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Short communication

Highly permissive subclone of the porcine kidney cell line for porcine circovirus type 2 production

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A B S T R A C T

This study established a highly permissive and decontaminated cell line for growing porcine circovirus type 2 (PCV2). A porcine kidney-15 cell line (PK-15) contaminated with porcine circovirus type 1 (PCV1) was decontaminated by neutralizing with rabbit anti-PCV1 hyperimmune serum. Subsequently, by limiting dilution and cell subcloning, four PCV1-free monoclonal cells were grown to monolayers. Each cell clone and PK-15 cell were infected with PCV2. The PKKC cell clone yielded up to $10^{6.8}$ $TCID_{50}$/ml at 6 days post-infection. In addition, PKKC was free of extraneous viral contamination and exhibited a cytopathic effect (CPE) to PCV2 at 6 days post-infection. The advantages of the PKKC cell are that it can grow a high PCV2 titer and exhibit CPE; therefore, it can be used for PCV2 cultivation, vaccine production, and diagnostic purposes.

The *Circoviridae* family is divided into two genera: (i) the genus *Gyrovirus*, consisting of the chicken anemia virus; and (ii) the genus *Circovirus*, consisting of the transfusion transmitted virus, psittacine beak and feather disease virus, circoviruses of pigeon, duck, and goose, as well as porcine circovirus type 1 (PCV1) and porcine circovirus type 2 (PCV2) (Meehan et al., 1998). Currently, the cultivation of PCV2 in heterogeneous porcine kidney cells (PK-15) yields viral titers of approximately 10^4–10^5 $TCID_{50}$/ml, which is unsuitable for vaccine production. Previous studies have reported a method to improve the virus susceptibility of cell lines, such as MARC-145, which was cloned from the MA-104 cell to grow higher titers of the porcine reproductive and respiratory syndrome virus (Kim et al., 1993). Zhu et al. (2007) reported that PK15-C1, a highly permissive daughter cell of PK-15, can grow a titer of approximately 10^4–10^5 $TCID_{50}$/ml of PCV2 at primary cultivation and subsequently produce a titer of 10^8 $TCID_{50}$/ml after 5 passages of the virus. The purpose of this study is to establish a highly permissive and decontaminated cell line for growing PCV2.

PK-15 cells contaminated with PCV1, which were acquired from the Bioresource Collection and Research Center in Taiwan (passage 137), originally from American Tissue Culture Collection (passage 132) were used in this study. The PK-15 cells were maintained and subcultured in a minimal essential medium (MEM) (Gibco) supplemented with 5% heat-inactivated fetal bovine serum (FBS; PAA, Austria) and penicillin/streptomycin at 37 °C in a 5% CO_2 incubator. The PK-15 cells were grown in large quantities to obtain purified PCV1, and a total of 1 L of cell culture supernatant was harvested. The supernatant was treated with polyethylene glycol (PEG) 6000 to precipitate and concentrate PCV1. The PCV1 was purified further by ultracentrifugation at 180,000 × *g* for 3 h at a 20–45% sucrose gradient in an SW 41 Ti rotor. The purified PCV1, *E. coli*-expressed PCV1 ORF2 recombinant proteins without the first 16 N-terminal amino acids (AA) and Freund's complete adjuvant were mixed to inoculate the rabbits subcutaneously. A booster immunization of PCV1 mixed antigens with Freund's incomplete adjuvant was administered after 3 weeks. A second booster was administered after 2 weeks. The rabbit serum was collected and the anti-PCV1 titer was measured using an immunofluorescence assay (IFA) 6 weeks after primary immunization.

To eliminate PCV1 in the PK-15 cells, 10^4 PK-15 cells were grown in a 6-well dish with MEM containing 5% FBS and 5% rabbit anti-PCV1 hyperimmune serum to neutralize PCV1. After 6 days of incubation, the PK-15 cells were trypsinized and 10^4 cells were seeded into a 6-well plate for a second treatment with anti-PCV1 hyperimmune serum. After the third treatment, the cells were used for limiting dilution and cell cloning to obtain PCV1-free PK-15 cells.

