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A multiplex real-time PCR assay for the detection and differentiation of the newly emerged porcine circovirus type 3 and continuously evolving type 2 strains in the United States

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ABSTRACT

A multiplex quantitative real-time polymerase chain reaction (mqPCR) assay was developed and validated for the detection and differentiation of porcine circovirus type 3 (PCV3) and type 2 (PCV2) strains. The assay coverage was 97.9% (184/188) for PCV3 and 99.1% (1889/1907) for PCV2 sequences that were available from the current GenBank database. The PCR amplification efficiencies were 98–99% for plasmids, and 92–96% for diagnostic samples, with correlation coefficients all greater than 0.99. The limit of detection (LOD) determined as plasmid copies per reaction was 17 for PCV3 and 14 for PCV2. The assay specifically detected the targeted viruses without cross reacting to each other or to other common porcine viruses. Among 336 swine clinical samples collected in 2018, 101 (30.1%) were PCV3 positive, 56 (16.7%) were PCV2 positive and 18 (5.4%) were co-positives. Sixty selected PCV3 positives were confirmed by Sanger sequencing, and 53 of the 56 PCV2 positive samples were tested positive by another validated PCR assay.

1. Introduction

Different from the non-pathogenic PCV1 strains (Tischer et al., 1974), PCV2 is considered a major swine pathogen causing porcine circovirus-associated diseases (PCVAD) including post-weaning multi-systemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), enteritis and reproductive failure (Opriessnig et al., 2007; Tischer et al., 1986). Since its first identification in 1998, PCV2 has been reported worldwide and has caused significant economic losses to the swine industry (Allan et al., 1998).

In 2015, another porcine circovirus, named PCV3, was found in the US that can cause PCVAD-like clinical symptoms similar to those caused by PCV2. The symptoms include PDNS and reproductive failure, and cardiac and multi-organ inflammation (Palinski et al., 2017; Phan et al., 2017). PCV3 has also been detected in several other countries including Brazil, China, Thailand, Sweden, Denmark, Italy, Poland and Spain (Chen et al., 2017; Franzo et al., 2018; Kedkovid et al., 2018; Stadejek et al., 2017; Tochetto et al., 2018; Xu et al., 2018; Ye et al., 2018).

Porcine circoviruses are small non-enveloped viruses with a circular single-stranded DNA genome, belonging to genus *Circovirus* in the family of *Circoviridae*. In the viral genome, ORF1 codes for the replicase (Rep) protein and ORF2 for the capsid (Cap) protein. The Rep protein is a non-structural protein and functions for the viral replication, while the structural Cap protein dominates immunogenicity (Cheung, 2003; Mankertz et al., 1998a, b; Nawagitgul et al., 2002). As a DNA virus, PCV2 has high evolutionary mutation rate of 1.2×10^{-3} substitutions/site/year, and has been constantly evolving. With the emerging viral strains, PCV2 has been divided into 5 genotypes, namely PCV2a–2e strains, according to the diversity level of the ORF2 nucleotide sequences (Franzo et al., 2016; Segales et al., 2008; Xiao et al., 2016). The continued mutation in the PCV2 genome (Eddicks et al., 2015; Yang et al., 2018) made it more difficult to identify, especially by some older molecular detection methods.

Although the PCV3 genomes are different from PCV2 genomes, they cause similar syndromes and it is difficult to differentiate clinical symptoms caused by the two circoviruses. The objective of this study was to develop a multiplex quantitative real-time PCR (mqPCR) assay

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to rapidly detect and differentiate the two important circoviruses, with significantly improved diagnostic coverage to current field strains.

2. Materials and methods

2.1. Viral isolates

Cell culture isolates of PCV2a, PCV2b and PCV2d, porcine reproductive and respiratory syndrome virus type 2 (PRRSV-2) and Seneca Valley virus 1 (SVV-1) that were previously confirmed by culture and sequencing were used in this study.

2.2. Multiplex real-time PCR assay design

According to the 188 PCV3 whole genome sequences that were available from the GenBank database, one set each of primers and probes were designed from ORF1 and ORF2. Both PCV3 probes were labeled with 5′-FAM and 3′-BHQ1. Based on 1907 PCV2 whole genome sequences from the GenBank database, two sets of primers and probes were chosen from ORF1 and ORF3 respectively. The PCV2 probes were labeled with 5′-VIC and 3′-BHQ1. A conserved swine gene, serum beta-2-microglobulin (SB2M) was applied as internal control in the assay, coding for a small membrane protein that may be involved in immune system regulation (Xie et al., 2003). The SB2M probe was labeled with 5′-Cy5 and 3′-BHQ2. Information of all primers and probes are given in Table 1.

