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Journal of Virological Methods 286 (2020) 113971

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Development of a differential multiplex real-time PCR assay for porcine circovirus type 2 (PCV2) genotypes PCV2a, PCV2b and PCV2d

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ARTICLE INFO

Keywords:
PCV2
Genotype
PCV2a
PCV2b
PCV2d
Multiplex real time PCR

ABSTRACT

A multiplex quantitative real-time polymerase chain reaction (mqPCR) assay was developed and validated for detection and differentiation of porcine circovirus type 2 (PCV2) genotypes, PCV2a, PCV2b and PCV2d. Single nucleotide polymorphism in primers or probes was deployed for different genotype detections, while conserved sequence in the 3' end of a primer and in the middle of a probe was used for the targeted genotype. *In silico* analysis of 2601 PCV2 ORF2 sequences showed that the predicted strain coverage of the assay was 93.4 % (409/438) for PCV2a, 95.1 % (1161/1221) for PCV2b and 93.6 % (882/942) for PCV2d strains. The PCR amplification efficiencies were 94.5 %, 100.2 %, and 99.2 % for PCV2a, PCV2b and PCV2d, respectively, with correlation coefficients >0.995 for all genotypes. The limits of detection (LOD) were 1.58×10^{-4} TCID50/mL for PCV2a, 5.62×10^{-4} TCID50/mL for PCV2b, and 3.16×10^{-3} TCID50/mL for PCV2d. Sanger sequencing of 74 randomly selected PCV2 positive clinical samples confirmed the genotypes of strains identified by the mqPCR. Validation with clinical samples co-positive for target and non-target pathogens demonstrated that the mqPCR assay specifically detected targeted viruses without cross reacting to each other or to other common porcine viruses.

1. Introduction

Porcine circovirus type 2 (PCV2) plays a significant role in porcine circovirus associated diseases (PCVAD) and is one of the most economically important porcine viral pathogens (Gillespie et al., 2009). Since it was first isolated as the causative agent of postweaning multisystemic wasting syndrome in 1998 (Ellis et al., 1998), PCV2 has been a highly prevalent disease worldwide, including in Asia, America and Europe (Saporiti et al., 2020; Wang et al., 2019a; Zheng et al., 2020).

With a high substitution rate, 5 major genotypes of PCV2 have been identified based on ORF2 sequences: PCV2a, PCV2b, PCV2c, PCV2d and PCV2e (Davies et al., 2016). Among the five genotypes, PCV2a, PCV2b and PCV2d are the most commonly circulating genotypes in the USA (Wang et al., 2019b; Xiao et al., 2016), in China (Hou et al., 2019; Lv et al., 2020) and in European countries (Saporiti et al., 2020). There are two major genotype shifting events that have occurred globally: PCV2b replaced PCV2a as the dominant genotype around 2003, and PCV2d became the most prevalent genotype beginning in 2012 (Xiao et al., 2016). The first shift is related to increased severity of clinical PCVAD (Beach and Meng, 2012), while the second shift may be related to inappropriate vaccine applications (Karuppannan and Opriessnig, 2017).

ORF2-targeted gene sequencing is widely used as a gold standard for genotyping of PCV2 strains (Wang et al., 2019b). Other methods are developed as well for the differentiation of PCV2a and PCV2b strains, such as real time PCR, loop-mediated isothermal amplification method (LAMP) and restriction fragment length polymorphism (RFLP) (Guo et al., 2010; Qiu et al., 2012; Wozniak et al., 2019; Xiao et al., 2016). However, there's no assay that can simultaneously identify genotypes PCV2a, PCV2b and PCV2d. Therefore, based on the current PCV2 ORF2 sequences available from GenBank database, we have developed a multiplex quantitative real time PCR for rapid detection and differentiation of the three genotypes with high sensitivity, specificity and strain coverage.

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https://doi.org/10.1016/j.jviromet.2020.113971
Received 16 July 2020; Received in revised form 7 September 2020; Accepted 8 September 2020
Available online 12 September 2020

2. Materials and methods

2.1. Sequence dataset and phylogenetic analysis

The sequence dataset was established from our previous study (Wang et al., 2019b). Briefly, all available PCV2 ORF2 sequences were downloaded from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The sequence alignment was performed with MAFFT (Katoh, Misawa, Kuma, & Miyata, 2002). Low quality sequences, as indicated by the presence of premature stop codons, and short sequences were removed from the analysis. The sequence identity matrix calculation was performed using BioEdit 7.2.5 (https://bioedit.software.informer.com/).

