The presence and genetic characteristics of porcine circovirus 3 from pigs in Southern and Central provinces of Vietnam

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ABSTRACT

Porcine circovirus type 3 (PCV3) is an emerging circovirus species that has recently been reported in different countries around the world, suggesting a widespread circulation. This study was carried out in order to investigate the presence and further genetic characteristics of PCV3 from swine herds in Southern and Central provinces of Vietnam. A duplex PCR assay for rapid detection of PCV3 in pigs was established with a pair of specific primers designed between *rep* and *cap* gene segment to amplify full-length ORF2 and another set of primers binding to COX1 gene serving as an internal amplification control (IAC). The resulting duplex PCR was used to examine PCV3 presence in 94 tissue and serum samples. Subsequently, PCV3 was detected in 10 out of 94 cases (10.6%). The infection rate in sows (14.3%) was higher than that in grower pigs (7.7%). Regarding nucleotide sequence comparison, 10 ORF2 genes were selected for nucleotide sequencing and their alignment showed 97.2% - 99.5% homology. According to the phylogenetic analysis and sequence alignment of *cap* gene, all the sequences were clustered into group PCV3a, including 9 strains of sub-group PCV3a1 and only one strain of sub-group PCV3a2. These findings indicated that the PCV3a group is circulating in swine farms in Vietnam. This study provides better insights into epidemiology of this pathogen in the national swine industry.

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1. Introduction

Porcine circovirus 3 (PCV3) is a small non-enveloped virus with single-stranded circular DNA belonging to the genus *Circovirus* in the family *Circoviridae*. Similar to other circoviruses, the capsid protein is considered major structural protein and antigenic characteristics. In 2016, the virus was initially described in sows in the North Carolina (USA) with manifestation of porcine dermatitis and nephropathy syndrome (PDNS) and reproductive failure (Palinski et al. 2017). Despite the novelty, a number of studies associated with the prevalence of PCV3 have been conducted in different pork-producing countries, namely Brazil (Saraiva et al. 2019), China (Guo et al. 2019), Korea (Kim et al. 2018), Thailand (Kedkovid et al. 2018), etc. However, up to date, PCV3-related studies are still limited in Vietnam. Therefore, the objective of this investigation was to establish of duplex PCR assay for detection and amplification of full-length ORF2 of PCV3 in order to determine the presence and genetic characteristics of the virus in pig populations in

Southern and Central areas of Vietnam.

2. Materials and Methods

2.1. Establishment of duplex PCR assay

2.1.1. Primer design and selection

One set of primers designed for PCV3 detection and ORF2-region genome sequencing was generated based on 70 sequences available in the Genbank by Primer3plus software, and validated by NCBI BLAST, OligoAnalyzer 1.0.2, Clustal Omega tool, PCV3-F: 5'-ATGCGAGGGCGTTTACCTG-3', PCV3-R: 5'-TCCCTACAGACCTCCGTGG-3'. Another primer pair designed in COX1 gene (Genbank ID: KY661881.1) was selected to serve as an internal amplification control (IAC), IAC-F : 5'-GCACTGCCTTGAGCCTACTAAT-3', IAC-R: 5'-AACAGGGGTGTTTGGTATTGAG-3'.

2.1.2. Positive and negative DNA controls

DNA template of PCV3 was originally obtained from a field sample followed by sequencing confirmation to be PCV3. The resultant sequence exhibited 99.69% identity to the previously reported PCV3 (Gene ID MN583577.1). The DNA was then quantified to 5×10^6 copies/µL by Biodrop µLITE Spectrophotometer (BiochromTM 80-3006-51, England) and used as positive control for subsequent PCR reactions.

Unrelated DNA used to determine specificity of the primers was DNA viruses or bacteria that are frequently found in swine farm environment and potentially contaminate the samples, including *Salmonella* spp., *Escherichia coli* (*E. coli*) (ATCC 25922), *Streptococcus suis* (*S. suis*), *Staphylococcus aureus* (*S. aureus*) (ACTC 6338), *Clostridium perfringens* (*C. perfringens*); *Pasteurella multocida* (*P. multocida*), *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*), *Haemophilus parasuis* (*H. parasuis*), *Mycoplasma hyopneumoniae* (*M. hyoneumoniae*); Aujeszky's disease virus (ADV), porcine circovirus type 2d (PCV2d). Nuclease free water was used as negative control for all PCR reactions.

