Rapid Detection of Porcine Circovirus Type 2 by TaqMan-based Real-time Polymerase Chain Reaction Assays

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ABSTRACT

PCV2 is the primary causative agent of porcine circovirus-associated disease (PCVAD). In this study, a TaqMan-based real-time polymerase chain reaction (PCR) assay targeting ORF2 gene of PCV2 was developed and their sensitivities and specificities were investigated. The results indicated that the standard curve had a wide dynamic range (102-107copies/μL) with a linear correlation (R2) of 0.997 between the cycle threshold (Ct) value and template concentration. The real-time PCR assay is highly sensitive and able to detect 2.5×101 copies/μL of PCV2 DNA, as no cross-reaction was observed with other viruses. These data suggested that the real-time PCR assay developed in this study will be suitable for future surveillance and specific diagnosis of PCV2-infection.

INTRODUCTION

- Porcine circovirus 2 (PCV2), a member of the genus Circovirus in the family Circoviridae, is a very small single-stranded negative-sense DNA virus of approximately 1.7 kb. The genome of PCV2 encodes three major open reading frames (ORFs) encoding the replicase

Table 1. *Primers and probe used in real-time PCR assay for PCV2*

Type	Sequence (5'-3')	Position[a]
Forward	AAGTAGCGGGAGTGGTAGGA	1253-1272
Reverse	GGGCTCCAGTGCTGTTATTC	1362-1381
Probe	FAM -TCCCGCCATACCATA-ACCCAGC-TAMRA	1278-1299

proteins (ORF1), the viral capsid protein (ORF2), and a protein with suggested apoptotic activity (ORF3) (Timmusk et al, 2008). PCV2-infection is widespread and essentially all pig herds are infected with PCV2 in China. It is associated with distinct syndromes and diseases in swine, collectively known as porcine circovirus associated diseases (PCVAD), which include:

- post-weaning multi-systemic wasting syndrome (PMWS)
- PCV2-associated pneumonia as a part of the porcine respiratory disease complex (PRDC)
- PCV2-associated enteritis,PCV2-associated reproductive failure, and porcine dermatitis and nephropathy syndrome (PDNS) (Segales et al, 2005; Meng, 2013).

The current methods of detecting PCV2 include virus isolation, polymerase chain reaction (PCR) , and enzyme linked immunosorbent assay (ELISA) (Caprioli et al, 2006; Liu et al, 2004).

In contrast to conventional assays, real-time PCR offers rapid results with potentially increased sensitivity and specificity of detection. It is also less prone to false positive results from amplicon contamination, and is more amenable to the quantitative estimation of viral load.

In addition, A PCV2-induced cytopathic effect is typically not observed and in order to determine viral replication, immunofluorescent, or immunoperoxidase staining has to be performed. In this study, we developed a highly sensitive and specific TaqMan-based real-time PCR method to target the ORF2 gene for the rapid detection and quantitation of PCV2-infection in clinical specimens.

MATERIALS AND METHODS

Virus

PCV2 used for the study was isolated and identified in our laboratory (data not shown). PK-15 cells used for virus propagation were maintained in GIBCOTM Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Auckland, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., South Logan, UT) at 37 ^{o}C under 5% CO_2.

Design of Primers and Probes

ORF2 genes are highly conserved in the genome of PCV2. Primers and TaqMan probes were selected and designed from conserved ORF2 genes using the Primer Express Software (version 3.0; Applied Biosystems, USA) to generate a 129 bp amplicon. The probe was labeled with 5-carboxyfluorescein (FAM) at the 5'-end and N, N, N', N'-tetramethyyl-6-carboxyrhodamine (TAMRA) at the 3'end. Nucleotide information of each primer or probe is summarized in Table 1.

DNA Extraction

Viral DNA was extracted from 150μL of supernatant from virus-infected PK-15 cells or tissue samples using the DNA extraction kit (Qiagen Inc., USA) following the manufacturer's instruction. The extracts were resuspended in 20μL of distilled water, aliquoted, and stored at -20 ^{o}C before real-time PCR amplification was carried out.

Real-time PCR Assay

PCV2 DNA was extracted as a template. The real-time PCR assay was performed in a 25 μL reaction mixture containing 1 μL extracted DNA, 12.5μL FastTaqMan Mixture (with ROX) (Cwbiotech, Beijing, China),

Table 2. *Comparison of PCR and real-time PCR methods for detection of PCV2 from clinical samples*

Type of tissue or samples	No.positive/no.tested samples(%)	
	PCR	Real-time PCR
Serum	6/80	8/80
Tonsils	13/20	15/20
Total	19/100	23/100

400 nM each of forward and reverse primer, and 200 nM of probe. Amplification and detection were performed with an Bio-Rad iQ5 real-time PCR detection system under the following conditions: PCR activation at 95 °C for 3 min and 40 cycles of amplification (5 sec at 95 °C and 40 sec at 60 °C). Analysis of each assay was conducted with iQ5 Standard Edition Optical System Software (version2.1; Bio-Rad).

