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Detection of PCV2 DNA by SYBR Green I-based quantitative PCR

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Abstract: We developed an assay for the detection and quantitation of porcine circovirus type 2 (PCV2) with the SYBR Green I-based real-time PCR. The real-time PCR provides a broad dynamic range, detecting from 10^3 to 10^{11} copies of DNA per reaction. No cross-reactions were found in specimens containing PCV1. Because of the high sensitivity and specificity of the assay with a relatively rapid and simple procedure, real-time PCR can be used as a routine assay for the clinical diagnosis of PCV2 infection. In this study we applied real-time PCR assay to 80 clinical samples, collected from 40 pigs with postweaning multisystemic wasting syndrome (PMWS) and 40 healthy pigs in comparison with conventional PCR assay. In 56 of 80 samples, PCV2 DNA was detected by conventional PCR assay. All samples positive for PCV2 DNA in conventional PCR assay were also positive in real-time assay, and 12 of 24 samples that tested negative for PCV2 DNA in the conventional assay were tested positive in real-time PCR assay. Real-time PCR assay increased the number of samples in which PCV2 was detected by 15%. It is, therefore, considered to be a useful tool for the detection of PCV2.

INTRODUCTION

Porcine circovirus (PCV) is a small, naked DNA virus with diameter of 17 nm (Tischer *et al*., 1982), and is a member of the *Circoviridae* family. PCV isolated as a persistent contaminant from a porcine kidney cell line is nonpathogenic and is designated as PCV1 (Allan *et al*., 1995; 2000). Postweaning multisystemic wasting syndrome (PMWS**)** is an emerging disease in pigs and was first described in 1991 (Harding, 1997). The causative agent of PMWS is believed to be a pathogenic strain of PCV, named PCV2 (Allan *et al*., 1999). PMWS is a disease that can affect nursery and fattening pigs, and is being reported in pigs throughout the world (Allan *et al*., 1998; Kennedy *et al*., 1998; Segales and Domingo, 2002; Wen *et al*., 2005). The disease is characterized by weight loss, dyspnoea and jaundice as well as the pathological findings of interstitial pneumonia, generalized, enlarged lymph nodes, hepatitis and nephri-

tis (Ladekjær-Mikkelsen *et al*., 2002). In addition, PCV2 was found to be associated with porcine dermatitis and nephropathy syndrome (PDNS) (Wellenberg *et al*., 2004), respiratory disease complex (Kim and Chae, 2003) and reproduction failure (Sanchez *et al*., 2001). Several qualitative PCR methods have been described for the detection of PCV2 in fresh and fixed material (Allan *et al*., 1999; Hamel *et al*., 2000; Kim and Chae, 2003; Larochelle *et al*., 1999; Ouardani *et al*., 1999). However, as PCV2 is so common within the swine population that conventional PCR can only be used as reference for PMWS diagnosis (Brunborg *et al*., 2004). Conventional PCR is time-consuming and prone to sample contamination by DNA amplified previously. This increases the potential for false-positive results. Furthermore the load of PCV2 is closely related to PMWS (Brunborg *et al*., 2004; Liu *et al*., 2000; Olvera *et al*., 2004; Rovira *et al*., 2002), so quantifying the number is important for diagnosing PCV2 in suspected pig.

Real-time PCR is an excellent diagnostic tool

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with high sensitivity and specificity and fast turn-around time. This system is so-called because the accumulated amplicons can be monitored directly during the DNA amplification process. The quantitation of DNA is based on the determination of the threshold cycle when the amplified PCR product is first detected.

The higher the initial DNA copy number input, the sooner the product of amplification is detected. Reports on virus detection based on real-time PCR technology have been described for various human and animal viruses. There are only five published studies of real-time PCR assays for the detection of PCV2 (Brunborg *et al*., 2004; Chung *et al*., 2005; Ladekjær-Mikkelsen *et al*., 2002; Olvera *et al*., 2004; Rovira *et al*., 2002). But all of them used a hybridization probe method in the TaqMan system and are mostly applied on serum samples and the primers were mostly based on their local PCV2 strains. Although some papers (Gilpin *et al*., 2003; Fenaux *et al*., 2004) used an SYBR Green I to quantify the PCV2 DNA, but a regression lines between the C_T values and the input concentrations were not given.