2.1.3. Single PCR construction

Initially, to survey annealing temperature of PCV3 primer, the single PCR reactions were conducted in a 20 µL mixture including 10 µL Dream Taq Green PCR Master Mix 2X (Cat#K1081, Thermo Fisher Scientific, USA), 1 µL of each 10 µM PCV3 primer, 6 µL of DNA and nuclease free water was added to reach final volume of 20 µL/reaction. The amplification was performed in a GENE Q Thermal Cycler (Bioer, China) under the following conditions: pre-denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 sec, annealing temperature optimized in the range of 50°C to 62°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 5 min. Subsequently, the 10 µL PCR products were visualized by 1.5% agarose gel electrophoresis (Cat#16500100, Invitrogen, Thermo Fisher Scientific, USA) with Midori Green Advance DNA stain (Cat#MG04, NIPPON Genetics Europe, Germany) and ultraviolet light (UV Transilluminators, Korea), using 1Kb DNA Plus ladder (Cat#10787018, Invitrogen, Thermo Fisher Scientific, USA) as the molecular weight markers to indicate the sizes of amplification products. The range of appropriate annealing temperature of PCV3 primers was compared with those of IAC primers to decide the final thermal condition in the mPCR.

2.1.4. Optimization of primer concentration of duplex PCR assay

Entering the upcoming step, to assess mutually compete interfering level of two pairs of primers in duplex PCR assays, the 20 µL duplex PCR reaction encompassed 10 µL Dream Taq Green PCR Master Mix 2X (Cat#K1081, Thermo Fisher Scientific, USA), proper ratio of PCV3 and IAC primer concentration, 4 µL and 1 µL DNA of pig and target pathogen, respectively, nuclease-free water was added to reach the designed volume.

2.1.5. Specificity and sensitivity

In order to prove the specificity, DNA of different viral and bacterial agents was used as unrelated template in the duplex PCR reactions. Regarding the sensitivity, defined as the minimum detectable DNA molecules in a reaction, the PCV3 template was diluted 10-fold serially in nuclease-free water and used in the duplex PCR to determine the detection limit. The applicability of this assay was basically validated by using PCV3-positive samples.

2.2. Application of the duplex PCR to detect PCV3 in field samples

From July 2019 to November 2019, a total of 94 serum and tissue samples (lymph nodes and spleen) were collected from sows (n = 42) and grower farms (n = 52) in 12 provinces of Southern and Central Vietnam. Viral DNA of these samples was extracted using a 'DNeasy Blood and Tissue' Kit (Cat#69504, Qiagen, Germany), and was detected by the duplex PCR assay under optimized conditions as constructed above.

2.3. Genetic characteristics

The full-length ORF2 of PCV3-positive samples was purified and submitted to Nam Khoa Biotek (Vietnam) for sequencing in both directions. The sequences were then assembled by BioEdit version 7.2.5.

To investigate the evolutionary relationship of PCV3 strains detected, full-length cap genes downloaded from GenBank were used together with ORF2 sequences in this study to analyse the similarity by Clustal Omega software and BLAST tool. The genetic distance among strains was also calculated using MEGA X software.

Phylogenetic tree of PCV3 was built based on ORF2 sequences using the neighbor-joining (NJ) method in MEGA X software with bootstrap value of 1000 replicates. The classification of PCV3 in this study was based on four amino acid positions 24, 27, 77 and 150 in cap protein (Fux et al., 2018).

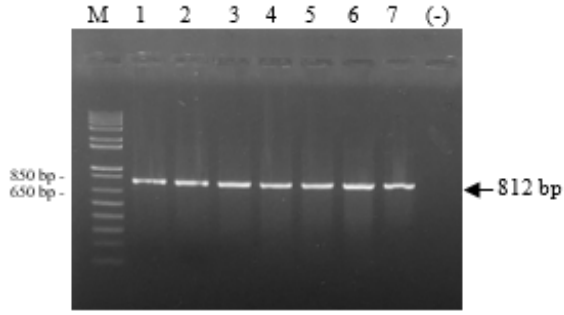

Figure 1. Annealing temperature optimization for single PCR. M: 1Kb plus DNA ladder; (1): 50°C; (2): 52°C; (3): 54°C; (4): 56°C; (5): 58°C; (6): 60°C; (7): 62°C; (-): negative control with nuclease-free water. PCR products were analyzed in 1.5% agarose gel at 90 Volt for 30 min.