The phylogenetic analysis was conducted with MEGA 7.0.26 (Kumar et al., 2016). The best fit model was selected based on Bayesian information criterion (BIC). The maximum likelihood phylogenetic trees were constructed using the best substitution pattern with the lowest BIC scores. The reliability of clusters formed in the tree was evaluated by performing 500 bootstrapping reiterations.

2.2. Multiplex real-time PCR assay design

Based on the sequences from the dataset, one set of primers and probe was designed for each genotype (PCV2a, PCV2b and PCV2d). The PCV2a, PCV2b and PCV2d probes were labeled with 5′-Texas Red and 3′-BHQ1, 5′-VIC and 3′-MGBNFQ, and 5′-FAM and 3′-MGBNFQ, respectively (Table 1).

2.3. Viral isolates and viral DNA or RNA extraction

Cell culture isolates of PCV2a, PCV2b and PCV2d from the Virology Section of Kansas State Veterinary Diagnostic Laboratory were propagated as previously described (Pogranichniy et al., 2002), which were genotyped by sequencing. The viral DNA was extracted from 140 µl of clinical samples or cell culture by QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations, and stored at −80 °C until use.

2.4. Construction of standard plasmid as positive amplification control

For positive assay controls applied in routine testing, PCV2 fragments carrying the target of each genotype were amplified and cloned into the pCR™2.1 vector using the original TA Cloning kit according to the manufacturer's instruction (Invitrogen/ThermoFisher, Waltham, MA). The ligated products were then transformed in Mix & Go competent cells (Zymo Research, Irvine, CA), propagated in LB broth (Teknova Inc, Hollister, CA), and the plasmid construct was then extracted using QIAprep Spin Miniprep Kit (Qiagen). The presence of cloned inserts was confirmed by gel electrophoresis and Sanger sequencing (Genewiz, South Plainfield, NJ). The primers used for cloning are also listed in Table 1.

2.5. Multiplex real-time PCR reaction composition and condition

All PCR reactions were performed in a 20 µL total reaction volume composed of 5 µL of DNA samples prepared as described (Wang, Das, et al., 2019), 0.25 µM each of forward and reverse PCR primers, 0.25 µM each of probes, and 10 µl of 2X iQ™ Multiplex Powermix (Bio-Rad, Hercules, CA). Thermocycler running conditions consisted of initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 45 s. The cycle threshold (Ct) values were generated with CFX96 Touch™ Real-Time PCR Detection System and standard curve results were analyzed with Bio-Rad CFX Manager 3.0 (Bio-Rad) and GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

2.6. Assay sensitivity and specificity analysis

Standard curves were generated with triplicates of 10-fold serial dilutions of the cell culture isolates of PCV2a, PCV2b and PCV2d to evaluate the analytical sensitivity of the assay. The median tissue culture infectious dose (TCID50) of the highest dilution that still generated positive Ct values was considered the limit of detection (LOD) for cultured viruses.

The specificity of the assay was first evaluated *in silico* with the online NCBI primer designing tool, Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), followed by confirmation with Sanger sequencing of PCV2 ORF2. The conserved primers flanking the ORF2 were designed and listed in Table 1. Furthermore, the cell culture

Table 1
Primers and probes used in PCV2 genotyping real-time PCR assays and construction of positive standards.