Sensitivity and Specifity of the Real-time PCR

To examine the sensitivity of real-time PCR for PCV2 amplification, PCR, and real-time PCR reactions were conducted using various concentrations of PCV2 DNA as template. The DNA was quantified by NanoDrop 1000 (Thermo Scientific, USA) and was diluted serially 10-fold from 2.5×107 to 2.5×101 copies/μL as template for two methods. Real-time PCR were performed using the optimized reaction parameters. PCR was performed using PCV2-specific primers (5'-GCT GAT TTC TTT TGT TGT TTG GT-3'), and the reverse primers (5'-TGC CCT TTG AAT ACT ACA GGA TAA-3') (data not shown). Briefly, PCR was performed by using 1μL of diluted DNA template and 10 μmol of each primer in a 25 μL reaction volume by following the manufacturer's protocol with the following cycling times and temperatures: 94 °C for 3 min and 30 cycles of 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 20 sec. Three microliters of PCR products were analyzed by agarose gel electrophoresis and subjected subsequently to automated sequencing reactions (Invitrogen, Beijing, China). The size of fragment amplified by PCR was 276 bp .

Reactions with different viruses including PCV2, classical swine fever virus (CSFV), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), and encephalomyocarditis virus (EMCV) were performed to determine the specificity of the real-time PCR assay.

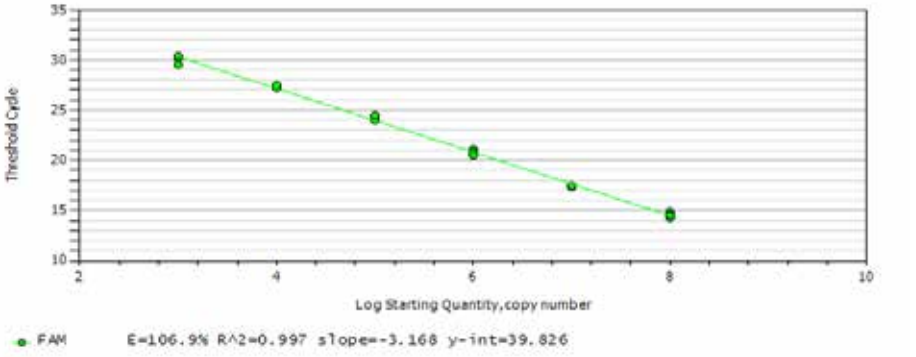
Clinical Specimens

Clinical specimens were collected from different swine farms in the Hebei province. The samples mainly included serum and tonsils of healthy chickens, and tonsils of diseased chickens. Tissue samples were homogenized and centrifuged at 4,000 x g for 15 min to obtain a cell-free supernatant. The sample DNAs were extracted as described above. The real-time PCR were performed using the optimized reaction parameters. Conventional PCR were preformed simultaneously.

RESULTS AND DISCUSSION

The optimization of the real-time PCR reaction was performed by evaluating different concentrations of components and cycling conditions using DNA standards and PCV2 strain. Primers and probe were titrated to determine optimum concentrations and different annealing, and data acquisition temperatures were also evaluated (data not shown). The optimum fluorescence and the lowest Ct values were defined in the absence of primer dimer or nonspecific amplification. The real-time PCR assay was performed by the optimized reaction parameters in a 25 μL reaction mixture containing 1 μL of extracted DNA, 12.5 μL of 2×FastTaqMan Mixture,1.0 μL of each primer (10 μm), 1.0 μL of Probe (5 μm), and 9.0 μL of Dnase/Rnase-free water.

Figure 1. *Standard graph of the TaqMan-based real-time PCR assay for PCV2 detection. The assay was performed using the TaqMan method on serial 10-fold dilutions of PCV2 DNA (2.5×107 to 2.5×102 copies/µL). The standard curve was linear, with a correlation (R2) of 0.997 between the cycle threshold (Ct) value and template concentration, and a slope of -3.168.*



Serial 10-fold dilutions of PCV2 DNA were used to construct a standard curve by plotting the logarithm of the plasmid copy number against the measured Ct values (Fig.1). The standard curve had a wide dynamic range of 102-107 copies/µL with a linear correlation (R2) of 0.997, and a slope of -3.168 between the Ct value and the logarithm of the plasmid copy number.

