2023

Differential diagnosis of dna viruses related to reproductive disorder on sows by multiplex-pcr technique

T N. Nguyen

H T T Le

H L X Vu

P X. Dinh

Differential diagnosis of dna viruses related to reproductive disorder on sows by multiplex-pcr technique

T N Nguyen[1], H T T Le[1], H L X Vu[2], P X Dinh[1]*
[1]Department of Biotechnology, Nong Lam University, Ho Chi Minh City, Vietnam
[2]Department of Animal Science, University of Nebraska - Lincoln, United States

*dinhxuanphat@hcmuaf.edu.vn

Abstract. The newly emerged diseases caused by ASFV and PCV3 and their confirmed prevelance in Vietnam whereas most of available common commercial methods such as ELISA or realtime PCR designed for detecting single pathogen per reaction, highlighted a necessity for another diagnostic method to simultaneously detect and differentiate DNA viruses that are related to reproductive failures in sow herds including PCV2, PCV3, PPV, ASFV. In this communication, a diagnostic multiplex-PCR (mPCR) was established with pathogen-specific primers selected from previous studies and another set of primers designed for *COX1* gene serving as an internal amplification control (IAC). The predicted products of PCV2, PCV3, PPV, ASFV and IAC were 702 bp, 223 bp, 380 bp, 278 bp and 463 bp, respectively. After optimization, the mPCR functioned specifically at 62°C. Results revealed the consistent detection limit at 100 copies/gene/reaction. In application, 185 serum samples from sows were used to examine the presence of the related pathogens. mPCR results showed that the mono-infection rate of PCV2, PCV3, PPV, and ASFV was 0% (0/185), 40% (74/185), 28.1% (52/185), and 48.1% (89/185), respectively. Regarding coinfection rate, the data indicated that coinfections of 2, 3 and 4 pathogens were 20%, 8.1% and 0% accordingly. In conclusion, the mPCR assay was successfully established and ready to serve for diagnosis of PCV2, PCV3, PPV and ASFV infection in reality with high specificity and sensitivity. It is a good contribution to a better understanding of the epidemiology of these diseases in swine.
Keywords: ASFV, DNA viruses PCV2, multiplex PCR, PCV3, PPV, reproductive failures

1. Introduction

Porcine circovirus type 2, porcine circovirus type 3 (PCV2, PCV3) together with porcine parvovirus (PPV), and African swine fever virus (ASFV) are important viruses that significantly affect the national pig industry [1-4]. PCV2 has been associated with various disease syndromes, so called porcine circovirus-associated disease (PCVAD), which is a common terminology used in North America to refer to the different clinical signs associated with PCV2 infection. PCVAD consists of both clinical and subclinical disease conditions [5]. Porcine circovirus type 3 has been recently confirmed to be pathogenic in sows exhibiting porcine dermatitis and nephropathy syndrome. Even though PCV2 was not detected, PCV3 was present in skin and internal organs (kidneys, lung, and lymph nodes) of the diseased sows [6]. Afterwards, the pathogenesis of PCV3 has been documented to be experimentally involved in porcine dermatitis and nephropathy syndrome [7].

Another non-enveloped virus that carries a single-stranded DNA genome is porcine parvovirus (PPV), in the family of Parvoviridae. It is a relevant agent for reproductive disorders in swine. PPV was identified in the late 1960s [8], and was known to be associated with SMEDI syndrome, comprising stillbirths, mummification, embryonic death, and infertility [2].

Recently, ASFV, one of the most devastating swine viral pathogen, has emerged and posed a dramatically economic loss for pig industry in Vietnam [9]. ASFV is an enveloped virus belonging to Asfaviridae family with a double-stranded DNA genome. Disease caused by ASFV can be

described by high body temperature, systemic hemorrhage to bleeding, pulmonary edema and severe necrosis of lymphoid tissue [10].

These viruses can coexist in the same host and can aggravate severity of the diseases. There are numerous reports of co-infection between PCV2 and PPV [11, 12]; PCV2 and PCV3 [13]; as well as PCV2, PPV and ASFV altogether [14]. Co-infection between PCV2 and PCV3 did not result in significantly enhanced disease consequences, when it was compared to single infection with either of these two viruses [15]. However, co-infection of swine with PPV and PCV2 led to the escalated amount of PCV2 in blood [16]. In Vietnam, these viruses are presently circulating [9, 17, 21]. This has been leading to considerable economic losses [17, 18]. Several traditional and real-time PCRs have been generated for molecular diagnosis purpose of these viruses too [13, 19, 20]. However, those PCRs were designed for detection one or some but not these pathogens in a single reaction. That is still time-consuming and more costly for the diagnosing in high frequency cases. Besides, the main challenge in diagnosis of the subclinical signs is really difficult to evaluate or prove the no infectious causes of the reproductive problems if using methods without amplification such as serological based assays. To remedy the problem, in our study, we report the development and evaluation of an mPCR that contains an internal control and allows identification of these four viruses in specimens from pigs. This study is much high concern of the owners and high practical in diagnosis even in case of subclinical signs of reproductive failures; they dedicate to know frequently presence either of these viruses onto their farms, especially African swine fever virus.