In this study, the development of a quantitative real-time PCR for the detection of PCV2 based on SYBR Green I dye using LightCycler (LC) system is reported. To evaluate the usefulness of this real-time PCR for diagnosing and monitoring ill pigs with PCV2 infection, we compared results of real-time PCR and conventional PCR using clinical samples from 40 PMWS affected and healthy pigs, respectively.

MATERIALS AND METHODS

Samples

Tissue samples (liver, mesenteric lymph node) were collected from 40 living or dead pigs from 21 to 90 d of age suffering from PMWS. Thirty-three of them originated from five different farrow-to-finish large operations located in eastern and western Hangzhou. The rest of them were obtained from southern Hangzhou. The samples were collected from Jan. 2003 through Oct. 2005. Gross lesions consisted of lymph node and spleen enlargement and non-collapsed, rubbery Samples were stored at −70 °C until DNA isolation.

Liver and mesenteric lymph node samples were collected from 40 healthy randomly selected pigs aged 5 to 6 months from an abattoir in Oct. 2005 and stored at −70 °C until use.

PCV2 DNA isolation

Tissue samples were minced into small pieces and homogenized in phosphate buffered saline (PBS) and centrifuged at 10000×g for 5 min. The virus DNA was extracted from the supernatant by column chromatography using mini DNA extraction kit (Shanghai Shenergy Biocolor Bioscience & Technology Company, Shanghai, China). Cell lysis and precipitation of DNA was performed as recommended by the manufacturer. Briefly, 200 μl of supernatant was incubated with digestion buffer and a final proteinase K concentration of 0.2 mg/ml for 30 min at 55 °C. Then DNA was bound to spin columns and washed twice. Finally, the genomic DNA dissolved in 50 μl of distilled water was stored at −20 °C until use.

Primer designing for SYBR Green I-based quantitative PCR

The primer design for PCV2 quantitation using real-time PCR with SYBR Green I was based on nucleotide sequences of ORF2 (open reading frame 2) retrieved from GenBank (accession Nos. DQ231511~ DQ231519 and DQ235696), which were isolated from Hangzhou during 2003~2005. Nucleotide sequences were aligned to identify conserved regions using DNAStar software (DNASTAR, Madison, WI, USA). The primers were as follows: P1, 5′-ATAA CCCAGCCCTTCTCCTACC-3′; P2, 5′-GGCCTA CGTGGTCTACATTTCC-3′. The length of the amplified products was 145 bp.

Construction of standard plasmids for SYBR quantitative PCR

The plasmid (pPCV2) used as the standard DNA was constructed by ligation of a PCR fragment in pMD18-T simple vector according to the instructions of the manufacturer (TaKaRa, Dalian, China). The cloned fragment was comprised of a 702 bp region of the PCV2 ORF2 (capsid gene) amplified by primers P3: GCCGAGGTGCTGCCGCT and P4: CAGTTCG TCACCCTTTCCCC. Plasmid was propagated in *Escherichia coli* DH5α cells and was purified by using a Bio-Dev gel extraction kit (Bio-Dev, Beijing,

China) and quantified by measuring OD_{260} using spectrophotometer (Thermo Electron, USA). Ten-fold dilutions were made in order to get 10^{12}~10^1 plasmids per 2 μl sample for the real-time PCR. The dilutions were stored at −20 °C, while stock plasmid was stored at −70 °C.

Real-time PCR

Real-time PCR was done using the LC system with SYBR Green I detection and T_m analysis. SYBR® Premix Ex Taq™ (perfect real time) PCR kit was purchased from TaKaRa (Dalian, China). Five hundred nanograms DNA in an aliquot of 2 μl was used as template. Viral load was expressed as copies per 500 ng DNA. The procedure was optimized with regard to concentrations of primers, and denature/extension temperature.

