

Identification of porcine circovirus type 2 (PCV2), type 3 (PCV3) and porcine parvovirus (PPV) in swine by multiplex PCR test

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ABSTRACT

This study aimed to simultaneously detect three important viruses reported to be involved in the reproductive problems of sows. A multiplex PCR (mPCR) test was developed to provide rapid diagnosis of porcine circovirus type 2 and 3 (PCV2, PCV3) and to illustrate parvovirus (PPV) prevalence in sow herds. Three pairs of specific primers were designed to target PCV2 *Cap* gene, PCV3 *Cap* gene and PPV NS1 gene, with predicted mPCR products of 702 bp, 267 bp and 380 bp, respectively. The detection limit of mPCR was 100 copies/reaction per target gene. The mPCR was run against a panel of 94 swine serum samples whose infection status had been pre-determined by commercial real-time PCR kits. Sequencing of mPCR products performed with clinical serum samples accurately confirmed the results. Overall, the results indicated that the mPCR functioned accurately and specifically and matched 100% with the single-target real-time PCRs. The mPCR was developed successfully and can be used in routine diagnosis of PCV2, PCV3 and PPV.

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1. Introduction

Reproductive disorders are among the most concerned syndromes in sow herds, causing huge economic losses to swine producers. Many infectious pathogens are involved in the problems, including porcine parvovirus (PPV), porcine circovirus type 2 (PCV2) and porcine circovirus type 3 (PCV3), a newly discovered viral pathogen (Palinski et al., 2016).

Porcine circovirus type 2 and type 3 are non-enveloped and single-circular DNA viruses that belong to the family *Circoviridae* (Tisciier et al., 1986; Palinski et al., 2016). Porcine circovirus

type 2 has been demonstrated to play significant roles in different swine syndromes such as postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), or porcine circovirus associated diseases (PCVAD) in general (Allan et al., 1999; Allan et al., 2000; Rosell et al., 2000). The virus was first identified in western Canada in a case of wasting disease using electron microscopy, immunohistochemical, and in-situ hybridization methods (Ellis et al., 1998). Later, various publications illustrated the circulation and the pathogenesis of PCV2 in abortion cases or weak piglets at birth (West et al., 1999; Calsamiglia et al.,

2007; Oropeza-Moe et al., 2017). Porcine circovirus type 3 was recently detected in a case of PDNS syndrome in a sow farm in North Carolina (USA) (Palinski et al., 2016). The virus was found in the skin, kidneys, lungs, and lymph nodes of the affected pigs without the existence of PCV2. From this observation, PCV3 was also suspected to play a pathogenic role in swine, especially in the sow herds, although the evidence of its pathogenesis remains to be elusive. In addition, another virus in the list of causative agents for reproductive disorders in swine is PPV, a member of the *Parvoviridae* family, that was identified at the end of the 1960s (Cartwright et al., 1967; Cartwright et al., 1969; Johnson & Collings, 1969). Typical manifestations associated with a PPV infection in pregnant sows are collectively identified as SMEDI syndrome which includes stillbirths, mummification, embryonic death, and infertility. These viruses are currently circulating in Vietnam and cause significant losses (Huynh et al., 2014; Dinh et al., 2021). Co-infection among these viruses may result in a different impact on the swine farms. Co-infection of swine with PCV2 and PCV3 does not result in significantly enhanced disease outcomes compared to individual infection with one of these two viruses (Wozniak et al., 2019). However, co-infection of swine with PCV2 and PPV led to the increased viremia level of PCV2 (Milek et al., 2020).

Several conventional and real-time PCRs have been developed for molecular diagnosis of these three viruses (Chen et al., 2009; Zhao et al., 2010; Yang et al., 2019). However, those PCRs are designed for the detection of a single or double pathogens at a time. In the present study, we report the development and validation of a mPCR that allows simultaneous detection of these three viruses in field samples.

2. Materials and Methods

2.1. Controls and clinical samples

Positive controls: DNA fragments of PCV3 *Cap* gene and PPV *NS1* gene were synthesized by Integrated DNA Technologies (IDT - USA) based on the sequences of PCV3 (MH184542.1) and PPV (MK993540.1), respectively. The DNA template of PCV2 was originally obtained from a field isolate followed by sequencing confirmation. The resulting sequence exhibited 99.72% identity to the previously reported PCV2 (Gene Accession

no. LC383449.1).

