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RESEARCH ARTICLE

Isolation, Genome Phylogenetic Analysis and *In vitro* Rescue of a Newly Emerging Porcine Circovirus Type 2

Weijuan Zhu and Xiaofeng Ren*

College of Veterinary Medicine, Northeast Agricultural University, 59 Mucai Street, Xiangfang District, 150030 Harbin, China
*Corresponding author: rxfemail@yahoo.com.cn; renxf@neau.edu.cn

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ABSTRACT

Porcine circovirus type 2 (PCV2) is the major causative agent of post-weaning multisystemic wasting syndrome (PMWS). Infection by PCV2 may cause heavy losses in pig industry. In this study, we report the isolation of a newly emerging PCV2 from northeastern China. The complete genome of the PCV2 isolate named PCV2-LJR contains 1766 nucleotides and was compared with reference sequences published in GenBank followed by topology analysis of the resulting phylogenetic tree. The data indicated that the prevalent PCV2 isolates in the northeastern China had close relationship, although various genotypes of PCV2 existed. In addition, by gene recombination and transfection techniques, the PCV2 infectious clone was achieved and was able to rescue virus *in vitro* determined by indirect immunofluorescence assay and PCR. The obtained biological materials may be used for biological characterization of PCV2.

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INTRODUCTION

Porcine circovirus type 2 (PCV2) is the primary causative agent of postweaning multisystemic wasting syndrome (PMWS). Since PMWS was recognized in Canada in 1997, this disease has become a serious economic problem for the pig industry in many countries including China (Wellenberg *et al*., 2004; Shuai *et al*., 2011). PCV2 is a member of family *Circoviridae*, genus *Circovirus* and it is a small non-enveloped virus with a single-stranded circular DNA. The viral genome is approx. 1.8 kb and contains two major open reading frames (ORFs). ORF1 encodes two replication-associated proteins (Rep and Rep') and ORF2 encodes a viral major antigen protein (capsid, Cap) (Harding *et al.*, 2010; Fu *et al.*, 2011).

Although PCV2 has been identified in many countries, its infection and transmission mechanisms remain unclear. Herein, we report the isolation of a local PCV2 named PCV2-LJR in Jilin Province, northeastern China. The sequence comparison and phylogenetic relatedness of the newly identified PCV2 isolate with reference PCV2 isolates were investigated based on the available complete genomes. In addition, the infectious clone for the PCV2 was constructed and used to rescue PCV2 *in vitro*, providing a platform for function analysis of this virus.

MATERIALS AND METHODS

Virus isolation: Superficial inguinal lymph node samples from 15 pigs with PMWS were submission of a pig farm in Taonan area Jilin Province, China to College of Veterinary Medicine, Northeast Agricultural University, China. For virus isolation, the lymph nodes were frozen and thawed three times, homogenized in RPMI1640 medium, and centrifuged at 4000 rpm for 30 min at 4°C. A portion of the filtered supernatants were subjected to DNA extraction and the rest were used to inoculate onto a porcine kidney cell line, PK15, to make PCV2 stock. Briefly, The PK15 cells were free of PCV1 contamination and maintained at 37°C with 5% CO_2 in RPMI1640 and 10% fetal bovine serum (FBS). When the cells were semi-confluent, they were trypsinized, mixed with the PCV2-containing supernatant at a ratio of 10:1 and cultured at 37°C with 5% CO_2 for 24 h to form cell monolayers. Then the cells were washed with PBS and the medium was changed to RPMI1640 containing 3% FBS plus D-glucosamine (300 mM). Three days later, the infected cells were frozen and thawed three times and the resulting suspension containing cell debris and membrane was clarified by centrifugation at 3000 rpm for 10 min. A panel of positive sera against PCV2, Porcine Parvovirus (PPV), Porcine Reproductive and Respiratory Syndrome

Virus (PRRSV), Porcine Pseudorabies Virus (PrV), Swine Transmissible Gastroenteritis Virus (TGEV), Porcine Epidemic Diarrhea Virus (PEDV) & Porcine Rotavirus (PRV) was used as primary antibody in an indirect immunofluorescence assay to confirm the existence of PCV2.