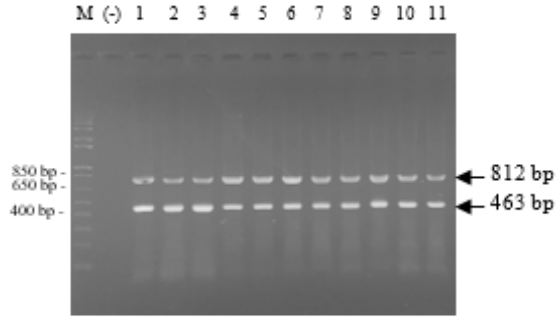

Figure 2. Optimization of primer concentration in duplex PCR assay. M: 1Kb plus DNA ladder; (-): negative control with nuclease-free water; lanes 1-11 are equivalent to ratio of 0.4:0.4, 0.3:0.4, 0.4:0.3, 0.4:0.2, 0.2:0.4, 0.2:0.5, 0.2:0.6, 0.3:0.6, 0.2:0.3, 0.3:0.5 and 0.3:0.7; PCR products were analyzed in 1.5% agarose gel at 90 Volt for 30 min.

3. Results and Discussion

3.1. Establishment of duplex PCR assay

3.1.1. Optimization of duplex PCR assay

Results of the single PCR assay indicated that PCV3 primers worked well in the range of 54°C to 60°C to generate a product of 812 bp as predicted (Figure 1). In addition, optimal annealing temperature for the IAC primers was determined in a separate study to be 60°C. For this reason 60°C was chosen for optimization of the primer ratio in the duplex PCR assay.

To determine the optimal proportion of the IAC/PCV3 primer, different ratios of primers were tested including 0.4:0.4, 0.3:0.4, 0.4:0.3, 0.4:0.2, 0.2:0.4, 0.2:0.5, 0.2:0.6, 0.3:0.6, 0.2:0.3, 0.3:0.5 and 0.3:0.7. Results showed that the ratio of 0.2:0.5 (lane 6) produced the clearest signal and easily distinguishable from each other (Figure 2). It indicates that the concentration of PCV3 primer should be 2.5 times as much as that of IAC primers.

3.1.2. Specificity and sensitivity

As illustrated in Figure 3, the PCV3 primers were specific for the target virus, not binding to DNA from other tested pathogens while the IAC primers were functioning well to confirm the samples to be from swine.

Additionally, Figure 4 showed that the minimum amount of target gene for successful amplification was consistently at 5×10^1. The as-

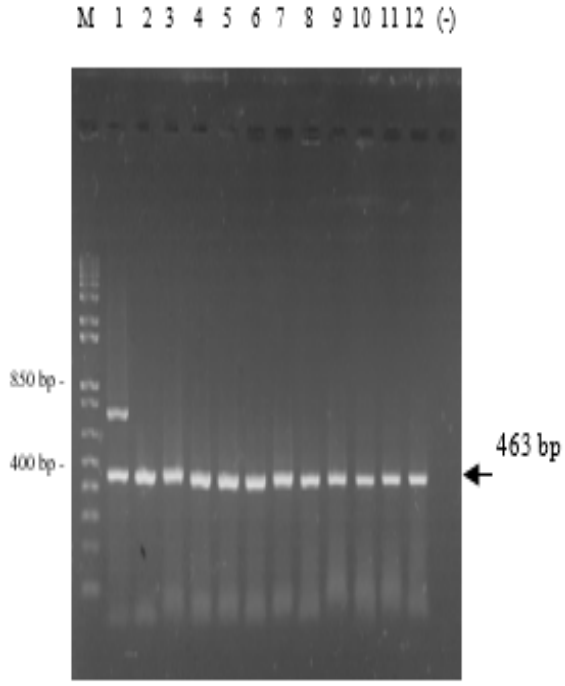

Figure 3. Specificity of duplex PCR assay. M: 1Kb plus DNA ladder; (1): positive control; (2): ADV; (3): PCV2; (4): *M. hyopneumoniae*; (5): *H. parasuis*; (6): *A. pleuropneumoniae*; (7): *P. multocida*; (8): *E. coli*; (9) *Salmonella* spp.; (10): *S. aureus*; (11): *S. suis*; (12): *C. perfringens*; (-): negative control with nuclease-free water. PCR products were analyzed in 1.5% agarose gel at 90 Volt for 30 min.

