

# The 6th amino acid mutation of Rep protein had no effect on PCV2b but enhanced PCV2d virus replication in vitro

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## Abstract

Porcine circovirus type 2 (PCV2) is the etiological agent that primary cause of post-weaning multisystemic wasting syndrome (PMWS). The major genotypes, PCV2a, PCV2b and PCV2d, are highly prevalent, but now replaced with 2b and 2d in swine population in worldwide. Rep protein is the key protein for viral replication. Compared a large number of Rep protein amino acid (aa) sequences, we found that there were three sites with regular changes between 2b and 2d. In order to analyze the effect of key sites on viral replication, we used site-directed mutagenesis to mutate the 6th aa of Rep (alternations with asparagine and serine) between PCV2b and PCV2d. Two wild-type and two mutant viruses infectious clones were rescued by non-contaminated porcine kidney-15 (PK-15) cells. Real-time quantitative PCR and a one-step growth curve were used to determine viral load to assess the replication of rescued viruses. The results showed that there was no significant difference between the PCV2b mutation and the wild-type PCV2b virus in vitro, while the mutation of PCV2d enhanced viral replication.

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**Abstract:** Porcine circovirus type 2 (PCV2) is the etiological agent that primary cause of post-weaning multisystemic wasting syndrome (PMWS). The major genotypes, PCV2a, PCV2b and PCV2d, are highly prevalent, but now replaced with 2b and 2d in swine population in worldwide. Rep protein is the key protein for viral replication. Compared a large number of Rep protein amino acid (aa) sequences, we found that there were three sites with regular changes between 2b and 2d. In order to analyze the effect of key sites on viral replication, we used site-directed mutagenesis to mutate the 6<sup>th</sup> aa of Rep (alternations with asparagine and serine) between PCV2b and PCV2d. Two wild-type and two mutant viruses infectious clones were rescued by non-contaminated porcine kidney-15 (PK-15) cells. Real-time quantitative PCR and a one-step growth curve were used to determine viral load to assess the replication of rescued viruses. The results showed that there was no significant difference between the PCV2b mutation and the wild-type PCV2b virus in vitro, while the mutation of PCV2d enhanced viral replication.

**Keywords:** Porcine circovirus type 2; Rep protein; mutate sites; viral replication

## 1. Introduction

Porcine circovirus is the smallest virus known to replicate autonomously in mammalian cells (Breitbart et al., 2017). Now, according to the antigenicity of porcine circovirus and the difference of its genome sequence, porcine circovirus is divided into three serotypes, porcine circovirus type 1 (PCV1), porcine circovirus type 2 (PCV2) and porcine circovirus type 3 (PCV3). PCV2 is the main pathogen of post-weaning multisystemic wasting syndrome (PMWS) and nephrotic syndrome (PDNS) (An et al., 2007). Similar to PCV2, PCV3 may cause reproduction disorder in sows and PDNS in adult pigs (Palinski et al., 2017).

Currently, PCV2 is divided into five different genotypes: PCV2a, PCV2b, PCV2c, PCV2d and PCV2e. PCV2a, PCV2b and PCV2d are the major genotypes. Before 2001, the most clinically prevalent genotype affected pigs was PCV2a. Since 2003, PCV2b genotype has essentially replaced the previously predominant PCV2a genotype in global. Recently, the worldwide prevalence of PCV2d has increased, and seems a tendency to replace PCV2b (Franzo et al., 2018; Wang et al., 2020).

Rep protein is the key protein for viral replication. Some special structures are essential to maintain Rep protein function, and mutations or deletions of related genes affect virus replication (Mankertz et al., 2001). Studies have shown that mutations in Cap protein from proline to alanine in 110<sup>th</sup> and from arginine to serine in 191<sup>th</sup> could enhance the replication capacity of PCV2 in vitro and reduce toxicity in vivo (Fenaux et al., 2004). It had been shown that altering the amino acid sequence at the Rep protein also altered the replication ability of the virus (Shi et al., 2015). However, information regarding the differential site mutation of different genotypes PCV2 Rep protein, as well as the correlation between the mutation and replication, remains limited.

In order to analyze the effect of the key sites on viral replication, we analyzed 1000 PCV2 genome, the alignment result showed the differences between PCV2b and PCV2d in 6<sup>th</sup>, 34<sup>th</sup>, 77<sup>th</sup> amino acids. Moreover, for the PCV2b and PCV2d, the mutation frequency of the 6<sup>th</sup> is 1.75% and 26.17%, respectively. Here we used site-directed mutagenesis to mutate the 6<sup>th</sup> aa of Rep between PCV2b and PCV2d infectious clone to assess the effect of key sites on viral replication.

