Isolation and Tissue Culture Adaptation of Porcine Deltacoronavirus: A Case Study

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Abstract

Porcine deltacoronavirus (PDCoV) has emerged as a novel, contagious swine enteric coronavirus that causes watery diarrhea and/or vomiting and intestinal villous atrophy in nursing piglets. PDCoV-related diarrhea first occurred in the USA in 2014 and was subsequently reported in South Korea, China, Thailand, Vietnam, and Lao People’s Democratic Republic, leading to massive economic losses and posing a threat to the swine industry worldwide. Currently, no treatments or vaccines for PDCoV are available. The critical step in the development of potential vaccines against PDCoV infection is the isolation and propagation of PDCoV in cell culture. This chapter provides a detailed protocol for isolation and propagation of PDCoV in swine testicular (ST) and LLC porcine kidney (LLC-PK) cell cultures supplemented with pancreatin and trypsin, respectively. Filtered clinical samples (swine intestinal contents or feces) applied to ST or LLC-PK cells produce cytopathic effects characterized by rounding, clumping, and detachment of cells. PDCoV replication in cells can be quantifiably monitored by qRT-PCR, immunofluorescence assays, and immune-electron microscopy. Infectious viral titers can be evaluated by using plaque assays or 50% tissue culture infectious dose (TCID50) assays. The ST or LLC-PK cells efficiently supported serial passage and propagation of PDCoV. After serial passage of PDCoV in either ST or LLC-PK cells, the virus can be purified further in ST cells by plaque assays.

Key words Porcine deltacoronavirus (PDCoV), Isolation, Propagation, TCID50, Plaque assay

1 Introduction

Porcine deltacoronavirus (PDCoV), a member of the genus Delta-coronavirus in the family Coronaviridae of the order Nidovirales, is a novel, contagious swine enteropathogenic coronavirus that causes watery diarrhea and/or vomiting, dehydration, and intestinal villous atrophy in nursing pigs [1]. PDCoV was first detected in pig feces during a molecular surveillance of coronaviruses in mammals and birds in Hong Kong in 2012 [2]. However, the first outbreak of PDCoV-related diarrhea in pigs was identified in the USA in 2014 [3]. Since then, PDCoV-related diarrhea has been reported in many countries, including China, Canada, South Korea, Lao People’s Democratic Republic, Thailand, and Vietnam [4–8].
PDCoV is enteropathogenic in young pigs [1]. Wild-type or cell culture–adapted strains of PDCoV caused severe gastrointestinal disease in gnotobiotic (Gn) and conventional, 5–19-day-old pigs [9–11]. Apart from pigs, PDCoV was also reported to have a limited ability to infect Gn calves [12]. This was evident by the detection of high titers of fecal PDCoV RNA and serum PDCoV-specific IgG antibody in inoculated calves (but with a lack of clinical signs and histological lesions). Similarly, PDCoV infected and replicated within various cell lines of human and chicken origin in vitro [13]. In in vivo settings, PDCoV could also infect and serially propagate in chicken embryos, and orally inoculated chickens showed mild diarrhea and low viral RNA titers in the feces, providing evidence of the possibility of interspecies transmission of deltacoronavirus between birds and pigs [14].

Cell culture–adapted and plaque-purified strains of PDCoV are useful for studies of PDCoV pathogenesis, and development of virological and serological assays and vaccines. Many cell culture–grown strains of PDCoV have been reported in the USA, China and Korea. The strains USA/IL/2014 [10], Michigan/8977/2014 [11], and OH-FD22 [15] were isolated in the USA, and the strain OH-FD22 has been serially passaged more than 100 times in LLC porcine kidney (LLC-PK) cells. The Chinese strains HNZK-02 [14], NH [16], CHN-HN-2014 [17, 18], CHN-GD-2016 [19], and CHN-HG-2017 [20] and Korean strain KNU16-07 [21] were all isolated in cell culture primarily utilizing methods first reported by our laboratory [15].

