



Short communication

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



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ARTICLE INFO

Article history:

Received 12 January 2015

Received in revised form 10 March 2015

Accepted 14 March 2015

Keywords:

Porcine circovirus type 2

N-Glycosylation

Mutation

Infectious clone

Rescued

ABSTRACT

Porcine circovirus type 2 (PCV2) Rep protein and the splice variant Rep' protein impact genome replication. The Rep protein contains three potential N-glycosylation at positions 23–25aa (NPS), 256–258aa (NQT) and 286–288aa (NAT). Three double copy infectious clones with Rep protein N-glycosylation at positions mutations 23–25aa (DPS), 256–258aa (DQT) and 286–288aa (DAT) were constructed and their function in virus replication in PK-15 cells was investigated. The results showed that the double copy infectious clone with N-glycosylation site mutation could be rescued in vitro and 23–25aa, 256–258aa mutation reduced virus replication but 286–288aa mutation enhanced virus replication.

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1. Introduction

Porcine circovirus type 2 (PCV2) has been identified as the primary causative agent of post-weaning multisystemic wasting syndrome (PMWS) that primarily affects pigs from 5 to 12-week-old pigs and has been associated with morbidities of 5–30%, resulting in substantial economic losses to the pig industry (Segalés et al., 2005; Bolin et al., 2001). PCV2 has a circular DNA genome that encodes three well-characterized open reading frames (ORFs). ORF1 encodes Rep protein that is involved in genome replication.

Rep contains three potential N-glycosylation sites 23–25aa (NPS), 256–258aa (NQT) and 286–288aa (NAT). ORF2 encodes the dominant immunogenic capsid protein, Cap, that contains one putative N-glycosylation site at amino acids 143–145 (NYS) (Nawagitgul et al., 2002). The ORF3 encodes a non-structural protein characterized as a pro-apoptotic protein (Liu et al., 2005).

N-Glycosylation promotes proper folding of envelope proteins in many viruses and plays an important role in viral pathogenesis and immune evasion (Vigerust and Shepherd, 2007). Deletion of the single putative N-glycosylation site of the PCV2 Cap protein enhances specific immune responses following DNA immunisation in mice (Gu et al., 2012). Rep protein and the splice variant Rep' protein impact PCV2 genome replication. There are no reports about the effects of PCV2 Rep protein N-glycosylation site mutagenesis on virus

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Table 1
Oligonucleotide primers used to generate PCV2 infectious clone.

Position	primer sequence (5' to 3')
PCV2 R	TGTCCGCGGGCTGGCTGAACCTTTTGAAGTGA (<i>Sac</i> II)
PCV2 F	GCCCCGCGAAATTTCTGACAAACGTTACA (<i>Sac</i> II)
PCV2 23–25aa R	TTCTTGCGCTCGTCTTCGGAAGGATcATTCAGA GTGAACA
PCV2 23–25aa F	TGTTCACTCTGAATgATCCTTCCGAAGACGAGCGCAAGAA
PCV2 256–258aa R	ATTCCAACGGGTCTGATcGCTGGTAATCAGAATA
PCV2 256–258aa F	TATTCTGATTACCAGcGATCAGACCCCGTTGGAAT
PCV2 286–288aa R	TGGATTGTCTGTAGCATcCTTCCAAAATACCAAG
PCV2 286–288aa F	CITGGTATTTTGAAGgATGCTACAGAACAATCCA
PCV2 (double) R	GCACTCGAGCCGAAATTT CTGACAAACGTTA (<i>Xho</i> I)
PCV2 (double) F	GAAGGATCCCGGGCTGGCTGAACCTTTT GAAAG (<i>Bam</i> HI)

replication. In this study, we constructed three double copy mutant infectious clones with Rep protein N-glycosylation positions 23–25aa (DPS), 256–258aa (DQT) and 286–288aa (DAT). The result show that 23–25aa, 256–258aa mutation reduced virus replication but 286–288aa mutation enhanced virus replication in PK-15 cells.