The optimized reaction was carried out in a 20 μl final reaction volume containing 0.4 μmol/L concentration of each forward and reverse primer, 2 μl DNA solution, 6.4 μl distilled water, 10 μl of kit-supplied SYBR® PCR master mix (including HotStart Ex Taq HS DNA polymerase, reaction buffer, dNTP mix, and SYBR Green I). Prior to cycling, the glass capillaries were sealed, and placed into the LC sample carousel, and centrifuged at 400×g for 10 s in the LC carousel centrifuge (Eppendorf, Germany). The thermal profile for the real-time PCR was 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s. Three concentrations of pPCV2-ORF2 (10^9, 10^5, and 10^3 copies per sample) were included in each run, and served as positive controls as well as to derive the standard curve used for quantitation of PCV2 DNA in tissue samples. Positive and negative reference samples were tested along with the unknown samples in each run.

Melting curve analysis of the PCR product

Melting curve analysis was performed to measure the specificity of PCR product. After PCR cycling, samples were heated to 95 °C for 0 s and 65 °C for 15 s and then heated to 95 °C at a linear transition rate of 0.1 °C/s. Fluorescence of the samples was monitored continuously while the temperature was increasing. SYBR Green I is released upon denaturation, which results in a decreasing fluorescence of the signal. The LC software calculates the T_m. All samples were analyzed once.

Conventional PCR

Viral DNA sequences were amplified by conventional PCR as previously described (Huang *et al.*, 2004) using the oligonucleotide primers PCV2-1 (5′-TAGGTTAGGGCTGTGGCCTT-3′) and PCV2-2 (5′-CCGCACCTTCGGATATACTG-3′). Amplicons of 264 bp were detected by electrophoresing 8 μl aliquots through 1% agarose gel containing 0.5 μg/ml ethidium bromide. To ensure quality of data, negative and positive reference samples were always applied in each PCR reaction. DNA extraction and amplification were performed in different rooms.

Statistics

The viral concentrations were expressed as the viral DNA copies per 500 ng DNA of tested tissues. The data obtained from PMWS and healthy pigs were analyzed using *t* test, considering a value of *P*<0.05 as statistically significant.

RESULTS

Primer design

The current assay leads to a PCR product of 145 bp, with primers covering 44 bp of the 145 bp. Alignment of the 10 Hangzhou PCV2 strains with the chosen primers showed that there were up to two mismatches with some PCV2 strains of Hangzhou. However, the present real-time PCR can efficiently amplify various PCV2 field strains of Hangzhou even if PCV2 nucleotide sequence showed up to two mismatches in the primers.

Optimization strategies

Real-time PCR offers continuous monitoring of the PCR reaction cycle by cycle by using fluorescence. The detection in our assay is based on the binding of the fluorescent dye SYBR Green I, which binds all double stranded DNA molecules. Since this process is sequence independent, the characteristic T_m of the target is determined by performing a melting curve analysis on the PCR products to test the formation of primer-dimers and non-specific products, which compete with formation of specific PCR products. T_m analysis of the real-time PCR product revealed that the denaturation/elongation temperature in the range of 55~65 °C in cycles had no effect. The distinct

temperature at which the double stranded amplicon was denatured was 82.1 °C (range: 81.9~82.4 °C). No increase in fluorescence signal from primer-dimers or other non-specific amplification products (distinguished from the specific product through their lower T_m) was monitored (Fig.1). In addition, concentrations of primers were essential for optimal working conditions. As shown in Fig.2, when the PCV2 primer concentrations were lowered from 1000 nmol/L to
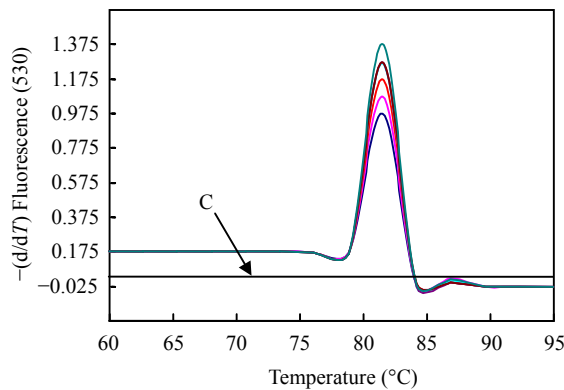
Fig.1 Melting curve analysis of PCR products obtained following 40 cycles of amplification
Melting peaks were produced by plotting the change in the relative intensity of fluorescence for 11 different Hangzhou PCV2 strains over temperature. Note that the T_m of the PCR products of PCV2 isolates was 82.1 °C. No peaks of amplification products from negative control (arrow C) were observed
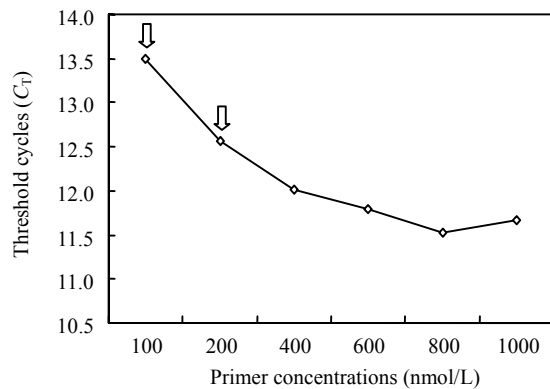