In our earlier studies, various cell lines of swine and monkey origin, including Vero cells, commonly used for isolation and propagation of porcine epidemic diarrhea virus, were tested for primary isolation of PDCoV using cell culture medium supplemented with different additives (small intestinal contents, trypsin, and pancreatin). However, only the LLC-PK or swine testicular (ST) cells of swine origin efficiently supported the isolation and serial propagation of PDCoV in cell cultures supplemented with exogenous trypsin or pancreatin, respectively [15]. Recently, porcine, human, and avian aminopeptidase N (APN) was identified as a major cell entry receptor for PDCoV in vitro [17, 22, 13]. Therefore, possible differences in APN expression levels among the cell lines used could influence their susceptibility to infection with PDCoV. In our studies, PDCoV replicated in LLC-PK cells without trypsin treatment, but it did not induce obvious cytopathic effects (CPE), and the virus titer was lower when compared with the trypsin supplemented cell cultures [15]. We also found that PDCoV could be serially propagated in ST cells supplemented with pancreatin or small intestinal contents from Gn piglets, but not with trypsin [15]. The addition of trypsin and pancreatin in PDCoV-inoculated LLC-PK and ST cells, respectively, might be essential for both growth of PDCoV and induction of CPE [15, 23]. Similarly, a
porcine small intestinal epithelial cell line, IPEC-J2, was also susceptible to PDCoV infection, accompanied by CPE, when the cell culture medium was supplemented with 10 μg/ml of trypsin [24]. The cell culture systems currently available for PDCoV are useful for an understanding of the mechanisms related to PDCoV infection and generation of live or inactivated vaccine strains of PDCoV.

This chapter is focused on the description of basic protocols for the isolation and propagation of PDCoV in LLC-PK or ST cells, titration of infectious virus by TCID$_{50}$ or plaque assays, and purification of PDCoV by plaque assay, based on our experience in isolating and adapting US PDCoV OH-FD22 or Chinese HNZK-02 strains in these cell lines. Details regarding quantification of viral RNA by TaqMan real-time quantitative RT-PCR (qRT-PCR), immunofluorescence assay for detection of PDCoV antigen in cells, and immune-electron microscopy for identification of viral particles in the culture medium have been published previously [15].

2 Materials

2.1 Sample Collection and Preparation

1. Clinical swine samples, such as feces or intestinal contents, are collected from diarrheic pigs positive for PDCoV RNA by RT-PCR or qRT-PCR and stored at −80°C (see Note 1).

2.2 Cell Culture, Virus Propagation, and TCID$_{50}$

1. LLC-PK cell growth medium: Minimal essential media (MEM) supplemented with 5% heat inactivated fetal bovine serum (FBS), 1% MEM nonessential amino acids (NEAA), 1% antibiotic–antimycotic, and 1% HEPES.

2. LLC-PK cell maintenance medium (LLC-PK cell MM): MEM is supplemented with 1% NEAA, 1% antibiotic–antimycotic, 1% HEPES, and 5 μg/ml of trypsin (see Note 2).

3. ST cell growth medium: Advanced MEM supplemented with 5% heat-inactivated FBS, 1% antibiotic–antimycotic, 1% HEPES, and 1% l-glutamine.

4. ST cell maintenance medium (ST cell MM): Advanced MEM supplemented with 1% antibiotic–antimycotic, 1% HEPES, and 1% (v/v) pancreatin (see Note 3).

5. Cell dissociation medium for LLC-PK cells: 0.05% trypsin with 0.02% EDTA (see Note 4).

6. Cell dissociation medium for ST cells: 0.25% trypsin with 0.02% EDTA (see Note 4).

7. Dulbecco’s modified Eagle’s medium (DMEM).

8. Phosphate buffered saline (PBS).
9. Tissue culture flasks.
10. 15 ml conical centrifuge tubes.
11. Benchtop centrifuge.
12. 6-well cell culture plates.
13. 96-well cell culture plates.
14. Cell culture incubator set at 37 °C with 5% CO₂.

### 2.3 Plaque Assay for Purification of PDCoV

1. 2% (v/v) of pancreatin (see Note 3).
2. Dulbecco’s PBS (DPBS) without Mg²⁺ and Ca²⁺.
3. 2× MEM: 2× MEM supplemented with 1% antibiotic–antimycotic, 1% HEPES, 1% NEAA, and 2% pancreatin.
4. 2% (w/v) agarose (see Note 5).
5. Microwave.
6. 0.33% neutral red.
7. 1.5 ml microcentrifuge tube.

### 3 Methods

#### 3.1 Isolation and Propagation of PDCoV in LLC-PK or ST Cells

##### 3.1.1 Passage or Preparation of LLC-PK Cells

1. Wash confluent LLC-PK cell monolayers grown in T75 cell culture flasks once with 3–5 ml of 0.05% trypsin–EDTA, and then immediately aspirate the wash fluid.