Amplification of the viral genome by PCR: PCV2-containing supernatants were frozen and thawed three times and centrifuged as above. The resulting supernatant used as a template and a pair of primers: sense primer H1 (5'-GGGAATTCAACCTTAACCTTTCTTA-3') and antisense primer H2: (5'-AAGAATTCTGGCCCTGCT CCCCAATC-3') were used to amplify the PCV2 genome. Both primers contained EcoR I sites, the PCR mixture included 100 ng of template (1 µl), 0.5 µM (0.5 µl) of each primer, 1 unit (1 µl) of high fidelity DNA polymerase (TaKaRa, Dalian, China), 0.2 mM (2 µl) of dNTP, 5 µl of PCR buffer (TaKaRa, Dalian, China) and 15 µl of sterile water. The PCR reaction was performed at 94°C for 5 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min; a final extension at 72°C for 15 min. The PCR product was subjected to 1% agarose electrophoresis and sequencing. The resulting sequence was compared with reference PCV2 sequences published in GenBank and a phylogenetic tree was constructed using Lasergene software package V5.0 (DNAStar, USA) (Ren *et al.*, 2009).

Construction of infectious clone bearing PCV2 genome: To construct a molecular DNA clone containing the PCV2 genome, the PCR product containing the complete PCV2 genome was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified product was ligated into the pMD18-T cloning vector (TaKaRa, Dalian, China) and transformed into *Escherichia coli* (*E. coli*) JM109 competent cells. Positive plasmids from different colonies were selected for sequencing by the commercial company (Sangon, Shanghai, China) and both strands of the insert were sequenced. A positive clone was selected and named as PCV2-T. The concentration of the recombinant DNA plasmid was determined spectrophotometrically. The PCV2-T was separated from the plasmid DNA after digestion with EcoR I by electrophoresis on 1% agarose gel, resulting in a linear approx. 1.8 bp fragment. The fragment was purified with a Gel-purification kit (Axygen, Hangzhou, China) according to the manufacturers' instructions and self-ligated with T4 DNA ligase (TaKaRa, Dalian, China) at 16°C overnight.

Cell transfection and immunofluorescence assay: The infectivity of the molecular DNA clone *in vitro* was tested by transient transfection. Briefly, the PK-15 cells were grown in 24-well plates to approx. 80% confluency as above-mentioned culture conditions. The cells were washed twice with PBS, and then were transfected with 1 µl (1 µg) of self-ligation DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturers' instructions. Mock-transfected cells with empty vector were used as controls. Three days post-transfection, the cells were harvested and subjected to blink passage and the existence of PCV2 in the passaged cells was identified by PCR using above-mentioned protocols.

In parallel, the rescued PCV2 infected cells were fixed with 4% paraformaldehyde in PBS followed by quenching with 0.1 M glycine in PBS. After three times washes with PBS, the cells were permeabilized with 1% Triton X-100 for 10 min, incubated with pig anti-PCV2 antibody (1:100 dilutions in PBS) for 1 h. After three times washes with PBS, the cells were incubated with fluoresceine isothiocyanate (FITC) conjugated rabbit anti-pig IgG (Zhongshan, China) in the dark for 1 h. The isolated PCV2-infected cells and mock-infected cells served as positive and negative controls, respectively. The green fluorescence signals were observed and recorded using a fluorescence microscope (Leica, Germany).

RESULTS

Isolation of PCV2 and sequencing of the viral genome: Five out of 15 clinical samples were PCR positive for PCV2. One PCV2 isolate was obtained and named as PCV2-LJR. After six blind passages of PCV2-LJR in PK-15 cells, the cells were subjected to a discrimination immunofluorescence assay using a panel of positive sera against PCV2, PPV, PRRSV, PrV, TGEV, PEDV and PRV. Green positive signals were able to be observed, only if the anti-PCV2 antiserum was used as primary antibody (data not shown). After DNA extraction and PCR, the genome of the isolated virus was identified. The sequencing result showed that the PCV2 genome was 1766 nucleotide (nt) in length; the sequence has been submitted to GenBank database and allocated an accession number, JN388690.