2.3. Viral DNA extraction and standard control constructs preparation

The viral DNA was extracted from 140 µl of clinical samples or cell culture by QIAamp Viral RNA Mini Kit (Qiagen, MD) or ZR Viral DNA/RNA Kit (Zymo Research, CA) according to the manufacturer's recommendations, and stored at −80 °C until use. To ensure that the obtained PCV3 genome was from a single genome, and not amplified from more than one genome, a full length PCV3 genome was amplified by a single pair of tail-to-tail primers that overlapped at a unique *Pst*I site in the PCV3 genome (illustrated in Fig. 1). The full-genome amplicon of PCV3 was then digested with the *Pst*I restriction enzyme and cloned into pACYC177 cloning vector. The PCV2 fragments containing the assay targets were also amplified, and the PCR product was cloned into the pCR™2.1 vector using the original TA Cloning kit (Invitrogen, CA). The presence of cloned inserts was confirmed by gel electrophoresis and subsequent DNA sequencing. The primers used for cloning are also listed in Table 1.

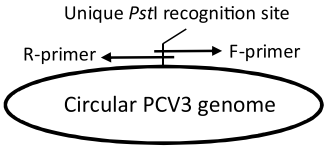

Fig. 1. Sketch of PCV3 genome amplification using tail-to-tail overlapping primers that were designed to have an unique type II restriction endonuclease, *Pst*I recognition site in the overlapping region that was later used for full-genome cloning. This is to ensure that the genome amplified is from the same genome, and not from separate genomes.

2.4. Multiplex real-time PCR reaction composition and condition

All PCR reactions were performed in a 20 µL reaction composed of 4 µL of DNA samples prepared as described (Shi et al., 2016), 0.4 µM each of forward and reverse PCR primers, 0.2 µM each of probes, and 10 µl of 2X iQ™ Multiplex Powermix (Bio-Rad, CA). The parameters for thermocycling start with an initial denaturation at 94 °C for 10 min, followed by 45 cycles of 94 °C for 15 s and 60 °C for 45 s. The cycle threshold (Ct) values were generated with CFX96 TouchTM Real-Time PCR Detection System and standard curve results were analyzed with Bio-Rad CFX Manager 3.0.

2.5. Assay sensitivity and specificity analysis

The analytical sensitivity was determined by generating standard curves with triplicates of 10-fold serial dilutions of control constructs and positive clinical samples, and a cultured PCV2d isolate. To obtain accurate LODs, 2-fold serial dilutions were prepared from the last 10-fold dilution to fine-tune the least concentration that still amplifies. The assay specificity was evaluated with cell cultures (PCV2a, PCV2b, PCV2d, PRRSV-2 and SVV-1) and clinical samples positive to specific pathogens (PCV3, PRRSV-2, groups A, B and C swine rotaviruses, swine

Table 1
Information of primers and probes used in this study.