2. Materials and methods

2.1. Positive and negative DNA Controls

DNA fragments based on *cap* gene (PCV3), *ns1* gene (PPV), and *p72* (ASFV) were synthesized by Integrated DNA Technologies (IDT - USA); PCV2-positive control was adopted from a previous study [17]. DNA of pig used as an internal amplification control (IAC) was extracted from a pig showing negative real-time PCR results for the tested viruses. All genes were quantified to 10^8 copies/µL by Biodrop µLITE Spectrophotometer (BiochromTM 80-3006-51, England) before uses.

Unrelated DNA from different bacteria or virus such as *Escherichia coli* (*E. coli*) (ATCC 25922), *Clostridium perfringens* (*C. perfringens*) (ATCC 51880), *Streptococcus suis* (*S. suis*) (ATCC 43765), *Staphylococcus aureus* (*S. aureus*) (ATCC 6338), Aujeszky's disease virus (ADV), stored at Gene technology lab (Faculty of Bio-Sciences, Nong Lam University), were used to verify the specificity of the developed mPCR assay.

2.2. Primer design

In this study, the primers for polymerase chain reaction (PCR) are shown in Table 1. Primers for PCV2 were constructed based on the consensus sequence of ORF2 (702 bp) from 66 PCV2 strains. Primers for PCV3 and PPV were obtained from a previous study, targeting *ns1* gene for PPV and ORF2 for PCV3 [21]. Primer sequences for ASFV were obtained from a previous study and were specific for the *p72* gene [22]. Besides, for internal control, one primer set was designed in this study to amplify *COX1* gene of pig (accession no. KY661881.1). The PCR products of PCV2, IAC, PPV, ASFV, and PCV3 were 702, 463, 380, 278, 223, respectively.

Table 1. Primer sequences and estimated product sizes

Primers	Primer sequences (5' – 3')	Accession no/target gene	Location (nt)	Products (bp)
PCV2-F	ATGACGTATCCAAGGAGGCG	PCV2 (GenBank: AY691679.1)/ Cap	1734 - 1715	702
PCV2-R	TTAAGGGTTAAGTGGGGGGTC		1033 – 1053	
PCV3-R	CATTCGTTTAGGCGGGTAAT	PCV3 (GenBank:	1773 - 1792	223
PCV3-F	GCACCAAAATGAGACACAGAG		1995 - 1975	

		MN431644.1) /Cap		
PPV-F PPV-R	GCTTTAGCCTTGGAGCCGTGGA	PPV (GenBank: KY994646.1) /ns1	1971 -1992	380
	CGTGTTCTTTTGCTGCGGCGTC		2350 -2329	
	AACAGGGGTGTTTGGTATTGAG		501 – 480	
ASFV-F ASFV-R	ATGGATACCGAGGGAATAGC	ASFV (GenBank: AF511455.1) /P72	356 – 337	278
	CTTACCGATGAAAATGATACGCA		79 - 101	
Sus scrofa-F Sus scrofa-R	GCACTGCCTTGAGCCTACTAAT	Sus scrofa (GenBank: KY661881.1) /COX1	39 – 60	463
	AACAGGGGTGTTTGGTATTGAG		501 – 480	

2.3. Single PCR construction

The PCR was conducted in a 25 µL reaction mixture: 12.5 µL of 2X DreamTaq master mix (Cat#K1081, ThermoFisher scientific), 1.0 µL primer (10 µM), 0.5 µL DNA (10^8 copies/µL), and distilled water was added to 25 µL. The amplifications were operated under these conditions: initial denaturation at 94°C for 3 min, followed by 36 cycles set at 94°C for 30 s, from 54°C to 64°C for 30 s and 72°C for 60 s, and a final extension at 72°C for 5 min. Subsequently, the 3 µL PCR products were analyzed in 1.5% agarose gel (Cat#16500100, ThermoFisher Scientific, USA), stained with Midori Green Advance DNA (Cat#MG04, NIPPON Genetics Europe, Germany) and observed under ultraviolet light (UV Transilluminators, Korea). 1Kb Plus DNA ladder (Cat#10787018, ThermoFisher Scientific, USA) was used to indicate the size of PCR products.