Sequence comparison and phylogenetic analysis: Using DNAStar software, the genome sequence of PCV2-LJR was compared with 23 PCV2 reference sequences. The results showed that all the PCV2 had a high homology

Table 1: Information on the PCV2 isolates used in this study

Isolate	Origin	Year	Accession #
Porcine circovirus type Canada II	Canada	2009	AF055392
Porcine circovirus type II	France	2009	AF055394
BJ-HB	China	2003	AF538325
PCV2-Y65	China	2007	EU266592
PCV2-WJXXL	China	2007	EU266593
PCV2-WJLJ	China	2007	EU266598
PCV2-FH	China	2007	EU266599
871	China/Heilongjiang	2008	EU420015
PCV2-WJXW	China	2007	EU266594
XS1106	China	2010	GU325755
PCV2-SJ	China	2007	EU266595
SCcd	China/Sichuan	2009	HM102350
SY4	China	2008	GU325754
AH	China/Heilongjiang	2008	HM038030
MDJ	China	2007	HM038031
YJ	China/Heilongjiang	2008	HM038032
QZ0906	China	2009	GU325758
XS090305	China	2009	GU325756
PCV2-JianL	China	2007	EU266597
JX0803	China	2008	GU325753
Porcine-derived pepsin product	USA	2004	AY699793
HZ0903	China	2009	GU325757
PCV2-JS	China	2007	EU266596
PCV2-LJR	China/Jilin	2010	JN388690

The information on the isolate name, origin place (country/province), isolating year as well as GenBank accession number of the PCV2 isolates used in this study.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
1		99.6	99.6	97.4	98.6	99.5	99.7	99.6	99.6	99.4	99.4	99.4	98.6	99.5	96.7	96.6	99.5	95.9	95.7	98.5	96.6	99.7	96.6	96.5	1	871
2	0.3		99.6	97.5	98.6	99.5	99.7	99.6	99.6	99.4	99.4	99.4	98.6	99.5	96.8	96.7	99.5	96.2	96.0	98.5	96.8	99.7	96.8	96.7	2	AF055394.1
3	0.4	0.3		97.4	98.6	99.5	99.7	99.6	99.6	99.4	99.4	99.4	98.6	99.5	96.9	96.8	99.5	96.0	95.8	98.5	96.7	99.7	96.7	96.6	3	BJ-HB
4	3.1	2.9	3.1		98.5	97.5	97.6	97.5	97.4	97.4	97.2	97.4	98.2	97.5	96.2	96.1	96.9	97.2	97.0	98.3	97.7	97.6	97.7	97.7	4	HZ0903
5	1.6	1.5	1.7	1.8		98.6	98.8	98.7	98.6	98.5	98.4	98.5	99.3	98.6	96.6	96.6	98.3	96.0	95.8	99.3	96.7	98.8	96.7	96.6	5	JX0803
6	0.6	0.5	0.6	3.1	1.7		99.6	99.5	99.5	99.3	99.2	99.2	98.5	99.4	96.6	96.5	99.3	95.8	95.6	98.5	96.6	99.6	96.6	96.5	6	PCV2-FH
7	0.3	0.3	0.4	2.9	1.5	0.5		99.7	99.7	99.5	99.4	99.4	98.7	99.5	96.7	96.6	99.7	95.9	95.8	98.7	96.7	99.8	96.7	96.6	7	PCV2-JianL
