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Short communication

TaqMan-based quantitative real-time polymerase chain reaction assay to detect porcine circovirus-like virus

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Abstract

The aim of this study was to develop a rapid, sensitive and highly specific TaqMan quantitative real-time polymerase chain reaction PCR (qPCR) assay for porcine circovirus-like virus (PCLV). The primers and probe were designed based on the conserved regions of the PCLV ORF4 gene. The assay has a good detection performance ($y=-3.3257x+ 1.482$, $R^2=0.9905$), with a limit of detection of 10 copies, which was 100 times more sensitive than conventional PCR (cPCR). No cross-reactivity was observed with other common viruses. The intra- and inter-assay coefficients of variation were less than 1.25%. 36 fecal samples were analyzed using this method, detecting a positivity rate of 8.33% (3/36) that was higher than the cPCR detected. In summary, the established assay for PCLV detection has high specificity, sensitivity, and reproducibility and can be used as a tool for clinical diagnosis and epidemiological investigation.

Keywords: porcine circovirus-like virus, detection, ORF4 gene, TaqMan quantitative real-time PCR

Introduction

Since porcine circovirus-like virus (PCLV) was first identified in the United States in 2011, it has gradually begun to be widespread in China (Liu et al. 2021, Sun et al. 2021, Yang et al. 2021, Hu et al. 2022, Ji et al. 2023). Similar to PCV, PCLV is a closed circular, non-enveloped virus with single-stranded DNA of approximately 3.9 kb, belonging to the family Kirkoviridae (Zhao et al. 2019). However, it differs markedly from PCV in its genetic structure, containing only an open reading frame (ORF) and lacking the classical capsid (Cap) gene (Shan et al. 2011, Guo et al. 2018, Liu et al. 2021). PCLV is mainly associated with severe diarrhea and hemorrhagic enteritis in piglets (Yang et al. 2021). As this virus undergoes continuous evolution, the prevalence of PCLV in swine herds is anticipated to increase. There are several available assays to detect PCLV, including conventional polymerase chain reaction (cPCR) (Ji et al. 2023), SYBR green–based quantitative real-time PCR (qPCR) (Zhang et al. 2022), but none of them can achieve both high sensitivity and efficiency for the PCLV. Meanwhile, as this virus undergoes continuous evolution, the prevalence of PCLV in swine herds is anticipated to increase. Therefore, an accurate and rapid TaqMan qPCR assay with better specificity and sensitivity was established in this study to rapidly detect PCLV, which will contribute to epidemiological surveillance and lay the foundation for additional studies on the biological characteristics and pathogenesis of PCLV.

Materials and Methods

All the viruses – involved PCLV, PCV2 and porcine epidemic diarrhea virus (PEDV) – were kept by the laboratory. Live swine fever vaccine (tissue culture origin), live pseudorabies vaccine (strain Bartha-K61), inactivated swine parvovirus vaccine (strain WH-1), live porcine reproductive, and respiratory syndrome vaccine (strain R98) were purchased from the China Animal Husbandry Industry Co., Ltd. (Beijing, China).

Two primers and a probe were designed based on the highly conserved region of the PCLV ORF4 gene (Table 1). The length of the amplification product was 581bp. The product was then purified, cloned into the pMD-19-T vector, and transformed into DH-5α competent cells. The positive plasmid (pMD19-T-F2-R2) DNA was extracted and the concentration was detected. The following formula was used to determine the plasmid copy number: (concentration in ng × 6.02×10^{23})/(plasmid length × 10^9 × 660 Da/bp).

The amplification reaction was conducted in a 20 μL total volume mixture, comprising 10 μL of 2 × TaqMan Fast qPCR Master Mix (Sangon Biotech), 2 μL of DNF Buffer, 0.4 μL of forward primer (10 μmol/L), 0.4 μL of reverse primer (10 μmol/L), 0.2 μL of TaqMan probe (10 μmol/L), 1 μL of the DNA sample as template, and 6 μL of ddH_2O). The thermal cycling conditions were: 94°C for 3 min, followed by 40 cycles of 94°C for 5 s, 45.8°C for 15 s, and 72°C for 30 s.

To evaluate and validate the specificity of this TaqMan qPCR method, DNA/complementary DNA (cDNA) from PCLV, PCV2, PEDV, CSFV, PRV, PPV and PRRSV were used as templates and ddH_2O was used as the negative control for amplification. A 10-fold serial dilution of standard plasmid DNA (ranging from 1×10^8 to 1×10^0 copies/μL) and ddH_2O were prepared as templates to amplify using the TaqMan qPCR assay and cPCR to determine the limit of detection. Moreover, four different PCLV standard plasmid DNA concentrations (1×10^5 to 1×10^2 copies/μL) was used for reproducibility of qPCR assays. Each reaction was repeated three times in the same assay to determine the intra-assay coefficient of variation (CV) and every 7 days for three consecutive times to determine the inter-assay CV. The stability of the assay was evaluated based on the above CVs.

