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SHORT COMMUNICATION

Development of a Real-Time Quantitative PCR Assay for Detection of Porcine Circovirus Type 2e

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ABSTRACT

PCV2e shows high sequence similarity to other PCV2 genotype, causing greater difficulties in the molecular biological identification of pathogens. In this study, we developed a TaqMan-based RT-qPCR for efficient detection of PCV2e in clinical samples. We designed specific primers and a probe for the PCV2e ORF2 gene and then identified target genes using the optimized PCR reaction system and reaction conditions. The results indicated that the cycle threshold (*Ct*) and the standard DNA template had a linear relationship between 10^3-10^8 copies/μL, and the determination coefficient was 0.998. The assay has a detection limit of 100 copies per reaction and a standard deviation of cycle thresholds below 1.00. The PCR method we established can specifically identify PCV2e, did not cross-react with other genotypes of PCV, as well as PRV and PPV. The RT-qPCR assay can be used for the special diagnosis and epidemiological investigation of PCV2e.

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INTRODUCTION

PCV is a small non-enveloped virus with a circular single-stranded DNA genome, which belongs to the genus Circovirus of the family Circoviridae (Poulsen Nautrup *et al*., 2021). PCV2 has been demonstrate to cause PMWS, PDNS, reproductive disorders, pneumonia, piglets congenital tremors and enteritis associated with cyclovirus (Han *et al*., 2021). Moreover, PCV2 is often co-infected with pathogens such as porcine reproductive and respiratory syndrome virus, Mycoplasma hyopneumoniae, Haemophilus parasuis, hemolytic streptococci, and swine influenza virus, resulting in decreased breeding efficiency of sows, piglets growth performance, feed remuneration, and increase in case fatality rate due to immune suppression (Saade *et al*., 2020; Dinh *et al*., 2021). The PCV2 genome is 1,767-1,777 bp nucleotides long. There are two main open reading frames (ORFs) in its antisense direction. ORF1 encodes its replication protein (Rep) and ORF2 encodes its capsid protein (Cap) (Wu *et al*., 2021). In 2011, PCV2e (PCV2a-like) was detected in clinical samples in China and was rapidly prevalent between Chinese pig farms (Zhai *et al*., 2011). However, a significantly different PCV2e was found in North America, and was also reported in China (Davies *et al*., 2016; Liu *et al*., 2018). The identification of the emerging PCV2e is very difficult in clinical samples, especially excluded from PCV1, PCV2 (a-h), PCV3, PRV and PPV. Furthermore, the diagnosis of PCV2e in the early stage of infection will help prevent the prevalence of PCV associated diseases by eliminating and purifying ill pigs. Therefore, the veterinary sector needs to establish an effective, rapid and easy to use detection method. Here, we established a reliable and rapid RT-qPCR assay to detect the epidemiology and pathogenesis of PCV2e.

MATERIALS AND METHODS

Viruses, positive plasmids and clinical samples: Positive recombinant plasmids of PCV2a to PCV2e (pUC47-PCV) were achieved from Sangon Biotech (Shanghai, China), PCV2e representative strain was

Table 1: Sequences of the three sets of probes and primers

Name	Forward primer PCV 2e-F	Reverse primer PCV 2e-R	Probe PCV 2e-P
PCV2e-81	5'- TCTCCAATATTAAATCTCATCA-3'	5'- CTCCTATGTATATACTGTTGTAA-3'	5'- TCCACAGTCACACCGCCATC-3'
PCV2e-117	5'- GGTCTTTCATATTAAATTCTCTAAA-3'	5'- CTGGTAATGTGGACCATA-3'	5'- TTCAACACAGTACCACTGCC-3'
PCV2e-133	CAGGTTATTGGTTTTGTTTAC	CTTCGAATACTACAGAATAAGAA	CTAAGATTACAGCACTGGAGCCTACT

selected from existing strains in GenBank (accession number: KT795287). PCV1, PCV3, PRV and PPV were preserved by the Institute of Animal Health of the Guangdong Academy of Agricultural Sciences (Guangzhou, China). Clinical samples were collected from markets of Guangzhou, China.

Design and screening of the probes and primers: According to the ORF2 gene of PCV2e published in GenBank (accession number: KT795287), three sets of specific primer and probe sequences were designed according to the conserved region of the ORF2 gene using OligoArchitect software (Sigma-Aldrich, USA) (Table 1). The final concentration of the probes in the system was optimized using the single factor control variable test.