8	0.4	0.3	0.5	3.0	1.5	0.6	0.4		99.6	99.5	99.4	99.5	98.7	99.6	96.8	96.7	99.5	95.9	95.8	98.7	96.7	99.8	96.7	96.6	8	PCV2-JS
9	0.4	0.3	0.5	3.1	1.7	0.6	0.4	0.5		99.4	99.5	99.4	98.6	99.5	96.7	96.6	99.5	95.9	95.7	98.5	96.6	99.7	96.6	96.5	9	PCV2-SJ
10	0.6	0.6	0.7	3.1	1.8	0.9	0.6	0.6	0.7		99.2	99.3	98.6	99.4	96.6	96.5	99.3	95.8	95.6	98.4	96.6	99.6	96.6	96.5	10	PCV2-WJLJ
11	0.7	0.6	0.7	3.4	2.0	0.9	0.7	0.7	0.6	1.0		99.1	98.4	99.2	96.5	96.3	99.2	95.6	95.5	98.3	96.5	99.5	96.5	96.3	11	PCV2-WJXW
12	0.7	0.6	0.7	3.2	1.8	0.9	0.7	0.6	0.7	0.9	1.0		98.5	99.3	96.6	96.4	99.2	95.8	95.6	98.4	96.6	99.6	96.6	96.4	12	PCV2-WJXXL
13	1.6	1.5	1.7	2.2	0.8	1.8	1.6	1.5	1.7	1.7	2.0	1.8		98.6	96.6	96.5	98.3	95.9	95.7	99.2	96.6	98.8	96.6	96.5	13	PCV2-Y65
14	0.6	0.5	0.6	3.0	1.7	0.7	0.6	0.5	0.6	0.7	0.9	0.8	1.7		96.7	96.6	99.3	95.9	95.8	98.5	96.7	99.7	96.7	96.6	14	PCV2-LJR
15	4.0	3.7	3.7	4.6	4.1	4.1	4.0	3.8	4.0	4.1	4.3	4.2	4.1	4.0		99.6	96.0	95.2	95.1	96.5	96.1	96.8	96.1	96.0	15	Porcine_circovirus_Type_II_Canada
16	4.1	3.9	3.9	4.7	4.2	4.3	4.1	4.0	4.2	4.3	4.5	4.4	4.3	4.2	0.5		95.9	95.1	94.9	96.4	96.0	96.7	96.0	95.8	16	porcine-derived_pepsin_product
17	0.4	0.3	0.5	3.1	1.7	0.6	0.3	0.5	0.5	0.6	0.7	0.7	1.7	0.6	4.0	4.1		95.8	95.6	98.2	95.9	99.6	95.9	95.8	17	YJ
18	4.1	3.8	4.1	2.8	4.1	4.3	4.1	4.1	4.2	4.3	4.4	4.3	4.2	4.1	4.8	5.0	4.2		99.7	95.7	99.8	96.0	99.8	99.6	18	AH
19	4.3	4.0	4.3	3.0	4.3	4.4	4.3	4.3	4.4	4.4	4.6	4.5	4.4	4.3	5.0	5.2	4.4	0.3		95.5	99.6	95.9	99.6	99.4	19	MDJ
20	1.7	1.7	1.8	2.1	0.8	1.8	1.6	1.6	1.8	1.9	2.1	1.9	0.9	1.8	4.3	4.4	1.8	4.4	4.6		96.5	98.7	96.5	96.5	20	QZ0906
21	4.1	3.8	4.0	2.8	4.0	4.2	4.1	4.1	4.1	4.2	4.4	4.3	4.1	4.1	4.7	4.9	4.1	0.2	0.3	4.3		96.8	99.8	99.7	21	SCcd
22	0.3	0.2	0.3	2.9	1.4	0.5	0.3	0.2	0.3	0.5	0.6	0.5	1.4	0.4	3.8	4.0	0.3	4.0	4.2	1.5	4.0		96.8	96.6	22	SY4
23	4.1	3.8	4.0	2.8	4.0	4.2	4.1	4.1	4.1	4.2	4.4	4.3	4.1	4.1	4.7	4.9	4.1	0.2	0.3	4.3	0.2	4.0		99.8	23	XS1106
24	4.3	4.0	4.2	2.8	4.2	4.4	4.3	4.3	4.3	4.4	4.6	4.4	4.3	4.3	4.9	5.1	4.3	0.3	0.5	4.4	0.4	4.1	0.3		24	XS090305
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		

