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Development of a Quantitative, Competitive-PCR (QC-PCR) Assay to Determine the DNA Load of Porcine Circovirus Type 2 (PCV2) in Blood and Fecal Swabs

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ABSTRACT

PCV2 is an essential causative agent of post-weaning multisystemic wasting syndrome (PMWS). The detection of PCV2 is not the definitive diagnosis since PCV2 infection does not always lead to PMWS; however, viral load may relate to PCV2-induced PMWS. In this study, a quantitative, competitive-polymerase chain reaction (QC-PCR) assay was developed to determine the amount of PCV DNA in whole blood and fecal swabs of pigs from PMWS-affected and PMWS-nonaffected farms. The QC-PCR was based on competitive co-amplification of a 345 bp fragment of the PCV 2 in the samples with a known concentration of the competitor DNA, which produced a 513 bp fragment. Blood and fecal swabs were collected from 140 pigs from 11 PMWS-affected and 14 PMWS-nonaffected farms. The results demonstrated that the PCV2 DNA from fecal swabs of pigs in the PMWS-affected farms ranged from less than 1 fg.μL^{-1} to 100 pg.μL^{-1} with a mean PCV2 DNA concentration of 6.42×10^7 copies.mL^{-1}, which was significantly higher than that from PMWS-nonaffected farms (3.8×10^5 copies.mL^{-1}). The results indicate correlation of PCV2 viremia and shedding to the development of PMWS. Therefore, the QC-PCR technique developed here could be applied as a tool to predict trends of the emergence and spread of the disease.

Keywords: Porcine circovirus; post-weaning multisystemic wasting syndrome; QC-PCR

INTRODUCTION

Porcine circovirus (PCV) belongs to the family *Circoviridae*. The virion is non-enveloped with icosahedral symmetry. It has a diameter of 17 nm and contains single-stranded, closed circular, ambisense genomic DNA of 1.7 kb. Both the viral DNA genome and the complementary DNA strand encode proteins (Meehan *et al*., 1997). The two major open reading frames (ORFs), ORF1 and ORF2, are oriented in opposite directions. ORF1 encodes replication-associated proteins (Rep)

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(Mankertz *et al*., 1998), while ORF2 encodes a viral capsid protein (Cap) (Nawagitgul *et al*., 2000). This arrangement creates two intergenic regions, between the 3´ends of the *rep* and *cap* genes and between their 5´ends. The later intergenic region contains the origin of viral genome replication. In addition to ORF1 and ORF2, ORF3 was later found to be responsible for induction of apoptosis by the activation of caspase 3 and 8 pathways (Liu *et al.,* 2005). In addition, an ORF3-deficient PCV2 mutant was found to be less pathogenic in mice than the wild type PCV2 (Liu *et al*., 2006).

PCV was divided into two genotypes—PCV1 and PCV2 (Meehan *et al*., 1998). PCV1 was discovered in 1974 (Tischer *et al*., 1974) and found to be non-pathogenic to pigs (Tischer *et al.,* 1982, 1986) while PCV2 is pathogenic when co-infected with other pathogens. Since its emergence in 1991, PCV2 has been associated with various clinical symptoms such as porcine dermatitis and nephropathy syndrome (PDNS) and post-weaning multisystemic wasting syndrome (PMWS). Subsequently, in 2006, the American Association of Swine Veterinarians approved the name "porcine circovirus associated disease" (PCVAD) for the clinical manifestation caused by PCV2 (Opriessnig *et al*., 2007). PMWS is currently considered as an important swine disease and potentially has a serious economic impact on the global swine industry. In particular, PMWS causes more than 50% case fatality in epidemic herds despite low morbidity (Harding, 1997). Clinical signs of the disease include progressive weight loss, lymph node enlargement, emaciation, diarrhea, pallor and jaundice. Various reports also demonstrated that PCV1 and PCV2 existed as early as 1985 since pig sera collected in Canada and United State during 1985 – 2000 were positive for both viruses (Magar *et al*., 2000; Nawagitgul *et al*., 2002). Although PCV2 is the primary causative agent of PMWS, a number of studies have suggested that other swine pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV) or porcine parvovirus (PPV) are usually required to increase pathogenesis of PMWS (Allan *et al.*, 1999, 2000; Kennedy *et al.*, 2000; Pogranichniy *et al.*, 2002; Rovira *et al.*, 2002; Opriessnig *et al.*, 2004; Dorr *et al*., 2007).

