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Construction and biological characterisation of recombinant porcine circovirus type 2 expressing the V5 epitope tag

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ABSTRACT

Porcine circovirus type 2 (PCV2) is a major causal agent of post-weaning multisystemic wasting syndrome in piglets. To investigate the feasibility of PCV2 expressing an exogenous epitope, a 14-amino-acid V5 epitope derived from simian parainfluenza virus type 5, was inserted into the C terminus of the capsid protein. Recombinant PCV2 expressing the V5 epitope, recPCV2/CL-V5, was rescued by transfecting an infectious clone into PK-15 cells and was characterised by an immunoperoxidase monolayer assay (IPMA), a serum neutralisation assay (SNA), a capture enzyme-linked immunosorbent assay (ELISA) and immunoelectron microscopy. The V5 epitope was detected in the recombinant marker virus by IPMA and capture ELISA. Furthermore, there was no detectable difference in the antigenicity of the recombinant marker virus compared with the parental virus by IPMA and SNA using PCV2-positive serum and the neutralising monoclonal antibody 1D2. However, recPCV2/CL-V5 marker virus could be differentiated from the parental virus by PCR, IPMA and capture ELISA. The recombinant marker virus was stable on multiplication through 10 passages in PK-15 cells, with a maximum titre of $10^{6.25}$ 50% tissue culture infective dose ($TCID_{50}$)/ml. BALB/c mice were inoculated with the recombinant or parental virus via the intranasal and intraperitoneal routes. The parental and recombinant viruses both could replicate in mice, cause microscopic pathological changes, and induce mice to generate anti-PCV2 antibodies. Furthermore, the recombinant marker virus could also induce anti-V5 epitope tag antibodies. These results indicated that V5 epitope could be displayed on the surface of the capsid protein by inserting its gene just before stop codon of open reading frame 2. More importantly, insertion of the V5 epitope did not seem to interfere with biological characterisation of the recPCV2/CL-V5 marker virus.

1. Introduction

To date, two types of porcine circovirus have been described, porcine circovirus type 1 and 2 (PCV1 and PCV2) (Meehan et al., 1998; Tischer et al., 1982). PCV1 is a persistent contaminant of PK-15 cells (Tischer et al., 1982) and it is not considered pathogenic (Tischer et al., 1986). By contrast, PCV2 has been detected consistently in pigs with PCV-associated diseases, such as post-weaning multisystemic wasting syndrome (PMWS) (Chae, 2005).

PCV2 belongs to the *Circovirus* genus of the *Circoviridae* family, and is a small non-enveloped virus with icosahedral symmetry and a diameter of 17 nm. Its genome comprises a closed circular, single-stranded DNA, which contains 1766–1768 nucleotides (Allan et al., 1998; Shang et al., 2009). PCV2 has two major open reading frames (ORFs). ORF1 is essential for viral DNA replication, whereas ORF2 encodes a major capsid protein that is required for viral assembly, infection and immune responses (Nawagitgul et al., 2000; Mankertz et al., 2004). As a result of the extremely small size of the PCV2 genome, its genetic manipulation mainly involves nucleotide substitution and mutation (Fenaux et al., 2003, 2004; Lekcharoensuk et al., 2004; Liu et al., 2007; Gillespie et al., 2008). Fenaux et al. (2003) have shown that a chimeric PCV1–2 infectious DNA clone (with the immunogenic capsid gene of PCV2 cloned into the backbone of the nonpathogenic PCV1) is infectious when injected directly into the lymph nodes of specific-pathogen-free piglets, and induces a strong antibody response to PCV2 capsid antigen. Lekcharoensuk et al. (2004) have used PCV1/PCV2 chimeric viruses and PCV2 monoclonal antibodies (mAbs) to map the antigenic epitopes of the PCV2 capsid protein. Liu et al. (2007) have mutated two nucleotides at the end of ORF1 to introduce a *SalI* site, thereby rescuing a PCV2 strain that carries a molecular marker. Recently, Beach et al. (2011) report that short epitope tags were inserted into the C terminus of the capsid protein of the chimeric PCV1–2 vaccine virus, which resulted in a tractable marker virus.

The N terminus of the PCV2 capsid contains residues rich in basic amino acids (De Castro et al., 2008; Grierson et al., 2004; Hanada et al., 2004) and thus may be involved in the formation of the interior surface of the virion, and interact with the negative charges of

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genomic DNA during virus assembly, as reported for many icosahedral viruses (Johne et al., 2006). In addition, the nuclear localisation signal is located in this region. After synthesis in the cytoplasm, capsid proteins enter the nucleoli to support viral transcription or influence the cell cycle (Finsterbusch and Mankertz, 2009). Therefore, one foreign epitope should be expressed on the interior of the virion if this epitope is inserted into the N terminus of the PCV2 capsid.

In this study, a 14-amino-acid V5 epitope derived from simian parainfluenza virus type 5 (Southern et al., 1991) was inserted into the C terminus of the capsid protein (before the stop codon of ORF2) to construct a recombinant marker virus. We demonstrated that the V5 epitope was displayed on the surface of the capsid protein. Furthermore, the recombinant marker virus behaved similarly to the parental virus *in vitro* and in mice, and could be differentiated from the parental virus via polymerase chain reaction (PCR) and serological methods.