Primer/ Probe	ORF location	Sequence (5′-3′)	Tm (°C)	Amplicon size (bp)	% Coverage (matched/ total[a])	References
Real-time PCR primers and probes						
PCV2a-F1	ORF2	CGGTGGACATGMTGAGATTTA	58.4−61.0	118	75.3~93.4[b] (330/438~409/438)	This study
PCV2a-R1	ORF2	GGCCAGAATTCAACCTTAACYT	58.7−60.7			
PCV2a-Pr1	ORF2	Texas Red-CAAAGGGTATAGAGATTTTGTTGGTCCC-BHQ2	65.7			
PCV2b-F1	ORF2	TTCTCCTACCACTCCCGCTA	59.8	78	86.3~95.1[b] (1054/1221~1161/1221)	This study
PCV2b-R1	ORF2	TTTGTTGTTTGGTTGGAAGTAATC	59.3			
PCV2b-Pr1	ORF2	VIC-CTGTCCTAGATTCCACTAT-MGBNFQ	66			
PCV2d-F1	ORF2	AGCCCTTCTCCTACCACTCM	58.9−59.7	88	78.0~93.6[b] (736/942~882/942)	This study
PCV2d-R1	ORF2	TTTCTTTTGTTATTGGGTTGGAA	59.8			
PCV2d-Pr1	ORF2	FAM-TGATRGGACAATCGATTAC-MGBNFQ	66			
Cloning primers						
PCV2-cF	ORF1	TGGTGACCGTTGCAGAGCAG	65.9	1093		Wang et al., 2019a
PCV2-cR	ORF2	TGGGCGGTGGACATGATGAG	67.7			
Sanger sequencing primers						
PCV2-sF	ORF1	CCCATGCCCTGAATTTCCATATG	66.4	862		This study
PCV2-sR	Untranslated Region	CATGTTGCTGCTGAGGTGCT	62.6			

a: Indicates number of matched sequences over total sequences used in the analysis.
b: The lower boundary of the range of percentage match is generated from perfect matches in a primer pair and its corresponding probe against total sequences analyzed; the higher boundary of the range is generated by allowing presence of single nucleotide variation in each primer (but keep the 5bp conserved in the 3 end), or in the probe (but keep the 5pb conserved in the middle).

isolates and clinical samples that were positive to specific swine pathogens were tested to evaluate the specificity of the assay (Table 2). Those include PCV2a, PCV2b, PCV2d, porcine circovirus type 3 (PCV3), porcine reproductive and respiratory syndrome virus type 2 (PRRSV-2), swine influenza virus (SIV), porcine parainfluenza virus (PPIV), rotavirus A (RVA), rotavirus B (RVB), rotavirus C (RVC), porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis coronavirus (TGEV).

3. Results

3.1. Analysis of strain coverage of the assay using sequence dataset

Based on our previous study (Wang et al., 2019b), PCV2 strains were separated into 12 clusters, namely Cluster 1–12, by phylogenetic analysis; Cluster 8, Cluster 3 and Cluster 1 corresponded to genotypes, PCV2a, PCV2b and PCV2d, respectively. The three genotypes were identified as common strains currently circulating in the USA (Wang et al., 2019b; Xiao et al., 2016). An unrooted phylogenetic tree generated with PCV2 ORF2 sequences indicated that the three genotypes grouped into three separate clusters (Fig. 1). The triangle markers in the tree indicated the ORF2 sequences generated in our lab that were used to validate this genotyping real time PCR assay; they share 99.4–100 % identity with the sequences collected from the GenBank.

More complete analysis with 438 PCV2a strains, 942 PCV2b ORF2 strains and 1221 PCV2d strains has identified one set of primers and probe for each genotype (Illustrated in Fig. 2). *In silico* analysis showed the PCR set can perfectly match 75.3 % of PCV2a strains, 86.3 % of PCV2b strains, and 78.1 % of PCV2d strains. Assuming a single nucleotide mismatch to a primer (excluding the more critical 5 bp in the 3′ end) or a probe (excluding the more critical 5 bp in the middle) in the assay can still amplify and generate signal (Wang et al., 2020b), the strain coverages would be increased to 93.4 %, 95.1 % and 93.6 % for the three genotypes, respectively. Information of all primers and probes are shown in Table 1.

3.2. Analytical sensitivity of the mqPCR assay on cell culture isolates

Analytical sensitivity of the mqPCR assay was analyzed using standard curves generated by three replications of 10-fold serial dilutions of the cell culture isolates. The data was presented by plotting the Ct values against log dilution factors. The PCR amplification efficiencies were 94.5 % for PCV2a, 100.2 % for PCV2b and 99.2 % for PCV2d, with correlation coefficients (R^2) all greater than 0.995 (Fig. 3). The LODs were 1.58×10^{-4} TCID50/mL for PCV2a, 5.62×10^{-4} TCID50/mL for PCV2b, and 3.16×10^{-3} TCID50/mL for PCV2d.

