Journal of Virological Methods 273 (2019) 113706

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Porcine circovirus type 2 induces a strong cytopathic effect in the serum-free culture cell line CPK-NS

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ARTICLE INFO

Keywords:
Porcine circovirus type 2
Cytopathic effect
CPK-NS cells

ABSTRACT

When the adherent stable serum-free porcine kidney cell line CPK-NS were inoculated with porcine circovirus type 2 (PCV2) and passaged, viral titre concentration-dependent cell detachment was observed. The copy number of viral genes in supernatants of the infected CPK-NS cells decreased as cell detachment progressed. Furthermore, cell detachment was completely inhibited via neutralisation of the virus using antisera collected from PCV2-infected specific pathogen-free pigs. These results indicated that detachment of CPK-NS cells is a cytopathic effect (CPE) caused via infection with PCV2. Only a single round of cell passaging was required to observe clear a CPE when the inoculated viral titre was significantly high [$\geq 10^{4.5}$ median tissue culture infectious dose ($TCID_{50}$)/mL]. Our study confirms that PCV2, which is normally non-cytopathogenic, is capable of inducing a distinct CPE in CPK-NS cells. Application of CPK-NS cells for detection of viruses may contribute towards the diagnosis and control of PCV2-mediated infectious diseases.

Porcine circoviruses (PCVs) (genus *Circovirus*; family *Circoviridae*) are compact, spherical, nonenveloped viruses; they have approximately 17-nm diameter and a 1.76-kb single-stranded circular genomic DNA (Tischer et al., 1982). PCV is divided into the following three genotypes: PCV type 1 (PCV1), type 2 (PCV2), and type 3 (PCV3) (Ouyang et al., 2019). PCV1 was found as a persistently infected contaminant in porcine-derived PK-15 cell lines; further, it shows no pathogenicity in pigs (Tischer et al., 1986; Allan et al., 1995). In contrast, PCV2 is pathogenic in pigs and is considered the causative agent of postweaning multisystemic wasting syndrome (Allan et al., 1998; Ellis et al., 2000). Furthermore, PCV2 is involved in porcine dermatitis and nephropathy syndrome and in porcine respiratory disease complex. These diseases are collectively annotated as porcine circovirus-associated disease (PCVD/PCVAD) (Allan et al., 1998; Drolet et al., 1999; Opriessnig et al., 2007).

PCVs are generally considered non-cytopathogenic and persistently infect cultured cells (Marks et al., 2016); however, few reports have showed the cytopathic effect (CPE) of PCV2 in specific cells (Chen et al., 2013; Dvorak et al., 2013). The low growth efficiency of PCVs in cultured cells has become a major obstacle to the establishment of diagnostic methods for PCV (Tischer et al., 1987; Misinzo et al., 2008). If PCV2 becomes capable of exhibiting a clear CPE on cultured cells, its detection will become easier and faster, and the method may then be applied for the early diagnosis of PCVAD.

The CPK-NS cell line, which was established from porcine kidney-derived CPK cells, could be maintained with serum-free culture medium (Sakoda et al., 1998). The cells showed a clear CPE after infection with non-cytopathogenic classical swine fever virus (CSFV) (Sakoda et al., 1998; Aoki et al., 2004). Thus, we focused on CPK-NS cells for assaying PCV2 infection. In this study, we investigated whether PCV2 infection-induced CPE can be observed in CPK-NS cells.

Owing to the high possibility that PCV1 already exists in porcine-derived cell lines and field-infected pigs (Allan and Ellis, 2000; Hattermann et al., 2004), it is extremely difficult to isolate only PCV2 using these starting materials. Therefore, we used reverse genetics to artificially generate PCV2 (Fenaux et al., 2002). First, we amplified the full-length of PCV2 Yamagata strain (1,766 b) (Onuki et al., 1999) by PCR and inserted it into pBluescript II SK (+) (Agilent Technologies, Santa Clara, CA) using In-Fusion cloning technique (TaKaRa Bio, Shiga, Japan). The resulting plasmid was called pPCV2 × 1. Referring to the report of Fenaux et al., to increase the efficiency of viral production, we tried to construct pPCV2 × 2 in which two full-length PCV2 genomes were integrated in tandem (Fig. 1A). One more copy of the PCV2 genome (1,766 b) was inserted by In-Fusion cloning technique (TaKaRa Bio) to be associated with the full-length of PCV2 genome already integrated into pPCV2 × 1. This plasmid was called pPCV2 × 2 and used