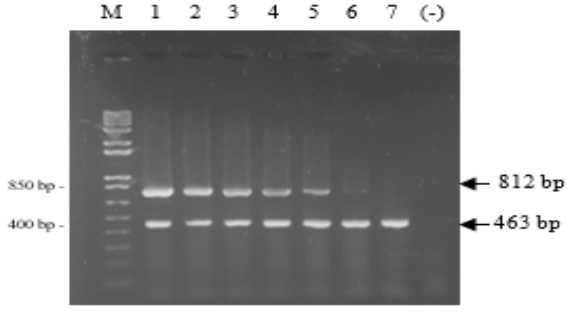

Figure 4. Sensitivity of duplex PCR assay. M: 1Kb plus DNA ladder; lanes 1 – 7 are: 5×10^6; 5×10^5; 5×10^4; 5×10^3; 5×10^2; 5×10^1; 5×10^0; (-): negative control with nuclease-free water.

say was repeated at least three times. The established duplex PCR assay in this study was comparatively sensitive to a SYBR Green-based Realtime quantitative PCR assay (1.73×10^2 copies/reaction) and a conventional PCR assay (1.5×10^2 copies/reaction) reported previously (Wang et al., 2017; Chen et al., 2018). Finally, to assess the applicability of this assay, 5 clinical samples confirmed positive with PCV3 by sequencing method were used. Results showed that the duplex PCR could detect PCV3 in all the five samples as expected (data not shown).

3.2. Application of the duplex PCR in detection of PCV3 in field sample

The established duplex PCR was used to detect PCV3 in 94 field samples collected from pigs. These results revealed 10.6% (10/94) samples positive for PCV3, doubling that reported in Nguyen et al. (2018)'s study conducted in the North of Vietnam which showed 4.44% (6/135) positivity.

3.3. Genetic characteristics

To characterize the genetic information of PCV3 strains detected in this study, the capsid gene of 10 positive samples (BP1_150719, BP2_250919, DN1_300819, DN2_230719, DN3_160719, DL_290819, VT_151119, BD1_160919, BD2_281119, and LD_061119) was sequenced and aligned. Nucleotide sequence analysis of these 10 cap genes demonstrated high sequence similarity, up to of 97.2% to 99.5%. In other words, the genetic distance of these strains was 0.005 to 0.028, which indicated a similar genetic variations compared to the previous study in the North of Vietnam (0.005-0.031) (Nguyen et al., 2018). Furthermore, alignment analysis together with 40 PCV3 ORF2 sequences from the Genbank revealed 97% to 100% sequence identity. Within the 10 strains sequenced in this study, four of them showed high identity to strains detected in Northeast China, Brazil, Italy and Spain at the level of 98.6% - 99.8%. Especially, BP1_150719_Vietnam strain displayed the highest homology to strains reported in Southern China (99.1%). It is in a branch far away from other strains in the phylogenetic tree (Figure 6). Besides, the remaining strains showed a high identity to strains found in Northeast China, Brazil, Italy and Spain.

To classify the subgroup for the 10 strains investigated in this study, amino acid sequences of ORF2 were compared. Based on the subgroup definition recommended by Fux et al. (2018), 9 strains were classified into PCV3a1 subgroup and the other one belonged to PCV3a2 sub-group (Figure 5). These 10 strains differed at 33 nu-

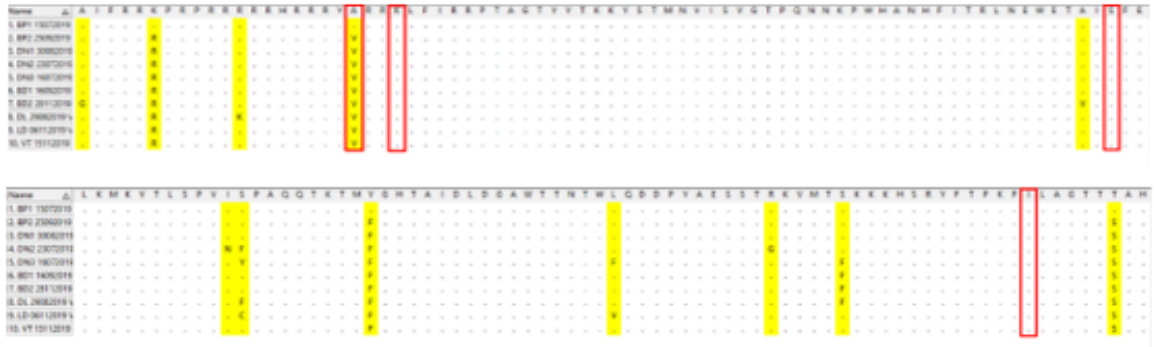

Figure 5. Alignment of the deduced amino acid sequences. Genetic marker at codon positions 24, 27, 77 and 150 were in red frames. Twelve mutations of aa at positions 5, 10, 16, 24, 94, 95, 104, 121, 132, 137 and 156 were shown.