Primer/ Probe	Target Gene	Sequence (5′–3′)	Tm (°C)	Amplicon Size (bp)	Coverage	Location on JX535288 (PCV2), KX778720 (PCV3) or AK398890 (SB2M)
Real-time PCR primers and probes						
PCV3-F1	ORF2	GGTGAAGTAACGGCTGTGTTTT	60.4	86	92.5%	1550–1571 nt
PCV3-R1		ACACTTGGCTCCARGACGAC	60.3		(174/188)	1635–1616 nt
PCV3-Pr1		FAM-ATGCGGAAAGTTCCACTCGK-BHQ1	62			1592–1611 nt
PCV3-F2	ORF1	TATAATGGGGAGGGTGCTGT	59.3	76	89.4% (168/	820–839 nt
PCV3-R2		CCCCAATTCTCAGCAATTCA	61		188)	895–876 nt
PCV3-Pr2		FAM-TGATTTTTATGGGTGGGTTCCATTT-BHQ1	65.2		(97.9% combined)	849-873 nt
PCV2-F1	ORF1	GARACTAAAGGTGGAACTGTACC	57-58	118	94.8%	762–784 nt
PCV2-R1		TCCGATARAGAGCTTCTACAGC	59		(1808/1907)	879–858 nt
PCV2-Pr1		VIC-AGGAGTACCATTCCAACGGGG-BHQ1	62.5			823–843 nt
PCV2-F2	ORF3	CGGGCTGGCTGAACTTTTG	59.5	87	90.5% (1726/	494-512 nt
PCV2-R2		CCAGGTGGCCCCACAAT	59		1907)	580–564 nt
PCV2-Pr2		VIC-TCACGCTTCTGCATTTTCCCGC-BHQ1	64.5		(99.1% combined)	520-541 nt
SB2M-F	SB2M	TGATGTTACCACAAATGTTGTCTTC	60.2	88		684-708 nt
SB2M-R		CCTCTACATCTACCTGCTCAGACA	60			771-748 nt
SB2M-Pr		Cy5-ATTCTACCTTGGGTGTAGTCTCCATGT-BHQ2	63.4			715–741 nt
Cloning and sequencing primers						
PCV3-cF	ORF1	GCCTGCAGTATTTATACGCTATGGGC	66.5	2000		618–643 nt
PCV3-cR	ORF1	TACTGCAGGCATCTTCTCCGCAACTTC	71			601–627 nt
PCV2-cF	ORF1	TGGTGACCGTTGCAGAGCAG	65.9	1093		445–464 nt
PCV2-cR	ORF2	TGGGCGGTGGACATGATGAG	67.7			1517-1536 nt

influenza virus (SIV), porcine epidemic diarrhea virus (PEDV), porcine delta coronavirus (PDCoV), porcine parvovirus (PPV) and porcine parainfluenza virus type 1 (PPIV-1) (summarized in Table3).

2.6. Comparison of multiplex and singular assays

To compare the performances of the singular PCV2 and PCV3 assays with the multiplex assay, standard curves were also generated for both singular assays and at the multiplexed condition that composed PCV2, PCV3 and the internal control. The performance of multiplex and singular assays was compared using PCV3 and PCV2 standard control constructs and a PCV3 positive sample and a cultured PCV2 isolate. The individual viral DNA was subjected to singular assays while a mixture of equal molarity of PCV2 and PCV3 DNA was used for the multiplex assay. Standard curves were generated and PCR efficiency analysis between the singular and the multiplex assays was performed.

2.7. Evaluation with clinical samples

A total of 336 clinical porcine samples collected in 2018 with different sample types including serum, oral fluid, feces and stomached organ tissue homogenates of heart, lung, thymus, tonsil, liver, spleen, kidney, fetus, placenta, lymph nodes and intestine, were collected from Kansas State Veterinary Diagnostic Laboratory (KSVDL). The mqPCR assays were performed on these samples for PCV3 and PCV2 detections, and for potential coinfection identifications.

3. Results

3.1. Analysis of assay coverage to GenBank sequence database

Based on 188 PCV3 full genome sequences in the GenBank, two sets of primers and probes, targeting the respective *cap* gene and *rep* gene, were designed for PCV3 detection. The *cap* gene set matched 92.6% (174/188) and the *rep* gene set matches 89.4% (168/188) of the strains with an overall coverage of 97.9%. The four mismatching strains all had a single nucleotide variation in the primers or probes (data not shown). The PCV2 sets were designed based on 1907 PCV2 whole genome sequences from the GenBank, including five genotypes, PCV2a–2e. To ensure high coverage of divergent PCV2 genomes, two sets of primers and probes were designed in ORF1 and ORF3 genes with coverages of 94.8% (1808/1907) and 90.5% (1726/1907), respectively. The combined coverage of the two PCV2 sets was 99.1% (1889/1907) based on an *in silico* analysis. Information of all primers and probes are shown in Table 1.

3.2. Analytical sensitivity of the multiplex real-time PCR assay on cloned positive controls

Analytical sensitivity of the mqPCR assay was analyzed using standard curves generated by three replications of 10-fold serial dilutions of the template, and analyzed by plotting their Cts against dilution factors. The results indicated that the PCR amplification efficiencies were 98.9% for PCV3 and 98.5% for PCV2 with correlation coefficient (R^2) both greater than 0.995 (Fig. 2A). For more accurate determination of limit of detection (LOD) of the assay, the cloned standard controls from the last 10-fold dilution were further diluted by 2-fold serial dilutions. The results indicated that LODs were 17 copies per PCR reaction for PCV3 and 14 copies per reaction for PCV2.