3.3. Specificity of the mqPCR assay on clinical samples

The specificity of primers and probes was first tested by *in silico* analysis using Primer-Blast, which determined sequences were unique to their respective assay targets. Then, assay specificity was evaluated by comparison with Sanger sequencing results. Seventy-four PCV2 positive samples were tested by the genotyping real-time PCR; results showed 8 PCV2a, 6 PCV2b and 60 PCV2d strains. Genotypes of all 74 samples were confirmed by Sanger sequencing (Table 2). Specificity was also tested with clinical samples that previously tested positive to non-target pathogens. The results demonstrated that the assay specifically detected positive samples and identified PCV2a, PCV2b or PCV2d genotypes without cross-detecting each other. Furthermore, no positive signals were generated from clinical samples that were positive to PCV3 (n = 3), PRRSV-2 (n = 6), SIV (n = 2), PPIV (n = 5), RVA (n = 2), RVB (n = 1), RVC (n = 1), PEDV (n = 16), and TGEV (n = 2), indicating a good specificity of the assay (Table 3).

Table 2
Threshold cycle (Ct) distribution and specificity of the PCV2 genotyping real-time PCR compared with the Sanger sequencing results.

Sample #	Ct value of Real-time PCR			Genotype by PCR	Genotype by Sanger sequencing
	PCV2a	PCV2b	PCV2d		
1	0	0	22.2	d	d
2	11.2	0	0	a	a
3	23	0	0	a	a
4	0	27.2	0	b	b
5	0	0	15.7	d	d
6	10.6	0	0	a	a
7	0	20	0	b	b
8	0	0	27.4	d	d
9	0	0	26.7	d	d
10	0	0	17.1	d	d
11	0	0	15.1	d	d
12	0	0	15.7	d	d
13	0	0	19.5	d	d
14	0	0	11	d	d
15	0	0	31.6	d	d
16	0	0	27.8	d	d
17	34	0	0	a	a
18	0	0	25	d	d
19	0	21.3	0	b	b
20	0	0	23.3	d	d
21	0	0	30.6	d	d
22	0	0	30.9	d	d
23	0	0	19.8	d	d
24	0	0	34.3	d	d
25	0	0	31.3	d	d
26	0	0	10.7	d	d
27	0	0	32.9	d	d
28	0	0	15	d	d
29	0	0	28.7	d	d
30	0	0	9.9	d	d
31	0	30.1	0	b	b
32	0	0	5.1	d	d
33	0	38.2	5.1	d	d
34	0	0	8.2	d	d
35	0	0	5.5	d	d
36	0	38.9	5.7	d	d
37	0	38.8	6.8	d	d
38	0	0	6.5	d	d
39	0	0	7.7	d	d
40	0	0	4.7	d	d
41	0	0	5.8	d	d
42	0	0	7.5	d	d
43	0	0	6.1	d	d
44	0	6	0	b	b
45	0	7	0	b	b
46	0	0	5.9	d	d
47	0	0	5.1	d	d
48	0	0	6.2	d	d
49	0	38.8	6	d	d
50	0	36.4	29.7	d	d
51	0	0	30.8	d	d
52	0	0	27.8	d	d
53	22.6	0	0	a	a
54	0	0	25.5	d	d
55	0	0	27.9	d	d
56	0	0	28.6	d	d
57	0	0	6.9	d	d
58	0	0	30.6	d	d
59	0	0	5.7	d	d
60	0	0	10.7	d	d
61	0	0	28.1	d	d
62	0	0	28.5	d	d
63	0	0	31.4	d	d
64	27.2	0	0	a	a
65	25.1	0	0	a	a
66	0	0	31.8	d	d
67	0	0	31.2	d	d
68	0	0	29.1	d	d
69	0	0	29.1	d	d
70	23.4	0	0	a	a
71	0	0	10.3	d	d
72	0	0	21.1	d	d

(continued on next page)

Table 2 (*continued*)

Sample #	Ct value of Real-time PCR			Genotype by PCR	Genotype by Sanger sequencing
	PCV2a	PCV2b	PCV2d		
73	0	0	22.1	d	d
74	0	0	28.1	d	d

