Journal of Virological Methods 250 (2017) 11–16

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Multiplex real-time polymerase chain reaction for the differential detection of porcine circovirus 2 and 3

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ARTICLE INFO

Keywords:
Porcine circovirus 2
PCV3
Capsid gene
Multiplex
Real-time PCR

ABSTRACT

A multiplex quantitative real-time polymerase chain reaction (mqPCR) assay was developed for the rapid and differential detection of porcine circovirus 2 (PCV2) and PCV3. Each of the capsid genes of PCV2 and PCV3 were amplified using specific primers and probe sets, while no other porcine pathogen genes were detected. Limit of detection of the assay was below 50 copies of the target genes of PCV2 and PCV3, and was comparable to that of previously described methods The assay showed high repeatability and reproducibility, with coefficients of intra-assay and inter-assay variation of less than 4.0%. Clinical evaluation using tissue samples from a domestic pig farm showed that PCV2 and PCV3 co-circulated at the farm. Moreover, singular infection rates of PCV2 or PCV3 were 21.7% (10/46) or 6.5% (3/46), respectively, while the co-infection rate of PCV3 with PCV2 was 28.3% (13/46). PCV3 DNA was detected by the mqPCR in respiratory diseased piglet tissue samples and aborted fetal tissue samples, suggesting that PCV3 infection is associated with porcine respiratory disease and reproductive failure in the pig farm. This mqPCR method is a rapid and reliable differential diagnostic tool for the monitoring and surveillance of PCV2 and PCV3 in the field.

1. Introduction

Porcine circovirus (PCV), which belongs to the genus *Circovirus* of the family *Circoviridae*, is a non-enveloped, spherical, single-stranded DNA virus (Tischer et al., 1982). Before 2016, two types of PCV were reported to infect pigs: PCV1 and PCV2 (Allan et al., 2012). PCV1 was initially discovered in 1974 as a permanent contaminant of continuous cell culture PK-15 and was considered non-pathogenic (Tischer et al., 1982). In contrast, PCV2 was first identified from post-weaning multi-systemic wasting syndrome-affected pigs in Canada in the early 1990s. PCV2 is now considered a major pathogen of porcine circovirus associated disease (PCVAD), which is characterized by several clinical conditions, including post-weaning multi-systemic wasting syndrome, porcine dermatitis and nephropathy syndrome (PDNS), reproductive disorders, enteritis, proliferative and necrotizing pneumonia, and porcine respiratory disease complex (Opriessnig et al., 2007; Segalés, 2012).

Recently, a novel porcine circovirus, designated as PCV3, was identified in pigs with PDNS, reproductive failure, and cardiac and multi-systemic inflammation in the US and China (Palinski et al., 2017; Phan et al., 2016). More recently, PCV3 was identified in pen-based oral fluid samples from Korean pig farms (Kwon et al., 2017b). Based on additional prevalence studies, PCV3 was suggested to commonly circulate within pig populations in the US, China, and Korea (Ku et al., 2017; Kwon et al., 2017b; Palinski et al., 2017) and that the new circovirus might cause clinical disease on swine farms.

The clinical presentations of PCV3 are similar to those of PCV2 and to coinfection with PCV2 and PCV3 in pig populations in the US, China, and Korea; therefore, a rapid and reliable diagnostic assay is needed for the differential detection of PCV2 and PCV3 in the field (Ku et al., 2017; Kwon et al., 2017b; Palinski et al., 2017). However, there is no specific single assay capable of differentiating PCV2 infection from PCV3 infection. Such an assay would enable the accurate diagnosis of suspected clinical cases and encourage further epidemiological studies for its control. Therefore, in the present study, we developed and evaluated a rapid multiplex quantitative real-time polymerase chain reaction (mqPCR) assay using primer sets capable of detecting and typing PCV2 and PCV3 in clinical samples.

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http://dx.doi.org/10.1016/j.jviromet.2017.09.021
Received 17 August 2017; Received in revised form 19 September 2017; Accepted 19 September 2017
Available online 21 September 2017
0166-0934/

Table 1
Specificity of, multiplex quantitative real-time PCR using PCV2 or PCV3-specific primers and probe set.

Pathogen	Strain	Source[a]	Amplification of target gene	
			PCV2 (FAM)	PCV3 (ROX)
Porcine circovirus 1	Field strain	ADIC	–	–
Porcine circovirus 2	PCK0201	ADIC	+	–
Porcine circovirus 3	Field strain	ADIC	–	+
PRRS virus, genotype 1	Lelystad virus	APQA	–	–
PRRS virus, genotype 2	LMY strain	APQA	–	–
Classical swine fever virus	LOM strain	APQA	–	–
Porcine parvovirus	NADL-2	APQA	–	–
ST cell	–	ADIC	–	–
PK-15 cell	–	ADIC	–	–

[a] APQA, Animal and Plant Quarantine Agency, Korea; ADIC, Animal Disease Intervention Center, Kyungpook National University, Korea.

2. Materials and methods

2.1. Viruses and samples

A PCV2 Korean field isolate (PCK0201 strain) (Park et al., 2004) and a PCV3-positive clinical sample were used to optimize the mqPCR conditions. The PCV3-positive tissue sample was collected from a PCV3-infected pig farm and confirmed as PCV3-positive using a previously described qPCR assay (Palinski et al., 2017). Other porcine viral pathogens, including PCV1 (positive PK-15 cell culture), type 1 porcine reproductive and respiratory syndrome virus (PRRSV, Lelystad virus), type 2 PRRSV (LMY strain), classical swine fever virus (LOM strain), and porcine parvovirus (NADL-2 strain) were obtained from the Animal and Plant Quarantine Agency or Animal Disease Intervention Center for the assay's specificity test (Table 1). All pathogen samples were allocated and stored at −80 °C until use.