2. Materials and methods

2.1. Viruses, plasmids, cells and antibodies

PCV2/CL was originally isolated from a pig that was clinically manifesting PMWS. Plasmid pMD18/PCV2-CL that contained the entire genomic sequence of PCV2/CL was constructed (Guo et al., 2010) and recPCV2/CL was rescued (data not shown). PK-15 cells, free of PCV1 and PCV2, were grown in minimal essential medium (MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus 100 μg/ml penicillin and streptomycin at 37 °C. Three mmol D-glucosamine (Sigma–Aldrich, NY, USA) was added to the medium for PCV2 propagation. PK-15 cells were used for plasmid DNA transfection, virus propagation and titration. PCV2-positive serum (against PCV2a/LG), PCV1-positive serum (against recPCV1/G), and PCVs-negative serum, along with mAb 1D2 (against capsid protein of PCV2) were all derived from Huang et al. (2011).

2.2. Construction of recombinant plasmid

The recognized V5 epitope represents ^{95}GKPIPNPLLGLDST^{108} of RNA polymerase α subunit of simian parainfluenza virus type 5. This short peptide sequence was chosen because high-affinity antibodies can be reliably produced in many different species (Southern et al., 1991; Zhou et al., 2005). To construct a recombinant plasmid containing the sequence of the V5 epitope, fusion PCR was performed. The pMD18/PCV2-CL template was PCR-amplified using primers A1 and A2 (Table 1) according to the instructions with the KOD-plus kit (Toyobo, Shanghai, China). The PCR products were gel purified, and subsequently served as the template for fusion PCR using primers B1 and B2 (Table 1), which inserted the V5 epitope gene (GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACG) into ORF2, just before the stop codon. The fusion PCR product was then used to transform *Escherichia coli* strain Top 10 according to the manufacturer's recommendations (Takara, Dalian, China). The obtained plasmid (pMD18/PCV2-CL-V5) was subjected to sequence analysis by the dideoxy chain termination method using an automated sequencer (A.L.F. Express; Pharmacia, Uppsala, Sweden). The sequence data were then compared with the data of parental virus.

Table 1
Primers used for the construction and identification of the recombinant virus.

Primer	The nucleotide position	Sequence
A1	F1037	5′-GGGTTTAAGTGGGGGGTCT-3′
A2	R1033	5′-ATGAATAATAAAAACAATTACGAAG-3′
B1	F1009	5′-CTTCGTAATTGTTTTTATTATTCATTTACGTAGAATCGAGACCGAGGAG AGGGTTAGGGATAGGCTTACCGGGTTTAAGTGGGGGGTCTTTAAGA-3′
B2	R1061	5′-TCTTAAAGACCCCCCACTTAAACCCGGTAAGCCTATCCCTAACCCTC TCCTCGGTCTCGATTCTACGTAAATGAATAATAAAAACAATTACGAAG-3′
C1	F920	5′-GTCGACGGAGGAAGGGGGCCAGTT-3′
C2	R925	5′-GTCGACTGTTCTGTAGCATTCTTCCA-3′
D1	F959	5′-CCCATGCCCTGAATTTCCATA-3′
D2	R1311	5′-TAAACTACTCCTCCCGCCATAC-3′

2.3. In vitro transfection

Plasmid pMD18/PCV2-CL-V5 was digested with *Sal*I, and purified *Sal*I fragments were self-ligated for 30 min at 16 °C using T4 DNA ligase (Takara, Dalian, China). This DNA was then transfected into PK-15 cells (80–90% confluency) in each well of a 24-well plate using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Empty plasmid-transfected PK-15 cells were included as a negative control. After incubation for 6 h at 37 °C, 400 μl MEM containing antibiotics and 10% FBS were added to each well and incubated at 37 °C with 5% CO_2. At 48 h post-transfection, the recombinant marker virus was detected using an immunoperoxidase monolayer assay (IPMA) as described previously (Liu et al., 2004). The recombinant marker virus was recovered from the transfected cells for serial passage as described previously (Tischer et al., 1987). The 5th and 10th generations of recombinant virus were cloned into the pMD18-T vector with primers C1 and C2 (Table 1), and three independent clones of both generations were sequenced by above-mentioned method.

2.4. Differentiation of the recombinant marker virus from the parental virus

A pair of primers, D1 and D2 (Table 1), were designed to PCR amplify the fragment containing the V5 epitope gene. An Ex-taq DNA polymerase (Takara, Dalian, China) was used to amplify fragments of recombinant and parental viruses with the following cycling programme: 2 min at 94 °C, 35 cycles consisting of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, and a final extension step at 72 °C for 5 min. An amplified fragment of 395 bp was obtained using the recombinant marker virus as a template, and a fragment of 353 bp was obtained using the parental virus as a template.

IPMA plates for the parental and recombinant marker virus were prepared as described previously (Liu et al., 2004), and then tested with the PCV2-positive serum and anti-V5-horseradish peroxidase (HRP) antibody (Invitrogen, Carlsbad, CA, USA), respectively. The plates were stained and examined under a light microscope.

To develop a PCV2 antigen-capture enzyme-linked immunosorbent assay (ELISA), the PCV2-positive serum and mAb 1D2 were respectively purified using a protein A Sepharose CL-4B column (GE Healthcare, Uppsala, Sweden). The purified mAb 1D2 was labelled using a peroxidase labelling kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. ELISA plates (Nunc, Glostrup, Denmark) were coated with purified PCV2-positive porcine serum (1 μg/ml) in 0.05 M carbonate buffer (pH 9.6) overnight at 4 °C. After three washes in PBS containing 0.05% Tween20 (PBS-T), the plates were blocked with 100 μl PBS containing 10% horse serum for 1 h at 37 °C. Aliquots of 100 μl of the PCV2 strain cultures diluted in PBS-T to a final concentration of 10^5 50% tissue culture infective dose ($TCID_{50}$)/ml, were distributed in each well and incubated at 37 °C for 1 h. After washing with PBS-T, 100 μl mAb 1D2 conjugated with HRP and anti-V5-HRP antibody diluted (1:1000) in PBS-T, were added, and the plates were