2.4. Optimize reaction conditions for the multiplex PCR (mPCR)

To achieve the optimal reaction conditions, the parameters were adjusted one by one. The optimization was done in a 25 µL PCR reaction mixture: 2X DreamTaq master mix 12.5 µL (Cat#K1081, ThermoFisher scientific), initial molar concentration of each primer is 2.5 µM or 5 µM, list primer described in Table 1, to get final molar concentration tested from 0.05 to 0.2 µM corresponding to 0.5 - 0.8 µL of each primer taken into a reaction, the DNA template 0.5 µL (10^8 copies/µL), and distilled water was added to the final volume of 25 µL. The reactions were amplified in a thermal cycler (Bioer, China), with the thermal conditions as follows: initial denaturation at 94°C for 3 min; 36 cycles conducted at 94°C for 30 s, the appropriate anneal temperature chosen in optimized single PCRs for 30 s, 72°C for 30 - 90 s; followed by a 5 min final elongation at 72°C. When it comes to detail, one final molar concentration of primer changed range from 0.05 to 0.2 µM, the remaining others would be fixed until 5 interest bands were visible clearly and separately in the agarose gel performance. That is called optimized mPCR assay in case of ratio of between primers.

2.5. Evaluation of the specificity and detection limit of the mPCR

Specificity of the established mPCR was assessed by using the DNA of other viruses or bacteria that are commonly found in the field samples. All templates were tested in triplicate. Furthermore, the mPCR product of positive controls were verified by sequencing.

Regarding the limit of detection, the template of PCV2, PCV3, PPV and ASFV were diluted ten-fold serially from 10^8 to 10^0 copies/reaction in nuclease free water and used in the mPCR to ascertain the detection limit. The lowest number copies of each gene called a detection limit of this assay. It could not be identical in between pathogens.

2.6. Field sample collection and DNA preparation

From January 2019 to July 2020, a total of 185 serum samples were assembled from sow farms in some Southern provinces of Vietnam. Each serum sample was pooled from five separate sows. All samples were sent to the laboratory and the owners signed a settlement between both sides about using the result data for the teaching and publication purposes. The infectious condition of these 185 samples was predetermined through commercial real-time PCR kits or master mix, as per the manufacturers' instructions. The real-time PCR kits used for finding of PCV2 based on 3' non-coding region, PPV based on structural protein VP2 gene, ASFV based on p72 gene and PCV3 were PowerChekTM PCV2 real-time PCR kit (Cat#path-pcv2-standard, Primerdesign), PowerChekTM ADV/PPV real-time PCR kit (Cat#path-ppv-standard, Primerdesign), VDx® ASFV qPCR (Cat# NS-ASF-31, Median Diagnostics), and Luna® Universal qPCR Master Mix (Cat#M3003S, NEB), respectively. The thermal conditions were followed sharply on the instruction of manufacturers. Briefly, in cases of detecting PCV2 or PPV, the amplification conditions is followed: initial denaturation at 95°C for 2 min; 50 cycles conducted at 95°C for 10 s, annealing and elongation at 60°C for 60 s; while that of detecting ASFV, the thermal amplification is conducted: uracil-N-glycosylase step at 50°C for 2 min, followed by initial denaturation at 95°C for 10 min, subsequent to 40 cycles: denaturation at 95°C for 15 s, annealing and elongation at 58°C for 60 s.

Viral DNA were extracted using the DNeasy ® Blood and Tissue (Cat#69504, Qiagen, Germany). Total amount of DNA was eluted with 200 µL of buffer and used immediately or kept at -80 °C for later usages.

2.7. DNA sequencing

Sequencing of amplified targets was performed using both forward and reverse primers, except for IAC product which was sequenced with only the forward primer (Table 1). The sequencing results were reconfirmed with the database of Genbank and were compared with the reference sequence: PCV2 (accession no. MF169716.1), PCV3 (accession no. MN431644.1), PPV (accession no. KY994646.1), ASFV (accession no. AF511455.1), IAC (accession no. KY661881.1).

3. Results

3.1. Optimization of the mPCR assay

The parameters of the mPCR were optimized for agent concentrations and thermal conditions. Each reaction was 25 µL in total, containing 12.5 µL volume of DreamTaq master mix (2X), 0.5 µL DNA template (10^8 copies/µL), optimal ratio of primers PCV2 : IAC : PPV : ASFV : PCV3 of 0.07 : 0.05 : 0.08 : 0.09 : 0.15 µM, and distilled water. The cycling temperature protocol consisted of a 3 min for initial denaturation at 94°C, and 36 cycles of amplification steps (denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and elongation at 72°C for 60 s), followed by an extension at 72°C for 5 min. DNA bands of PCV2, IAC, PPV, ASFV, PCV3 were detected at 702bp, 463bp, 380bp, 278bp and 223bp accordingly. Those bands size met our expectation, described as below (Figure 1).

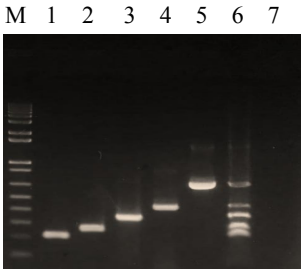

Figure 1. Electrophoresis of mPCR products in optimized conditions. (Lane M): 1 Kb Plus DNA ladder; (Lane 1): PCV3; (Lane 2): ASFV; (Lane 3): PPV; (Lane 4): IAC; (Lane 5): PCV2; (Lane 6): mPCR of the all five targets; (Lane 7): negative control with pure water.