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https://doi.org/10.1016/j.jviromet.2019.113706
Received 8 May 2019; Received in revised form 23 July 2019; Accepted 23 July 2019
Available online 13 August 2019

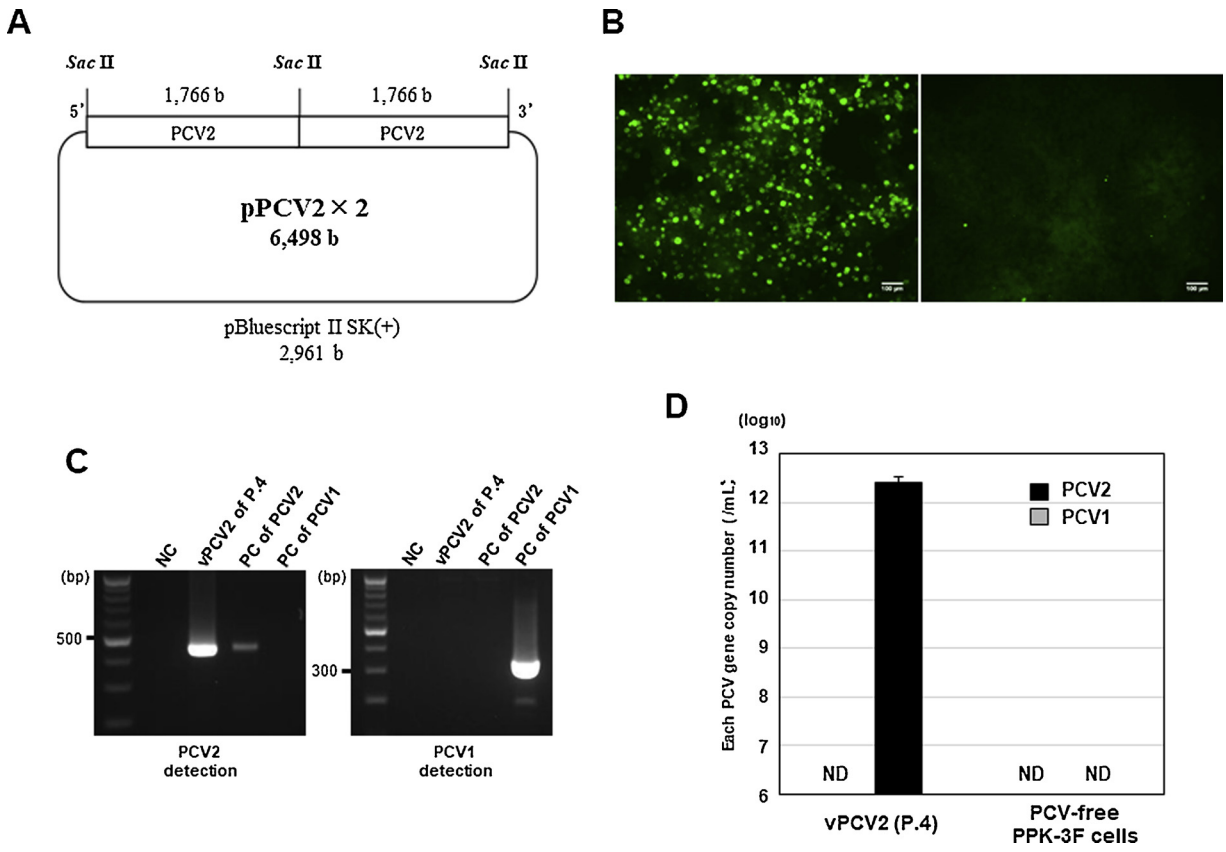

Fig. 1. Production of infectious particles of PCV2.
(A) Plasmid map of pPCV2 × 2 constructed in this study. (B) Detection of PCV2 antigen-positive cells in pPCV2 × 2-transfected PPK-3 F cells. Transfected cells were passaged 4 times (P.4) and analysed using the IFA method (left panel). Empty vector-transfected PPK-3 F cells were used as the negative control (right panel). (C) PCV type-specific PCRs were performed using vPCV2 (P.4). The primer pair for the detection of PCV2 amplified a 481-bp product, whereas that for PCV1 amplified a 347-bp product. The corresponding gene from each virus was used as the positive control (PC). Total DNA extracted from PCV-free PPK-3 F cells was set as the negative control (NC). (D) PCV type-specific qPCR was conducted using vPCV2 (P.4) to measure each viral gene copy number. ND means not detected.