## 2. Materials and Methods

The plasmid containing full-length PCV2b was mutated into PCV2b-6M with primers b-d-F and b-d-R, and the PCV2d plasmid was mutated into PCV2d-6M with primers d-b-F and d-b-R. The sequence of the primers was shown in Table 1. Methods and primers for construction of double copy infectious cloning were referred with articles published in our laboratory (Zhang et al., 2018). The four dual-copy plasmids constructed were

named as 2PCV2b, 2PCV2b-6M, 2PCV2d and 2PCV2d-6M. Genome sequence was used to determine the success of site-specific mutations.

The four different recombinant plasmids were transfected into porcine kidney-15 (PK-15) cells free of PCV using Lipofectamine 3000 (Invitrogen). The four viruses were harvested 72 hour (72h) post transfection and store at -80°C. The empty pEASY-Blunt vector was included as negative controls. The indirect immunofluorescence assay (IFA) and mRNA amplification were performed to determine the success of virus rescued (Cheung et al., 2003). Viral load of 10<sup>th</sup> passage was calculated using a real-time quantitative PCR. The viral titer and one-step growth curve of the 10<sup>th</sup> generation virus were calculated at different harvest time points. All assays were repeated at least three times, with each experiment performed in triplicate. One-way ANOVA and Student's *t* tests were used to compare results between different groups. All statistical analysis was performed using GraphPad Prism.  $P < 0.05$  was considered to be statistically significant.

Table 1 Site-directed mutagenesis primer name and sequence

Primer	Sequence (5'-3')	References
d-b-F	CAGCAAGAAGAATGGAAGAAGCGGAC	This study
d-b-R	CTTCTTCCATTCTTCTTGCTGGGCAT	This study
b-d-F	CCAGCAAAAAGAGTGAAGAAGCGGA	This study
b-d-R	CTTCTTCCACTCTTTTTGCTGGGCATG	This study

### 3. Results and discussion

The sequence results showed that the 6<sup>th</sup> aa of PCV2b-6M Rep protein was mutated from asparagine (N) to serine (S), and the 6<sup>th</sup> aa of PCV2d-6M Rep protein was mutated from serine (S) to asparagine (N). The results were shown in Figure 1.

Majority	MPSKKSGRSGPQPHKRWFTLNNPSEDERKKI RELPI SLFDYFI VGEEGN	
	10                      20                      30                      40                      50	
PCV2b.seq	..... N.....	148
PCV2b-6M.seq	..... S.....	148
PCV2d.seq	..... S.....	148
PCV2d-6M.seq	..... N.....	148
Majority	EEGRT.PHLQGFANFVKKQTFNKVKWVFGARCHI EKAKGTDQQNKEYCSKE	
	60                      70                      80                      90                      100	
PCV2b.seq	..... S.....	298
PCV2b-6M.seq	..... S.....	298
PCV2d.seq	..... S.....	298
PCV2d-6M.seq	..... S.....	298

IFA with PCV2 specific antibody (Gene Tex) confirmed that the four different infectious clones were infectious (Fig.2). 486bp specific bands were detected for all the four viruses through RT-PCR, indicating that the Cap gene mRNA had normal specific splicing and expression, so the four viruses were successfully rescued (Fig.3).