Unrelated DNA from other pathogens used for specificity evaluation: bacteria and viruses that are commonly present in swine farm environment and potentially contaminated into the samples were used to confirm the specificity of the mPCR. *Staphylococcus aureus* (ATCC 6338) and *Escherichia coli* (ATCC 25922) were offered by Sanphar Vietnam laboratory (belonging to Erber group, Austria); *Streptococcus suis* and *Clostridium perfringens* were obtained from a previous study (Nguyen et al., 2018). The bacterial DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) solution (Cat#P1037, Sigma; Cat#25666, Merck). Pseudorabies virus *gE* gene was synthesized by Integrated DNA Technologies (IDT - USA).

Field samples: Ninety-four serum samples from sows were collected and used to evaluate this mPCR procedure. These sow farms were located in the South of Vietnam. Each serum sample was pooled from five individual sows. All samples were submitted to the laboratory and the senders signed an agreement for the laboratory to use the result data for teaching and publication. The infectious status of these 94 samples was predetermined by using commercial real-time PCR kits or master mix, as per the manufacturers' instructions. The real-time kits used for detection of PCV2, PPV and PCV3 were PowerChek™ PCV2 real-time PCR kit (Cat#R0809, Primerdesign), PowerChek™ ADV/PPV real-time PCR kit (Cat#R0832, Primerdesign) and Luna® Universal qPCR Master Mix (Cat#M3003S, NEB), respectively.

2.2. DNA preparation from clinical samples

The DNA was extracted from test serum samples by using the advanced phenol-chloroform method. The extracted DNA was stored at -20°C for subsequent assays.

2.3. Primers

The primers used in this study are listed in Table 1. For PCV2, primers were designed based on the consensus of 65 PCV2 *Cap* gene sequences in the NCBI GenBank with a PCR product of 702 bp. For PPV, primers were designed based on the consensus of 67 PPV *NS1* sequences in the NCBI GenBank with an expected product of 380 bp. Lastly, for PCV3, primers were obtained from

Table 1. Primer sequences and estimated product sizes

Virus	Primers	Gene	Primer sequences (5' – 3')	Product size (bp)	Reference
PCV2	PCV2	<i>Cap</i>	F: ATGACGTATCCAAGGAGGCG R: TTAAGGGTTAAGTGGGGGGTC	702	Present study
PCV3	PCV3	<i>Cap</i>	F: TTCCGGGACATAAATGCT R: GGGCACACAGCCATAGAT	267	Chen et al., 2017
PPV	PPV	<i>NS1</i>	F: GCTTTAGCCTTGGAGCCGTGGA R: CGTGTTCCTTTTGCTGCGGCGTC	380	Present study

Chen et al. (2017). They were designed based on the PCV3 *Cap* gene with a product size of 267 bp.

2.4. Multiplex PCR (mPCR)

After multiple rounds of optimization, a primer mix containing three primer pairs were created at a mixing ratio of 1 PPV : 2 PCV2 : 4 PCV3. Accordingly, the final concentrations of the PPV, PCV2 and PCV3 primer pairs in the mixture were 0.2 µM, 0.4 µM, 0.8 µM, respectively. The mPCR was performed in a 20 µL reaction volume containing 10 µL of DreamTaq 2X (Cat#K1072, No MAN0012702, Thermo Scientific), 1.4 µL of the primer mix, 3 µL DNA template, and 5.6 µL of nuclease free water (Thermo Fisher). After initial denaturation at 94°C for 5 min, 35 cycles were conducted at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 40 sec, followed by a final extension at 72°C for 5 min. The amplified products were then analyzed by electrophoresis in a 1.5% (w/v) agarose gel in 1X Tris-Boric-EDTA (TBE) containing Midori Green Advance DNA stain (Cat#MG04, Nippon Genetics). The standard DNA (Cat#10787018, Invitrogen) was included in each DNA gel electrophoresis to indicate the PCR product size.

2.5. Evaluation of the specificity of the mPCR

The target viruses of this study can be found in blood, saliva, feces, vulval discharge in live pigs. In disease surveillance, these types of samples are favored due to the less invasive advantages. However, sampling procedures in practice are prone to contamination with other bacteria or viruses popularly distributed in pig farms. Therefore, genomic DNA of *S. aureus*, *E. coli*, *S. suis*, *C. perfringens* and PRV *gE* gene were used as templates in the mPCR to determine the specificity of the test. The concentration of DNA templates used

in the reaction was 30 ng per reaction in the cases of bacterial target and 10⁸ copies per reaction in the case of *gE* gene.