Optimization of the RT-qPCR reaction system and conditions of RT-qPCR: The RT-qPCR reaction system was prepared according to the instruction of FastFire qPCR PreMix kit. The volume of reaction system was 25 µl: 12.5 µl of 2×FastFire qPCR PreMix, 1.0 µl of PCV2e-F, 1.0 µl of PCV2e-R, 1.0 µl of PCV2e-Probe, 2.0 µl of PCV DNA template, and 7.5 µl of ddH_2O. The RT-qPCR condition was: 2 min at 95°C, followed by 45 cycles (5 s at 95°C and 15 s at 58°C) and scanning for the fluorescence signal in each cycle.

The PCR reaction system and and PCV2e reaction conditions were optimized using the following methods. The primer and probe concentrations were first optimized to 0.2, 0.4, 0.6, 0.8 and 1.0 µM, initial annealing temperature was 58°C, the initial probe concentration was 0.2 µM and 10^2 copies of pUC47-PCV2e. The determination of optimum reaction was based on cycle threshold (CT) value and FAM signals in the positive results. The optimized reaction conditions were used in the following tests.

Specificity and sensitivity test: To investigate the specificity of RT-qPCR, DNA from PCV1, PCV2(a-e), PCV3, PRV and PPV was selected as a template for RT-qPCR, respectively. To detect the sensitivity of the established method, the positive plasmids (pUC47-PCV2e, the initial concentration was 40 ng/µl, i.e., 10^{10} copies/µl) were 10-fold diluted. The fluorescence signals corresponding to the melting peaks were obtained between 10^0 and 10^7 copies per reaction for each concentration, and the detection limit was established as the lowest copy number of pUC47-PCV2e for which at least one was replicate tested positive.

Reproducibility test and standard curve: Reproducibility tests were performed by performing the assay in three dilutions of 10^4, 10^6 and10^8 copies/µl, with three replicates for each dilution in one run. The assay was tested three replicates on a range of pUC47-PCV2e dilutions from 10^1 to10^8 copies/µL, established a standard curve.

Detection of biological samples: A total of one hundred Pork samples were collected from markets in Guangzhou province, China. Viral DNA was first extracted from the collected samples using the PureLink Viral RNA/DNA Mini Kit (ThermoFisher Scientific, Massachusetts, USA) followed by detection of PCV2e infection using the RT-qPCR method established in this study. Positive (RT-qPCR system with pUC47-PCV2e plasmid as template) and negative (RT-qPCR system without template) controls were included in each run.

RESULTS AND DISCUSSION

In this study, the ORF2 gene of MEX/41238/2014 (accession number: KT795287) was selected as a template to design primer and probe based on the sequence alignment results of each PCV2e and other PCV genotypes (Fig. 1A). Although the PCV2e-133 primer sequence binding site was highly specific, the lack of suitable position allowed the probe to avoid the repeats, and the GC content was so high that the primer and probe set was abandoned. The other two sets of primers and probe (PCV2e-81 and PCV2e-117) were used for feasibility testing. Positive PCV2(a-d) recombinant plasmids and pUC47-PCV2e were used to test the sensitivity and specificity of the two sets of primers and probe. PCV2e-81 did not respond specifically to the PCV2e positive plasmid, however, and its amplification curve did not fit the amplification kinetics and one of the repeats lacked an exponential amplification period (Fig. 1B). PCV2e-117 could react specifically with PCV2e positive plasmid, and the amplification curve fits the amplification kinetics and no nonspecific reactions were observed (Fig. 1C). The alignment of PCV2e-117 primer and probe sequences with the sequences of each PCV genotype is shown in Fig. 1D. Therefore, PCV2e-117 was used as the primer and probe of the RT-qPCR method for evaluation experiments. The optimal concentration of the PCV2e-117 primers and probe were 0.4 µM based on the comparison results of C_T values (Table 2). The reproducibility of SDs with CT values below 1.00 is acceptable. Regarding the specificity test, the primers we designed showed admirable specificity, which could identify PCV2e from samples (Fig. 1E). Fig. 1F showed that the maximum dilution for generating the amplification curve is 10^2 copies/µl, i.e., the method sensitivity was 10^2 copies/µl (4×10^{-4} pg/µl). The reproducibility test at three concentration levels showed that standard deviations (SD) were less than C_T values lower than 1.00 (Supplementary Table S1). Thus, this detection method we established meets the requirements. A linear standard curve was generated as templates from 10^3 to 10^8 copies/µl, the standard equation is $y=-3.87x+43.04$ (x is the logarithm of template concentration based on 10, and $3.0 \leq x \leq 8.0$). The coefficients of PCR efficiency and determination were 81% and 0.998,