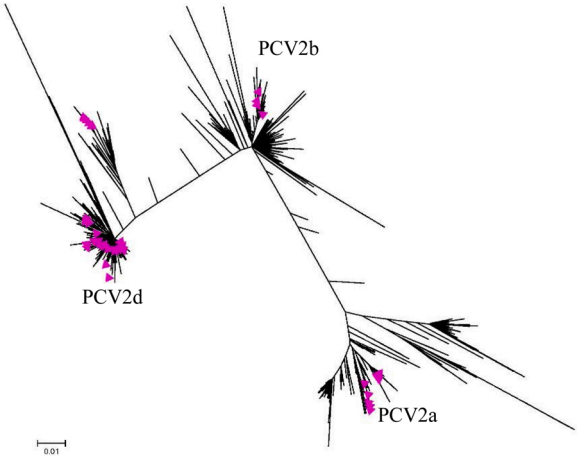

Fig. 1. Phylogenetic tree of PCV2a, PCV2b and PCV2d. The triangle markers indicate ORF2 gene sequences used for PCV2 genotyping by sequencing and validation of genotyping results by real time PCR.

4. Discussion

PCV2 is a small DNA virus with a circular genome of 1767–1768 nucleotides. The ORF2 gene (702 nucleotides) encodes the Capsid protein, which dominates immunogenicity (Nawagitgul et al., 2002). Extensive investigations have been done on genetic variations caused by point-mutations and recombinations (Firth et al., 2009; Franzo et al., 2016a), association of genotypes and disease severity (An et al., 2007; Opriessnig et al., 2006, 2008), and viral evolution under the selection pressure of vaccination (Franzo et al., 2016b; Karuppannan and Opriessnig, 2017; Xiao et al., 2016). Apparently genotyping of PCV2 is an important diagnostic tool for the study of viral pathogenesis and epidemiology of the disease, which will provide key information towards formulating strategies for disease management and vaccine development and applications.

By analyzing whole genome sequences, sequence variations among different genotypes were found mostly in the capsid gene, ORF2, which is consistent with previous studies (Cheung et al., 2007; Olvera et al., 2007; Wang et al., 2019b). In this study, all PCV2 ORF2 sequences were downloaded from the current GenBank database and included in the genetic analysis to ensure high strain coverage. Due to the high rate of nucleotide substitution in the genome, PCV2 displays substantial genetic variations (Firth et al., 2009). In our previous study, the inter-cluster and intra-cluster identities were as low as 83.6 % and 87.9 %, respectively. (Wang et al., 2019b). Because of this high sequence variation within

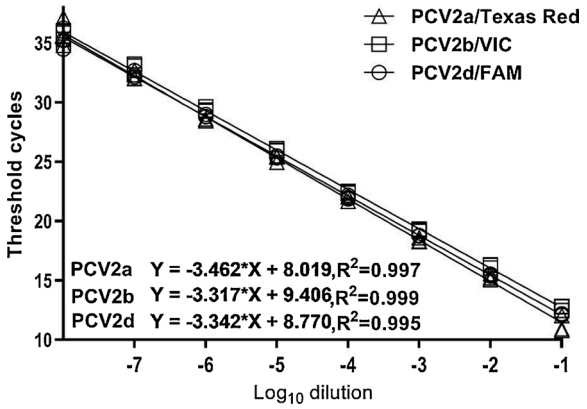

Fig. 3. Standard curves of PCV2a, PCV2b and PCV2d by serial dilutions of the cell culture isolates.

Table 3
Assay specificity tested on PCV2 cell cultures and diagnostic samples positive to PCV2 and other common swine pathogens.

Pathogen	Source	No. tested	Target gene		
			PCV2a	PCV2b	PCV2d
PCV2a	Clinical sample	8	+	–	–
	Cell culture	1	+	–	–
PCV2b	Clinical sample	6	–	+	–
	Cell culture	1	–	+	–
PCV2d	Clinical sample	60	–	–	+
	Cell culture	1	–	–	+
PCV3	Clinical sample	3	–	–	–
PRRSV-2	Clinical sample	6	–	–	–
SIV	Clinical sample	2	–	–	–
PPIV	Clinical sample	5	–	–	–
Rotavirus A	Clinical sample	2	–	–	–
Rotavirus B	Clinical sample	1	–	–	–
Rotavirus C	Clinical sample	1	–	–	–
PEDV	Clinical sample	16	–	–	–
TGEV	Clinical sample	2	–	–	–

+: Positive; -: Negative.