2.6. Evaluation of the detection limit of the mPCR

Ten-fold serial dilutions of each positive control DNA templates ranging from 10⁷ to 10⁰ copies were used as the DNA template in the mPCR to determine its limit of detection. The minimum number of template copies that enable successful amplification of all products was considered as the limit of detection of the mPCR.

2.7. DNA sequencing

Sequencing of each PCR product was performed by using both forward and reverse primers used in the corresponding PCR (Table 1). The sequencing results were aligned with the reference PCV2 (accession no. MH470234.1), PCV3 (accession no. MK058529.1) and PPV (accession no. MK378155.1) using Clustal Omega program.

3. Results

3.1. Optimal conditions for the mPCR

Before the optimal ratio of primers for mPCR was achieved, each primer pair was tested for its performance in single PCR (sPCR) at different annealing temperatures. Gel analysis indicated that only one DNA product was generated in each sPCR, with the product sizes of 267 bp for PCV3, 380 bp for PPV and 702 bp for PCV2 (Figure 1). It appeared that the three primer pairs worked well at the annealing temperatures between 52°C - 58°C.

Subsequently, the annealing temperature of 56°C was chosen for mPCR. As shown in Figure 2, the mPCR produced three amplicons that were

clearly visible and easily distinguishable. Importantly, the sizes of the amplicons were as expected and were similar to the ones observed in the corresponding sPCRs (Figure 2). These three products in mPCR were recovered from agarose gel and subjected to sequencing. The results confirmed that the three PCR amplicons from the mPCR aligned well with the three respective target DNA sequences, indicating that the primer pairs functioned as anticipated.

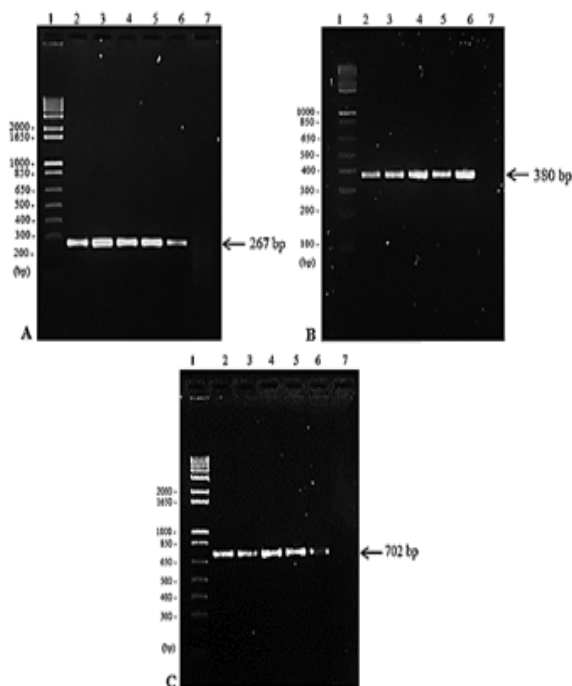


Figure 1. Optimization of annealing temperature in s-PCR. (A) PCV3, (B) PPV, (C) PCV2. (Lane 1): DNA ladder 1 Kb plus, (Lane 2): 50°C, (Lane 3): 52°C, (Lane 4): 54°C, (Lane 5): 56°C, (Lane 6): 58°C, (Lane 7): negative control with pure water. The thermal cycling conditions were 94°C/5 min; 35 cycles of 94°C/30 sec, 50-58°C/30 sec and 72°C/40 sec, a final extension at 72°C/5 min.

3.2. Specificity and detection limit of the mPCR

Next, we wanted to evaluate the specificity of this mPCR by running the assay with DNA templates extracted from bacteria and viruses commonly present in swine farm environment including *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus suis*, *Clostridium perfringens* and Pseudorabies virus. Results showed that the three primer pairs did not cross-react with any of

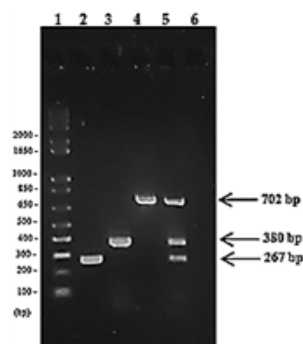


Figure 2. Products of sPCRs and mPCR. (Lane 1): DNA ladder, (Lane 2): PCV3 267 bp, (Lane 3): PPV 380 bp, (Lane 4): PCV2 702 bp, (Lane 5): mPCR of all three targets, (Lane 6): negative control with pure water. The thermal cycling conditions were 94°C/5 min; 35 cycles of 94°C/30 sec, 56°C/30 sec and 72°C/40 sec, a final extension at 72°C/5 min.