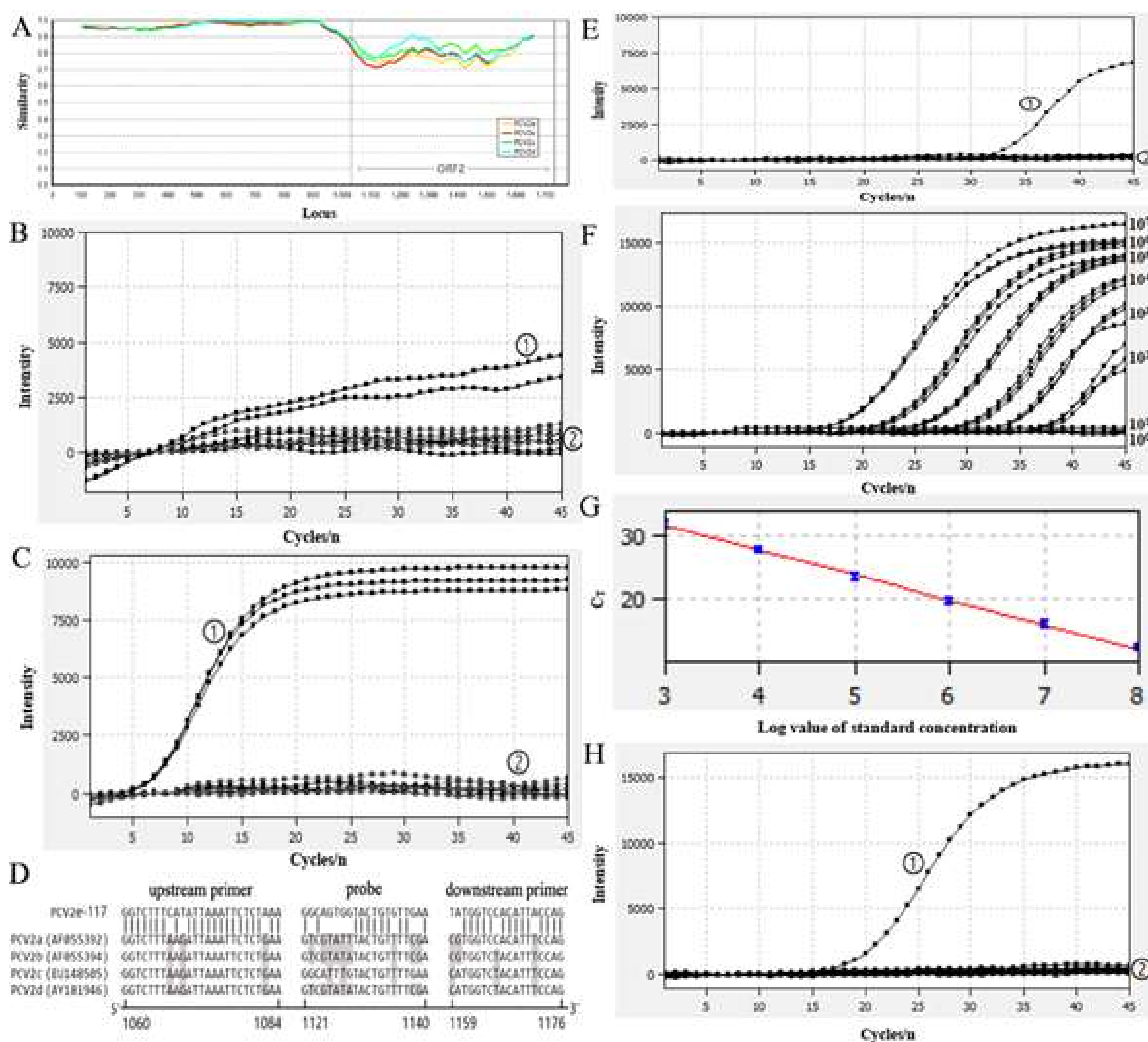

Fig. 1: Similarity curves of PCV2e to other PCV genotypes (**A**). Feasibility analysis of the PCV2e-81 (**B**) and PCV2e-117 (**C**). ①: RT-qPCR amplification curves with PCV2e plasmid as a DNA template; ②: RT-qPCR amplification curves with PCV2a, PCV2b, PCV2c, and PCV2d plasmid as a DNA template, respectively. **D:** Comparison of the primer and probe sequences of PCV2e-117 with the sequences of each genotype of PCV. **E:** Specificity of the established RT-qPCR method. ①: RT-qPCR amplification curves with PCV2e as a DNA template; ②: RT-qPCR amplification curves with PCV1, PCV2a, PCV2b, PCV2c, PCV2d, PCV3, PPV and PRV as a DNA template, respectively. **F:** Sensitivity of the established RT-qPCR method. **G:** Standard curve of the RT-qPCR. **H:** Sensitivity of the established RT-qPCR method. ①: PCV2e positive control; ②: Clinical samples and negative control.

Table 2: Comparison of C_T values for different concentrations of primers and probe combinations.

Final concentration (μM)	Primers	Probe
	Mean C_T ± SD (n=3)	Mean C_T ± SD (n=3)
0.2	28.73±0.34	29.63±0.26
0.4	28.35±0.13	28.77±0.63
0.6	28.47±0.08	29.18±0.36
0.8	28.64±0.07	29.01±0.17
1.0	28.58±0.15	29.03±0.49

Supplementary Table S1: Reproducibility of the RT-qPCR.

Concentration of pUC47-PCV2e(copies/μL)	Mean C_T ± SD
10^4	27.78±0.62
10^6	19.45±0.76
10^8	12.63±0.50

respectively (Fig. 1G). One hundred pork samples collected from the market were tested for PCV2e using the assay established in this study (Fig. 1H). The results showed that all samples were negative for PCV2e, indicating that these pigs may not be infected with PCV2e. In summary, these results suggested that the developed RT-qPCR is practical and dependable for PCV2e detection, and it is expected to be of benefit in epidemiological and pathogenesis studies of PCV2e.