Currently, at least five subtypes of PCV2 have been identified worldwide—namely, PCV2a, PCV2b, PCV2c, PCV2d and PCV2e (Dupont *et al*., 2008; Segalés *et al*., 2008; Guo *et al*., 2010; Juntafong *et al*., 2011). PCV2a and PCV2b have been associated with clinical PCVAD with varying degrees of severity. Since 2005, PCV2b has been a dominant subtype found in pig populations and appears to be more pathogenic than other subtypes (Wiederkehr *et al*., 2009). The pathogenicity of PCV2c is unclear as it was only reported in non-diseased herds in Denmark (Dupont *et al*., 2008). More recently, PCV2d and PCV2e were discovered in Asia (Guo *et al*., 2010; Jantafong *et al*., 2011) suggesting continual evolution of PCV2 worldwide.

To date, the "gold standard" diagnostic techniques for PMWS and other PCVAD are *in situ* hybridization (ISH) and immunohistochemistry (IHC) (Sorden, 2000). Both techniques must be performed on *post mortem* specimens and can be time consuming (Chang *et al*., 2010). Alternatively, molecular techniques such as polymerase chain reaction (PCR) are used to detect PCV2 DNA in samples from live pigs since PCR is a sensitive, specific and rapid detection technique (Larochelle *et al*., 1999; Shibata *et al*., 2003; Caprioli *et al*., 2006). In addition, the PCV2 viral load seems to be different between PMWS-affected and PMWS-nonaffected pigs (Liu *et al.*, 2000; Quintana *et al*., 2001). Therefore, PCV2 viral quantification may be useful for PMWS diagnosis. A competitive PCR was described (Liu *et al.*, 2000) to detect PCV2 DNA in blood. Moreover, real-time PCR was developed to detect the amount of PCV2 and rapidly distinguish between PCV1 and PCV2 (Chang *et al*., 2010). However, the cost of the diagnosis is very expensive and a real-time

thermocycler is required to perform the assay. Therefore, in the current study, a quantitative, competitive-PCR (QC-PCR) assay was developed to determine the PCV2 viral load and shedding from pigs in PMWS-affected and PMWS-nonaffected farms in Thailand.

MATERIALS AND METHODS

Experimental design and sample collection

The samples were collected from 11 PMWS-affected and 14 PMWS-nonaffected farms. The farms were located in eastern (Chonburi province), northeastern (Buri Ram and Chaiyaphum provinces), western (Ratchaburi province) and central (Lop Buri, Nakhon Pathom, Saraburi and Suphan Buri provinces) Thailand. The PMWS-affected farms were selected according to the following criteria: (1) clinical signs characterized by wasting, pale skin and respiratory distress; (2) lymph-node enlargement; and (3) more than 5 to 10% production loss in nursery and fattening groups. The PMWS-nonaffected farms were selected from those without the clinical presentation of PMWS. The numbers of samples were calculated based on a preliminary PCV2 survey study using PCR for which lung samples were collected from three 5–10 week-old pigs per farm on 30 farms. The sampling showed that 10% of the lung samples were PCV2 positive. Therefore, 10% prevalence was used to calculate the sample numbers in this study. Whole blood and fecal swabs were collected from piglets aged 5–10 wk, with 5 piglets from each PMWS-nonaffected farm (n = 70) and 5 to 10 piglets from each PMWS-affected farm (n = 70). Whole blood was collected in tubes containing Ca-EDTA, the blood tubes were centrifuged at 2,000 rpm for 30 min and the plasma was stored at -80 °C until used. The fecal swabs were soaked in virus transport medium, vortexed and clarified by centrifugation. The suspension was kept at -80 °C until used. The plasma and fecal swabs were analyzed by conventional PCR.

DNA isolation and PCR analysis

Total DNA was extracted from the plasma and the suspension of fecal swabs using a commercial kit (Nucleospin® Blood), according to the manufacturer's instructions. The isolated DNA was amplified by PCR using PCV2 specific primers (Jantafong *et al*., 2011). In 20 µL of PCR reaction, 4 µL of the total DNA was added to the mixture containing 0.2 µM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 X PCR buffer and 0.4 units of Taq polymerase (Fermentas). The PCR cycle comprised denaturation of DNA at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, with final extension at 72 °C for 7 min. The amplified products were electrophoresed through 1.2% agarose gel and visualized by staining with ethidium bromide. One positive sample from each farm was selected for DNA sequencing to confirm the specificity of the primers.