to produce PCV2 without PCV1 contamination. The viral genome of pPCV2 × 2 was confirmed to completely matched with the nucleotide sequence of the parent Yamagata strain (data not shown) (Hamel et al., 1998). pPCV2 × 2 (20 μg) was introduced into porcine kidney (PK)-15–derived PPK-3 F cells (Hirai et al., 2003) by electroporation (10 ms at 360 V and 50 ms × 5 times at 20 V) using a pulse generator (CUY 21Vitro-Ex; BEX Corp., Japan). The cells were cultured on Eagle's minimum essential medium (MEM) supplemented with 5% inactivated foetal bovine serum (FBS), 0.295% tryptose phosphate broth (TPB), 0.1125% $NaHCO_3$ and 2 mM L-glutamine. The transfected cells were passaged 4 times at 4-day intervals, and the supernatant (vPCV2) was collected at each passage. PCV2 antigen-positive cells were detected using the indirect fluorescent antibody (IFA) method with PCV2 capsid antibody (Genetex; Irvine, CA) during each passage. A large number of cells showing green fluorescence in the nucleus were observed in passage no. 3 (P.3) and P.4 cells. In particular, the number of positive cells per microscopic field was most frequently observed in P.4 cells (Fig. 1B), and the viral titre in the P.4 supernatant was $10^{4.5}$ median tissue culture infectious dose ($TCID_{50}$). To rule out the possibility of contamination with PCV1 in the P.4 vPCV2 supernatant, PCV type-specific polymerase chain reaction (PCR) was performed (Ellis et al., 2000). Amplification of specific viral genes was observed when a primer pair for PCV2 was used but not when a primer pair for PCV1 was used (Fig. 1C). Furthermore, PCV gene copy numbers in the P.4 supernatant were measured using PCV-specific quantitative-PCR (qPCR) (Chang et al., 2010). Results revealed that the PCV2 gene copy number was approximately 10^{12} copies/mL; PCV1 gene was not detected (Fig. 1D). Thus, it was inferred that the vPCV2 generated in this study was not contaminated with PCV1 and that the generated PCV2 was genetically homogenous.

Next, to analyse the cytopathogenicity of PCV2 in CPK-NS cells, an infection experiment was performed using the cultured cells. PPK-3 F cells were used as the control. To this end, 1 mL of CPK-NS cells [cultured in MEM supplemented with 0.295% TPB, 0.5% bacto peptone (BP), 0.213% N,N-Bis (2-hydroxyethyl)-1-amin ethanesulfonic acid (BES), 0.225% $NaHCO_3$, 2 mM L-glutamine and antibiotics] and a PPK-3 F cell suspension (5×10^5 cells/mL) was mixed with vPCV2 ($10^{3.0}$ $TCID_{50}$) in a 12-well culture plate, and the cells were passaged thrice at 4-day intervals. Considerable cell detachment was observed in the CPK-NS cells at P.2 after inoculation (Fig. 2A; upper panels). In contrast, weak cell detachment was observed in P.3 PP K-3 F cells (Fig. 2B; upper panels). When the viral gene copy numbers in the supernatants were measured using qPCR, compared with the CPK-NS cell supernatant, the PPK-3 F cell supernatant was observed to contain a higher copy number of the PCV2 gene. No change in the amount of viral gene was observed in the PPK-3 F cells through P.0 to P.3 (Fig. 2B; lower panel). In contrast, the viral gene copy number in the CPK-NS cell supernatant was markedly reduced owing to cell detachment and decrease in adherent cells (Fig. 2A; lower panel).

To confirm the correlation between cell detachment and PCV2 infection, the influence of the viral titre in the PCV2 inoculum on cell detachment was analysed. Cell detachment was delayed when the vPCV2 viral titre decreased. When inoculation was performed using a high viral titre, detachment of the CPK-NS cells was observed after only one passage (Table 1). Moreover, when inoculation was performed using $10^{2.0}$ $TCID_{50}$ of vPCV2, no cell detachment was observed in the