Fig. 1: Homologous identity percentage of PCV2

The homologous identity percentage of the PCV2 genomes used in this study was analyzed using DNAStar software. The sequence numbers of the viruses are corresponding to the PCV2 isolate names (on the right of the figure).

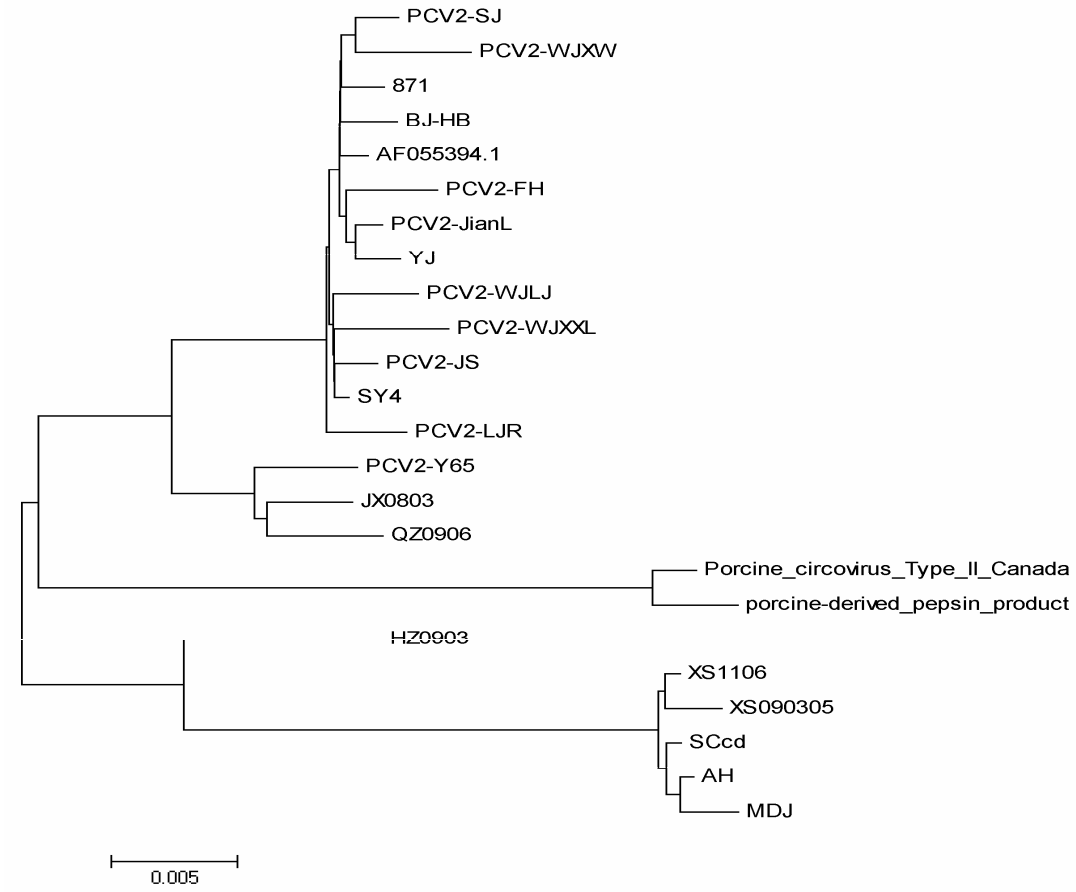

Fig. 2: PCV2 genome based phylogenetic tree. Using the genome sequences of the PCV2 isolates, the corresponding phylogenetic tree was generated with the Jotun Hein method. The isolate names are indicated. The bootstrap value is 1,000.

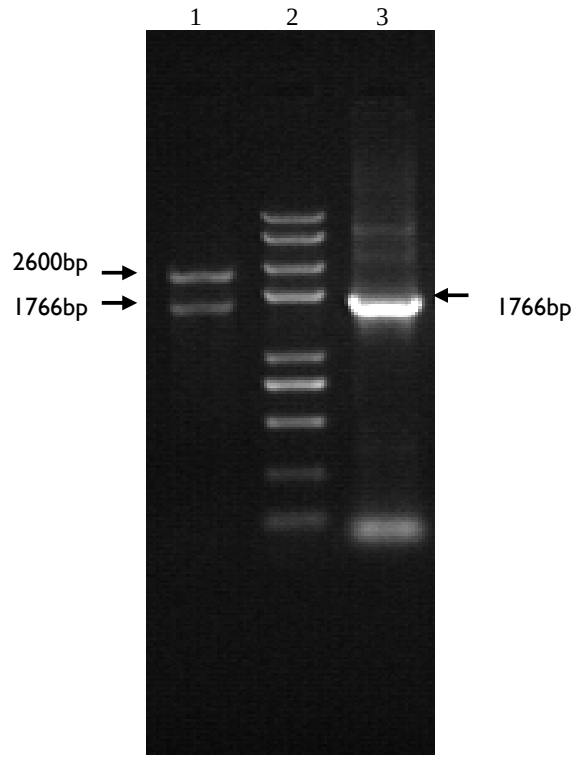

Fig. 3: Construction of infectious clone for PCV2-LJR. Using standard cloning technique, the complete genome of PCV2-LJR was cloned into pMD18-T vector and the resulting recombinant plasmid PCV2-T was digested by EcoR I and the size of the linearizing PCV2 genome and vector are indicated (lane 1). Lanes 2 and 3 are DNA marker and the PCR product containing the PCV2 genome used as a control, respectively.