3.3. Analytical sensitivity of the multiplex real-time PCR assay on a viral isolate and clinical samples

To make sure that the assay sensitivity was not compromised when tested on virus and diagnostic samples, a PCV2d cell culture isolate and PCV3 positive clinical samples (we have not been able to culture PCV3) were also used. The standard curves generated by 10-fold dilutions were plotted with Cts versus dilution factors and showed that the amplification efficiencies were 92.9% for PCV3 and 95.3% for PCV2 with R^2 greater than 0.995 for both viruses. The LOD of the PCV2d cell culture isolate was around 1.4 TCID50 per reaction. A more accurate Ct cutoff for detection limit was also determined by 2-fold serial dilutions starting with the last 10-fold dilution that still generated signals. The results indicated that the cutoff for both PCV3 and PCV2 positive for diagnostic samples was Ct 37. The standard curve of the internal control SB2M showed that there is no inhibition to PCR amplification (Fig. 2B).

3.4. Diagnostic sensitivity of the assay

From mqPCR data, 60 selected PCV3 positive samples with Ct values ranging from 18 to 35 were verified by Sanger sequencing, indicating a 100% diagnostic sensitivity. For PCV2, 53 of 56 positive samples were verified as positives by a previously validated PCV2 qPCR assay that is currently used at KSVDL. Thus for the 53 positives identified by the current KSVDL assay, the diagnostic sensitivity of the newly developed assay is also 100%. The new assay may have higher diagnostic sensitivity as it detected three more positive samples.

3.5. Comparison of multiplex and singular assays

As shown in Fig. 2, the mqPCR generated similar correlation coefficients and PCR amplification efficiencies to those generated by singular qPCR reactions. Both multiplex and singular assays had R^2 greater than 0.994 and PCR amplification efficiencies between 92.9% and 96.9%. The Ct values for serial dilutions of clinical positive samples in singular and multiplex reactions were nearly identical (Table 2) indicating that multiplexing is not reducing the assay's sensitivity.

3.6. Specificity of multiplex real-time PCR assay

The specificity of primers and probes was evaluated by an *in silico* analysis with NCBI primer design tool, which presented a unique viral target for each set of assay. Assay specificity was further tested experimentally with a PCV3 dominated clinical sample as confirmed by next generation sequencing, viral isolates (PCV2a, PCV2b, PCV2d, PRRSV-2, and SVV-1), and clinical samples that were previously tested positive to non-target, specific pathogens. The results demonstrated that assay specifically detected PCV2 and PCV3 positive samples without cross detection and no positive targets were detected from samples that were positive to other common porcine viruses that included 16 PEDV positive samples, 16 PRRSV positives, 7 SIV positives, 1 PPIV positive, 1 PDCoV positive, 1 PPV positive, and one positive sample each to group A, group B and group C rotaviruses. Results showed that there was no signal generated on those non-target positive samples indicating a good specificity of the assay (Table3).

3.7. Prevalence of PCV2 and PCV3 on clinical samples collected in 2018

The 336 clinical porcine samples collected in 2018 with different sample types were tested for PCV2 and PCV3 to determine assay performance and viral prevalence in the field. Of the 336 samples, 56 (16.7%) were PCV2 positives, 101 (30.1%) were PCV3 positives. Among the 139 PCV2 and PCV3 positive samples, 18 (5.4% of total number of samples, and 12.9% of positive samples) were positive for both PCV3 and PCV2 viruses (Table 4). The house keeping gene, SB2M, that was included as an internal control, generated Cts on these diagnostic samples mostly in between 20–30, indicating an efficient nucleic acid extraction and no PCR inhibition observed.

