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Optimal transfection methods and comparison of PK-15 and Dulac cells for rescue of chimeric porcine circovirus type 1-2

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A B S T R A C T

A chimeric porcine circovirus type 1-2 (PCV1-2) infectious DNA clone has low transfection efficiency and exhibits low levels of proliferation. Electroporation and lipofection parameters were optimized for PK-15 and Dulac cells with the purpose of increasing the efficiency for rescuing infectious PCV1-2. Titers of PCV1-2 in Dulac cells were 100-fold higher than those in PK-15 cells following transfection. The electroporation efficiency into Dulac cells was high when three 400 μs pulses at 250 V with 6 μg of plasmid DNA was used, lipofection efficiency was high when the ratio of DNA to transfection reagent was 1:3. The proportion of infected cells was 55.6% compared with 44.2%, for the electroporation and lipofection techniques respectively. Virus titers were higher in Dulac cells, from $10^{4.44}$ to $10^{5.32}$ $TCID_{50}$/mL compared with $10^{1.90}$–$10^{3.38}$ $TCID_{50}$/mL for PK-15 cells. Dulac cells were more permissive to PCV1-2 than PK-15 cells regardless of the transfection technique.

1. Introduction

Porcine circovirus type 2 (PCV2) was first identified in western Canada in 1991, and has since been recognized as the etiological agent of post-weaning multisystemic wasting syndrome (PMWS) (Allan and Ellis, 2000; Chae, 2005; Finsterbusch and Mankertz, 2009). Along with PMWS, PCV2 is associated with a number of diseases and other syndromes in pigs that are collectively referred to as porcine circovirus-associated diseases (PCVADs). These PCVADs have had a major impact on the global swine industry and are recognized as economically important diseases (Baekbo et al., 2012; Ge et al., 2012).

Before PCV2 vaccines were introduced to the world market, the goal was to control PCVADs by improving management strategies and controlling co-infections (Chae, 2012). Five commercially available PCV2 vaccines have been used in different parts of the world, three of these are subunit vaccines, one is an inactivated PCV2 vaccine, and the other one is an inactivated chimeric virus in which the ORF2 capsid gene of PCV2a was cloned into the genomic backbone of the non-pathogenic PCV1 (Fenaux et al., 2004; Segales et al., 2009). In a previous study, the chimeric PCV1-2b was constructed and effective against wild-type PCV2 challenge in conventional pigs (Liu et al., 2011). For vaccine production, PK-15 cell lines are widely used, and lipofection is now frequently employed for the rescue of PCV1-2b from PK-15 cells (Fenaux et al., 2003, 2004; Beach et al., 2011). However, lipofection results in low transfection efficiencies and higher cytotoxicity compared with electroporation, virus titers are low, approximately $10^{4.5}$ $TCID_{50}$/mL, which is too low to produce vaccine (Fenaux et al., 2003; Zhu et al., 2007; Beach et al., 2010; Krakowka et al., 2012). Therefore, it is necessary to increase the titer of PCV1-2 in cell lines for vaccine production. Dulac cells, a subtype of PK-15 cells (Wellenberg et al., 2000; Zhou et al., 2006), are highly permissive to PCV1-2 infection and exhibit few cytopathic effects following transfection. In the current study, the transfection efficiency of electroporation and lipofection were compared in PK-15 and Dulac cells.

2. Materials and methods

2.1. Cells lines, medium, plasmid

Dulac and PK-15 cells for PCV1-2 proliferation were purchased from the China Institute of Veterinary Drug Control. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, CA,

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USA) supplemented with 10% fetal bovine serum (FBS; HyClone, UT, USA). The recombinant pBSK(+)-dPCV1-2b plasmid was generated previously by cloning two tandem copies of the complete PCV1-2 genomes into pBluescript vector (Liu et al., 2011).

2.2. Optimization of electroporation conditions

Subconfluent Dulac cells in T-25 flasks were trypsinized and washed with phosphate-buffered saline (PBS; 0.01 M, pH 7.2) twice, resuspended in 400 μL of transfection buffer (5–10 × 10^5 cells), and then transferred to a sterile electroporation microcuvette containing plasmid DNA. Four electroporation parameters (pulse voltage, pulse length, number of pulses, and DNA concentration) were optimized. Following electroporation, fresh DMEM with 10% FBS was added and cells were transferred to six-well plates and incubated at 37 °C/5% CO_2. Mock-transfected cells and cells transfected with pBSK(+) were included as controls. Cell cultures were harvested after 48 h using three freeze-thaw cycles. Indirect immunofluorescence assays (IFAs) were conducted to determine the titer of PCV1-2.

2.3. Optimization of lipofection conditions

Cells were seeded in six-well plates 24 h prior to transfection and grown to 90–95% confluency, then transfected with PCV1-2 DNA using Lipofectamine® 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's recommended protocols. Briefly, DMEM (500 μL) was dispensed into six sterile polystyrene tubes, followed by the addition of 2, 4, 8, 12, 16, or 20 μL of transfection reagents. Solutions were mixed gently and incubated at room temperature for 5 min, then 500 μL of DMEM mixed with 4 μg of pBSK(+)-dPCV1-2b was added to each tube and incubated for 20 min at room temperature. These mixtures were added to the appropriate culture wells and incubated for 5 h at 37 °C/5% CO_2. The medium containing transfection mixtures was removed from each well and replaced with 2 mL of DMEM containing 10% FBS. Untransfected cells and cells transfected with pBSK(+) were used as controls. All transfected cells were incubated for 48 h, harvested, and then subjected to IFAs to determine virus titers.