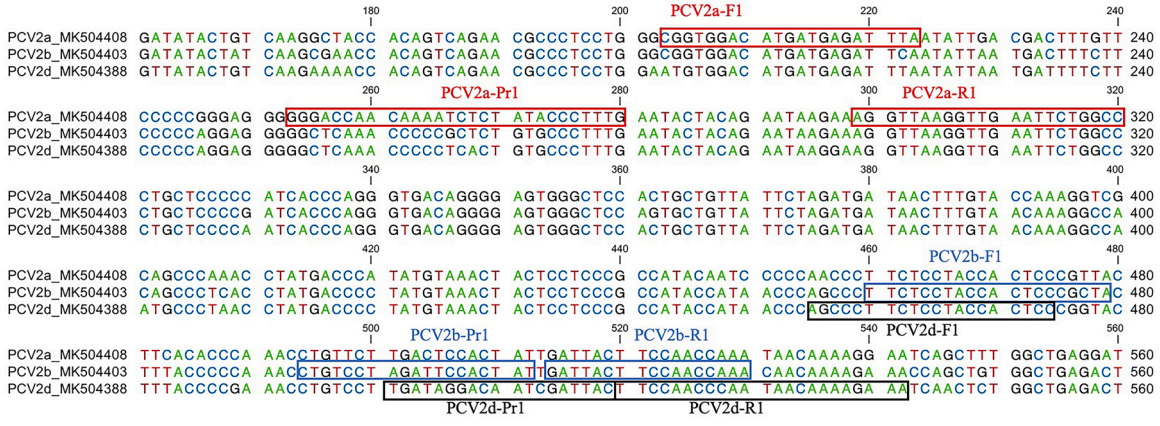

Fig. 2. Primer and probe locations of the PCV2 genotyping real-time PCR assay.

each genotype and relatively high genome homology between genotypes, the development of a molecular genotyping assay with high strain coverage can be challenging. In our design, the three sets of primers and probes can match 75.3–86.3 % strains of each genotype. In general, an assay with single nucleotide mismatches occurring in the middle, especially in the 5′ end of a primer, or in the two ends of a probe may still amplify and generate a signal (Bru et al., 2008; Forney et al., 2004; Wang et al., 2020a). Therefore, when single nucleotide mismatch in such situations is considered in the design of the primers and probes, strain coverage can potentially increase to 93.4 %–95.1 %. With that said, this genotyping assay should not replace general detection assays that may have higher strain coverage (Wang et al., 2019a).

Although some PCV2 genotyping assays have been published, these assays only differentiate between PCV2a and PCV2b strains (Wozniak et al., 2019; Xiao et al., 2016). For that reason, Sanger sequencing of the ORF2 gene, which is a labor intensive and time-consuming process (Lv et al., 2020; Zheng et al., 2020), is still widely used for PCV2 genotyping. We have developed this genotyping mqPCR assay that can use a single PCR reaction to differentiate the three major PCV genotypes, (PCV2a, PCV2b and PCV2d) circulating in the US. This simplified method is a low-cost and faster turnaround protocol, and should facilitate the genotyping process for field PCV2 strains. We have already developed a PCV2 general assay using the conserved ORF1 gene as the target (Wang et al., 2019a), which has enabled us to have a much higher strain coverage (99.1 %) for PCV2 detections. By utilizing that generic test for general detection and surveillance, followed by the genotyping PCR reported here, we should be able to detect and differentiate the majority of field PCV2 strains.

In conclusion, the newly developed and validated mqPCR assay allows for rapid, sensitive and specific detection and differentiation of the most common PCV2 genotypes, (PCV2a, PCV2b and PCV2d) in clinical samples. *In silico* analysis and clinical sample testing indicated that the assay has relatively high strain coverage. It can be used as an alternative method for PCV2 genotyping.

Author statement

All authors have read the revision and response to reviewers' comment document, and are agreed with the submission of this revised manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgement

This project is partially funded by the Swine Health Information Center, Ames, Iowa, USA, with grant number: 16-257 SHIC.

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