4. Discussion

PCVAD is a common disease in swine production systems and has

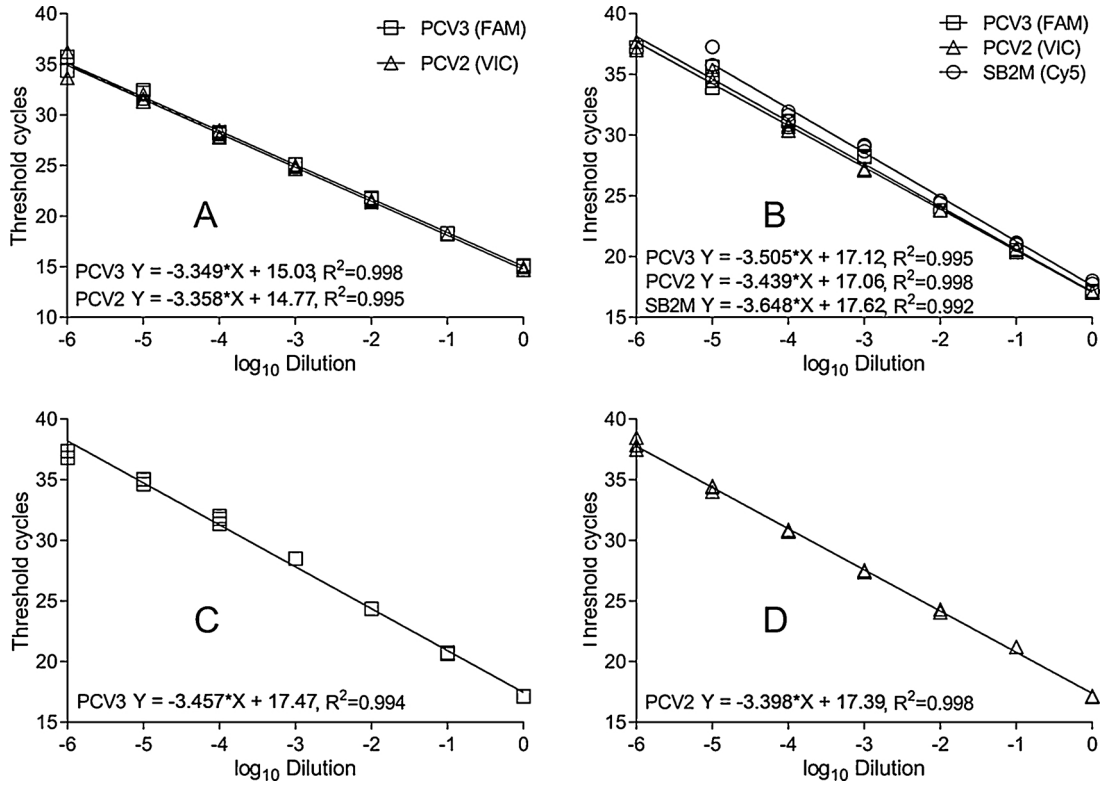

Fig. 2. Standard curves of (A) PCV3 and PCV2 mqPCR by serial dilutions of cloned control constructs (whole genome of PCV3 and half genome of PCV2); (B) PCV3 and PCV2 mqPCR by serial dilutions of mixture of PCV3 clinical positive samples and PCV2d cell culture with the inclusion of the internal control, SB2M; (C) PCV3 singular qPCR by serial dilutions of a clinical PCV3 positive sample; (D) PCV2 singular qPCR by serial dilutions of a PCV2d cell culture isolate.

Table 2
Analytical sensitivity comparison of the multiplex assay and singular assays.

		PCV3		PCV2	
		Singular	Multiplex	Singular	Multiplex
Efficiency (E)		94.7%	92.9%	96.9%	95.3%
Correlation coefficient (R^2)		0.994	0.995	0.998	0.998
Mean Cts of 3 replicates of clinical samples at different dilutions	10^0	17.14	17.13	17.14	17.07
	10^{-1}	20.69	20.70	21.24	20.62
	10^{-2}	24.37	23.95	24.16	23.86
	10^{-3}	28.54	27.23	27.48	27.17
	10^{-4}	31.73	30.74	30.81	30.64
	10^{-5}	34.92	34.89	34.30	34.79
	10^{-6}	37.10	37.44	37.94	37.36

caused significant economic losses. PCV2 is a major pathogen involved in PCVAD (Segales et al., 2008). A new porcine circovirus, PCV3, was identified in 2015 and is causing PCVAD-like clinical signs, including PDNS and reproductive failure, and cardiac and multi-organ inflammation, and thus has attracted researchers' and producers' attention (Chen et al., 2017; Palinski et al., 2017; Phan et al., 2017; Stadejek et al., 2017). Considering the similar clinical symptoms caused by PCV2 and PCV3, and that PCV2 assays built many years ago are no longer covering the majority of field strains, there was a need to develop a mqPCR assay that was based on the most current sequencing data and capable of detecting the two viruses with high diagnostic coverage to field strains.