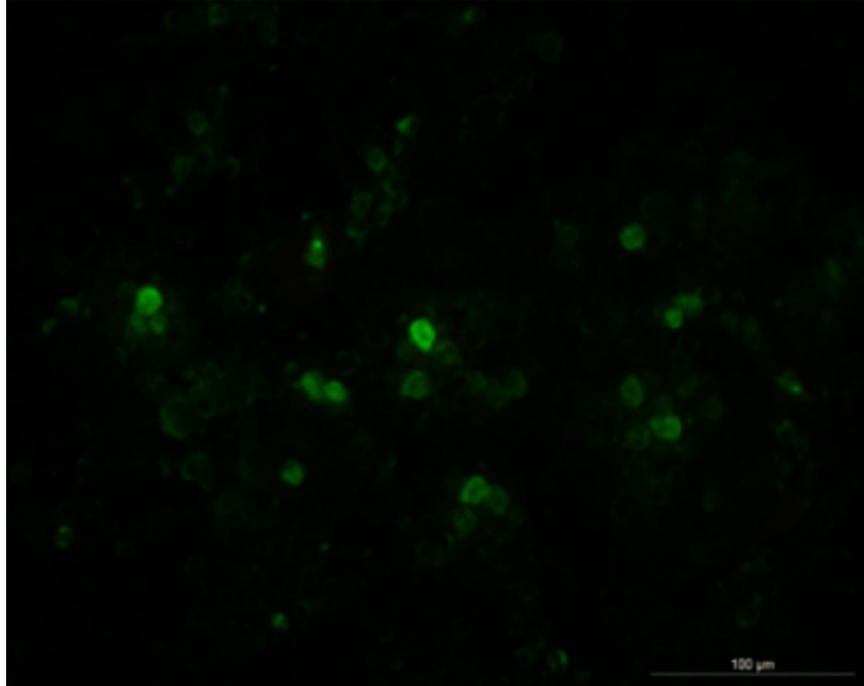


Fig.2 IFA results of PCV2 double copy infectious clone transfected in vitro in PK-15 cells (200×). There was number same with transfected cells. (A) 2PCV2b infectious clone. (B) 2PCV2b-6M infectious clone. (C) 2PCV2d infectious clone. (D) 2PCV2d-6Minfectious clone. (E) Empty vector.

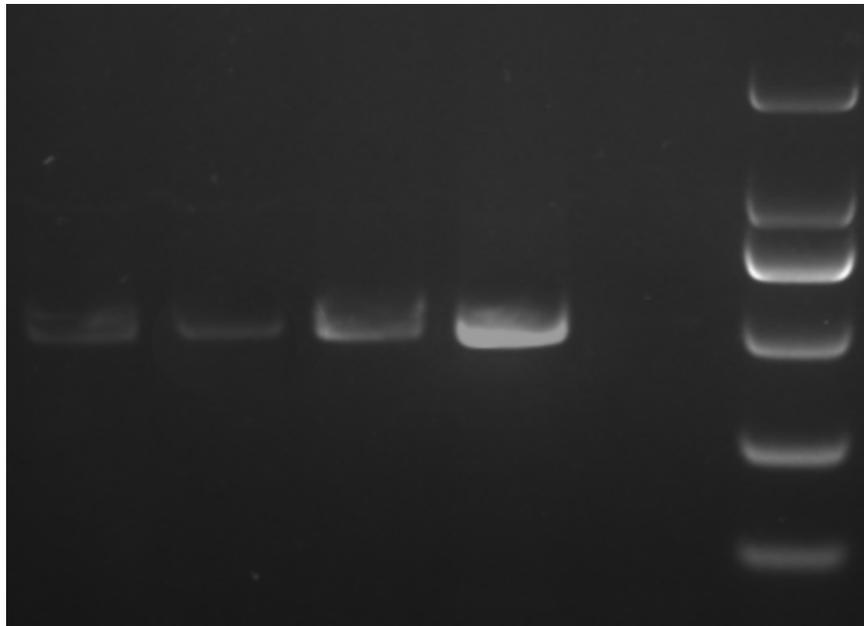


Fig.3 Result of mRNA RT-PCR amplification. A: 2PCV2b; B: 2PCV2b-6M; C: 2PCV2d; D: 2PCV2b-6M; M: DL 2000 Marker.

The real-time quantitative PCR result showed that the viral load of 2PCV2b group was similar to that of 2PCV2b-6M. However, the viral load of 2PCV2d-6M group was higher than that of 2PCV2d group, with significant difference ( $P < 0.05$ ). Similarly, 2PCV2b and 2PCV2b-6M are significantly higher than 2PCV2d ( $P < 0.01$ ) (Fig.4). The infectious titers for each virus at different time points were determined by IFA. The infectious titers increased from 24 to 72h but decreased from 72 to 84h for three viruses. By 72h post-infection, they have the highest viral titer. And the viral titer of mutant virus 2PCV2b and 2PCV2b-6M are highest of four viruses at 72h after infection (Fig.5).

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Fig.5 The one-step growth curve of 2PCV2b, 2PCV2-6M, 2PCV2d and 2PCV2d-6M) produced by transfection of PK-15 cells with DNA clones. The infectious titers were determined by IFA according to the Student's *t* tests methods.