Fig.2 Amplifications of PCV2 DNA. The effect of the PCV2 forward and reverse primer concentrations on PCV2 amplification
The experiment was carried out with forward and reverse primer concentrations varying from 100 to 1000 nmol/L, The C_T value obtained from each set of amplification conditions was plotted against the concentrations of the forward or reverse primer. Left open arrow, when the reverse primer concentration was lowered from 1000 nmol/L to 100 nmol/L, the PCV2 C_T increased markedly by 1.8 cycles; the C_T only increased by 0.9 cycles if the reverse primer was adjusted to 200 nmol/L (right open arrow)

100 nmol/L, the PCV2 C_T increased markedly by 1.8 cycles. The C_T only increased by 0.9 cycles if the forward and reverse primers were adjusted to 200 nmol/L. To maintain PCV2 amplification at a moderate and reproducible level, we chose the combination of 200 nmol/L for the forward and reverse primers.

Detection and quantitation limit of the assay

Ten-fold serial plasmid dilutions were tested and used to construct the standard curve by plotting the plasmid copy number logarithm against the measured C_T values. Samples were repeated three times. The generated standard curve covered a linear range of 1×10^3 to 1×10^{11} copies per 2 μl for PCV2. The efficiency was 2.02 (A perfect amplification reaction would produce a standard curve with an efficiency of "2", because the amount of target DNA would double). The amount of 1×10^{12} copies per 2 μl was outside the linear range of the standard curve, amounts between 10~100 copies per 2 μl were not detected (Fig.3).
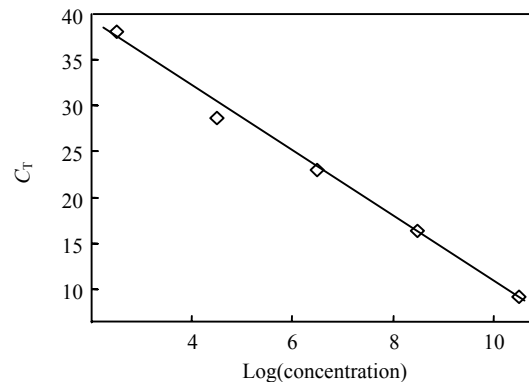
Fig.3 Regression lines between the C_T values and the input concentrations of PCV2 plasmid DNA in real-time PCR detected by using SYBR Green I

Specificity of the assay

The specificity of the assay was examined with regard to PCV1. Duplicates of PCV1 DNA, isolated from 200 μl of a suspension of clinical samples infected with PCV1, were run under the optimal conditions of the assay, and no increase in fluorescence being observed.

Reproducibility of the real-time PCR for PCV2

When the standard PCV2 plasmid DNA was used for the evaluation of the coefficients of variation (CVs) of the real-time PCR, the intra- and inter-assay CVs for C_T values ranged between 0.29% and 0.82%, and 0.29% and 1.24%, respectively (Table 1).