The ability of the TaqMan qPCR and cPCR assays were evaluated to detect PCLV in 36 clinical samples which were collected from diarrheic and non-diarrheic clinical fecal samples from five pig farms in Anhui province between June 2018 and October 2022. All positive samples were sequenced to confirm the results.

Results and Discussion

The assay generated a linear standard curve (y=-3.3257x+41.482) with a high R^2 value of 0.9905 and an efficient amplification efficiency of 99.8%, showing that the Ct value was linearly related to the logarithm of the plasmid copy number (Fig. 1). The detection limit of the TaqMan qPCR method is 10 copies (Fig. 2A), which is 100 times more sensitive than that of the cPCR assay (Fig. 2B), indicating that the assay has high sensitivity. Moreover, it exceeded the lowest sensitivity the reported SYBR green qPCR method could test (Zhang et al. 2022).

The specificity analysis result demonstrated that the TaqMan qPCR assay has a high specificity to detect PCLV; the negative control and the other viral samples (PCV2, PEDV, CSFV, PRV, PPV, and PRRSV) were negative (Fig. 3). The reproducibility results showed the intra-assay CV and inter-assay CV were 0.31%-1.13% and 0.25%-1.25% respectively, demonstrating good stability and reliability of the assay (Table 2). Evaluation of the clinical samples showed that the assay was effective in detecting PCLV infection, with a positive

Table 1. Primers and probe used in TaqMan qPCR assay.

Primer/probe	Sequence	Length (bp)
Primer-PCLV-F1	CCATACTGACTGAAGTCT	71bp
Primer-PCLV-R1	CTTCATTTTATGTCTCAGGA	
Probe-PCLV	FAM-5-ATGTATTCGACCCTTTCAGTGACT-3'-BHQ1	
Primer-PCLV-F2	GAATACTTTACTGTTTTTACACGGC	581bp
Primer-PCLV-R2	ATTTCTCACTTCATTTTATGTCTCA	

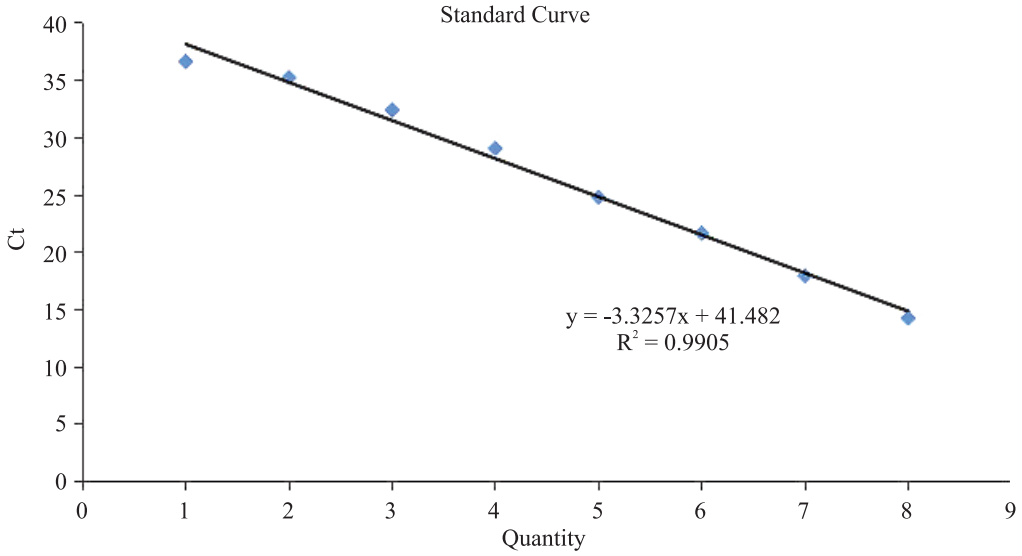

Fig. 1. Standard curve. There is a good linear correlation between the threshold and the plasmid concentration between 1×10^1 to 1×10^8 copies/µL. Experiments were conducted in triplicate.