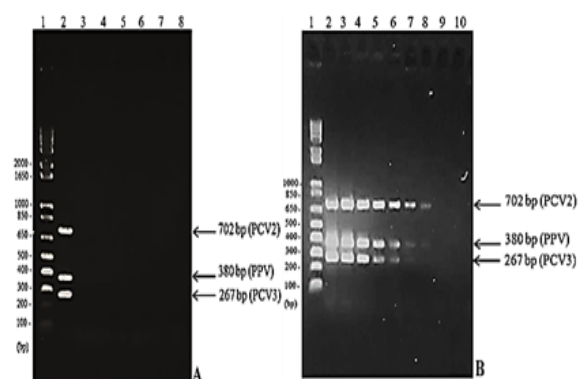


Figure 3. Specificity and detection limit of the mPCR. (A) Result of specificity test. (Lane 1): DNA ladder, (Lane 2): positive control, (Lane 3): *E. coli*, (Lane 4): *Streptococcus suis*, (Lane 5): *Staphylococcus aureus*, (Lane 6): *C. perfringens*, (Lane 7): Pseudorabies virus, (Lane 8): negative control. (B) Detection limit of the mPCR. (Lane 1): DNA ladder, (Lane 2-9): DNA template of PCV2, PCV3 and PPV at 10^7 - 10^0 copies/reaction; (Lane 10): negative control with pure water.

these DNA templates (Figure 3A), indicating the mPCR established in this study was highly specific.

We then evaluated the limit of detection of the mPCR by performing the assay with a set of 10-fold serially diluted positive control DNA samples with the copy numbers ranging from 10^7 to 10^0 copies per reaction. As shown in Figure 3B, three distinct DNA bands at the expected size were observed at the concentration of 10^2 copies per reaction. It demonstrated that this mPCR

could detect the viral genes at the limit of 100 copies/gene/reaction.

3.3. Performance of the mPCR with clinical samples

The ultimate aim of this experiment was to evaluate the diagnostic performance of the mPCR using field samples. A total of 94 sow serum samples whose infectious status was predetermined by commercial single-target real-time PCR kits were used to evaluate the established mPCR. The DNA electrophoresis of mPCR results was partially illustrated in Figure 4 and the sample that was infected with one single pathogen such as PPV (Figure 4A, lane 6) or samples that were dually infected with two pathogens (Figure 4B, lane 4 and 11) or three pathogens (Figure 4A, lane 8 and 10). The mPCR results matched completely with the results generated by commercial real-time PCR. The infection rates for PCV2, PCV3 and PPV in these 94 samples were 43.6%, 39.4% and 55.3%, respectively. Combined infection of the three tested viruses (PCV2, PCV3 and PPV) was 17.0% while the dual infection between PCV2 and PCV3, PCV3 and PPV, or PCV2 and PPV were 7.4%, 5.3%, and 11.7%, respectively.

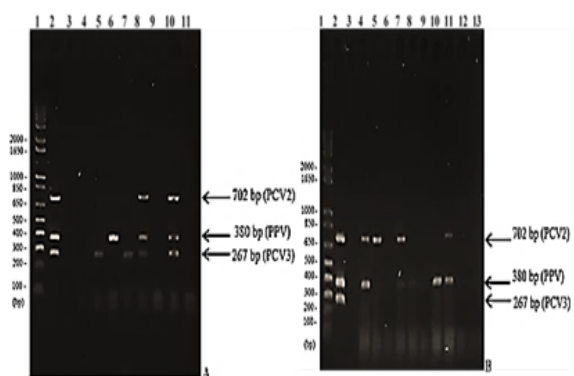


Figure 4. Diagnosis of PCV2, PCV3, PPV in clinical samples. (A) mPCR result of samples 1-9. (Lane 1): DNA ladder; (Lane 2): positive control; (Lane 3): negative control. (Lane 4 - 11): samples 1 to 9. (B) mPCR result of samples 10-19. (Lane 1): DNA ladder; (Lane 2): positive control; (Lane 3): negative control with pure water. (Lane 4 - 13): samples 10-19.