2. Add 3 ml of 0.05% trypsin–EDTA and incubate cells for 5–20 min at 37 °C, dependent on the extent of cell detachment.

3. Terminate digestion by adding 3 ml of FBS-containing growth medium and transfer cells to a sterile 15 ml conical centrifuge tube.

4. After centrifugation of the cell suspension medium at 200 × g for 5 min, remove the medium and resuspend the cell pellet in 6 ml of growth medium.

5. 1 ml of precipitated cells (approximately 1–2 × 10⁶ cells/flask) and 14 ml of growth medium are added to a new T75 cell culture flask that is then incubated at 37 °C in 5% CO₂.

##### 3.1.2 Passage or Preparation of ST Cells

1. Wash confluent ST cell monolayers grown in T75 cell culture flasks once with 3–5 ml of 0.25% trypsin–EDTA, and then immediately aspirate the wash fluid.

2. Add 3 ml of 0.25% trypsin–EDTA and incubate cells for 10–20 min at 37 °C, dependent on the extent of cell detachment.

3. Aspirate 2 ml of trypsin–EDTA when cell detachment begins.
4. Digest cells in the remainder (1 ml) of trypsin–EDTA until they are completely detached.

5. Terminate digestion by adding 5 ml of FBS-containing growth medium and transfer to a sterile 15 ml conical centrifuge tube.

6. After centrifugation of the cell suspension medium at 200 × g for 5 min, remove the medium and resuspend the cell pellet in 6 ml of growth medium.

7. 1 ml of precipitated cells (approximately 1–2 × 10^6 cells/flask) and 14 ml of growth medium are added to a new T75 cell culture flask that is then incubated at 37 °C in 5% CO_2.

3.1.3 Isolation of PDCoV in LLC-PK or ST Cells

1. Prepare 1–2-day-old, 80–90% confluent cell monolayers in 6-well cell culture plates for inoculation with filtered clinical samples.

2. Wash cells twice with LLC-PK or ST cell MM (without 5 μg/ml of trypsin or 1% pancreatin) or DPBS (see Note 6).

3. To prepare inoculum for viral isolation, tenfold serially dilute PDCoV RNA-positive intestinal contents or feces in PBS or DMEM. Vortex for 2 min, and then centrifuged at 1847 × g at 4 °C for 10 min.

4. Filter supernatants through 0.22 μm filters. These filtered supernatants are used as the inoculum for viral isolation in cell culture.

5. Add 300 μl of filtered samples to each well and incubate for 1 h at 37 °C in 5% CO_2 (see Note 7).

6. Remove the inoculum, and wash cells three times with MM or DPBS (see Note 6).

7. Add 2 ml of MM supplemented with 5 μg/ml of trypsin (LLC-PK cells) or 1% pancreatin (ST cells) to each well.

8. Incubate cells at 37 °C in 5% CO_2 until CPE, such as rounding, clumping, or detachment of cells (Fig. 1), is observed usually by 5 days postinoculation (see Note 8).

9. Freeze the cell plates at −80 °C once CPE is observed in 80% of the cell monolayer.

10. Thaw and freeze the cells twice at −80 °C and harvest the cell supernatants for qRT-PCR or titration of infectious virus.

3.1.4 Serial Passage of PDCoV in LLC-PK or ST Cells

1. Prepare 1–2 day-old, 80–90% confluent cell monolayers in T25 cell culture flasks for inoculation with the cell supernatants.

2. Remove the old growth medium and wash cells twice with LLC-PK or ST cell MM (without 5 μg/ml of trypsin or 1% pancreatin) (see Note 9).

3. If the titer of infectious virus in the inoculum is known, a desired multiplicity of infection (MOI) of the virus
should be inoculated. If not, a sufficient volume (0.5–0.6 ml) of inoculum is added to a T25 cell culture flask (see Note 10).

4. Incubate cells at 37 °C for 1 h.
5. Remove the inoculum, and wash cells three times with MM or DPBS.
6. Add 5 ml of MM supplemented with 5 μg/ml of trypsin (LLC-PK cells) or 1% pancreatin (ST cells) per T25 cell culture flask and incubate at 37 °C in 5% CO₂ until CPE, such as rounding, clumping, or detachment of cells, is observed.
7. Freeze the cell flasks at −80 °C once CPE is observed in more than 80% of the cell monolayer.
8. Thaw and freeze the cells once at −80 °C and harvest the cell supernatants for qRT-PCR or titration of infectious virus by TCID₅₀ or plaque assays.