The sensitivity of the real-time PCR assay was evaluated by testing 10-fold serial dilution of DNA templates (2.5×107 to 2.5×101 copies/µL). The detection limit of real-time PCR was 2.5×101 copies/µL, whereas that of PCR was 2.5×103 copies/µL (Fig.2A and B). Comparisons between the real-time PCR and PCR amplification indicated that real-time PCR is 100-fold more sensitive than PCR. The specificity of the TaqMan PCR assay was evaluated using other animal viruses, and a water negative control. Strong fluorescent signals were obtained only in the detection of PCV2, whereas the signals from other four virus samples and the water control were equivalent to baseline levels under the optimized reaction conditions (Fig.3). Thus, PCV2 was clearly differentiated from other viruses by comparing the signal strengths at different levels.

Real-time PCR and conventional PCR were preformed simultaneously on 100 clinical samples. The results are shown in Table 2. Nineteen of 100 samples (19%) were positive by PCR analysis, whereas 23 of 100 samples (23%) were positive by real-time PCR (Table 2). Nineteen samples (19%) were positive by two methods. Four sample (4%) were positive by real-time PCR, but negative by PCR analysis. No sample (0%) was positive by PCR and negative by real-time PCR. The results showed that real-time PCR was more sensitive than the conventional PCR assay.

PCV2 is an emerging swine pathogen causing significant economic losses in the global swine industry. The current methods of detecting PCV2 include virus isolation, serology, and PCR (Caprioli et al, 2006; Liu et al, 2004). Virus isolation is one of the conventional methods, but it is time-consuming and laborious. Serology represents the most popular technique, however, this method has some disadvantages because antibody titres can fall rapidly after infection (Large et al, 1997). Conventional PCR is less time-consuming, but prone to sample contamination occurring during PCR processing steps, which increase the potential for false-positive results.

The development of real-time PCR technology presents an opportunity for more rapid, sensitive, and specific detection of nucleic acids and, is becoming widely used