3.2. Specificity and detection limit of the proposed mPCR assay

The target viruses in this study can be detected in blood, saliva, body discharges, feces… in live pigs. Thus, sampling live pigs for the purpose of disease monitoring is prone to potential contamination with unrelated microorganism commonly circulating in the farm environment. Therefore, genomic DNA of some particular bateria as well as virus: *S. aureus*, *S. suis, E. coli*, *C. perfringens* and PRV *gE* gene were used as negative templates in the mPCR reaction in order to determine its specificity. The amount of DNA templates used in the reaction was 30 ng per reaction for bacterial targets and 10^8 copies per reaction for PRV *gE* gene. As shown in Figure 2, the multiplex PCR assay was absolutely specific for target DNAs and no products amplified from DNA templates of unrelated pathogens (lanes 2 - 6) while the internal control confirmed the successful reaction. In contrast, the target fragments were specifically amplified using the five defined primer pairs (lane 1).

In the next step, we evaluated the minimum amount of template needed for a successful assay. The assay was performed with a set of 10-fold serially diluted positive control DNA samples with the copy numbers ranging from 10^8 to 10^0 copies per reaction. As shown in Figure 3, five distinct DNA bands at expected size were observed at the concentration of 10^2 copies per reaction. It illustrated that this mPCR could detect these viral genes at 100 copies/gene/reaction. The tests were performed consistently in triplicate.

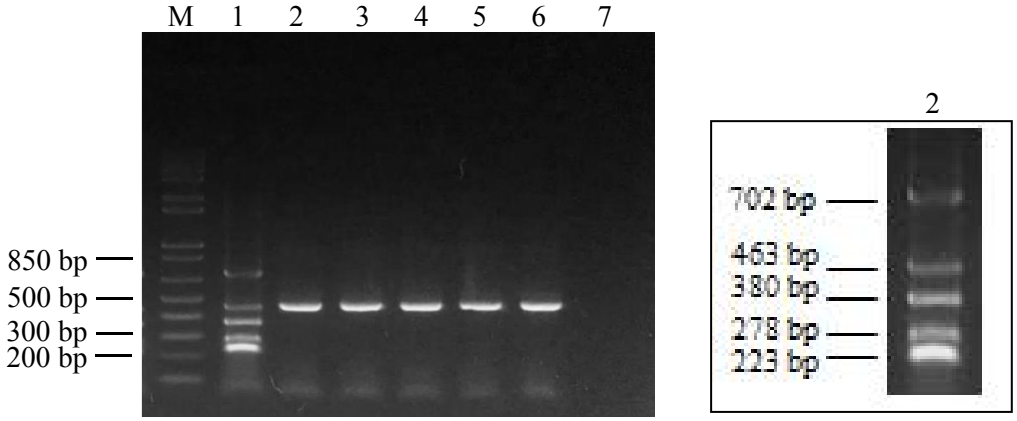

Figure 2. Specificity of the mPCR. (Lane M): 1 Kb Plus DNA ladder; (Lane 1): positive control; (Lane 2): *E. coli*; (Lane 3): *C. perfringens*; (Lane 4): *S. aureus*; (Lane 5): *S. suis*; (Lane 6): PRV; (Lane 7): negative control with pure water. The thermal cycling conditions were: 94°C /3 min; 36 Cycles of 94°C /30 s, 62°C /30 s and 72°C /60 s, a final extension at 72°C /5 min; Gel electrophoresis was 1% agarose and performed at 90 Volt/30 minutes.

3.3. Detection of viruses in field samples

The final purpose of this experiment was to validate the mPCR assay in practical diagnosis using field samples. A total of 185 serum samples whose infectious condition was acknowledged by commercial single-target real-time PCR kits. A part of mPCR results was displayed in Figure 4. Some samples were positive for single pathogen including PPV (lane 9, 10, 13), ASFV (lane 6, 7, 24), or PCV3 (lane 19, 20). While samples in lane 39, 40 were dually infected with ASFV and PCV3; and there was a coinfection of 3 viruses (PPV/ASFV/PCV3) in lane 29, 30, 33. All tested samples showed the precise band of the internal control, 463 bp.

In 185 samples tested, the mono-infection rate of PCV2, PPV, ASFV, and PCV3 detected was 0% (0/185), 28.1% (52/185), 48.1% (89/185), 40% (74/185), respectively (Table 2). It was quite high for ASFV, in agreement with the recent nationwide outbreaks of ASF in Vietnam. Regarding coinfection rate, our mPCR detected the presence of two and three pathogens together in 20% and in 8.1% of the samples examined but none of the case was infected with all four viruses (Table 2). The total co-infection rate was 28.1% (52/185) in all of the assayed samples.

