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

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\section{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 (Wang et al., 2019b; 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., 2016; 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.

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 \times 10^{-5} \text{TCID50/mL} for PCV2a, 5.62 \times 10^{-4} \text{TCID50/mL} for PCV2b, and 3.16 \times 10^{-3} \text{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.

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 (Saporti 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).
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/). 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 (Pogranichnyi 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, Valencia, CA). The ligated products were then transformed in Mix & Go competent cells (Zymo Research, Irvine, 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.

2.4. 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™/TOPO™. 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 et al., 2019b), 0.25 μM each of forward and reverse PCR primers, 0.25 μM each of probes, and 10 μl of 2X iQ™ 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

| Primer/Probe | ORF Location | Sequence (5′-3′) | Tm (°C) | Amplicon size (bp) | % Coverage (matched/total) | References |
|--------------|--------------|-----------------|---------|--------------------|---------------------------|------------|
| Real-time PCR primers and probes | | | | | | |
| PCV2a-F1 ORF2 | | GGGTGCGATGATGATAGTTA | 58.4-61.0 | | 75.3-93.4a | This study |
| PCV2a-R1 ORF2 | | GCCCAGATGTCACTTAACTT | 58.7-60.7 | 118 | (330/438–409/438) | |
| PCV2a-P1 ORF2 | | Texas Red-CAAAGGATAGATGATGGTTTGGTCC-BHQ2 | 65.7 | | | |
| PCV2b-F1 ORF2 | | TCTTCTCTACACTCCCGCTA | 59.8 | | 86.3-95.1a | |
| PCV2b-R1 ORF2 | | TGTGTGTGTTGAGTGAGTATC | 59.3 | 78 | (1054/1221–1161/1221) | This study |
| PCV2d-R1 ORF2 | | TACACATTTTTTTTTTGTGGTCA | 59.8 | 88 | (736/942–882/942) | This study |
| PCV2d-P1 ORF2 | | FAM-TACAGAGAATGATGATAGTTAC-MGBNFQ | 66 | | | |
| Cloning primers | | | | | | |
| PCV2-cF ORF1 | | TGGTGACCCCTGACAGACG | 64.9 | 1093 | | Wang et al., 2019a |
| PCV2-cR ORF2 | | TGGGCGGATGACATGATGAC | 67.7 | | | |
| Sanger sequencing primers | | | | | | |
| PCV2-cF ORF1 | | CCCATGCGGTCAATCATGATGAG | 66.4 | 862 | | This study |
| PCV2-cR Untranslated Region | | CATGGTCTGCTGAGTCTGCT | 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 5bp 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 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 (excluding the more critical 5 bp in the middle) in 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.

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 (R²) 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 × 10⁻⁴ 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).

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

(continued on next page)
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

Table 2 (continued)

| Sample # | Ct value of Real-time PCR | Genotype by PCR | Genotype by Sanger sequencing |
|----------|--------------------------|----------------|-----------------------------|
| 73       |                          |                |                             |
| 74       |                          |                |                             |

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.

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

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          | –     | –     | +     |
|          | Clinical sample   | 3          | –     | –     | +     |
| PRSV-2   | Clinical sample   | 6          | –     | –     | +     |
| SIV      | Clinical sample   | 2          | –     | –     | +     |
| PIV      | 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.
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%–95.1% with relatively high genome homology between genotypes in United States swine herds. Arch. Virol. 152, 1035–1046. Wang, Y., Feng, Y., Zheng, W., Noll, L., Porter, E., Potter, M., Cino, G., Peddireddi, L., Li, X., Liu, L., Liu, L., Liu, X., Dodd, K., Jia, W., Bai, J., 2020b. Development of a real-time PCR assay for detection of African swine fever virus with an endogenous internal control. Virot. J. 17, 122.

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