Construction of PCV2 DNA competitor

Plasmid pKU14NS6 was amplified to generate a 168 bp DNA fragment with primers containing an *Nco*I site at the 5'end; NS1_403*Nco*I (5′-CGC GCC ATG GAT AGT GGA GCG GAT TCT G-3′) and NS1_571*Nco*I (5′-GCG GCC ATG GCA TTA TTG CCT GGT CCA T-3′). The PCR product was then purified using a QIAEX II gel extraction kit (QIAGEN) and cut with the *Nco*I restriction enzyme. The *Nco*I DNA fragment was dephosphorelated and cloned into the *Nco*I site, the unique recognition site at position 668/673 within the ORF1-PCV2 of the plasmid p31/31. It should be noted that the plasmid p31/31 is a plasmid pBluescript II KS+ containing the whole genome of PCV2 (Lekcharoensuk *et al*., 2004) (Figure 1). The primers for the competitive PCR, PCV2_563F (5´-CAG ACC CGG AAA CCA CAT ACT-3´) and PCV2_907R (5´-GGG AAA GGG TGA CGA ACT-3´), were resided on the conserved

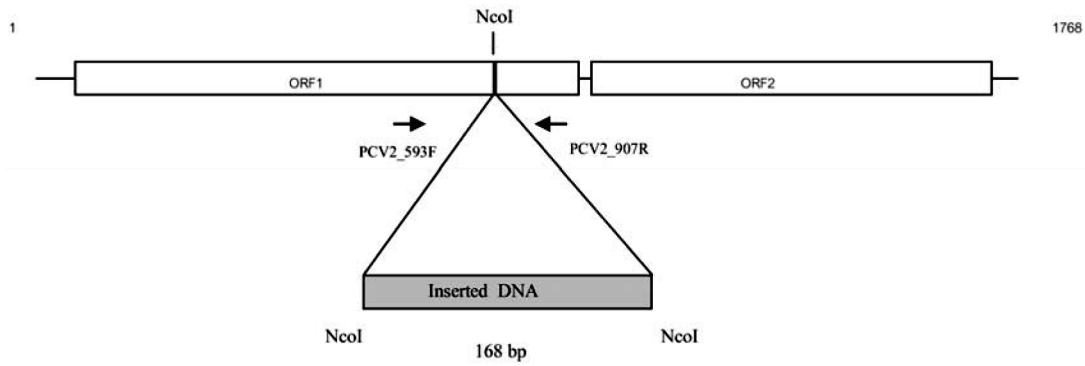

Figure 1 Schematic diagram of PCV2 genome and strategy for construction of the competitor plasmid DNA. The largest two open reading frames (ORFs) in the PCV2 genome are indicated by non shaded boxes. Arrows represent the primer positions. The inserted DNA for the construction of the competitor plasmid is demonstrated by the shaded box.

sequence within the PCV2 genome that flanks the *Nco*I insertion site to distinguish between PCV2 and the competitor DNA. The concentration of the competitor plasmid, so called p31/31NS1, was measured using a spectrophotometer at wave length 260/280 nm and its concentration was adjusted to be 100 ng.μL^{-1} prior to being used as the competitive DNA. When the competitor and PCV2 DNAs were co-amplified using the same set of primers, the amplified products of the competitor and PCV2-DNA were 513 and 345 bp, respectively.

Quantitative, competitive-PCR (QC-PCR)

QC-PCR was developed and standardized. The competitor plasmid DNA (p31/31NS1) and the wild type target plasmid DNA (p31/31) were co-amplified in one tube PCR reaction. Initially, these two plasmids were adjusted to have a concentration of 100 ng.μL^{-1}. Then, both competitor and target DNAs were tenfold serially diluted from 2.5 ng.μL^{-1} to 0.0025 fg.μL^{-1}. The various sets of QC-PCR were performed with each set containing a fixed amount of the target DNA (p31/31) and varied amounts of the competitor DNA (p31/31NS1). Equal volumes (1 μL) of the target DNA and diluted competitor DNA were used as the template in each QC-PCR reaction mixture. Positive and negative controls were included in each run. The target and competitor DNA were co-amplified using primers PCV2_563F and PCV2_907R. The PCR reactions were prepared to a final volume of 25 μL which contained 0.2 μM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 X PCR buffer and 0.4 units of Taq polymerase (Fermentas). The PCR cycle included denaturation of DNA at 95 °C for 3 min, followed by 30 cycles of 94 °C for 20 s, 48 °C for 20 s and 72 °C for 40 s, with the final extension at 72 °C for 7 min. The PCR products were separated by gel electrophoresis through 1.2% agarose gel and visualized by staining with ethidium bromide. The amplified competitor DNA (513 bp) can be distinguished from the target DNA (345 bp) by the size of the PCR products.