Analysis of data generated by the mPCR with that of Realtime-PCR demonstrated 97.2% (180/185 samples), 98.9% (183/185 samples), 100% (185/185 samples), 97.2% (180/185 samples) matching for PCV2, PPV, ASFV, and PCV3 respectively. It indicated that the mPCR could be used in replacement of the single-target real-time PCR with high reliability.

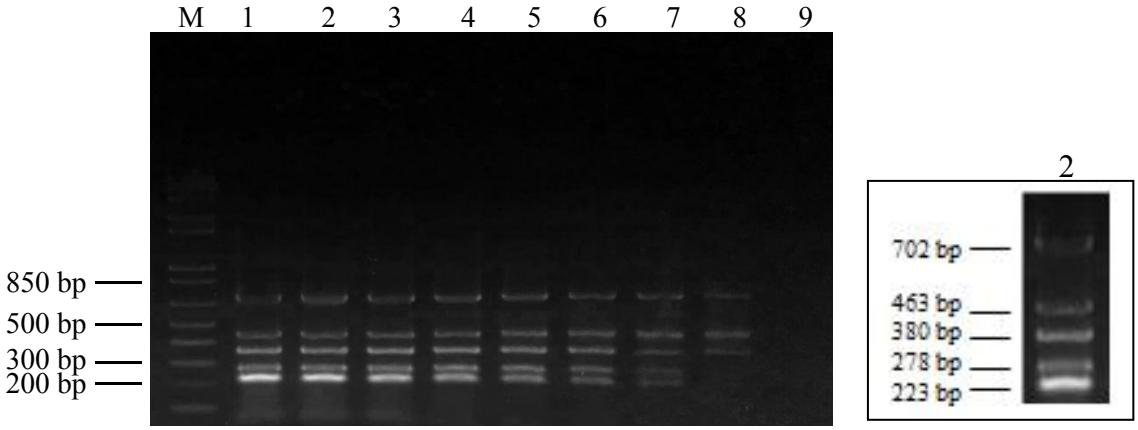

Figure 3. Detection limit of mPCR. (Lane M): 1 Kb Plus DNA ladder; (Lanes 1 – 9) are: 1, 10^8; 2, 10^7; 3, 10^6; 4, 10^5; 5, 10^4; 6, 10^3; 7, 10^2; 8, 10^1; 9, 10^0 copies/reaction. The thermal cycling conditions were: 94°C /3 min; 36 Cycles of 94°C /30 s, 62°C /30 s and 72°C /60 s, a final extension at 72°C /5 min; Gel electrophoresis was 1% agarose and performed at 90 Volt/30 minutes.

Table 2. Mono-infection and coinfection in field specimens by multiplex PCR (n = 185)

	Result of mPCR			Referential result of realtime-PCR		
Infection/Coinfection	Number of positive cases	Percentage of positivity (%)	Percentage of positivity each co-combination (%)	Number of positive cases	Percentage of positivity (%)	Percentage of positivity each co-combination (%)
PCV2	0	0		3	1.6	
PPV	52	28.1		52	28.1	
ASFV	89	48.1		89	48.1	
PCV3	74	40		79	42.7	
Combination of 2 pathogens	**37**		**20**	**39**		**21.1**
ASFV + PCV3	11	5.9		11	5.9	
PPV + PCV3	26	14.1		26	14.1	
PCV2 + PPV	0	0		2	1.1	
Combination of 3 pathogens	**15**		**8.1**	**15**		**8.1**

PPV + ASFV + PCV3	15	8.1		15	8.1	
Combination of 4 pathogens	**0**		**0**	**0**		**0**
PCV2 + PPV + ASFV + PCV3	0	0		0	0	

3.4. Sequence analysis of the PCR amplicons

To further confirm the mPCR assay's specificity, positive PCR products of PPV, PCV3, PCV2, and ASFV from the field samples, were recovered from the agarose gel and submitted for DNA sequencing. The resulting sequences showed 99.76% (compared with accession no. KY661881.1), 99.39% (compared with accession no. KY994646.1), 97.83% (compared with GenBank no. AF511455.1), and 99.1% (compared with accession no. MN431644.1) in term of identity to the reference sequences for IAC, PPV, ASFV, and PCV3 respectively. The results explicitly demonstrated that the mPCR could specifically detect the viral nucleic acid in field samples.