Table 1 Variance analysis of C_T values quantified by real-time PCR in serially diluted standard plasmid solutions

Concentration of standard plasmid (copies/ml)	n	Intra-assay variability			Inter-assay variability		
		C_T		CV (%)	C_T		CV (%)
		Mean	SD		Mean	SD	
10^9	10	14.2	0.08	0.56	14.5	0.18	1.24
10^7	10	20.8	0.06	0.29	20.7	0.06	0.29
10^5	10	26.7	0.22	0.82	26.8	0.16	0.59

Quantitation of viral load in tissue samples

The assay was tested on lymph nodes and livers from both healthy and PMWS affected pigs. There was a significant difference between PCV2 load in healthy and PMWS affected pigs ($P<0.01$) with a cut-off between healthy and PMWS pigs at 10^6 (liver) or 10^7 (lymph node samples) PCV2 genomes per 500 ng DNA. The virus load of specimens that were tested positive for PMWS by the real-time PCR was 1×10^6 to 1×10^{10} (liver) and more than 1×10^7 per 500 ng DNA (lymph node), while the virus load of specimens that were tested positive for the healthy pigs was $10^3\sim10^6$ per 500 ng DNA (liver or lymph node samples).

Comparison of results obtained by real-time PCR and conventional PCR

Results of the real-time PCR and conventional PCR assays are shown in Table 2. All liver and lymph node samples positive for PCV2 DNA in conventional PCR were also positive in real-time PCR assay, whereas half of the samples negative for PCV2 DNA in the conventional PCR assays were positive in the real-time PCR assays. The copy number of PCV2 DNA in tissue samples which were positive in both methods ranged from 1×10^5 to 1×10^{12} copies per 500 ng DNA. The copy number of PCV2 DNA of twelve lymph node samples, which were positive by the real-time PCR assay but negative by the conventional PCR assay, was 1×10^3 to 1×10^5 copies per 500 ng DNA.

Table 2 Comparison of real-time PCR and conventional PCR

Conventional PCR assay result (n)	Real-time PCR assay result (n)		
	Positive	Negative	Total
Positive	56	0	56
Negative	12	12	24
Total	68	12	80

DISCUSSION

The established real-time PCR assay for PCV2 DNA quantitation was found to be in the range of 10^3 to 10^{11} with excellent linearity. The new assay increased the number of samples in which PCV2 were detected by 15% more than conventional PCR.

Several PCV2 specific serological assays were established, but they could not differentiate between maternal antibodies and antibodies due to infection. Furthermore, presence of PCV2 antibodies is not relevant to the disease symptoms. Also seroconversion is commonly detected around 20 d after infection, so these methods cannot detect latent infection or early infection. PCV2 infection is common in pig herds and its shedding has been reported in PCV2-infected pigs without any clinical symptoms. The ability to quantify viral loads with the quantitative PCR assay is a valuable tool to gain further understand of mechanisms of PCV2 infection.

For precise estimation of viral loads in samples, a competitive quantitative PCR was applied that can detect and quantify target DNA simultaneously (Liu *et al.*, 2000), but it requires an exogenous competitor as a control that needs to be constructed and characterized, and requires post-PCR electrophoresis. Real-time PCR is performed in a closed tube and requires no post-PCR electrophoresis. Therefore, contamination with amplicons can be avoided. This assay is more reproducible and convenient than competitive quantitative PCR tests. TaqMan real-time PCR requires an efficient probe with double-labelled fluorescent reporter dyes to be incorporated in each reaction. And sometimes the probe cannot bind with target sequence when gene sequence variation occurs.

The primers used in this study were positioned in ORF2 that were conserved between 11 different Hangzhou strains on which sequence data were available. Our system has advantages in having wider

range than the fluorogenic probe and competitive quantitative PCR system. The established real-time PCR for PCV2 DNA quantitation were found to be in the (3~11)-log10 dynamic range with excellent linearity (Fig.3). This compares favorably with other methods described previously: a competitive PCR (Liu *et al*., 2000) or real-time PCR based on SYBR Green I (Fenaux *et al*., 2004; Gilpin *et al*., 2003). The former have given linear ranges of 4- to 6-log10, whereas the latter have not presented the range. The detection of PCV2 over a wide range of concentrations allows us to measure viral loads in animals with various levels of infection without further sample concentration or dilution. Furthermore, our method assay was reproducible, as indicated by the low intra- and inter-assay CVs for C_T values obtained with the standard plasmids. The intra-assay CVs were equal or less than 0.82%. The limitation of the comparison between conventional and real-time PCR is the absence of a gold standard. There were 12 samples that tested negative by conventional PCR but positive by real-time PCR. Another approach to increase the sensitivity of conventional PCR is to perform nested PCR (Kiatipattanasakul-Banlunara *et al*., 2002), which has been reported to be more sensitive than single-round PCR. However, a major disadvantage of nested PCR is the high risk of carryover and cross-contamination. Since the real-time PCR uses a completely sealed capillary system, the risk of contamination is much lower.