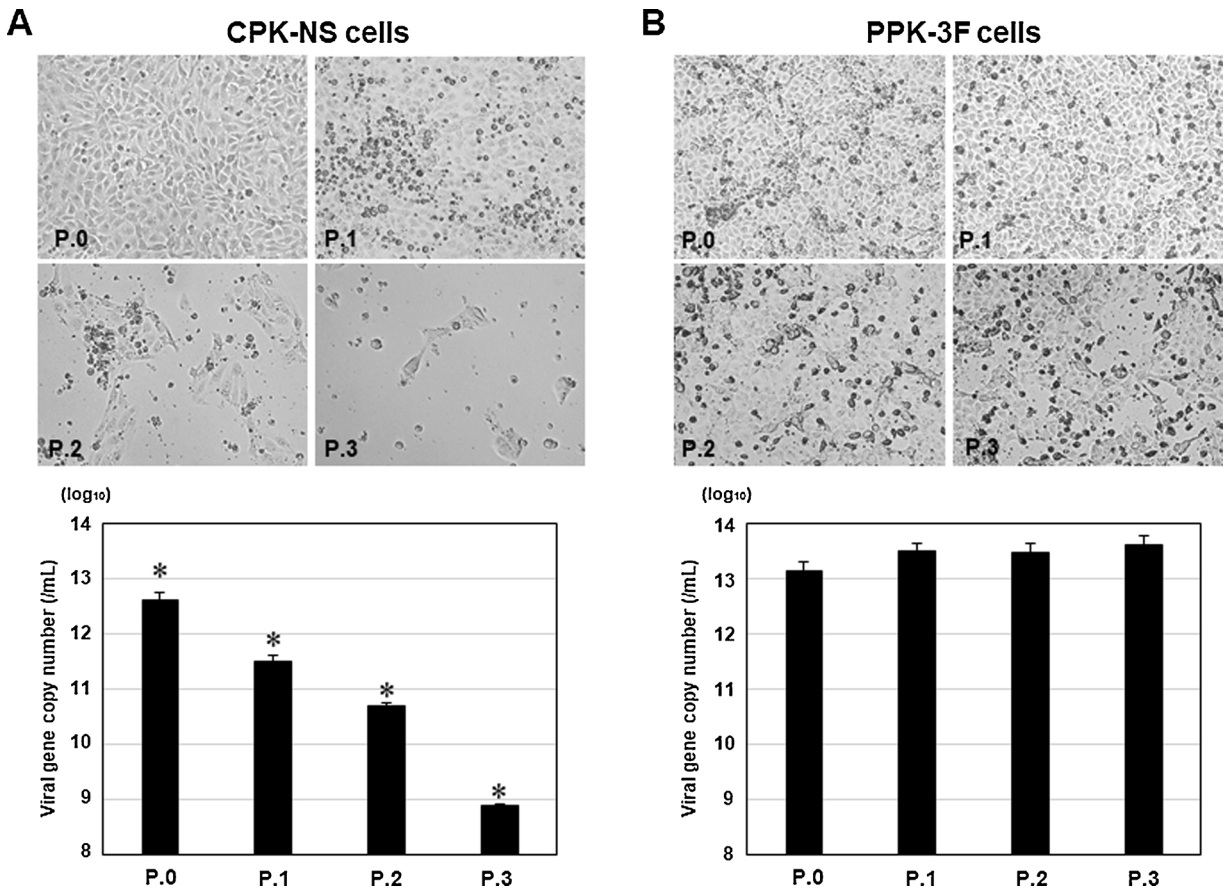

Fig. 2. Relationship between cell detachment and PCV2 propagation.
CPK-NS (A) and PPK-3 F cells (B) were inoculated with vPCV2 ($10^{3.0}$ $TCID_{50}$) and each infected cell was passaged thrice at 4-day intervals. The supernatants of P.0, P.1, P.2 and P.3 cells were collected during cell passaging and used for qPCR to measure copy numbers of viral genes (bottom panels). The pictures of the infected cells were captured at 4 days after cell passaging (top panels). Student-*t* test was performed to compare PCV2 DNA copy numbers between CPK-NS and PPK-3 F cells (* $P < 0.01$).

Table 1
Correlation between the titre of inoculated virus and the frequency of cell detachment.

Cells	Viral titre (log_{10} $TCID_{50}$)	Passage No.				
		0	1	2	3	4
CPK-NS	4.5	–	+++ *1	+++	+++	NT
	4.0	–	++ *2	+++	+++	+++
	3.0	–	–	+++	+++	+++
	2.0	–	–	+ *3	+++	+++
	mock	–	–	– *4	–	–
PPK-3F	4.5	–	++	+++	+++	NT
	4.0	–	+	++	+++	+++
	3.0	–	–	–	+	+
	2.0	–	–	–	–	–
	mock	–	–	–	–	–

*1: Cell detachment was observed thrice during a 3 times trial.
*2: Cell detachment was observed twice during a 3 times trial.
*3: Cell detachment was observed once during a 3 times trial.
*4: No cell detachment was observed during the 3 times trial.
NT: Not tested.