Individual daughter cells were selected and grown in 10% FBS MEM. Four selected daughter cells (C10, 10G, H10 and PKKC) were grown to monolayers in a 25-cm^2 flask.

A PCV2 strain (H strain, genotype PCV2d) (GenBank ID: JQ390467) isolated from the lung tissue of a pig naturally infected by PCV2 in Hsin-Chu County, Taiwan was used. Each monolayer

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of the C10, 10G, H10, PKKC, and PK-15 cells was infected with PCV2 at a multiplicity of infection (MOI) of 0.5. The monolayers were stained at Day 3 post-infection using IFA to evaluate the percentage of the infected cells. The percentage of IFA-positive cells was counted in a square area under a fluorescent microscope. Each count contained at least 300 cells with three repeats at various locations to compare the susceptibility of cells to PCV2.

The PK-15 and the most susceptible subclone cell, PKKC, were infected with PCV2 at 0.1 and 0.5 MOIs, respectively. The PCV2 titers in supernatant, intracellular phase, and in supernatant plus intracellular phase were measured daily from Day 0 to Day 7 post-infection.

The IFA was performed as described previously (Chen et al., 2011) with a minor modification. The PCV2 infected cells were fixed with 80% acetone at 4 °C and washed and incubated with a swine polyclonal antibody against PCV2 (VMRD, Inc.) (1:1500 in PBS) as the primary antibody for 1 h at 37 °C. The cells were subsequently incubated with goat anti-swine IgG conjugated with FITC (KPL) (1:1000 in PBS) for 1 h at 37 °C.

For the karyotyping analysis, the PK-15 and PKKC cells were cultured with 5% FBS MEM and treated with 1 mg/ml colchicine for 12 h. The cells were subsequently treated with hypotonic saline and fixed with glacial acetic acid/methanol (1:3). The treated cells were dropped on the slides then dried and stained with Giemsa stain. The karyotypes of chromosomes were analyzed in 100 cells of each (1000× magnification). Furthermore, contamination of PKKC with other porcine viruses were also tested by using PCR and RT-PCR according to primer sequences described in previous publications (Fouchier et al., 2000; Giammarioli et al., 2008; Nunez et al., 1998; Ogawa et al., 2009; Weinstock et al., 2001). A forward primer 5′-ATGACGTGGCCAAGG-3′ and a reverse primer 5′-TTTATTTAGAGGGTCTTT-3′ designed in this study was used for PCV1 detection by PCR.

PCV1 was detected in the supernatant of the PK-15 cells before treatment. The amplified PCR product of PCV1 was 699 bp (Fig. 1). However, PCV1 was not detected in the PK-15 cells after the second treatment with rabbit anti-PCV1 hyperimmune serum (IFA titer >10,000×; data not shown).

The various morphologies of each clone (PK-15, C10, 10G, H10, and PKKC) were observed at Day 1 after seeding (Fig. 2). The susceptibility of each cell to PCV2 detected using IFA is shown in Fig. 3. The results showed that the 10G, H10, and PKKC clones had higher permissivity than the PK-15, whereas the C10 clone had the lowest permissivity. The percentage of IFA-positive cells in PK-15 was 4.52%, whereas in C10, 10G, H10, and PKKC were 3.29%, 10.39%, 9.25%, and 24.07%, respectively. Among the tested cells, PKKC exhibited the highest susceptibility to PCV2.