After standardization of the assay, the QC-PCR was used to quantify the PCV2 DNA in the fecal and blood samples by the method described previously. However, the competitor DNA was tenfold serially diluted in DNase- and RNase-free H_2O, from 10 ng.μL^{-1} to 1 fg.μL^{-1} instead of from 2.5 ng.μL^{-1} to 0.0025 fg.μL^{-1}.

Statistical analysis

The Wilcoxon rank-sum test, a non-parametric analytic method, was used to determine the significant differences of the data. The concentrations of the PCV2 DNA in blood and fecal swabs were transformed from nanograms (ng) or picograms (pg) or femtograms (fg) per microliter or from copies per milliliter to log 10. The Wilcoxon Rank-Sum test was used to calculate differences between the means of 1) PCV2 DNA in feces of pigs from the PMWS-affected and PMWS- nonaffected farms and 2) PCV2 DNA in feces and in whole blood of PMWS-affected farms.

RESULTS AND DISCUSSION

Co-amplified competitor with a fixed amount of wild-type PCV2 plasmid DNA

To examine whether there was competition between the competitor and PCV2 DNAs when they were co-amplified in one reaction tube, the QC-PCR was carried out with a fixed amount of the wild-type PCV2 plasmid DNA (p31/31) and various quantities of the competitor plasmid DNA. The results showed that at a high concentration of the competitor DNA, only a high density of the 513 bp DNA band was observed, because it can compete for more primer binding compared to the target DNA, p31/31. When the concentration of the competitor was gradually decreased while the concentration of p31/31 remained stable, the intensity of the 513 bp DNA band was progressively decreased while that of the 345 bp DNA band was observed and gradually increased. When the amounts of the PCV2 DNA and the competitor DNA were equal, the 513 bp and 345 bp bands appeared to have comparable intensities. Figure 2 shows the progressive competition between p31/31 at fixed amounts (2.5 pg.μL^{-1}, 250 fg.μL^{-1} or 25 fg.μL^{-1}) and varied amounts of the competitor DNA ranging from 2.5 ng.μL^{-1} to 0.025fg.μL^{-1}. The equivalent points determined the amount of the competitor DNA correctly as

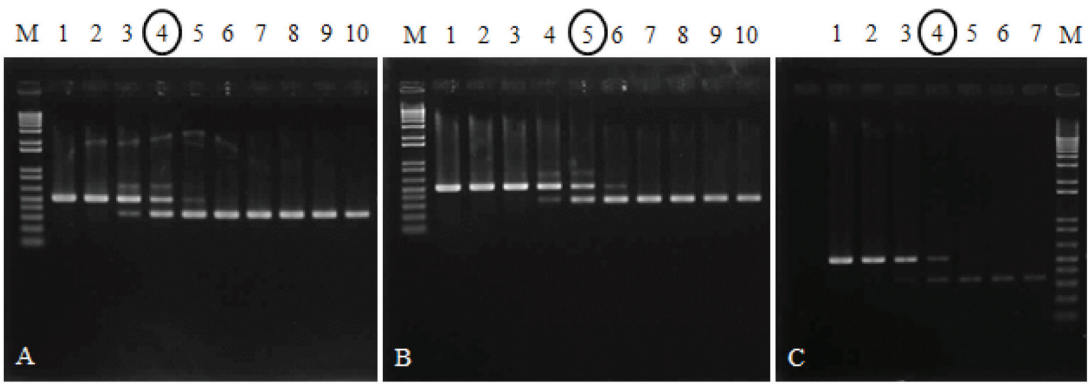

Figure 2 Quantification of wild-type PCV 2 plasmid DNA (p31/31) using QC-PCR. The QC-PCR was performed with a serial tenfold dilution of the competitor DNA (Figures 2A and 2B, lanes 1 to 10 are 2.5 ng.μL^{-1}, 250 pg.μL^{-1}, 25 pg.μL^{-1}, 2.5 pg.μL^{-1}, 250 fg.μL^{-1}, 25 fg.μL^{-1}, 2.5 fg.μL^{-1}, 0.25 fg.μL^{-1}, 0.025 fg.μL^{-1} and 0.0025 fg.μL^{-1}, respectively; Figure 2C, lanes 1 to 7 are 25 pg.μL^{-1}, 2.5 pg.μL^{-1}, 250 fg.μL^{-1}, 25 fg.μL^{-1}, 2.5 fg.μL^{-1}, 0.25 fg.μL^{-1}, 0.025 fg.μL^{-1} and 0.0025 fg.μL^{-1}, respectively). The competitor DNA was allowed to compete against either 2.5 pg (A), 250 fg (B) or 25 fg (C) of wild-type PCV2 plasmid DNA. The circled lane numbers in Figures A to C show the equivalent points which are 2.5 pg, 250 fg and 25 fg, respectively.