PPK-3 F cells thorough four cell passages; however, cell detachment was observed in the CPK-NS cells at P.2 or P.3. The appearance of cell detachment observed in both cells in an infection dose-dependent manner suggested that the cell detachment was caused by PCV2 infection and that the CPK-NS cells were strongly affected by PCV2 infection.

Finally, to determine the cytopathogenicity of PCV2 in the CPK-NS cells, cell detachment was examined via viral neutralisation test. After $10^{3.0}$ $TCID_{50}$/100 µL of vPCV2 were neutralised with 100 µL of antiserum against PCV2 (antibody titre by IFA test; 6400-12800-fold), the viral suspension treated with antisera was inoculated into the CPK-NS and PPK-3 F cells, and cell detachment was examined during cell passages. Because the CPK-NS cells inoculated solely with vPCV2 had increased number of dead cells in the P.2, only few cells remained adherent after passage.3. In contrast, the CPK-NS cells infected with neutralised vPCV2 did not show any cell detachment, and most cells survived at P.3 (Fig. 3A). Similar to the CPK-NS cells, cell detachment was inhibited when neutralisation was performed using PPK-3 F cells (Fig. 3B). Thus, the neutralisation of vPCV2 was concluded to prevent cell detachment. Thus, cell detachment was dependent on the initiation titre of PCV2 infection, and the appearance of cell detachment was inhibited by neutralisation with the PCV2 antiserum. Our study also revealed that PCV2, which is normally non-cytopathogenic, induces a clear and strong CPE in CPK-NS cells.

It has been also reported that CPK-NS cells exhibit a clear CPE after inoculation with the non-cytopathogenic CSFV GPE^- strain (Sakoda et al., 1998). It can be hypothesized that CPK-NS cells have a mechanism owing to which they strongly respond to non-cytopathogenic viral infections resulting in CPEs. The GPE^- strain of CSFV is used as a live-attenuated vaccine for CSFV infection in Japan and is known to significantly induce innate immune responses in hosts (Fukusho et al., 1976). Moreover, cytopathogenicity of GPE^- virus in CPK-NS cells is associated with apoptosis (Sakoda et al., 1998). The innate immune responses and/or apoptosis induced by PCV2 infection may also be involved in the expression of CPE in CPK-NS cells.