For clinical evaluation of the mqPCR, 46 tissue samples (17 aborted fetuses and 29 respiratory diseased piglets) were collected from a PCV2 and PCV3-infected pig farm located in Kyungpook province, in the southern part of Korea, where PCV2 was endemically infected and regularly vaccinated with commercial PCV2 vaccine in the sow and piglet groups, and PCV3 infection was confirmed early in 2017 by qPCR, as previously described (Palinski et al., 2017). The tissue samples were homogenized and diluted 10-fold with phosphate-buffered saline (0.1 M, pH 7.4). All samples were frozen and thawed twice, vortexed for 5 min, and centrifuged at 10,000 × *g* (Hanil, Korea) for 10 min at 4 °C. The supernatants were used for DNA extraction immediately or stored at −80 °C until use.

2.2. Reference gene construction

The complete capsid gene of PCV2 or PCV3 was amplified by PCR from a Korean field isolate (PCK0201 strain) (Park et al., 2004) or a PCV3 isolate (PCK3-1701 strain, GenBank accession number MF611876.1) using a pair of specific primers (forward, 5′- ATG ACG-TATCCAAGGAGGCG-3′ and reverse, 5′-TTAGGGTTTAAGTGGGGGG TC-3′ for PCV2, or forward, 5′-TGAGACA CAG AGCTATATTC-3′ and reverse, 5′-TTCACTTAGAGAACGGACTT-3′ for PCV3, respectively). PCR was carried out using a commercial kit (Excel TB 2X Taq premix; Inclone, Korea) in 25-μL reaction mixtures containing 12.5 μL of 2 × premix, 0.4 μM of each primer, and 5 μL of PCV2 or PCV3 DNA as template, according to the manufacturer's instructions. Amplification was carried out using a thermal cycler (Applied Biosystems, USA) under the following conditions: initial denaturation at 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and a final extension at 72 °C for 10 min. Each amplified product was purified and cloned into the pTOP TA V2 vector (TOPcloner™ TA core Kit; Enzynomics, Korea), which was transformed into *Escherichia coli* competent cells according to the manufacturer's instructions (DH5α Chemically Competent *E. coli*; Enzynomics, Korea). Plasmids containing the PCV2 or PCV3 capsid gene were purified using a commercial kit (GeneAll Expin™ Combo GP 200 miniprep kit, GeneAll, Seoul, Korea). The concentrations of each plasmid sample were determined by measuring the absorbance at 260 nm using a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA), and the copy numbers of each cloned gene were quantified as previously described: copies/μL = concentration of plasmid (g/μL)/[(plasmid length × 660) × (6.022×10^{23})] (Parida et al., 2011). Ten-fold dilutions of the standard PCV2 or PCV3 DNA sample (from 10^6 to 10^0 copies/μL) were stored at −80 °C and used as standards for PCV2 or PCV3 quantitation of diagnostic samples.

2.3. Primers and probes for mqPCR

For the differential detection of PCV2 and PCV3, two sets of primers and probes were used for mqPCR. Primers and probes for PCV2 were used as described in a previous report (Olvera et al., 2004), with some base modifications to reflect the genetic variation of the target gene sequences among the different genotypes of PCV2 strains in Korea (Kwon et al., 2017a). Two qPCR assays have been developed to detect and quantify PCV3 DNA in previous studies (Palinski et al., 2017; Wang et al., 2017). These qPCR assays used a primers/probe set designed based on a limited number of US and Chinese PCV3 sequences that were available in GenBank when the assay was developed. In this study, primers and the probe for PCV3 were newly designed using Primer Express software (version 3.0) (Applied Biosystems, USA) based on a total of 32 PCV3 genome sequences, including five US, 16 Chinese, 9 Korean, and two Brazilian strains, available in National Center for Biotechnology Information (NCBI). To facilitate the establishment of multiplex qPCR, the sequences of the primers/probe for PCV3 were carefully selected so that their melting temperatures were similar to those of the PCV2 primers and probe. The lengths of amplicons for PCV2 and PCV3 were 99 and 118 base pairs (bp), respectively (Table 2). A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to check the specificity of the primers and probe. Each

Table 2
Primers and probes used in this study.

Virus	Primer/Probe	Sequence (5′−3′)[a]	Position[b]	Tm (°C)	Amplicon (bp)	Reference
PCV3	PCV3F	CGGTGGGGTCATATGTGTTG	1443–1462	62.5	118	In this study
	PCV3R	CACAGCCGTTACTTCACC	1543–1560	60		
	PCV3P	ROX-CTTTGTCCTGGGTGAGCGCTGGTAG-BHQ2	1496–1520	69.6		
PCV2	PCV2F	CCAGGAGGGCGTT**S**TGACT	1535–1553	61.3	99	Olvera et al. (2004), modified
	PCV2R	CG**Y**TACCG**Y**TGGAGAAGGAA	1614–1633	58.3		
	PCV2P	FAM-AATGGCATCTTCAACACCCGCCTCT-TAMRA	1612–1592	68.0		

[a] Bold text in sequences of PCV2F and PCV2R primers represent a degenerative base: S, C or G; Y, C or T, respectively.
[b] Genome position of primer- and probe-binding sequences according to the complete genome sequence of PCV2 KU-1601 strain and PCV3 29160 strain (GenBank accession no. KX828228.1 and NC-031753.1, respectively).