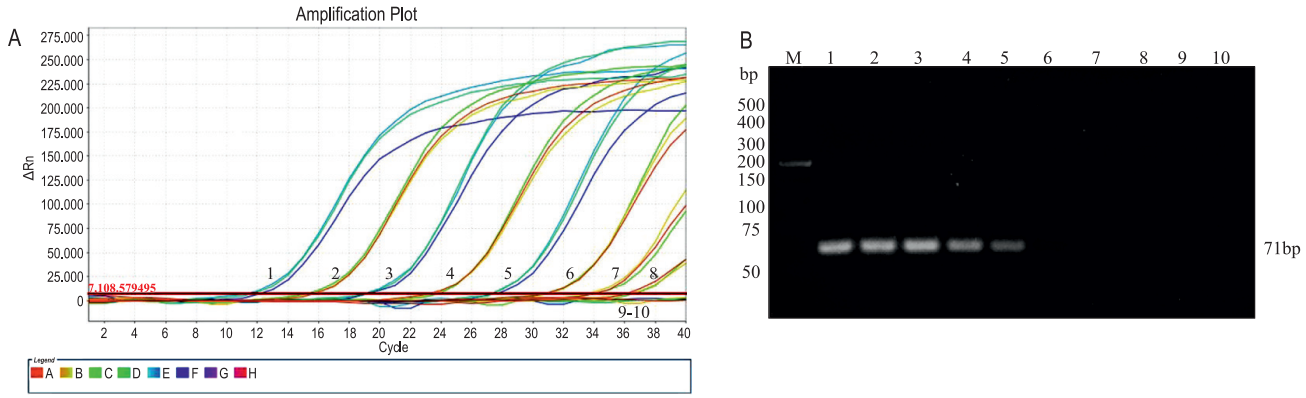

Fig. 2. (A) Sensitivity of porcine circovirus-like virus (PCLV) TaqMan qPCR assay. Lanes 1-9: serial dilutions of PCLV standard plasmid DNA (1×10^8 to 1×10^0 copies/µL), Lane 10: negative control.
(B) Sensitivity of PCLV conventional PCR assay. Marker: 500-bp DNA ladder; Lanes 1-9: serial dilutions of PCLV standard plasmid DNA (1×10^8 to 1×10^0 copies/µL), Lane 10: negative control.

rate of 8.33% (3/36) for the TaqMan qPCR assay compared to 2.78% (1/36) for the cPCR assay. This indicated that the detection rate of PCLV by TaqMan qPCR was about 5.55% higher than that by cPCR, which further demonstrated the higher sensitivity of the TaqMan qPCR assay in the detection of PCLV infection. Notably, all samples that were positive in the cPCR assay were also positive in the TaqMan qPCR assay. In addition, sequencing results showed an exact match with PCLV sequences, further confirming the accuracy and reliability of the assay.

In conclusion, a TaqMan qPCR assay with the advantages of high specificity, strong sensitivity and good reproducibility was established successfully in this study for rapid detection of PCLV. It is suitable for clinical diagnosis and quantitative detection of PCLV infection, and provides technical support for early epidemiological investigation and surveillance.

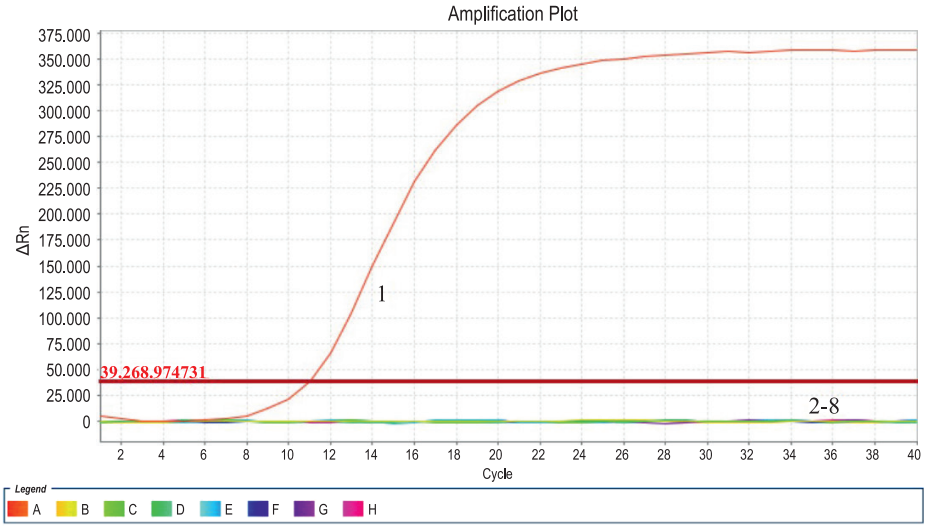

Fig. 3. Specificity of PCLV TaqMan qPCR assay for different viruses. Lanes 1-8: PCLV, PCV2, PEDV, CSFV, PRV, PPV, PRRSV and ddH_2O.

Table 2. Intra-assay and inter-assay variability analysis of TaqMan qPCR assay.

DNA standard (copies/μL)	n	Intra-assay			Inter-assay		
		Mean Ct	SD	CV/%	Mean Ct	SD	CV/%
1×10^5	3	24.82	0.28	1.13%	23.87	0.06	0.25%
1×10^4	3	29.07	0.17	0.58%	27.89	0.29	1.04%
1×10^3	3	32.42	0.10	0.31%	31.35	0.29	0.93%
1×10^2	3	35.24	0.19	0.54%	34.49	0.43	1.25%

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