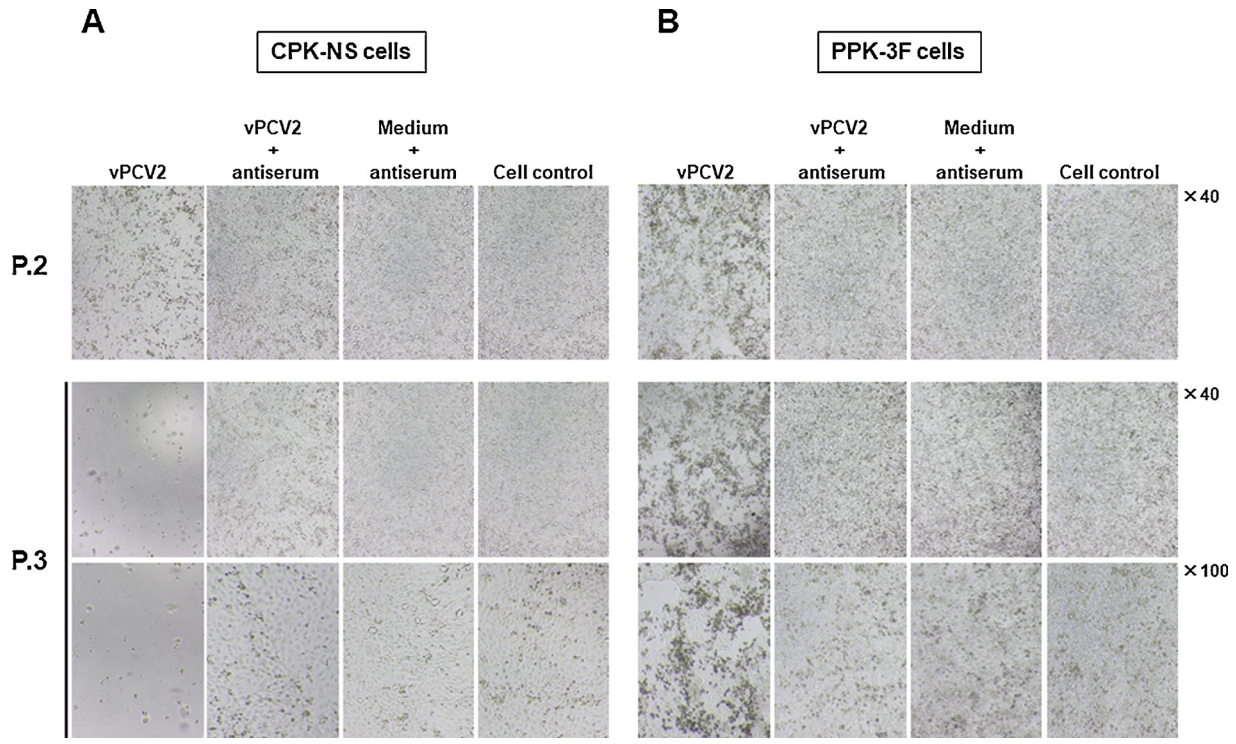

Fig. 3. Cell detachment is prevented by neutralisation of PCV2 in CPK-NS and PPK-3 F cells.
Antiserum prepared from PCV2-infected SPF pigs was used to neutralise vPCV2 ($10^{3.0}$ $TCID_{50}$). CPK-NS cells (A) and PPK-3 F cells (B) were infected solely with either vPCV2 or neutralised vPCV2. Experimental and control cells were passaged thrice at 5-day intervals. CPE was observed in P.2 (×40) and P.3 (×40 and ×100) cells. Each picture was captured at 5 days after cell passaging.

Our study indicated that PPK-3 F cells exhibit good PCV2 replication efficiency (Fig. 2B). For establishing PCV2 persistently infected cells, it would be better to use PPK-3 F cells. Because CPK-NS cells showed strong and distinct CPE by PCV2 infection compared with PPK-3 F cells and could detect a small amount of viruses, it is considered that the CPK-NS cells are more superior in detecting PCV2. Moreover, in the case of using CPK-NS cells, detachment of adherent cells occurs markedly; therefore, we could easily distinguish CPE exerted by CPK-NS cells compared with mock-infected cells. Therefore, the application of CPK-NS cells is useful for the diagnosis of PCVAD caused by PCV2 infection.

Owing to the low growth efficiency of PCV2, the induction of CPE needed at least once cell passage in spite of the high sensitivity of the CPK-NS cells to PCV2. Thus, if the growth efficiency of PCV2 can be promoted, it may be possible to induce CPE more quickly and without the inoculated cell passages, which will thereby contribute to the rapid diagnosis of PCVAD. It is also expected that the neutralisation test using CPE as an index becomes possible, and the measurement of neutralizing antibody titre becomes easy. Previous studies reported that an addition of interferon (IFN)-γ and -α increased the replication of PCV2 in vitro (Meets et al., 2005; Misinzo et al., 2008). The infection efficiency of PCV2 in the PK-15 cells was improved by temporal morphological changes upon treatment with Tween 20 (Hua et al., 2018), and the efficiency of viral DNA replication and protein synthesis in PCV2-infected cells was increased after treatment with D-glucosamine-HCl (Tischer et al., 1987). Applying these treatments to the CPK-NS cells to increase the propagation efficiency of PCV2 may render PCV2-induced CPE readily observable without any need for cell passaging. A further analysis of the CPK-NS cells would provide important insights towards understanding PCV2 replication and/or the innate-immunological characteristics of CPK-NS cells.

A porcine foetal retina cell line and a cell population obtained by cloning the PK-15 cells was reported to exhibit CPE after PCV2 infection (Chen et al., 2013; Dvorak et al., 2013). The PPK-3 F cells used in this study also exhibited the CPE of PCV2 via repeated passage of the infected cells (Fig. 2B). However, compared with all the other cell lines, the CPK-NS cells offer advantages such as culture capacity in serum-free conditions and detection of a minor amount of PCV2. Thus, our study demonstrated the capacity of the CPK-NS cells for the rapid and reliable detection of infectious particles of PCV2. Application of the CPK-NS cells for the diagnosis of PCVAD will be an important contribution for the prevention of PCV2-mediated infectious diseases.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

We would like to thank Dr Hidetoshi Ikeda for providing PCV2 Yamagata strain and National Institute of Animal Health (Tsukuba, Japan) for providing CPK-NS, PPK-3 F cells, and antiserum against PCV2.

We also would like to thank Aya Inagaki, who contributed to the finding of CPE by PCV2 infection.

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