2. Materials and methods

The primers used in this study are listed in Table 1. The PCV2 whole genome was amplified into two segments with primers PCV2 R/PCV2 23–25aa R, PCV2 F/PCV2 23–25aa F. The overlap extension PCR with primers PCV2 R/F was used to amplify the PCV2 whole genome with 23–25aa N-glycosylation site mutant. As a PCR template, the PCV2 whole genome A with the *Sac*II and *Xho*I restriction enzyme sites was amplified using primers PCV2 R/PCV2 (double) R and inserted into pEASY-Blunt vector. The PCV2 whole genome B with the *Sac*II and *Bam*HI restriction enzyme sites was amplified using primers PCV2 F/PCV2 (double) F and inserted into pEASY-Blunt vector with PCV2 whole genome A. The mutant double copy infectious clones with Rep protein N-glycosylation positions

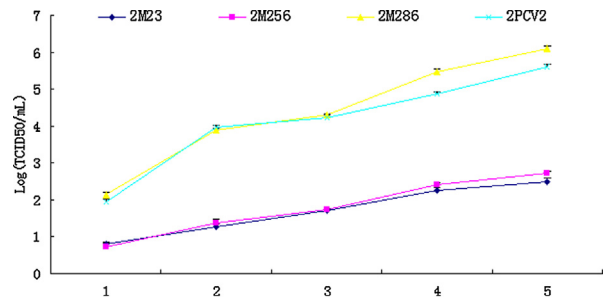


Fig. 2. Result of PCV2 double copy infectious clone infectious titer of rescued virus 1–5 passages in 72 h. The titer of 2M286 was $10^{6.0}$ TCID₅₀/mL, while 2PCV2 $10^{5.5}$ TCID₅₀/mL, which was higher than 2M23 and 2M256 ($10^{2.5}$ and $10^{2.7}$ TCID₅₀/mL).

23–25aa (DPS) designated 2M23 was confirmed by DNA sequence analysis. The mutant double copy infectious clone with Rep protein N-glycosylation positions 256–258aa (DQT) named 2M256 and 286–288aa (DAT) named 2M286 were constructed as above. The infectious clone was transfected into porcine kidney-15 (PK-15) cells free of PCV using Lipofectamine 2000 (Invitrogen) according to the protocol supplied by the manufacturer and harvested 3 days post transfection and stored at -80°C until use. The empty pEASY-Blunt vector and no mutant double copy infectious clone named 2PCV2 were included as negative and positive controls. An immunofluorescence assay (IFA) was performed to determine if the virus was rescued. The infectious PCV2 titer of the rescued virus after 5 passages was calculated and virus load of 5th passage was calculated using a fluorescent quantitative PCR (Li et al., 2013). 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 SPSS version 16.0. $P < 0.05$ was considered to be statistically significant.

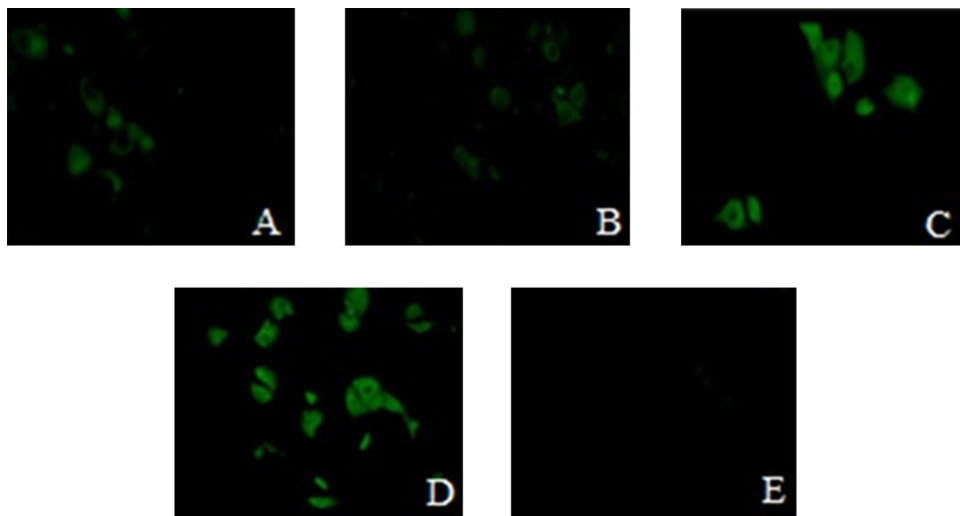


Fig. 1. IFA results of PCV2 double copy infectious clone transfected in vitro in PK-15 cells. There was number difference with transfected cells. (A) 2M23 rescued virus. (B) 2M256 double copy infectious clone. (C) 2M286 double copy infectious clone. (D) 2PCV2 double copy infectious clone. (E) Empty vector.