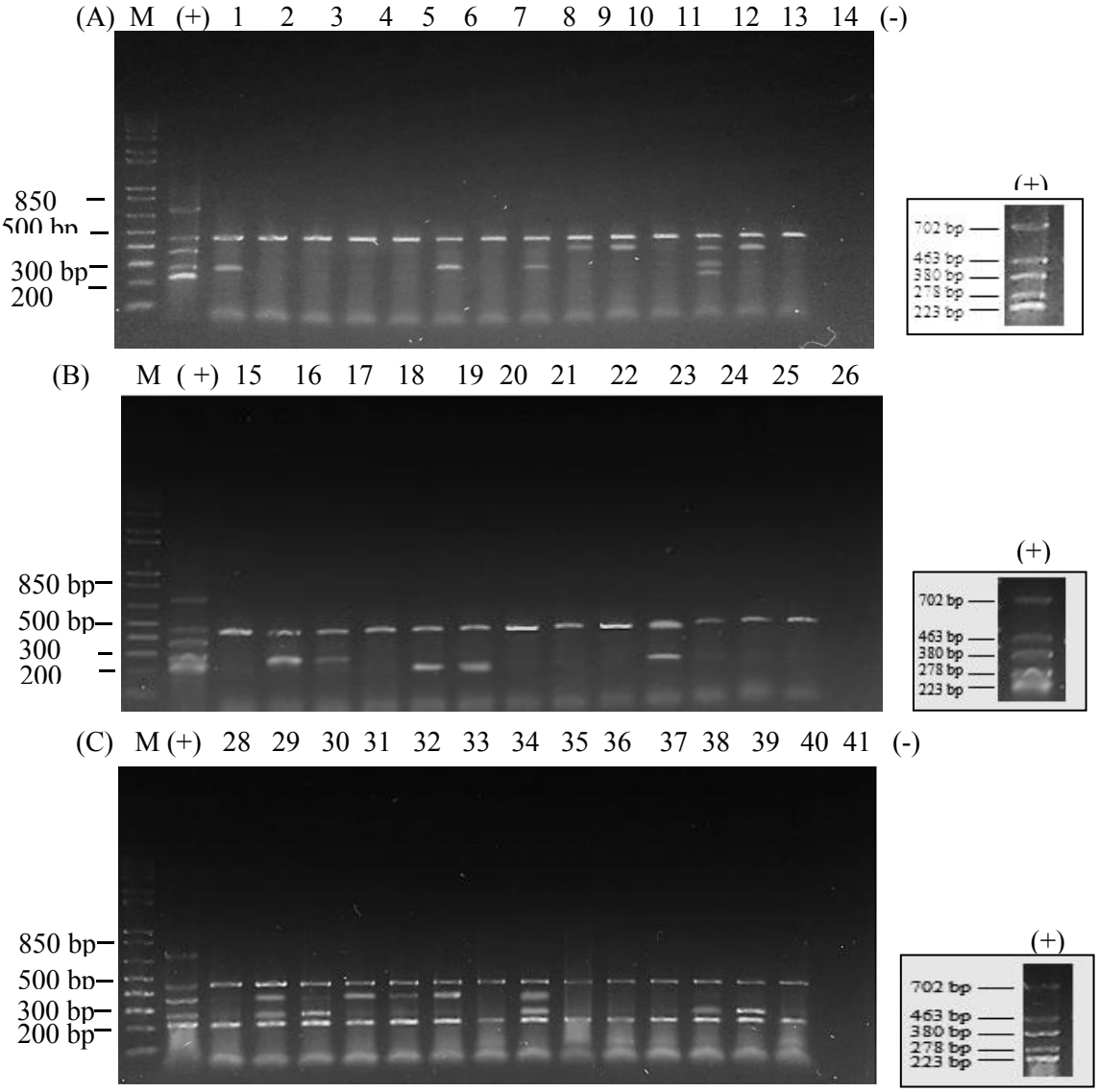

Figure 4. Detection results of partial clinical samples by the mRT-PCR. (Lane M): 1 Kb Plus DNA ladder; Lanes 1 – 41 are the results of mPCR conducted on collected sera samples.

4. Discussion

At present, PCV2, PCV3, PPV and ASFV are co-epidemic in Vietnam and cause huge economic losses to the pig production. Besides, the combination of these pathogens in the field are not easy to be ruled out clinically. In addition, use vaccine as a standard method to protect the farm. But PCV3 and ASFV have not been officially launched out yet. Taken together, the diagnosis is still a good method allowing the owner could control those pathogen in their farm efficiently.

PCR method has been widely used for research and routine diagnosis owing to its advantages as compared to other methods. Multiplex PCR has the potential to save time, labor and cost considerably without compromising test utility. In this study, we chose 4 set of primers to distinguish four pathogens at a time. In addition, IAC was also combined in the reaction, which helps further strengthen the reliability and specificity of the test. Initial optimizations confirmed the detectability of our mPCR at 100 copies/gene/reaction, lower than the recent 760 copies/reaction for the same pathogen reported by Liu et al. [23]. The optimized mPCR used in this study could be considered as a practical method for testing clinical samples.