shown in lane 4 of Figures 2A (2.5 pg) and 2C (25 fg) and lane 5 of Figure 2B (250 fg). In addition, the detection limit of the assay was determined by tenfold serially diluting the target plasmid DNA to the lowest concentration of 0.025 fg.μL^{-1}. Then, a fixed amount of the target DNA was allowed to compete with a set of the competitor plasmid DNA with the concentration ranging from 2.5 pg.μL^{-1} to 0.025 fg.μL^{-1}. The QC-PCR was able to determine the concentration of the target plasmid as low as 2.5 fg.μL^{-1}.

Concentrations of PCV2 DNA in the samples

A conventional PCR was performed on fecal and blood samples from 140 pigs in PMWS-affected (n = 70) and PMWS-non affected (n = 70) farms. The results demonstrated that PCV2 DNA was detected in 40 blood and 36 fecal swab samples from PMWS-affected farms. In addition, PCV2-DNA was also detected in five fecal swabs from two PMWS-nonaffected farms. Subsequently, the concentration of PCV2 DNA was determined from the PCV2 positive samples using the QC-PCR. Examples of the QC-PCR results performed on the clinical samples are shown in Figure 3. The results showed that the amounts of PCV2 DNA from the fecal swabs of four pigs on the two PMWS-nonaffected farms were less than 1 fg.μL^{-1} (the lowest concentration of the competitor DNA), but one pig from one of these two farms had 10 pg.μL^{-1} of PCV2 DNA (Table 1). The PCV2 DNA in the fecal swabs of pigs in the PMWS-affected farms ranged from less than 1 fg.μL^{-1} (4/36 samples) to 100 pg.μL^{-1}. The mean viral loads in the fecal swabs from both farm categories were significantly different, with a *P* value of 0.0294. The results suggested that pigs from PMWS-affected farms shed higher amounts of PCV2 than those from PMWS-nonaffected farms. The viral load in the blood samples of pigs