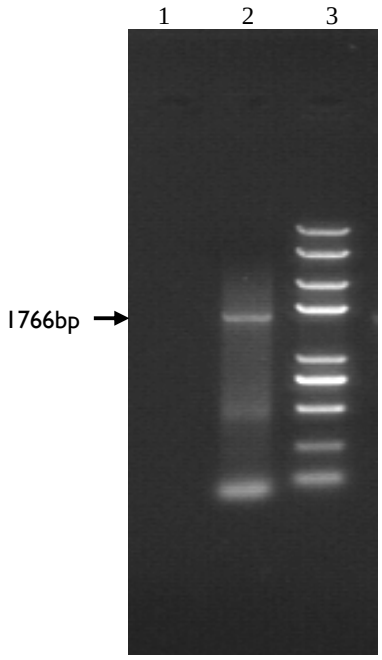

Fig. 4: Identification of PCV2 by PCR after virus rescue. PK15 cells were transfected with infectious clone for PCV2 and a rescued virus named tPCV2 was obtained. The tPCR2 were passaged in PK15 cells followed by PCR amplification of its genome DNA (lane 2). Lane 1: PCR negative control using water as template; lane 3: DNA marker.

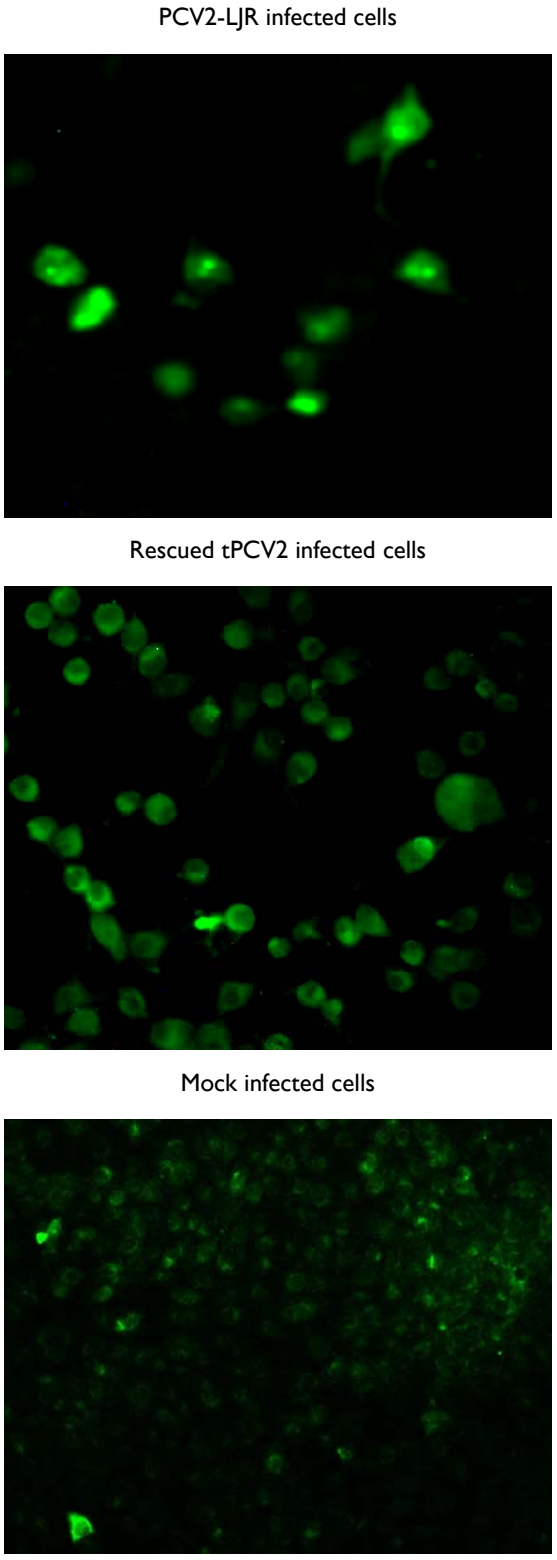

Fig. 5: Immunofluorescence assay for PCV2 infected cells. After genome transfection, the rescued tPCV2 or isolated PCV-LJR was used to infect PK15 cells. Pig anti-PCV2 antibody was used as primary antibody followed by a conventional immunofluorescence assay. Mock infected cells were used as control. The assay was performed in triplicate. A representative comparison is provided and the experimental group names are indicated above each image.

(Fig. 1). For example, PCV2-LJR had 99.7, 95, 95, 99 and 99% homology with the PCV2 isolates derived from neighboring areas such as SY4, AH, MDJ, YJ, and 871, respectively.