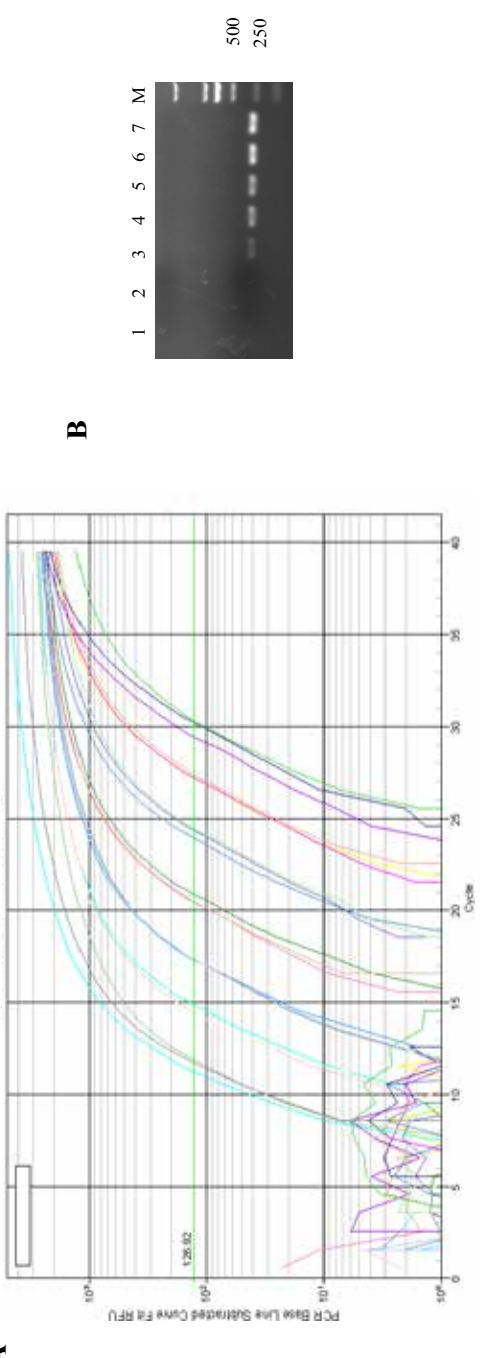


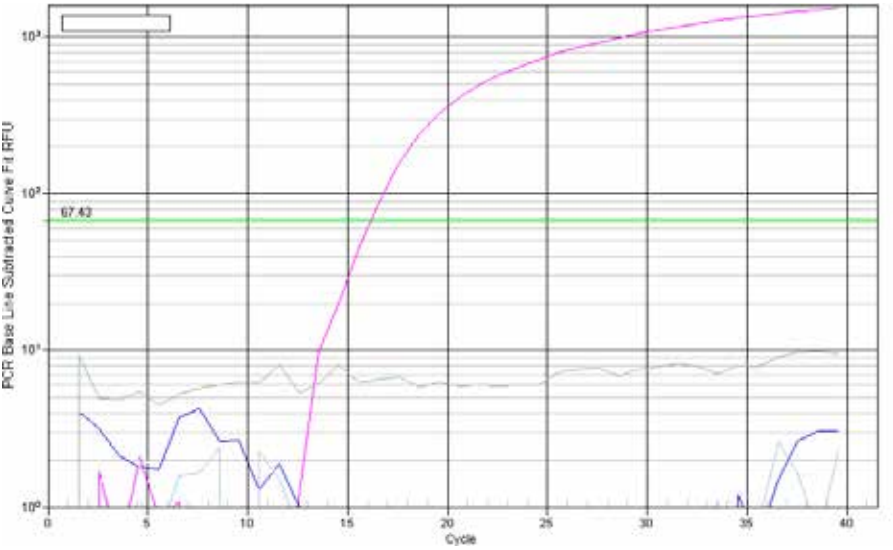
Figure 2. *The sensitivities of PCR and real-time PCR assays for PCV2 detection. A serial 10-fold dilution of PCV2 DNA was used in the PCR and real-time PCR assays. (A) Analysis of the real-time PCR assay by amplification curve graph. (B) Analysis of the PCR assay by agarose gel electrophoresis. Lane 1: 2.5×101 copies/μL; Lane 2: 2.5×102 copies/μL; Lane 3: 2.5×103copies/μL; Lane 4: 2.5×104 copies/μL; Lane 5: 2.5×105 copies/μL; Lane 6: 2.5×106copies/μL, Lane 7:2.5×107copies/μL. All experiments were repeated three times and similar results were obtained.*

because the accumulated amplicons can be detected directly during the nucleic acids amplification (Gibson et al, 1996). Real-time PCR has been used in detecting virus from animals due to the following:

- its simplicity and high sensitivity including influzenza A (H1N1) (Whiley et al, 2009)
- transmissible gastroenteritis virus (Vemulapalli et al., 2009)
- foot-and-mouth disease virus (Reid et al., 2009; Tam et al., 2009),
- dengue virus (Dos Santos et al., 2008),
- classical swine fever virus (Le Dimna et al., 2008; Zhao et al., 2008),
- and porcine reproductive and respiratory syndrome virus (Lurchachaiwong et al., 2008).

In this study, a highly efficient and practical method for the detection of PCV2 was established. Since ORF2 gene of PCV2 is among the most conserved regions and has been chosen as a preferred target region for the detection of PCV2 DNA by PCR (Brunborg et al, 2004), primers, and probe were designed to amplify target sequences at the ORF2 gene region of the PCV2 genome for the real-time PCR assay. The real-time PCR assay is highly sensitive and able to detect 2.5×101 copies/μL of PCV2 DNA, as no cross-reaction was observed with other viruses.

Figure 3. *Specific evaluation of the real-time PCR assay for the detection of PCV2. The line of PCV2 amplification was indicated (A). The other lines represented the amplifications using nucleic acids from other viruses (B), including PRRSV, EMCV, CSFV, and PEDV. The control reaction using DEPC-treated water as template was also included.*



The sensitivity of real-time PCR for PCV2 detection was 100-fold greater than PCR. Most of the amplification reactions could be finished within 60 min. Thus, the real-time PCR assay is faster than PCR. A real-time polymerase chain reaction with SYBR Green was developed for the detection and quantification of PCV2 in porcine tissues (Wang et al., 2012). Brunborg et al have used a TaqMan probe to detect an 84 bp fragment in ORF2 region and to quantify the viral load in different tissues and serum samples (Brunborg et al., 2004). Chung et al have established a TaqMan real-time PCR to detect a fragment of 269 bp (Chung et al., 2005). In a report by Zhao et al, PCV2 was quantified using a TaqMan real-time PCR that detected a fragment of 149 bp (Zhao et al., 2010).

Sequence analysis revealed that PCV2 shared only approximately 68% nucleotide sequence identity with that of porcine circovirus type 1(PCV1) (Ellis et al., 1998). Both PCV1 and PCV2 are classified in the genus Circovirus within the family Circoviridae. PCV1 was derived from PK-15 cell and not pathogenic in pigs. ORF2 region displays the highest diversity between PCV1 and PCV2 (Brunborg et al., 2004). The real-time PCR assay developed in this study can detect PCV2. Whether the developed method can detect PCV1, which need to be further studied.

CONFLICT OF INTEREST

No authors in this study have any conflict of interest.

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