2.4. Serial passaging of PCV1-2 in Dulac and PK-15 cells

To compare transfection efficiencies and the viability of subsequent progeny viruses, synchronized Dulac and PK-15 cells were subjected to electroporation and lipofection using the optimal conditions which had determined. Transfected cell cultures were grown to confluency and then serially passaged for 10 generations. At each passage, transfected cells were harvested and virus titers determined by IFA.

2.5. Morphological observations of rescued viruses

Dulac cells infected with PCV1-2 were harvested, subjected to three freeze-thaw cycles, and centrifuged (14,000 × *g*, 30 min). Virus cultures were mixed with a polyclonal porcine antiserum against PCV2 (1:1000 dilution; VMRD, Washington, USA), incubated overnight at 4 °C, and then centrifuged (14,000 × *g*, 30 min). Immune complexes coupled with viral particles were harvested and the morphology of viruses was observed using an electron microscope (Philips, Tecnai 12, Netherlands) (Guo et al., 2011).

2.6. IFA and flow cytometry analysis

Transfected cell cultures were grown to confluency, and after 48 h cells were harvested and then subjected to three freeze-thaw cycles. Virus was collected, serially diluted 10-fold (10^{-1}–10^{-9}, and used to inoculate PK-15 or Dulac cells growing in 96-well microtiter plates). After incubation at 37 °C for 72 h, IFAs were conducted as previously reported (Liu et al., 2011). Infected cells were fixed with acetone for 15 min at 4 °C. Fixed cells were washed with PBS three times, and then incubated with the PCV2 antiserum at 37 °C for 1 h. After washing three times with PBS, cells were incubated with a fluorescein isothiocyanate (FITC)-labeled rabbit anti-pig IgG (Southern Biotech, Birmingham, USA) at 37 °C for 45 min. Virus titers were determined using a fluorescence microscope (Olympus, IX51, Japan).

Dulac cells were transfected and incubated at 37 °C for 72 h, harvested by trypsinization, and processed for analysis using a fluorescence-activated cell sorter (FACS) to determine the number of PCV2-infected cells. Dulac cells were fixed with methanol and incubated with PCV2 antiserum, then incubated with the FITC-labeled rabbit anti-pig IgG. Stained cells were analyzed with a FACSAria flow cytometer (BD Biosciences, USA) (Dvorak et al., 2013).

2.7. Quantitative polymerase chain reaction (qPCR) assays

Specific oligonucleotide primers (5′-GCTGAACTTTT-GAAAGTGAGCGGG-3′ and 5′-TCACACAGTCTCAGTAGATCATCCCA-3′) targeting a segment of PCV1-2 were used to amplify a 220 bp fragment as previously described (Fenaux et al., 2004; Liu et al., 2011). Total DNA extracted from freeze-thawed cells and suspended in 300 μL of sterile water (Liu et al., 2011). The qPCR assays were performed on a Roche Real-Time PCR Detection System (Roche, LightCycler® Nano, Switzerland) using a Roche SYBR Green I Real-Time PCR Kit (Roche, Basel, Switzerland). Samples were amplified at least three times.

2.8. Statistical analysis

Statistical analyses for assays were conducted using Graphpad Prism v5.0 (Graphpad Software, La Jolla, CA). Values are expressed as the mean with standard deviation with nonparametric *t*-tests used to analyze these data. A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Comparative transfection efficiencies

To select appropriate cell lines for the rescue of PCV1-2, transfection efficiencies between Dulac and PK-15 cell lines were compared. Electroporation was conducted using two pulses at 200 V for 400 μs with 4 μg of DNA. Virus titers were significantly higher in Dulac cells ($10^{4.06}$ $TCID_{50}$/mL) compared with those in PK-15 cells ($10^{2.67}$ $TCID_{50}$/mL; $P < 0.0001$) (Fig. 1A). Lipofection was performed in six-well plates using 4 μg of DNA and 10 μL of transfection reagent, the virus titer was $10^{3.59}$ $TCID_{50}$/mL in Dulac cells and $10^{1.97}$ $TCID_{50}$/mL in PK-15 cells ($P = 0.0004$) (Fig. 1B).

3.2. Influence of electroporation conditions

The optimal electroporation conditions for Dulac cells were determined, when the voltage was increased from 150 V to 250 V, virus titers were significantly increased ($P < 0.0001$) (Fig. 2A). When the voltage exceeded 250 V, transfection efficiency decreased. Under the optimal voltage, the pulse length was adjusted from 100 μs to 400 μs. Pulse lengths had a significant effect on the transfection of pBSK(+)-dPCV1-2 into cells ($P = 0.0019$), however,