

# 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 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.

## 1. Introduction

Porcine circovirus is the smallest virus known to replicate autonomously in mammalian cells<sup>1</sup>. 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)<sup>2</sup>. Similar to PCV2, PCV3 may cause reproduction disorder in sows and PDNS in adult pigs<sup>3</sup>.

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<sup>4,5</sup>.

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<sup>6</sup>. Studies have shown that mutations in Cap protein from proline to alanine in 110th and from arginine to serine in 191th could enhance the replication capacity of PCV2 in vitro and reduce toxicity in vivo<sup>7</sup>. It had been shown that altering the amino acid sequence at the Rep protein also altered the replication ability of the virus<sup>8</sup>. 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 6th, 34th, 77th amino acids. Moreover, for the PCV2b and PCV2d, the mutation frequency of the 6th is 1.75% and 26.17%, respectively.

Here we used site-directed mutagenesis to mutate the 6th 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(b-d-F:5'-CCAGCAAAAAGAGTGGAAGAAGCGGA-3', b-d-R:5'-CTTCTTCCACTCTTTTTGCTGGGCATG-3'), and the PCV2d plasmid was mutated into PCV2d-6M with primers d-b-F and d-b-R(d-b-F:5'-CAGCAAGAAGAATGGAAGAAGCGGAC-3', d-b-R:5'-CTTCTTCCATTCTTCTTGCTGGGCATG-3'). Methods and primers for construction of double copy infectious cloning were referred with articles published in our laboratory<sup>9</sup>. 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<sup>10</sup>. Viral load of 10th passage was calculated using a real-time quantitative PCR. The viral titer and one-step growth curve of the 10th 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.

## 3. Results And Discussion

The sequence results showed that the 6th aa of PCV2b-6M Rep protein was mutated from asparagine (N) to serine (S), and the 6th aa of PCV2d-6M Rep protein was mutated from serine (S) to asparagine (N).

IFA with PCV2 specific antibody (Gene Tex) confirmed that the four different infectious clones were infectious (Fig. 1). 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. 2).

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.3A). 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.3B).

In conclusion, this study demonstrated that the 6th 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.

## Declarations

### 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, ZhePeng, 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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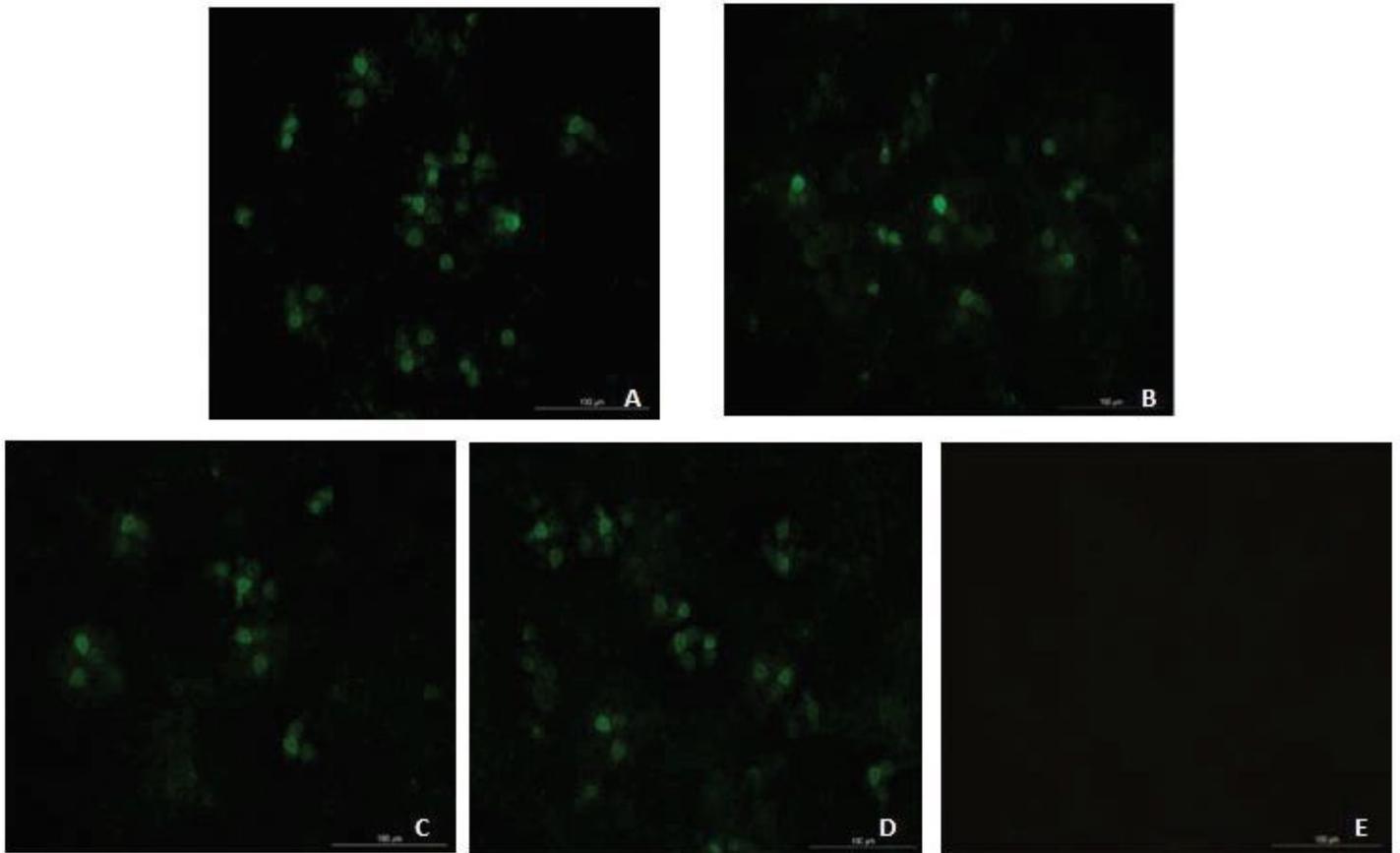
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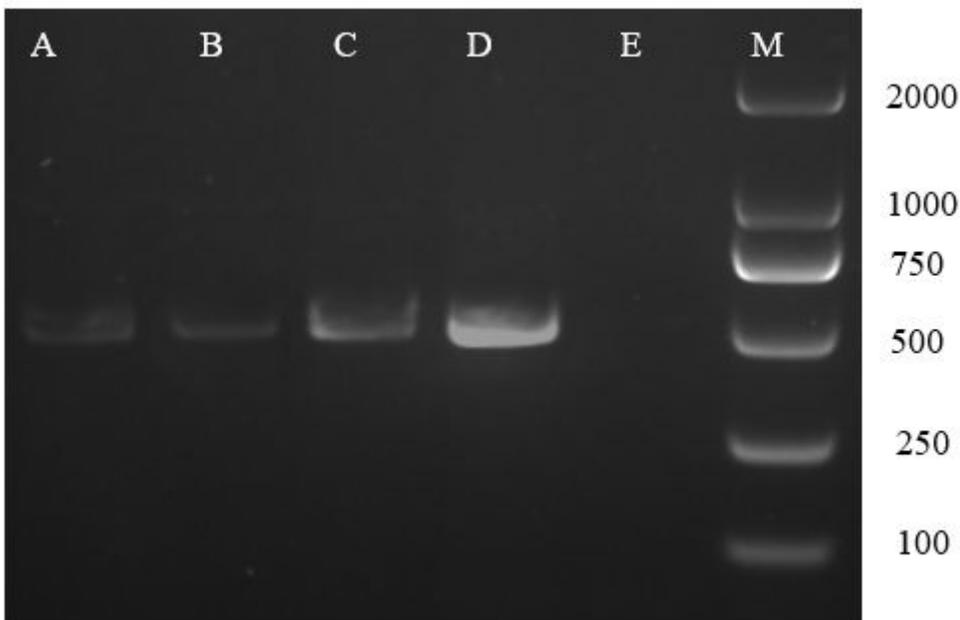
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## Figures