Brunborg *et al*.(2004) presented encouraging results with PCV2 Norwegian isolates by using TaqMan system, our study has the unique advantage of having the real-time assay validated with various PCV2 strains from Hangzhou. With these primer sets, our PCR assay detected all PCV2 identified by the conventional PCR method. Moreover, SYBR Green I can bind to any double-strand DNA, so the dye can also be used in diagnosis of other viruses [e.g. porcine reproductive and respiratory syndrome virus (PRRSV)], and most of real-time machines can detect the fluorescence emitted by SYBR Green I. These will lower the diagnosis costs and make the method more applicable and practicable than probe. The LightCycler 2.0 is very quick. It can reach 20 °C/s during cycles and the real-time PCR can finish within 30 min.

We cloned a plasmid containing viral ORF2 for the generation of standard PCV2 for quantitative PCR. The detection limit of this assay was 1000 copies of PCV2 DNA. Steps were taken to avoid false positives in quantitative PCR, because SYBR Green I dye can bind to any double stranded DNA, even nonspecific amplicons. A "hot start" *Ex Taq* HS polymerase, which requires incubation at 95 °C to activate just before the first denaturation step of thermocycling, was applied. Any nonspecific amplicon would be eliminated before the amplification, with no subsequent contamination detected on further runs. The specificity of PCR was verified by the performance of melting curve analysis for the PCR product, which depends on its GC content, length, and sequence. All the Hangzhou PCV2 strains were amplified and examined by melting curve analysis. The melting temperatures of PCR products were 82.1 °C indicating an amplification of a specific product. The specificity of the real-time PCR with SYBR for PCV2 was also determined by testing samples infected with PCV1. As expected, the PCV1 was not detected using the established real-time PCR. Changing the annealing temperature to 55 °C or 65 °C did not affect the efficiency and did not lead to detection of PCV1 in the assay. Also, this proved our primer was specific. Furthermore, this makes it easier to design other primer sets to detect two or more viruses simultaneously, which is our next goal, because Chung *et al*.(2005) have established a real-time PCR based on SYBR Green I to detect PRRSV. Finally, according to the instructions provided by TaKaRa we did routine analyses with an annealing/elongation temperature of 60 °C.

A high PCV2 load in tissues in an animal is a good indicator of its PMWS status. Significant differences in viral load of PCV2 in serum from sick and healthy animals have been described by other authors (Brunborg *et al*., 2004; Liu *et al*., 2000; Olvera *et al*., 2004; Rovira *et al*., 2002). Brunborg *et al*.(2004) reported a cut-off of 1×10^7 PCV2 genomes per 500 ng DNA for PMWS. We also found that animals with mild, moderate and severe symptom had PCV2 loads of $1 \times 10^6 \sim 1 \times 10^{10}$, $1 \times 10^9 \sim 1 \times 10^{10}$ and $1 \times 10^{10} \sim 1 \times 10^{12}$ copies per 500 ng DNA, respectively, while healthy pigs had only $1 \times 10^3 \sim 1 \times 10^5$ copies. There was a slight difference between our and Brunborg's results. One of the reasons for this difference may be the time of sample collection, if samples were collected from the

dead animals, or pigs were dead prior to sending to the laboratory and the collected tissue samples were in various states of degradation, they may have influenced the recovery of nonpackaged viral DNA, and an underestimation of viral load in tissues (Brunborg *et al.*, 2004). The other reason may be the inherent nature of different method, e.g. TaqMan, SYBR have different sensitivity and efficiency.

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