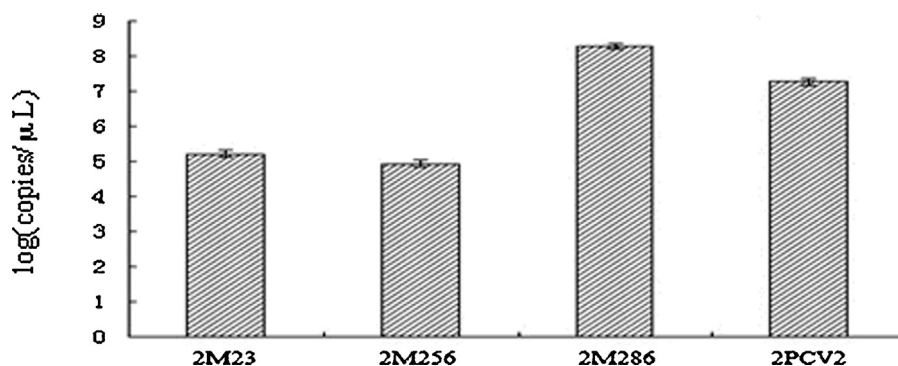


Fig. 3. Result of PCV2 double copy infectious clone virus load of 5th passage using fluorescent quantitative PCR in 72 h. The data showed that there was significant difference in the double copy infectious clones ($P < 0.05$), 2M286 had more copies than 2PCV2, 2M23 and 2M256 had fewer copies.

3. Results and discussion

IFA with PCV2 specific antibody (Anti cap Mab) confirmed that the double copy infectious clone was infectious. The number of 2M286 and 2PCV2 transfected cells was greater than 2M23 and 2M256. The cells that were mock transfected with the empty vector remained negative for PCV2 antigen (Fig. 1). The TCID₅₀ result showed that passage 1 and 2 with a low infectious titer and passage 5 with a stabilized infectious titer. The titer of 2M286 was $10^{6.0}$ TCID₅₀/mL, while 2PCV2 $10^{5.5}$ TCID₅₀/mL, which was higher than 2M23 and 2M256 ($10^{2.5}$ and $10^{2.7}$ TCID₅₀/mL) (Fig. 2). The fluorescent quantitative PCR result showed that there was a significant difference among the double copy infectious clones ($P < 0.05$), 2M286 had more copies than 2PCV2, 2M23 and 2M256 had fewer copies (Fig. 3). These results showed that the double copy infectious clone with N-glycosylation site mutation could be rescued in vitro and 23–25aa, 256–258aa mutation reduced virus replication but 286–288aa mutation enhanced virus replication. The main reason that N-glycosylation mutagenesis affected Rep protein post-translational modification and virus assembly requires more research.

In conclusion, this study demonstrated that the double copy infectious clones with N-glycosylation site mutation could be rescued in vitro and 23–25aa, 256–258aa mutation reduced virus replication but 286–288aa mutation enhanced virus replication. Based on these findings it may be possible to find a high titer virus to inactivate to protect pigs from porcine circovirus diseases.

Disclosure

No competing financial interests exist.

Acknowledgments

The study was supported by the National Natural Science Funds (31201889 and 31170146), Shandong Province Modern Agricultural Industry Technology System (SDAIT-06-021-07), Shandong Province Science and Technology Development Plan (2014GNC111011), Young Talents Training Program of Shandong Academy of Agricultural Sciences and the Youth Scientific Research Foundation of Shandong Academy of Agricultural Sciences (2014QNM40).

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