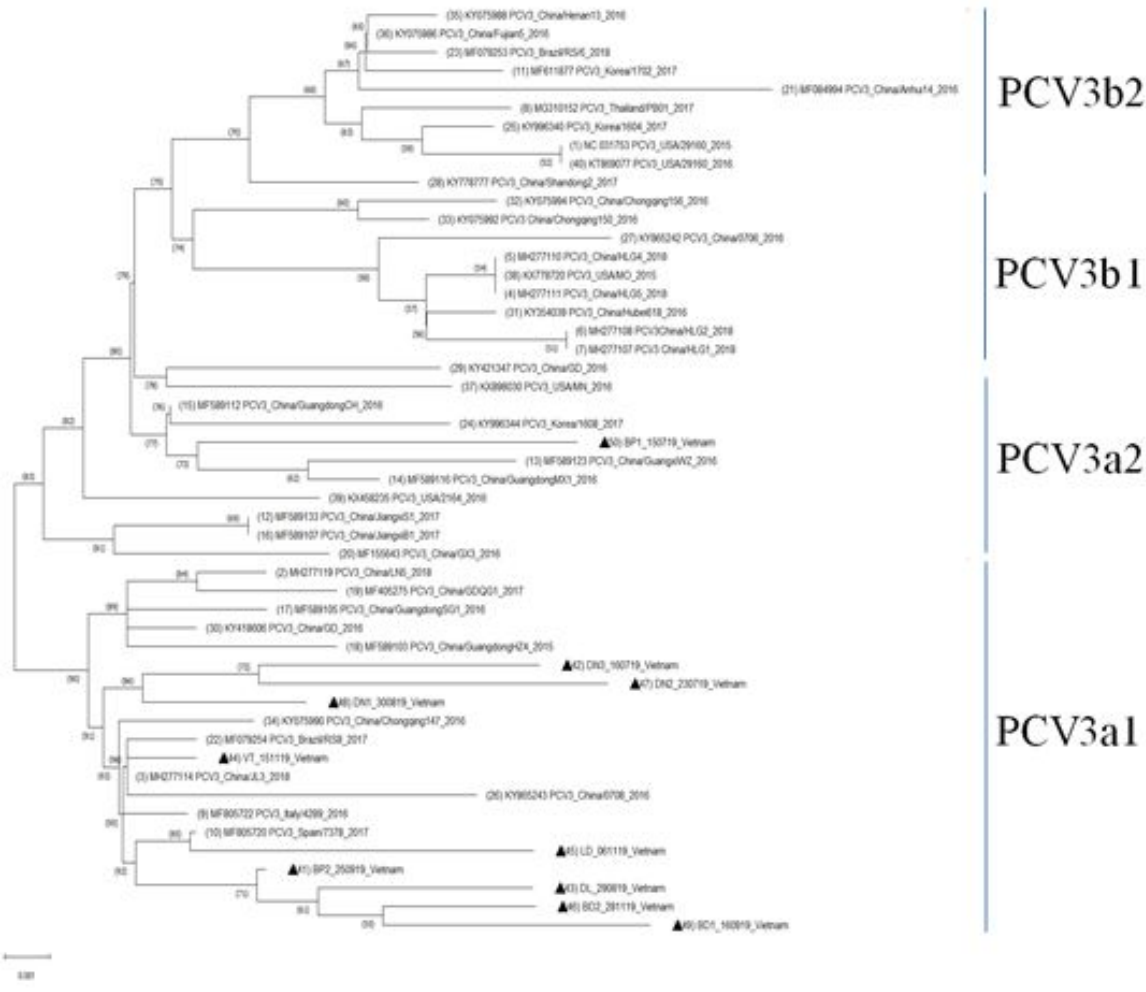

Figure 6. Phylogenetic tree of the full-length ORF2 gene sequences based on 10 different PCV3 strains from this study (black triangles) and 40 reference strains from Genbank. The subgroup of PCV3a1, PCV3a2, PCV3b1, and PCV3b2 was classified based on amino acid motif sequences proposed by Fux et al. (2018).

cleotide positions, leading to 12 non-synonymous mutations of the cap protein (Figure 5). How these nucleotide and amino acid polymorphisms affect the virus life cycle, pathogenesis and its interaction with the host immune responses requires further examinations.

4. Conclusions

The duplex PCR assay was successfully established for the diagnosis of PCV3. In this study, PCV3 was found to be present at a frequency of 10.6% in pigs in the Southern and Central provinces of Vietnam. Furthermore, the circulation rate of PCV3 in sows (14.3%) was twice as high as that in growers (7.7%). Strains of PCV3 found in this study belonged to PCV3a1 and PCV3a2 sub-group.

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