3.2 Titration of Infectious PDCoV by TCID₅₀ Assay

1. Add 200 μl of LLC-PK cells (approximately 5 × 10⁴ cells/well) to each well in 96-well cell culture plates and incubate cells at 37 °C in 5% CO₂ until reaching 80–90% confluence.
2. Wash 80–90% confluent cell monolayers once with LLC-PK cell MM either with or without 5 μg/ml of trypsin.
3. Serially dilute samples or virus (tenfold) with LLC-PK cell MM either with or without 5 μg/ml of trypsin.
4. Inoculate 100 μl of diluted samples or virus per well in eight replicates per dilution (see Note 11).
5. Incubate cells at 37 °C in 5% CO₂ for 1 h.
6. Add 100 μl of the MM supplemented with 5 μg/ml of trypsin (MMT) (see Note 12).
7. Monitor CPE for 5–7 days postinoculation. If CPE is observed, virus titers are calculated by using the Reed–Muench method [25] and expressed as TCID$_{50}$ per ml (see Note 13).

3.3 Titration and Purification of PDCoV in ST Cells by Plaque Assay (See Note 14)

1. Prepare 100% confluent ST cell monolayers in 6-well cell culture plates (see Note 15).

2. Discard and replace the old growth medium with ST cell MM without 1% pancreatin. Then, incubate cells for 1 h at 37 °C in 5% CO$_2$ (see Note 16).

3. Prepare viral inoculum by tenfold serial dilution in ST cell MM without 1% pancreatin.

4. Wash cells twice with ST cell MM or DPBS.

5. Add 300 μl of tenfold serially diluted viral inoculum in duplicate to each well and incubate for 1 h at 37 °C in 5% CO$_2$.

6. Prepare agarose. Warm 2× MEM in a water bath at 37 °C. Approximately 7 ml is needed per plate.

7. Heat the agarose gel in a microwave oven until fully melted, and then keep it in a water bath at 42 °C. Approximately 7 ml is needed per plate.

8. Mix equal volumes of 2× MEM (step 6) and agarose (step 7) (see Note 17).

9. Remove virus inoculum and wash cells twice with DPBS.

10. Add 2 ml of the agarose-MEM mixture to each well (see Note 18).

11. Cool the plates for 10 min to solidify the agarose.

12. Incubate the plates upside down at 37 °C in 5% CO$_2$ for 2–3 days postinoculation (see Note 19).

13. Prepare the working solution of neutral red (0.01%). Dilute 1 ml of the stock neutral red (0.33%) in 25 ml of DPBS.

14. Add 2 ml of 0.01% neutral red solution to each agarose-coated well.

15. Incubate the plates at room temperature for 2–4 h, and then remove the neutral red solution. Plaques are visualized as clean spots in the red background (Fig. 2).

16. Count plaques under oblique light. Plaques should be confirmed by light microscopy. The plaque titers are expressed as plaque forming units (PFU) per ml. The titer of PFU/ml is calculated by using the following formula: $Y$ (viral titer, PFU/ml) = $X$ (mean numbers of plaques from the duplicate wells)/300 × 1000 × viral dilution factor.

17. Pick a uniform and clear plaque by using a sterile pipette tip, and then place the agarose plug into a 1.5 ml microcentrifuge tube containing 0.5 ml of ST cell MM (see Note 20).
18. Inoculate the selected plaque clones (0.5 ml) onto ST cell monolayers prepared in a 6-well cell culture plate.

19. Incubate the plate for 1 h at 37 °C in 5% CO₂.

20. Add 1.5 ml of ST cell MM supplemented with 1% pancreatin and incubate the plate at 37 °C in 5% CO₂ for 4–5 days until CPE is observed.