To characterize the evolutionary relatedness among these PCV2 isolates, the genome-based phylogenetic tree was constructed. The results further showed that PCV2-LJR had closer relationship with PCV2 isolates SY4, YJ and 871. Several foreign PCV2 isolates from other continents were also located in the same clade with the Chinese isolates (Fig. 2).

Construction of PCV2 infectious clone and virus rescue: Using standard cloning technique, the full-length PCV2 genome was cloned into pMD18-T vector, resulting in a recombinant plasmid PCV2-T. The digestion of the plasmid by EcoR I led to two fragments: one is the PCV2 genome of 1766 bp, the other one is the linearized vector of approx. 2600 bp (Fig. 3).

The PCV2 infectious clone was achieved through the self-ligation of the viral genome following digestion of PCV2-T by EcoR I. After cell transfection with the infectious clone, a rescued virus named tPCV2 was obtained. The tPCR2 were able to be passaged in PK15 cells and amplification of its genome DNA by PCR was successful (Fig. 4). After genome transfection, the rescued tPCV2 was used to infect PK15 cells and was further confirmed by IFA. As shown in Figure 5, both PCV2-LJR infected cells and tPCV2-infected cells were able to react with PCV2 antiserum, in contrast, the mock control showed no staining, revealing the presence of rescued PCV2.

DISCUSSION

It has been reported that inguinal lymph node was the tissue where PCV2 detection rate was the highest compared with lung and tonsil samples (Rosell *et al.,* 2000); in addition, collection of inguinal lymph node is also simple (Calsamiglia *et al.,* 2002), therefore, this lymphoid organ was selected for PCR detection in this study. Five PCV2 positive samples were found among 15 samples. As the samples were from the same geographical area, we randomly selected one sample to isolate PCV2 followed by sequencing and functional analysis in the current study. Isolation of PCV2 from the other samples will be done in the future. PCV2 may cause PMWS, however, the virus alone developed mild clinical symptoms (Rosell *et al.,* 2000; Allemandou *et al.,* 2011). A recent report indicated that co-infection with PPV or PRRSV and PCV2 resulted in the microscopic lesions associated with PMWS and/or porcine dermatitis and nephropathy syndrome, and lead to the development of severe disease (Yi and Liu, 2010; Sinha *et al.,* 2010). Therefore, we will also try to isolate PRRSV or PPV from the same batch of samples in the future.

DNA synthesis of PCV requires DNA polymerase expressed during the S-phase of the cell cycle and the PCV replication may be induced by glucosamine and cell cycle dependent (Chen *et al.,* 2011). In our study, by keeping the final concentration of D-glucosamine at 3 mM, the titer of the passaged PCV2 was approximately 10^5 plaque-forming units/ml, indicating that such concentration is appropriate for the propagation of this virus.

Most of past isolated PCV2 had a genome of 1767 or 1768 nt. The genome of PCV2-LJR consists of 1766 nt. The data indicate that there was a nucleotide deletion during PCV2 evolution and 1766 nt PCV-2 strain is a newly emerging PCV2 in the clinic. The difference in deletion position of the viral genome may lead to the existence and prevalence of various PCV2 isolates. Recently, Dupont *et al.* (2008) and Shang *et al.* (2009) had also identified newly emerging PCV2 isolates with a genome of 1766 nt. Therefore, the PCV2 of 1766 nt may be a representative of newly emerging virus. There is a loss of G in the 1039 position in the genome and such loss may have impact on the C terminus of the viral Cap protein. The pathogenesis, antigenicity and virulence of the mutated PCV2 therefore need to be investigated in the future.

The topology of the PCV2 genome based phylogenetic tree indicates that there were two major groups and most Chinese PCV2 isolates located in the same clade of the phylogenetic tree. However, there were also French or Canadian PCV2 which grouped with the Chinese PCV2. These data imply that the Chinese may originate from either America or Europe; at the same time, most Chinese isolates have evolved into an independent PCV2 group during the environmental pressure. More epidemic investigations and large-scale phylogenetic analyses are required to delineate the evolution and transmission of PCV2.