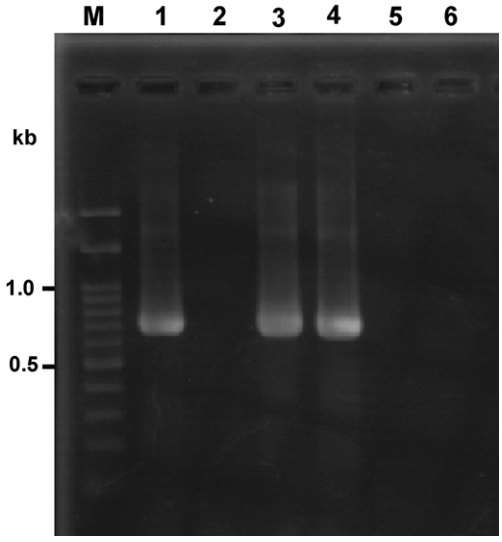

Fig. 1. Agarose gel analysis of PCV1 contamination in PK-15 cells. Total DNA were extracted from the cells and culture medium as the template for PCR. Lane 1: PCV1 DNA positive control (699 bp). Lane 2: negative control. Lane 3: before rabbit anti-PCV1 hyperimmune serum treatment. Lane 4: after first treatment. Lane 5: after second treatment. Lane 6: after third treatment.

The PCV2 growth titers in PK-15 and PKKC cells at MOIs of 0.1 and 0.5, respectively, are shown in Table 1. At a 0.1 MOI, the PKKC cells produced a PCV2 titer of $10^{5.83}$ $TCID_{50}$/ml in the intracellular phase, $10^{6.31}$ $TCID_{50}$/ml in the supernatant, and $10^{6.65}$ $TCID_{50}$/ml in the supernatant plus intracellular phase at Day 6 post-infection. At a 0.5 MOI, the virus titer was $10^{5.22}$ in the intracellular phase, $10^{6.67}$ in the supernatant, and $10^{6.83}$ in the supernatant plus intracellular phase at Day 6 post-infection. By contrast, at a 0.5 MOI, PK-15 produced a PCV2 titer of $10^{4.65}$ $TCID_{50}$/ml in the intracellular phase, $10^{4.37}$ $TCID_{50}$/ml in the supernatant, and

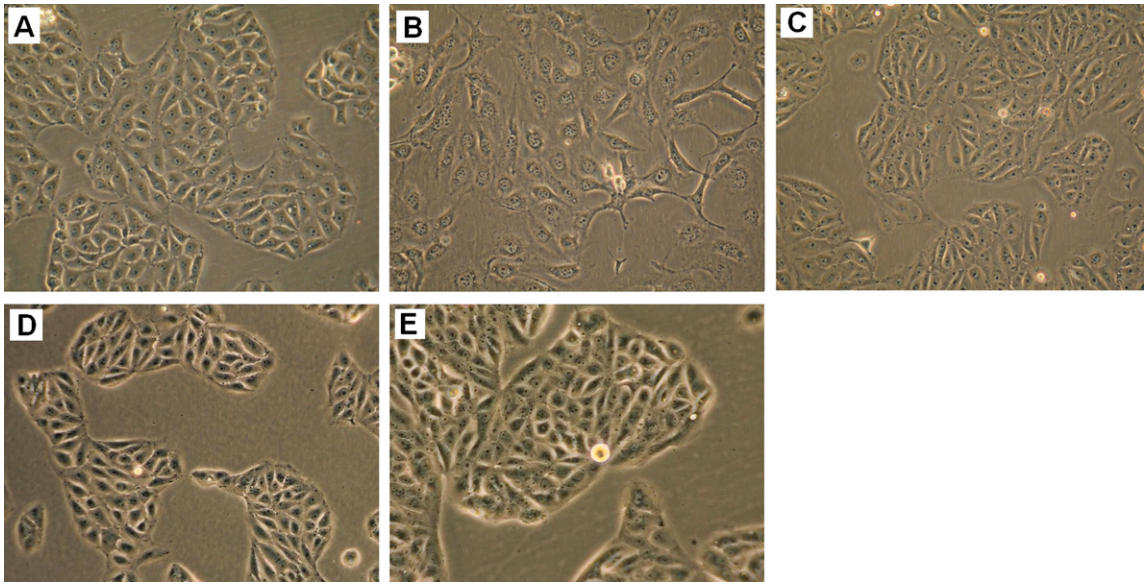

Fig. 2. Morphology of PCV1-free PK-15 and its subcloned cells at Day 1 after seeding. (A) PK-15 (B) C10, (C) 10G (D) H10 and (E) PKKC were observed under light microscopy (400× magnification).