In application using clinical sera samples, the mPCR provided similar results as compared to those of the real-time PCR. However, the mPCR detected a lower percentage of positive samples than the real-time PCR. That was likely to be because of the lower detection limit of mPCR than the real-time PCR (Table 2). Although some other studies reported quantitative real-time PCR and isothermal amplification assays with lower detection limits than the multiplex PCR developed in this study, our assay was much less expensive and can be applicable for detecting PCV2, PPV, ASFV, and PCV3 simultaneously in clinical samples. Several studies in Vietnam has found PCV3 at the rate of 14.3% and 39.4% [21, 24], quite comparable to 40% detection rate in our study. Similar detection rate were also reported in other countries such as 44.2% in Korea [25]; 31.18% in Central China [26]; 50% in Germany [27]; 56.4%, 37.4%, 14.8% in Denmark, Italy, Spain respectively [28]; 61.1% in United States [29], and 61.1% in Brazil [29, 30].

Concurrent infections between PCV2 and other viruses have been described broadly in pig farms. Surveillances from China have found PCV2/PCV3 coinfection in the range of 27.6 - 39.39% [31], compared to our previous report in Vietnam [21], and 0% in this study. Thus, we suggested that the co-infection rate between PCV2 and PCV3 or other viruses varies from farms to farms and depends on the epidemiological history of each farm.

PPV is the pathogen well-known to be associated with SMEDI syndrome and are often detected in PCV2 infected pigs [31]. Previous studies demonstrated that the replication and viremia of PCV2 was favored when coinfecting with PPV [16]. Thus, PPV was originally used in combination with PCV2 to induce PMWS in experimental studies [32]. Our previous study revealed 17% of sow samples to be simultaneously positive for PPV/PCV2/PCV3 [21]. In this study, the triple infection was lower (8.1%), possibly because the samples were collected from different farms.

ASFV causes lethal hemorrhagic disease in domesticated swine, with mortality approaching to 100% [33, 34]. Co-infections with other agents can also be a vital factor influencing the latency and shedding of ASFV [35]. Thus, it further highlighted the necessity for a good control strategy for these viruses in swine herds. Great control of infectious diseases requires highly sensitive diagnostic method which can detect and distinguish pathogens in clinical samples. In this study, we found the mono-infection of ASFV at 48.1% (89/185), while the rate of coinfection between ASFV and PCV3 was 5.9% (11/185). In addition, three pathogens together including ASFV, PPV, and PCV2 accounted for 8.1% (15/185). Nevertheless, to the best of our knowledge, similar to our result, there has been no report to date describing the simultaneous detection of these four viruses together.

5. Conclusion

In summary, an mPCR assay was optimized to be able to detect PCV2, PPV, ASFV, and PCV3 based on highly conserved regions of the cap/ns1/p72/cap gene. Additional studies will also be required to fully assess the mPCR assay by experiment with various type of samples from the fields.

This mPCR should provide a quick and reliable molecular method for the purpose of diagnosis and infectious diseases management in practical production.

References

[1] Dal Santo AC, Cezario KC, Bennemann PE et al 2020 Full-genome sequences of porcine circovirus 3 (PCV3) and high prevalence in mummified fetuses from commercial farms in Brazil *Microb Pathog* **141** 104027
[2] Dunne H, Gobble J, Hokanson J et al 1965 Porcine reproductive failure associated with a newly identified" SMEDI" group of picorna viruses *Am J Vet Res* **26** 1284-1297
[3] Schlafer D, Mebus C 1987 Abortion in sows experimentally infected with African swine fever virus: pathogenesis studies *Am J Vet Res* **48** 246-254
[4] Sirisereewan C, Thanawongnuwech R, Kedkovid R 2022 Current Understanding of the Pathogenesis of Porcine Circovirus 3 *Pathogens* **11** 64
[5] Opriessnig T, Meng X-J, Halbur PG 2007 Porcine circovirus type 2–associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies *J Vet Diagn Invest* **19** 591-615
[6] Palinski R, Piñeyro P, Shang P et al 2017 A novel porcine circovirus distantly related to known circoviruses is associated with porcine dermatitis and nephropathy syndrome and reproductive failure *J Virol* **91** e01879-01816
[7] Opriessnig T, Karuppannan AK, Castro AM et al 2020 Porcine circoviruses: Current status, knowledge gaps and challenges *Virus Res* **286** 198044
[8] Cartwright SF, Huck R 1967 Viruses isolated in association with herd infertility absortions and stillbirths in pigs *Vet Rec* **81** 196-+
[9] Le V, Jeong D, Yoon S et al 2019 Outbreak of African Swine Fever, Vietnam, 2019 *Emerg Infect Dis* **25** 1433-1435
[10] Dixon L, Sun H, Roberts H 2019 African swine fever Antiviral Res 165 34-41
[11] Cui J, Biernacka K, Fan J et al 2017 Circulation of porcine parvovirus types 1 through 6 in serum samples obtained from six commercial polish pig farms Transbound Emerg Dis 64 1945-1952
[12] Sharma R, Saikumar G 2010 Porcine parvovirus-and porcine circovirus 2-associated reproductive failure and neonatal mortality in crossbred Indian pigs Trop Anim Health Prod 42 515-522
[13] Yang K, Jiao Z, Zhou D et al 2019 Development of a multiplex PCR to detect and discriminate porcine circoviruses in clinical specimens BMC Infect Dis 19 1-10
[14] Pegu SR, Deb R, Das PJ et al 2022 Development of multiplex PCR assay for simultaneous detection of African swine fever, porcine circo and porcine parvo viral infection from clinical samples Anim Biotechnol 1-8
[15] Woźniak A, Miłek D, Bąska P et al 2019 Does porcine circovirus type 3 (PCV3) interfere with porcine circovirus type 2 (PCV2) vaccine efficacy? Transbound Emerg Dis 66 1454-1461
[16] Miłek D, Woźniak A, Podgórska K et al 2020 Do porcine parvoviruses 1 through 7 (PPV1-PPV7) have an impact on porcine circovirus type 2 (PCV2) viremia in pigs? *Vet Microbiol* **242** 108613
[17] Dinh PX, Nguyen MN, Tran VH et al 2021 Porcine circovirus genotypes and their copathogens in pigs with respiratory disease in southern provinces of Vietnam *Arch Virol* **166** 403-411
[18] Huynh T, Nguyen B, Nguyen V et al 2014 Phylogenetic and Phylogeographic analyses of P orcine Circovirus type 2 among pig farms in V ietnam *Transbound Emerg Dis* **61** e25-e34
[19] Chen H-Y, Li X-K, Cui B-A et al 2009 A TaqMan-based real-time polymerase chain reaction for the detection of porcine parvovirus *J Virol Methods* **156** 84-88