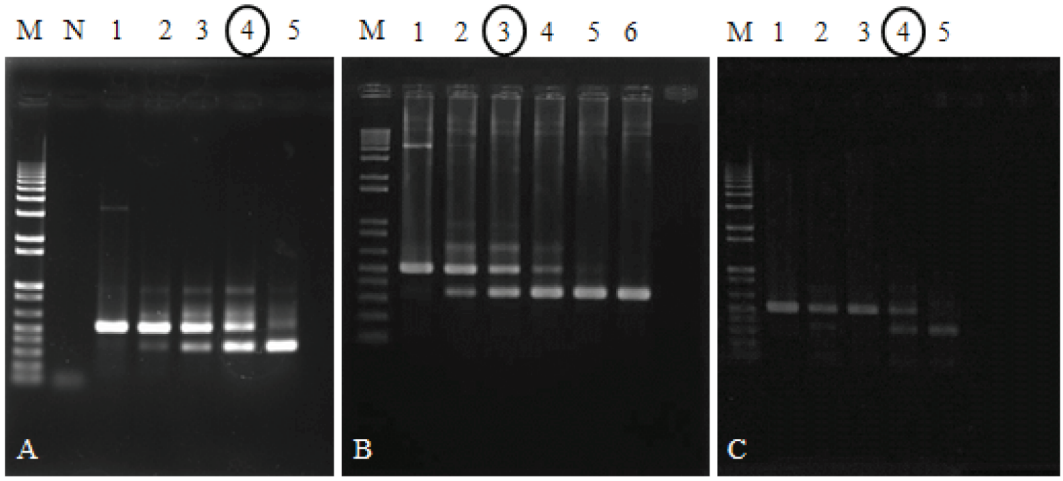

Figure 3 Examples of three sets of the QC-PCR assay. In each set, the competitive plasmid DNA was tenfold serially diluted to have the concentrations range from 10 ng.μL^{-1} to 10 or 1 fg.μL^{-1}. The competitor DNA in Figure 3A, lanes 1 to 5 are 10 ng.μL^{-1}, 1 ng.μL^{-1}, 100 pg.μL^{-1}, 10 pg.μL^{1} and 1 pg.μL^{1}, respectively and lane N is a negative control; Figure B, lanes 1 to 6 are 100 ng.μL^{1}, 10 ng.μL^{1}, 1 ng.μL^{1}, 100 pg.μL^{1}, 10 pg.μL^{1} and 1 pg.μL^{1}, respectively; Figure 3C, lanes 1 to 5 are 100 pg.μL^{1}, 10 pg.μL^{1}, 1 pg.μL^{1}, 100 pg.μL^{1}, 10 fg.μL^{1}, respectively. Each set contains a fixed amount of the target DNA from a fecal sample F49 (A) as well as whole blood samples B93 (B) and B91 (C). The circled lane numbers show the equivalent points which are 10 pg for F49, 1 ng for B93 and 100 fg for B91.

from the PMWS-affected farms ranged from less than 1 fg.μL^{-1} (16/40 samples) to 100 pg.μL^{-1}. The mean viral load in the fecal swabs and sera of pigs in the PMWS-affected group was not significantly different, with a correlation of 0.746 ($P < 0.05$). This indicated that PCV2-infected pigs with viremia generally shed viruses in the feces. The amount of DNA was calculated and the unit was transferred from the metric system to copy number per milliliter. For the pigs from PMWS-affected farms, the PCV2 DNA loads in the whole blood ranged from 1.9×10^2 to 1.9×10^9 copies.mL^{-1} (n = 40) with a mean of 6.05×10^7 copies.mL^{-1} while those in feces were from 1.9×10^2 to 1.9×10^9 copies.mL^{-1} (n = 36) with a mean of 6.42×10^7 copies.mL^{-1} (Table 1). There was no significant difference between the virus concentration in fecal swabs and whole blood samples. Interestingly, the mean PCV2 content in the feces obtained from the PMWS-affected farms (6.42×10^7 copies.mL^{-1}) was significantly higher than that obtained from PMWS-nonaffected farms (3.8×10^5 copies.mL^{-1}) with $P = 0.03$. This finding indicated that there might be a threshold of the PCV2 concentration required to trigger sufficient pathological changes resulting in clinical presentation of the PMWS (Liu *et al*., 2000). Although the amounts of PCV2 DNA from the fecal swabs of the four pigs in two PMWS-nonaffected farms were less than 1 fg.μL^{-1} or 1.9×10^2 copies.μL^{-1}, one pig from one of these farms had PCV2 DNA as high as 10 pg.μL^{-1} or 1.9×10^6 copies.μL^{-1}. Thus, PCV2 alone may not be sufficient to cause PMWS. However, it is possible that the pigs on this farm may have the potential to develop clinical presentation of PMWS later on.

CONCLUSION

In this study, a quantitative, competitive-PCR (QC-PCR) assay was developed to compare the viral load between PMWS-affected and PMWS-nonaffected farms in Thailand. The results showed that in the PMWS-non-affected farms, PCV2 DNA was detected in fecal swabs more frequently than in blood samples. It is likely that a fecal swab may be a suitable sample for PCV2 detection. However, in the farms that showed clinical presentation of PMWS, there was no significant difference of the viral concentration between blood and feces as determined by QC-PCR. This result was similar to a previous investigation (Caprioli *et al*., 2006). Although PCV2 DNA was also detected in the PMWS-nonaffected farms, it was detected in

Table 1 Results of QC-PCR performed from whole blood and fecal swabs of pigs from PMWS -affected and PMWS-nonaffected farms.

Farm type	Frequency distribution	Whole blood concentration	Fecal swab copy number (copy.mL^{-1})	Concentration	Copy number (copy.mL^{-1})
PMWS-affected farms	Min	<1 fg.μL^{-1}	$<1.9 \times 10^2$	<1 fg.μL^{-1}	$<1.9 \times 10^2$
	Mean	316 pg.μL^{-1}	6.05×10^7	337 pg.μL^{-1}	6.42×10^7
	Max	10 ng.μL^{-1}	1.9×10^9	10 ng.μL^{-1}	1.9×10^9
PMWS-nonaffected farms	Min	-	-	<1 fg.μL^{-1}	$<1.9 \times 10^2$
	Mean	-	-	2 pg.μL^{-1}	3.8×10^5
	Max	-	-	10 pg.μL^{-1}	1.9×10^6

Min = Minimum; Max = Maximum.