Conflict of interest: The authors state that there are no competing interests.

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Authors contribution: Conceived and designed the experiments: XW, DL, QZ, CJ and SLZ. Performed the experiments: XW, DL, QZ and XZ. Analyzed the data: XW, DL, MAG, MN and MUS. Contributed reagents/materials/analysis tools: XW, DL and QZ. Wrote the paper: XW, DL, QZ and XZ.

REFERENCES

Davies B, Wang X, Dvorak CM, *et al*. 2016. Diagnostic phylogenetics reveals a new Porcine circovirus 2 cluster. Virus Res 217:32-37.

Dinh P, Nguyen M, Nguyen H, *et al.,* 2021. Porcine circovirus genotypes and their copathogens in pigs with respiratory disease in southern provinces of Vietnam. Arch Virol 166:403-11.

Han L, Yuan G, Chen S, *et al*., 2021. Porcine circovirus type 2 (PCV2) infection in Hebei Province from 2016 to 2019: a retrospective study. Arch Virol 166:2159-71.

Liu J, Wei C, Dai A, *et al*. 2018. Detection of PCV2e strains in Southeast China. Peer J 6:e4476.

Poulsen Nautrup B, Van Vlaenderen I, Mah C, *et al*., 2021. Do high levels of maternally derived antibodies interfere with the vaccination of piglets against porcine circovirus type 2? A literature review and data analysis. Vaccines (Basel) 9:923.

Saade G, Deblanc C, Bougon J, *et al*., 2020. Coinfections and their molecular consequences in the porcine respiratory tract. Vet Res 51:80.

Wu X, Wu H, Wang H, *et al*., 2021. A new strategy to develop pseudorabies virus-based bivalent vaccine with high immunogenicity of porcine circovirus type 2. Vet Microbiol 255:109022.

Zhai S, Chen S, Wei Z, *et al*., 2011. Co-existence of multiple strains of porcine circovirus type 2 in the same pig from China. Virol J 8:517.