[20] Wang Y, Xu L, Noll L et al 2020 Development of a real‐time PCR assay for detection of African swine fever virus with an endogenous internal control *Transbound Emerg Dis* **67** 2446-2454
[21] Tran TK, Nguyen TTT, Vu HLX et al 2020 Identification of Porcine circovirus type (PCV2) and type 3 (PCV3), Porcine 2 parvovirus (PPV) in swine by multiplex PCR test *The Journal of Agriculture and Development* **20** 11-17
[22] Wilkinson P, Wardley R 1978 The replication of African swine fever virus in pig endothelial cells *Br Vet J* **134** 280-282
[23] Liu H, Shi K, Sun W et al 2021 Development a multiplex RT-PCR assay for simultaneous detection of African swine fever virus, classical swine fever virus and atypical porcine pestivirus *J Virol Methods* **287** 114006
[24] Trinh NPG, Nguyen NT, Dinh XP et al 2020 The presence and genetic characteristics of porcine circovirus 3 from pigs in Southern and Central provinces of Vietnam *The Journal of Agriculture and Development* **19** 26-31
[25] Kwon T, Yoo SJ, Park C-K et al 2017 Prevalence of novel porcine circovirus 3 in Korean pig populations *Vet Microbiol* **207** 178-180
[26] Xu PL, Zhang Y, Zhao Y et al 2018 Detection and phylogenetic analysis of porcine circovirus type 3 in central China *Transbound Emerg Dis* **65** 1163-1169
[27] Prinz C, Stillfried M, Neubert LK et al 2019 Detection of PCV3 in German wild boars *Virol J* **16** 1-7
[28] Franzo G, Legnardi M, Hjulsager C et al 2018 Full‐genome sequencing of porcine circovirus 3 field strains from Denmark, Italy and Spain demonstrates a high within‐Europe genetic heterogeneity *Transbound Emerg Dis* **65** 602-606
[29] Arruda B, Piñeyro P, Derscheid R et al 2019 PCV3-associated disease in the United States swine herd *Emerg Microbes Infect* **8** 684-698
[30] Saraiva GL, Vidigal PMP, Assao VS et al 2019 Retrospective detection and genetic characterization of porcine circovirus 3 (PCV3) strains identified between 2006 and 2007 in Brazil *Viruses* **11** 201
[31] Ouyang T, Zhang X, Liu X et al 2019 Co-infection of swine with porcine circovirus type 2 and other swine viruses *Viruses* **11** 185
[32] Allan G, Kennedy S, McNeilly F et al 1999 Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus *J Comp Pathol* **121** 1-11
[33] [33] Galindo I, Alonso C 2017 African swine fever virus: a review *Viruses* **9** 103
[34] [34] Lee HS, Bui VN, Dao DT et al 2021 Pathogenicity of an African swine fever virus strain isolated in Vietnam and alternative diagnostic specimens for early detection of viral infection *Porcine Health Manag* **7** 1-11
[35] [35] Abworo EO, Onzere C, Amimo JO et al 2017 Detection of African swine fever virus in the tissues of asymptomatic pigs in smallholder farming systems along the Kenya–Uganda border: Implications for transmission in endemic areas and ASF surveillance in East Africa *J Gen Virol* **98** 1806-1814