In conclusion, this study demonstrated that the 6<sup>th</sup> aa mutations within the PCV2 Rep protein are important for enhanced PCV2d replication ability in PK-15 cells, having no effect on the replication capability of PCV2b. Based on these findings, with other genes that contributes to changes the virulence and pathogenicity, it is possible to find an inactivated vaccine candidate strain of PCV2 with high viral titer to protect pigs from porcine circovirus diseases.

### Conflict of interest

The authors declare that they have no competing interests.(both financial and non-financial interests).

### Author contributions

Jun Li and Changxun Xin conceived the project; Changxun Xin designed the experiments; Xiaoyan Wu, Shuo Wang, Jianli Shi, Zhe Peng, Chang Liu, Hong Han performed most of the experiments; Chen Li, Yao Tian, Jiaxin Li, Shaojian Xu contributed materials and participated in discussion; Xiaoyan Wu wrote the manuscript; Jun Li and Fan Zhang supervised the work and edited the final version of the manuscript which was read and approved by all authors.

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### References

An, D., Roh, I., Song, D., Park, C.&Park, B. K. (2007). Phylogenetic characterization of porcine circovirus type 2 in PMWS and PDNS Korean pigs between 1999 and 2006. *Virus Res* , 129, 115-122. doi: 10.1016/i.virusres.2007.06.024

Breitbart, M., Delwart, E., Rosario, K., Segalés, J., Varsani, A.&ICTV Report Consortium. (2017). Ictv virus taxonomy profile: circoviridae. *Journal of General Virology* , 98(8), 1997-1998. doi:10.1099/jgv.0.000871

Cheung, A. K. (2003). Transcriptional analysis of porcine circovirus type 2. *Virology* , 305(1): 168-180. doi: 10.1006/viro.2002.1733

Franzo, G.&Segalés, J.(2018). Porcine circovirus 2 (PCV-2) genotype update and proposal of a new genotyping methodology. *PLoS one* , 13(12): e0208585. doi:10.1371/journal.pone.0208585

Fenaux, M., Opriessnig, T., Halbur, P., Elvinger, F., Meng, X.(2004). Two amino acid mutations in the capsid protein of type 2 porcine circovirus (PCV2) enhanced PCV2 replication in vitro and attenuated the virus in vivo. *Journal of Virology* , 2004, 78(24):13440-13446. doi:10.1128/JVI.78.24.13440-13446.2004.

Mankertz, A.&Hillenbrand, B. (2001). Replication of porcine circovirus type 1 requires two proteins encoded by the viral rep gene. *Virology* , 279(2), 429-438. doi: 10.1006/viro.2000.0730

Palinski, R., Pineyro, P., Shang, P., Yuan, F., Guo, R., Fang, Y., et al. (2017). A novel porcine circovirus distantly related to known circoviruses is associated with porcine dermatitis and nephropathy syndrome and reproductive failure.1. *Virology* , 91:e01879-16. doi: 10.1128/VI.01879-16

Shi, J., Peng, Z., Fu, F., et al. (2015). Mutant Rep protein of the porcine circovirus type 2 N-glycosylation: 23-25aa, 256-258aa mutation reduced virus replication but 286-288aa mutation enhanced virus replication in PK-15 cells. *Veterinary microbiology* , 2015, 177(3-4): 370-372. doi:10.1016/j.vetmic.2015.03.016.

Wang, S., Xin, C., Wu, X., Shi, J., Peng, Z., &Sun, P., et al. (2020). Genetic characterization of porcine circovirus type 2 from 2013 to 2018 in Shandong province, China. *Veterinary Medicine and Small Animal Clinician* , 6(1). doi:10.1002/vms3.196

Zhang, L. et al.(2018) .The correlation between the mutual deletions of amino acids within porcine circovirus rep protein and the discrepancy of replication. *Microbial pathogenesis* , 117: 327-334. doi: 10.1016/j.micpath.2018.02.045

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Majority	MPSKKS	GRSGP	QPHKR	WFTLN	NPSEDER	KKI	RELPI	SLFDYFI	VGEEGN	
		10	20	30	40	50				
PCV2b.seq	N									148
PCV2b-6M.seq										148
PCV2d.seq										148
PCV2d-6M.seq	N									148
Majority	E	EGRTP	PHLQGF	ANFV	KKQTF	NKVK	KWYF	GARCHI	EKAKGT	DQQNKEYCSKE
		60	70	80	90	100				
PCV2b.seq										298
PCV2b-6M.seq										298
PCV2d.seq										298
PCV2d-6M.seq										298

