If the RT-PCR assay is positive and the initial egg culture is negative for virus, then a second passage is recommended.

5. For short-term storage of virus cultures 4°C is satisfactory. Slow inactivation of infectivity occurs at 4°C permitting storage for several weeks (6). For long-term storage the virus cultures are stored at –70°C.

3.2.1. Egg Inoculation

1. All virus work should be done in a sterile biosafety cabinet that is cleaned and allowed to filter between samples to prevent cross-contamination. Add 0.5 mL of concentrated antibiotics to 50 mL sterile PBS.

2. Candle eggs to ensure viability of the embryo and to make a mark along the line of the air sac, preferably in an area free of blood vessels. Alternately, a small "X" may be used to indicate the site for injection.

3. Spray the eggs with a solution of 70% ethanol and pierce the shell in the area of the air sac just above the line (or at the "X").

4. For each egg to be injected, draw up 0.1 mL of PBS + antibiotics and 0.1 mL of sample into the syringe.

5. Hold the egg to a candling lamp to visualize the embryo (see Note 4) (Fig. 1). When inserting the needle into the amnion the embryo should move slightly. When this is observed inject 0.1 mL of inoculum, pause to allow the inoculum to finish flowing from the needle, and withdraw the syringe slightly (~1/4–1/2″) to inject 0.1 mL of inoculum into the allantois (see Note 5).

6. Seal the holes and incubate eggs at 35°C for approximately 3 days for influenza A and B, or 5 days for influenza C.

7. After incubation the eggs should be chilled at 4°C for 24 h (see Note 6).

3.2.2. Harvesting Virus from the Egg

1. Spray the eggs with 70% ethanol.

2. Use a forceps to crack the egg and remove the cap above the air sac. Sterility of the forceps can be maintained by placing them in a boiling pot between uses.

3. Use a sterile Scoopula™ to hold back the membrane of the egg and use a pipette to remove the allantoic fluid from the egg.

4. Locate the amnion by slowly inverting the egg over a beaker or another receptacle with a suitable disinfectant.

5. Use a 3-cm³ syringe with a 21 gauge 1″ needle to pierce the sac and draw as much amniotic fluid into the syringe as is possible. The fluid from each egg should be kept separate and tested individually for HA activity.

6. Any positive sample to be kept should be streaked on a blood agar plate (10% sheep blood) to check for sterility.

3.3. Isolation Procedures for Influenza Viruses in Tissue Cultures

Since many human influenza viruses cannot be isolated in embryonated chicken eggs it is necessary to utilize cell cultures for their isolation. A number of primary and continuous cell lines can be used for the isolation of influenza viruses. The most frequently used primary cultures are Cynomolgus or Rhesus monkey kidney cultures, while the most frequently used continuous cell lines are Madin–Darby Canine Kidney (MDCK) cells (19) or modified MDCK cells possessing an α 2-6 terminal sialic acid-Siat-1 gene (20). In addition to MDCK cells the continuous cell lines being used to produce influenza vaccines include Vero, PER.C6 (21), and EBx cells. Vero cells are a continuous cell line derived from African Green Monkey Kidney and have been used for over 30 years for the production of inactivated poliomyelitis vaccine, inactivated rabies vaccine, and oral poliomyelitis vaccine. PER.C6 cells were prepared from human fetal retinoblastoma and the cells were immortalized by transfection with an E1 minigenome of adenovirus (21). The EBx cell line developed from a duck embryonic stem cell line by Vivalis are licensed to GlaxoSmithKline.

A great advantage of using continuous cell lines is their flexibility. A qualified, fully validated cell line can be retrieved from the frozen state, expanded, and be available at any time. The difficulty of continuous tissue culture cell lines are that they are potentially tumorigenic or could potentially carry oncogenic agents that could theoretically transform host cells into cancer cells. Thus, for vaccine seed stocks for human vaccines, it has been necessary to obtain chicken egg isolated viruses. The WHO and the European Medical Evaluation Authority (EMEA) have set stringent requirements for the safety of continuous cell cultures for use in human vaccine preparation. Validated cell lines have now been approved in Europe for production of influenza vaccines.

Optimally, for influenza virus isolation, a universally available WHO validated cell line that replicates both α 2-3- and α 2-6-dependent influenza viruses (avian-like and human-like) to high titers without the selection of antigenic variants is what is needed. Investigations are ongoing both in WHO collaborating laboratories and in industry to achieve this goal.

Another issue with the use of cell cultures for isolation and production of influenza vaccines is the requirement for the addition

of trypsin in the medium for the cleavage activation of the HA molecule (22). The HA of most influenza viruses grown in tissue culture are not cleaved into HA1 and HA2 without the addition of exogenous trypsin and are therefore not infectious for subsequent cycles of replication. A limited number of influenza viruses including the highly pathogenic H5 and H7 strains do not require the addition of exogenous trypsin. For routine influenza virus isolation the source of the trypsin and animal sera used in influenza virus isolation is not an issue, but if the influenza viruses are to be used for human vaccine studies validated reagents must be used. Alternatively, synthetic tissue culture media can be used and crystalline trypsin produced by recombinant technology is an option.