21. Harvest CPE-positive clones and store them at −80 °C (see Note 21).

### 4 Notes

1. Detailed procedures, including isolation of viral RNA from clinical samples and qRT-PCR for the detection of the membrane (M) gene of PDCoV were published previously [15, 26]. The qRT-PCR was conducted by using Qiagen One-step RT-PCR kit (Qiagen Inc., Valencia, CA, USA) and a real-time thermocycler (RealPlex; Eppendorf, Germany) [15]. The forward and reverse oligonucleotide primers and probe used to detect the M gene of PDCoV are as follows: PDCoV MF: 5’-ATCGACCACATGGCTCCAA-3’, PDCoV MR: 5’-CAGCTCTTGCCCATGTAGCTT-3’, and PDCoV M-Probe: FAM-CACACCAGTGTTAAGCAGCATGGCAAGCT-IABkFQ (5 μM).

2. Trypsin (2.5%) without phenol red and EDTA is used. The presence of EDTA could enhance cell detachment, hindering infection and replication of PDCoV and observation of CPE.
The stock of trypsin (2.5%) is diluted (1:5000) in MEM (e.g., 10 μl of 2.5% trypsin is added to 50 ml of MEM supplemented with 1% NEAA, 1% antibiotic–antimycotic, and 1% HEPES), and the working concentration of trypsin in LLC-PK MM is 5 μg/ml.

3. A 10× pancreatin stock is made by dissolving 2.50 g of pancreatin and 0.85 g of NaCl in 100 ml of MilliQ water followed by sterilizing through a 0.22 μm filter. The 10× pancreatin solution is further diluted (1:10) in sterile PBS or MEM (1× pancreatin). Aliquot into 1 ml vials, and store at −20 °C until use. Store at 4 °C after thawing. Finally, 1 ml of the 1× pancreatin solution is added to 99 ml of the maintenance medium.

4. Aliquot into sterile 15 ml conical centrifuge tubes, and store at −20 °C until use. Prior to use, thaw and warm at room temperature.

5. 2 g agarose is added to 100 ml of distilled water, autoclaved, and stored at room temperature. Prepare the agarose overlay by melting the agarose gel in a microwave oven. Keep it in liquid form by incubation at 42 °C.

6. Trypsin-containing MM solutions can also be used for washing the LLC-PK cells.

7. Frequently, the filtered clinical samples can be toxic to cells, in which case the early detachment of cells is observed at 4–6 h postinoculation. To reduce their cell cytotoxicity, the samples can be diluted by 1:10, 1:100, or 1:1000 (v/v) in MM, or MM with trypsin (LLC-PK cells).

8. If CPE is not observed by 5 days postinoculation, the cell plates are frozen at −80 °C and thawed three times, and the cell culture medium is harvested and further inoculated onto fresh LLC-PK or ST cells according to the steps described. After three continuous passages of the samples, if there is no CPE and cell culture supernatants are negative for increased PDCoV RNA during the serial passages, the samples are considered as negative for growth in cell culture.

9. Cells should not be allowed to dry out before virus inoculation.

10. The MOI can also be estimated by the extent of CPE observed and is associated with the extent of adaptation of PDCoV to the LLC-PK or ST cells. After viral inoculation, flasks are also gently shaken by hand every 15 min.

11. Each plate should include one row of negative control (MM alone).

12. If the samples are already diluted with the MM supplemented with 5 μg/ml of trypsin, step 6 is not necessary. The final concentration of trypsin in the cell culture medium in each well should be 5 μg/ml.
13. The TCID$_{50}$ assay using ST cells is also similar to the TCID$_{50}$ assay using LLC-PK cells, except for use of ST MM supplemented with 1% pancreatin, instead of LLC-PK MM. The CPE observed can be further confirmed as PDCoV-specific by immunofluorescence staining with PDCoV-specific antibodies.

14. ST cells can be used to titrate PDCoV grown in either ST or LLC-PK cells by plaque assay.

15. For virus inoculation, 100% confluence cell monolayers are further allowed to attach to the plate for 24–36 h.

16. We believe that this step where cells are incubated without FBS will contribute to an enhanced adsorption of PDCoV to cells.

17. Prepare agarose only prior to use. Dissolve agarose completely. Be sure that the temperature of the agarose used for overlay is around 37°C.

18. Dispense the agarose mixture thoroughly throughout the well.

19. Duration of the incubation is dependent on the extent of plaque formation.

20. The plaques can be stored in the maintenance medium at −80°C until use or promptly mixed using a vortex mixer for 1 min and then used to inoculate cells prepared in 6-well cell culture plates.

21. Viral titers of the plaque clones can be determined by qRT-PCR, TCID$_{50}$, or plaque assays. Based on the titers, some clones can be selected for further passage.

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