- = Negative samples as determined by conventional PCR.

<1 fg = Samples positive by conventional PCR but negative in QC-PCR.

the PMWS-affected farms in greater quantities. The results of this study and the previous report suggest a correlation between PCV2 viremia as well as shedding and the development of PMWS. Therefore, assays which can determine the DNA concentration, such as the QC-PCR technique developed in this study and real-time PCR, are useful to predict potential emergence and the spread of the PMWS in swine herds. In addition, comparison between the results from QC-PCR and real-time PCR would elucidate the correlation of the two assays which may increase the assay reliability.

LITERATURE CITED

Allan, G.M., F. McNeilly, B.M. Meehan, J.A. Ellis, T.J. Connor, I. McNair, S. Krakowka and S. Kennedy. 2000. A sequential study of experimental infection of pigs with porcine circovirus and porcine parvovirus: Immunostaining of cryostat sections and virus isolation. **J. Vet. Med. B. Infect. Dis. Vet. Public Health.** 47(2): 81–94.

Allan, G.M., S. Kennedy, F. McNeilly, J.C. Foster, J.A. Ellis, S.J. Krakowka, B.M. Meehan and B.M. Adair. 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. **J. Comp. Pathol.** 121(1): 1–11.

Caprioli, A., F. McNeilly, I. McNair, P. Lagan-Tregaskis, J. Ellis, S. Krakowka, J. McKillen, F. Ostanello and G. Allan. 2006. PCR detection of porcine circovirus type 2 (PCV2) DNA in blood, tonsillar and fecal swabs from experimentally infected pigs. **Res. Vet. Sci.** 81(2): 287–292.

Chang, G.N., J.F. Hwang, J.T. Chen, H.Y. Tsen and J.J. Wang. 2010. Fast diagnosis and quantification for porcine circovirus type 2 (PCV-2) using real-time polymerase chain reaction. **J. Microbiol. Immunol. Infect.** 43(2): 85–92.

Dorr, P.M., R.B. Baker, G.W. Almond, S.R. Wayne and W.A. Gebreyes. 2007. Epidemiologic assessment of porcine circovirus type 2 coinfection with other pathogens in swine. **J. Am. Vet. Med. Assoc.** 230(2): 244–250.

Dupont, K., E.O. Nielsen, P. Baekbo and L.E. Larsen. 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case–control study supports a shift in genotypes with time. **Vet. Microbiol.** 128: 56–64.

Guo, L.J., Y.H. Lu, Y.W. Wei, L.P. Huang and C.M. Liu. 2010. Porcine circovirus type 2 (PCV2): Genetic variation and newly emerging genotypes in China. **Virol. J.** 7: 273–284.

Harding, J. C. 1997. Post-weaning multisystemic wasting syndrome (PMWS): Preliminary epidemiology and clinical presentation. **Proc. Am. Assoc. Swine. Pract.** 27: 503.

Jantafong, T., A. Boonsoongnern, P. Poolperm, K. Urairong, C. Lekcharoensuk and P. Lekcharoensuk. 2011. Genetic characterization of porcine circovirus type 2 in piglets from PMWS-affected and -negative farms in Thailand. **Virol. J.** 8: 88.

Kennedy, S., D. Moffett, F. McNeilly, B. Meehan, J. Ellis, S. Krakowka and G.M. Allan. 2000. Reproduction of lesions of postweaning multisystemic wasting syndrome by infection of conventional pigs with porcine circovirus type 2 alone or in combination with porcine parvovirus. **J. Comp. Pathol.** 122(1): 9–24.

Larochelle, R., M. Antaya, M. Morin and R. Magar. 1999. Typing of porcine circovirus in clinical specimens by multiplex PCR. **J. Virol. Methods.** 80(1): 69–75.

Liu, J., I. Chen and J. Kwang. 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. **J. Virol.**

79: 8262–8274.

_____. 2006. The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis in vivo. **J. Virol.** 80(10): 5065–5073.

Liu, Q., L. Wang, P. Willson and L.A. Babiuk. 2000. Quantitative, competitive PCR analysis of porcine circovirus DNA in serum from pigs with postweaning multisystemic wasting syndrome. **J. Clin. Microbiol.** 28:3474–3477.

Magar, R., P. Müller and R. Larochelle. 2000. Retrospective serological survey of antibodies to porcine circovirus type 1 and type 2. **Can. J. Vet. Res.** 64(3): 184–186.