Although several molecular detection methods were developed for rapid detection of PCV3 and PCV2 (Kim et al., 2017; Li et al., 2018; Zhang et al., 2018), our mqPCR assay showed apparent advantages. With the help of bioinformatics tools, 1907 PCV2 full genome sequences that were currently available in the GenBank were downloaded and analyzed to achieve high coverage in the assay design stage. We believe that the coverage of the primers and probes over available sequences in the designing stage is the best estimation of assay's future diagnostic sensitivity against field strains (Bai et al., 2018). Compared to the three published duplex assays, our assay has much higher coverages: 97.9% (184/188) for PCV3 and 99.1% (1889/1907) for PCV2. All 4 PCV3 strains that our primers or probes mismatch to are all single nucleotide variations, and very often these oligoes that have single nucleotide mismatches will not affect their ability of binding to the templates. Therefore, the actual diagnostic sensitivity for PCV3 can be higher than this estimation. Using two non-overlapping targets for a given virus may not increase the assay's analytical sensitivity, but it will increase field strain coverage, and will help to detect strains with additional mutations, as the chance for a strain to have mutations on both target sites is small. Also, compared to the LOD of 50 copies per reaction in Kim et al. (2017) and 90 copies per reaction in Zhang et al. (2018), our assay appeared to be more sensitive: 17 copies per reaction of PCV3 and 14 copies per reaction of PCV2. The LOD of PCV2 was also evaluated with cell culture, with an LOD of around 1.4 TCID50 per reaction. In addition, the internal control, swine SB2M gene, is included in our assay to monitor nucleic acid extraction efficiencies and potential PCR inhibitions in order to reduce the false-negative rate, which were not used in the previous studies.

In our study, different types of porcine samples were collected and subjected to the mqPCR testing. The viral targets were detected from porcine serum, oral fluid, feces, intestines and tonsil, indicating that our assay can be used for a wide range of sample types that are encountered in routine diagnostic operations. It is interesting to see, with our limited data, that the PCV3 positive rate was 30.1%, which was much higher than the PCV2 positive rate of 16.7%. We currently do not have the data to indicate whether the application of PCV2 vaccines in recent years play a role in reducing PCV2 prevalence. We will keep monitoring the field samples to see if this prevalence data holds true in the future.

In conclusion, the newly developed and validated mqPCR assay

Table 3
Assay specificity analysis using viruses or clinical samples.

Pathogen	Source	No. tested	Target gene		
			PCV3 (FAM)	PCV2(VIC)	SB2M(Cy5)
PCV3	Clinical sample[a]	1	+	–	+
PCV2a	Cell culture	1	–	+	+
PCV2b	Cell culture	1	–	+	+
PCV2d	Cell culture	1	–	+	+
PCV3 + PCV2a	Clinical sample + cell culture	1	+	+	+
PCV3 + PCV2b	Clinical sample + cell culture	1	+	+	+
PCV3 + PCV2d	Clinical sample + cell culture	1	+	+	+
PRRSV-2	Clinical sample/Cell culture	16/2	–	–	+
SVV-1	Cell culture	1	–	–	+
Rotavirus A	Clinical sample	1	–	–	+
Rotavirus B	Clinical sample	1	–	–	+
Rotavirus C	Clinical sample	1	–	–	+
SIV	Clinical sample	7	–	–	+
PEDV	Clinical sample	16	–	–	+
PDCoV	Clinical sample	1	–	–	+
PPV	Clinical sample	1	–	–	+
PPIV	Clinical sample	1	–	–	+

+: Positive; −: Negative.
[a] Next generation sequencing resulted strong positive to PCV3, and negative to other major swine viruses.

Table 4
Prevalence of PCV3 and PCV2 in 336 porcine samples used in this study.

PCV3 positive (%)	PCV2 positive (%)	PCV2-PCV3 co-positive (%)
101 (30.1)	56 (16.7)	18 (5.4)

enables us to perform rapid, sensitive and specific detection and differentiation of PCV3 and PCV2 strains in clinical samples. *In silico* analysis and clinical sample testing indicated that the assay has high strain coverage for both viruses. Our limited prevalence data indicated that PCV3 strains have been widely distributed, and may be more prevalent than PCV2 strains currently in the US.

Conflicts of interest statement

The authors declare that they have no competing interests.

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