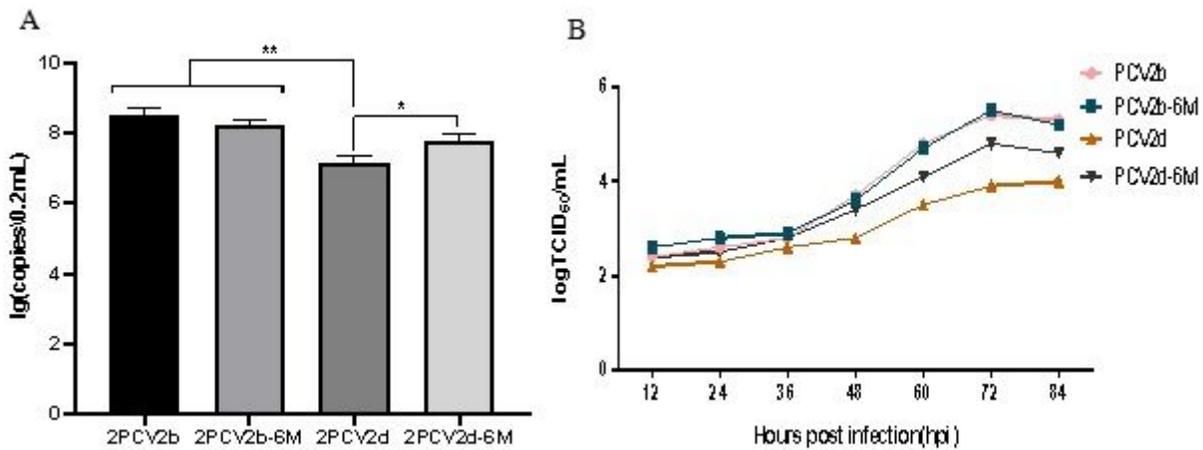
**Figure 1**

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.



**Figure 2**

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



**Figure 3**

The 6th amino acid mutation of Rep protein enhances the replication ability of PCV2 by increasing the level of viral RNA synthesis. A: Result of PCV2 double copy infectious clone viral load of 10th passage using real-time quantitative PCR in 72h.;B: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.