3.4. Sequence analysis of the PCR amplicons

To further confirm the specificity of the mPCR assay, PCR products of PPV, PCV3 and PCV2 amplified from the field samples were excised

from the agarose gel and subjected to DNA sequencing. The resulting sequences exhibited 100%, 98.88%, 98.95% identity to the reference sequences for PCV2 (MH470234.1); PCV3 (MK058529.1) and PPV (MK378155.1), respectively.

4. Discussion

Swine reproductive failure is a common disorder in sow herds in Vietnam at present. Various pathogens can be found in such cases including classical swine fever virus, Aujeszky's disease virus, PRRSV (Porcine reproductive and respiratory syndrome virus), PPV, PCV2, PCV3 and some bacteria (Christianson, 1992; Dinh et al., 2021). Currently, single real-time PCRs are available for molecular detection of these viruses. Nevertheless, single real-time PCRs are relatively expensive, thereby deterring swine producers from using these services. Multiplex PCRs can significantly reduce the cost spent in single PCR for each target. Technically, it is easier to develop a multiplex PCR for viruses that are in the same category of either RNA or DNA genome. Therefore, the goal of this study was to develop a quick and reliable mPCR that allows simultaneous detection of PCV2, PCV3 and PPV, the three DNA viruses that are commonly involved in reproductive failure.

Since its initial identification in the US (Palinski et al., 2016), PCV3 has been found in various pig-producing countries. The 39.4% detection rate for PCV3 in our study was also comparable to other reports elsewhere, such as 44.2% in Korea (Kwon et al., 2017); 31.18% in Central China (Xu et al., 2018); 50% in Germany (Prinz et al., 2019); 56.4%, 37.4%, 14.8% in Denmark, Italy, Spain, respectively (Franzo et al., 2018); 61.1% in United States (Arruda et al., 2019); and 47.8% in Brazil (Saraiva et al., 2019). Concurrent infections between PCV2 and other bacteria or viruses have been reported widely in swine herds (Ouyang et al., 2019; Dinh et al., 2021). One of the plausible reasons for this is that PCV2 can suppress the host immune system, therefore rendering the host more susceptible to infections with other microorganisms. The PPV is one of the common swine pathogens that are detected in PCV2 infected pigs (Ouyang et al., 2019). Previous studies showed that co-infections of pigs with PCV2 and PPV were more effective in inducing PMWS disease than coinfection between

PCV2 and other pathogens (Milek et al., 2020). Consequently, PPV could be used in combination with PCV2 to induce PMWS in experimental studies (Palinski et al., 2016). Coinfection of PCV2 and PCV3 is also commonly observed. Several surveillances from China have reported the PCV2/PCV3 co-infection rates varying from 27.6 - 39.39% (Ouyang et al., 2019). Those numbers were higher than what we found in this study (7.4%). In addition, it has been demonstrated that PCV2, PCV3 and PPV are endemic in Vietnam (Huyh et al., 2015; Pham et al., 2017; Nguyen et al., 2020), but the co-infection rates of these 3 viruses in sow herds have not been studied. Our preliminary demonstration of 17% of sow samples to be simultaneously positive for these three viruses illustrated the complex prevalence of various pathogens involved in porcine reproductive syndrome in Vietnam and highlighted the need for further investigations for a better understanding and control strategies.

The detection limit of our mPCR is 100 copies for each target gene per reaction. Liu et al. (2015) established a mPCR to detect and differentiate PCV2, PRRSV, PPV and Pseudorabies virus in pigs with PMWS manifestation. The authors reported that the sensitivity of their mPCR was 4.0×10^3 and 3.0×10^2 copies to PCV2 and PPV, respectively. Recently, Yang et al. (2018) developed a mPCR for simultaneous detection of PCV1, PCV2 and PCV3 with the detection limit of 50 copies of each target gene per reaction. Thus, our mPCR reported in this communication possesses a fairly good detection limit and certainly can be useful for pathogen monitoring in the field or used for predetermination of pathogen occurrence before further viral analysis of field samples.

5. Conclusions

In summary, we have successfully developed and validated a mPCR assay to simultaneously detect PCV2, PCV3 and PPV, the three common swine DNA viruses in field samples. This mPCR should provide a quick and reliable molecular diagnostic tool for reproductive failure in sow herds.

Conflict of interest

The authors declare no conflict of interest.

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