Cell cultures inoculated with samples for influenza surveillance are usually incubated at 35°C in a humidified CO_2 incubator. While the body temperature of the avian host is ~42°C, of the pig ~39°C, and of the human ~37°C (23), a temperature of 35°C is permissive for influenza viruses from all hosts and is used for influenza isolation.

A brief outline of influenza virus isolation in cell culture is given below. Detailed information is available at the WHO website in the document entitled "Laboratory-based surveillance of influenza virus infections—Part B Procedures" (http://whqlibdoc. who.int/publications/1982/a86910_partB.pdf).

3.3.1. Tissue Culture Maintenance

Preparing Flasks from Frozen Cells

1. Transfer a vial of frozen cells to a 37°C water bath to thaw. Warm growth media at the same time.
2. Place thawed cells into a 15-mL conical centrifuge tube and bring up to volume with cold growth media, and centrifuge at 134–314×g for 8 min.
3. Aspirate media and replace with fresh cold growth media, pipetting up and down to break up clumps and dilute DMSO used for nitrogen storage.
4. Centrifuge again at 134–314×g for 8 min.
5. Resuspend pellet in 5–10 mL of warm growth media, pipetting up and down to break up clumps, and use a hemacytometer to count.
6. Seed a flask according to the number of cells stored.

Preparing Flasks from Confluent Cultures

1. MDCK cells are passaged when confluent with trypsin–EDTA to provide new maintenance cultures in 75 cm² flasks, usually twice per week. Generally a 1:16–1:20 split will yield a confluent 75 cm² flask in 3 days. A 24-well plate seeded with a 1:5 split should yield a confluent plate overnight.
2. Remove growth media and rinse cells with sterile PBS or HBSS.
3. Place 1× trypsin–EDTA onto the monolayer and place in 37°C incubator (see Note 7).

4. Check occasionally to see when cells have lifted from the floor of the flask.

5. Pipette cells into a conical tube and add sufficient Trypsin Neutralizer solution or growth media with fetal calf serum to neutralize the trypsin–EDTA.

6. Centrifuge for 5 min at 1,200 rpm.

7. Resuspend in 5 mL growth media, pipetting up and down to break up cells.

8. Subculture according to the dilutions given above for preparing flasks or plates (see Note 8).

Inoculation of Cell Culture

1. Cells should be approximately 80–90% confluent when infected with influenza virus.

2. Wash monolayer three times with PBS-ABC to ensure the removal of any serum and any nonspecific inhibitors it may contain. Leave the third wash on until just before infection.

3. Add TPCK-trypsin to the infection media at 1:1,000.

4. Dilute the virus in infection media or sterile PBS with antibiotics, usually at 1:100 or 1:1,000 depending on the virus.

5. Remove the third wash from the monolayer and add enough virus dilution to cover the cells and prevent drying (see Note 9).

6. After infection wash with PBS-ABC.

7. Add an appropriate amount of infection media based on the plasticware being used.

8. Incubate at 37°C with 5% CO_2 for influenza A (influenza B generally grows better at 35° or 33°C).

9. Check daily for CPE under low power magnification on the microscope (see Note 10). When CPE is detected (3–7 days), the medium is removed and tested for hemagglutinating agents with red blood cells (selection of erythrocytes follows the same "rules" as discussed above for egg isolation—Subheading 3.2). If using multiple wells it is advisable to check each well separately for HA.

4. Notes

1. Ofloxacin and sulfamethoxazole may be eliminated in areas of low antibiotic usage. Glycerol-based media ensures better long-term stability when cooling is not readily available but is not suitable for use with tissue culture.

2. Wooden shafts may contain toxins and formaldehydes.

3. Cotton or calcium alginate swab residues may inhibit PCR.

4. Using a candling lamp that allows the egg to be held upright may make this easier (e.g., Kuhl Corporation Jiffy Egg Candler FRZ-210Z).

5. Angling the egg slightly on the candling lamp may allow extra light to escape and aid in seeing the volume being injected.

6. In case of time constraint, the eggs may be placed in ice until thoroughly chilled.

7. Trypsin–EDTA ($10\times$) may be aliquoted, stored at $-20°C$, and thawed and diluted to $1\times$ with sterile PBS as needed.

8. Alternately, cells may be counted and dilutions made as follows: 2×10^6 cells/25 mL for 75 cm^2 flask, 1×10^6 cells/7 mL for 25 cm^2 flask, and 2×10^5 cells/mL (200 µL per well) for TC96- wellplates.

9. When using TC24-wellplates for infection the fluid may be drawn to the outside of the wells if the volume of inoculum is low (≤100 µL), so care should be taken to use sufficient inoculum or rock the plates back and forth every 10 min to prevent drying.

10. If after 8 h the cell sheet is liberated from the plastic and the cells are rounded up, the usual cause is toxicity (e.g., fecal samples). The cells can be pelleted and immediately passed to new tissue culture.

Acknowledgments

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