Mankertz, J., H.J. Buhk, G. Blaess and A. Mankertz. 1998. Transcription analysis of porcine circovirus (PCV). **Virus Genes.** 16(3): 267–276.

Meehan, B.M., F. McNeilly, D. Todd, S. Kennedy, V.A. Jewhurst, J.A. Ellis, L.E. Hassard, E.G. Clark, D.M. Haines and G.M. Allan. 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. **J. Gen. Virol.** 79 (Pt 9): 2171–2179.

Meehan, B.M., J.L. Creelan, M.S. McNulty and D. Todd. 1997. Sequence of porcine circovirus DNA : Affinities white plant circovirus. **J. Gen. Virol.** 78: 221–227.

Nawagitgul, P., P.A. Harms, I. Morozov, B.J. Thacker, S.D. Sorden, C. Lekcharoensuk and P.S. Paul. 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. **Clin. Diagn. Lab. Immunol.** 9(1): 33–40.

Nawagitgul, P., I. Morozov, S.R. Bolin, P.A. Harms, S.D. Sorden and P.S. Paul. 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. **J. Gen. Virol.** 81: 2281–2287.

Opriessnig, T., M. Fenaux, S. Yu, R.B. Evans, D. Cavanaugh, J.M. Gallup, F.J. Pallares, E.L. Thacker, K.M. Lager, X.J. Meng and P.G. Halbur. 2004. Effect of porcine parvovirus vaccination on the development of PMWS in segregated early weaned pigs coinfected with type 2 porcine circovirus and porcine parvovirus. **Vet. Microbiol.** 98(3-4): 209–220.

Opriessnig, T., X.J. Meng and P.G. Halbur. 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis and intervention strategies. **J. Vet. Diagn. Invest.** 19(6): 591–615.

Pogranichniy, R.M., K.J. Yoon, P.A. Harms, S.D. Sorden and M. Daniels. 2002. Case-control study on the association of porcine circovirus type 2 and other swine viral pathogens with postweaning multisystemic wasting syndrome. **J. Vet. Diagn. Invest.** 14(6): 449–456.

Quintana, J., J, Segalés and C. Rosell. 2001. Clinical and pathological observations of pigs with postweaning multisystemic wasting syndrome. **Vet. Rec.** 149: 357–361.

Rovira, A., M. Balasch, J. Segalés, L. García, J. Plana-Durán, C. Rosell, H. Ellerbrok, A. Mankertz and M. Domingo. 2002. Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. **J. Virol.** 76(7): 3232–3239.

Segalés J., A. Olvera, L. Grau-Roma, C. Charreyre, H. Nauwynck, L. Larsen, K. Dupont, K. McCullough, J. Ellis, S. Krakowka, A. Mankertz, M. Fredholm, C. Fossum, S. Timmusk, N. Stockhofe-Zurwieden, V. Beattie, D. Armstrong, B. Grassland, P. Baekbo and G. Allan. 2008. PCV-2 genotype definition and nomenclature. **Vet. Rec.** 162(26): 867–868.

Shibata, I., Y. Okuda, S. Yazawa, M. Ono, T. Sasaki, M. Itagaki, N. Nakajima, Y. Okabe and I. Hidejima. 2003. PCR detection of

porcine circovirus type 2 DNA in whole blood, serum, oropharyngeal swab, nasal swab and feces from experimentally infected pigs and field cases. **J. Vet. Med. Sci.** 65(3): 405–408.

Sorden, S.D. 2000. Update on porcine circovirus and postweaning multisystemic wasting syndrome. **Swine Health Prod.** 8: 133–136.

Tischer, I., H. Gelderblom, W. Vettermann and M.A. Koch. 1982. A very small porcine virus with circular single-stranded DNA. **Nature** 295(5844): 64–66.

Tischer, I., R. Rasch and G. Tochtermann. 1974. Characterization of papovavirus-and picornavirus-like particle in permanent pig kidney cell lines. **Zentralbl. Bakteriol. [Orig A].** 226(2): 153–167.

Tischer, I., W. Mields, D. Wolff, M. Vagt and W. Griem. 1986. Studies on epidemiology and pathogenicity of porcine circovirus. **Arch. Virol.** 91(3-4): 271–276.

Wiederkehr, D.D., T. Sydler, E. Buergi, M. Haessig, D. Zimmermann, A. Pospischil, E. Brugnera and X. Sidler. 2009. A new emerging genotype subgroup within PCV-2b dominates the PMWS epizooty in Switzerland. **Vet. Microbiol.** 136(1–2): 27–35.