An inverted repeat forms a putative stem-loop structure with a nonamer (5'-T/AAGTATTAC-3') in its apex, which is conserved in all circoviruses (Todd *et al.,* 2001). The opening position of genome loop is crucial for construction of infectious clone for PCV2. Fenaux *et al.* linearized genome of PCV2 by utilizing a Sac II site in the ORF1 gene (Fenaux *et al.,* 2002). In our study, the primers contained the EcoR I sites and digestion of the sites may avoid destructing the rolling-circle replication sequences of PCV2 and the Rep protein encoded by ORF1 gene. Therefore, we selected this site to linearize the genome of PCV2-LJR prior to self-ligation. PCR and immunofluorescence assay indicated that the self-ligation genome may rescue infectious PCV2 by cell transfection. In the future, we will investigate the infection mechanism of PCV2 by using the infectious clone and site-direct mutagenesis.

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REFERENCES

Allemandou A, B Grasland, AC Hernandez-Nignol, A Kéranflec'h, R Cariolet and A Jestin, 2011. Modification of PCV-2 virulence by substitution of the genogroup motif of the capsid protein. Vet Res, 42: 54.

Calsamiglia M, J Segalés, J Quintana, C Rosell and M Domingo, 2002. Detection of porcine circovirus types 1 and 2 in serum and tissue samples of pigs with and without postweaning multisystemic wasting syndrome. J Clin Microbiol, 40: 1848-1850.

Chen HC, ST Chiou, JY Zheng, SH Yang, SS Lai and TY Kuo, 2011. The nuclear localization signal sequence of porcine circovirus type 2 ORF2 enhances intracellular delivery of plasmid DNA. Arch Virol, 156: 803-815.

Dupont K, EO Nielsen, P Baekbo and LE Larsen, 2008. Genetic analysis of PCV2 isolates from Danish archives and a current PMW S case Control study supports a shift in genotypes with time. Vet Microbiol, 128: 56-64.

Fenaux M, PG Halbur, G Haqshenas, R Royer, P Thomas, P Nawagitgul, M Gill, TE Toth and XJ Meng, 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. J Virol, 76: 541-551.

Fu F, X Li, Y Lang, Y Yang, G Tong, G Li, Y Zhou and X Li, 2011. Co-expression of ubiquitin gene and capsid protein gene enhances the potency of DNA immunization of PCV2 in mice. Virol J, 8: 264.

Harding JC, JA Ellis, KA McIntosh and S Krakowka, 2010. Dual heterologous porcine circovirus genogroup 2a/2b infection induces severe disease in germ-free pigs. Vet Microbiol, 145: 209–219.

Ren XF, JC Yin, DX Ma and GX Li, 2009. Characterization and membrane gene-based phylogenetic analysis of avian infectious bronchitis virus Chinese strain HH06. Virus Genes, 38: 39-45.

Rosell C, J Segale's, JA Ramos-Vara, JM Folch, GM Rodrı'guez-Arrioja, CO Duran, M Balasch, J Plana-Dura'n and M Domingo, 2000. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Vet Rec, 146: 40-43.

Shang SB, YL Jin, XT Jiang, JY Zhou, X Zhang, G Xing, JL He and Y Yan, 2009. Fine mapping of antigenic epitopes on capsid proteins of porcine circovirus and antigenic phenotype of porcine circovirus type2. Mol Immunol, 46: 327-334.

Shuai J, L Fu, X Zhang, B Zhu, X Li, Y He and W Fang, 2011. Functional exchangeability of the nuclear localization signal (NLS) of capsid protein between PCV1 and PCV2 in vitro: Implications for the role of NLS in viral replication. Virol J, 8: 341.

Sinha A, HG Shen, S Schalk, NM Beach, YW Huang, PG Halbur, XJ Meng and T Opriessnig, 2010. Porcine reproductive and respiratory syndrome virus infection at the time of porcine circovirus type 2 vaccination has no impact on vaccine efficacy. Clin Vaccine Immunol, 17: 1940-1945.

Todd D, JH Weston, D Soike and JA Smyth, 2001. Genome sequence determinations and analyses of novel circovirus from goose and pigeon. Virology, 286: 354-362.

Wellenberg GJ, N Stockhofe-Zurwieden, WJA Boersma, MF de Jong and ARW Elbers, 2004. The presence of co-infections in pigs with clinical signs of PMWS in the Netherlands: a case-control study. Res Vet Sci, 77: 177-184.

Yi JZ and CQ Liu, 2010. Molecular characterization of porcine circovirus 2 isolated from diseased pigs co-infected with porcine reproductive and respiratory syndrome virus. Virol J, 7: 286.