ĐẠI HỌC HUẾ
VIỆN CÔNG NGHỆ SINH HỌC

NGUYỄN TRẦN TRUNG

NGHIÊN CỨU ĐẶC ĐIỂM PHÂN TỬ GEN MÃ HÓA PROTEIN CẤU TRÚC CỦA VIRUS PARVO TRÊN LỢN (PORCINE PARVOVIRUS-PPV) Ở MỘT SỐ TỈNH MIỀN TRUNG VIỆT NAM

BỘ CÔNG TRÌNH KHOA HỌC
CÓ LIÊN QUAN ĐẾN LUẬN ÁN TIẾN SĨ

Người hướng dẫn khoa học:
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CÁC CÔNG TRÌNH CÔNG BỐ LIÊN QUAN ĐẾN LUẬN ÁN

1. Nguyen Thi Dieu Thuy, **Nguyen Tran Trung**, Tran Quoc Dung, Do Vo Anh Khoa, Dinh Thi Ngoc Thuy, Tanja Opriessnig (2021). First investigation of the prevalence of parvoviruses in slaughterhouse pigs and genomic characterization of ungulate copiparvovirus 2 in Vietnam. *Archives of Virology*, 166: 779-788. (SCIE/Q2)

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ORIGINAL ARTICLE

First investigation of the prevalence of parvoviruses in slaughterhouse pigs and genomic characterization of ungulate copiparvovirus 2 in Vietnam

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Abstract

Ungulate protoparvovirus 1, also known as porcine parvovirus 1 (PPV1), is considered to be one of the major causes of reproductive failure in pig breeding herds. Other parvoviruses have also been identified in pigs, including ungulate tetraparvovirus 3, or PPV2, ungulate tetraparvovirus 2, or PPV3, and ungulate copiparvovirus 2, or PPV4, but their significance for pigs is unknown. In the present study, the prevalence of PPV1-4 was investigated using a total of 231 lung and serum samples collected from slaughterhouses in 13 provinces throughout Vietnam. The overall prevalence was 54.5% (126/231) for PPV1, 28.0% (65/231) for PPV2, 17.7% (41/231) for PPV3, and 7.8% (18/231) for PPV4. While PPV1 and PPV2 were found in 11 provinces, PPV4 was detected in only three provinces. Co-circulation of PPV1, PPV2 and PPV3 was frequently observed, with PPV1/PPV2 coinfection predominating, with 20.8% (48/231). All four PPVs were detected together in only one sample from Thua Thien Hue. Three nearly complete PPV4 genome sequences of 5,453 nt were determined and deposited in the GenBank database. Alignment and comparison of the three genome sequences showed 99.5-99.6% nucleotide sequence identity, and the deduced amino acid sequences of open reading frames 1-3 were 99.6-99.9% identical to each other, 98.9-99.3% identical to those of other Vietnamese strains and 99.4-99.7% identical to those of Chinese strains). Phylogenetic analysis further confirmed a close relationship between Vietnamese and Chinese PPV4 strains. These results are the first to report the prevalence of PPV1, PPV2, PPV3, and PPV4 and nearly complete genomic sequences of PPV4 in pigs from slaughterhouses in Vietnam.

Introduction

Parvoviruses are small, non-enveloped viruses with a linear, single-stranded DNA genome of 4-6 kb [1]. Their genome is characterized by a hairpin structure at the 5' and 3' ends and contains two open reading frames (ORF) coding for non-structural protein (NSP) and viral coat and capsid protein (VP) [2, 3]. A small additional ORF3 located between ORF1 and ORF2 has been described for some parvoviruses [4]. Parvoviruses in vertebrates, including pigs, are members of the family *Parvoviridae* and the subfamily *Parvovirinae* [5]. There are 10 monophyletic genera in this subfamily, four of which include viruses that infect pigs. Specifically, these include *Bocaparvovirus* (species *Ungulate bocaparvovirus 2, 3, 4* and *5*), *Copiparvovirus* (species *Ungulate copiparvovirus 2* and *4*), *Protoparvovirus* (species *Ungulate protoparvovirus 1* and *2*), and *Tetraparvovirus* (species *Ungulate tetraparvovirus 2* and *3*) [6]. Ungulate protoparvovirus 1, or porcine parvovirus 1 (PPV1), was first isolated

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in 1965 in Germany [7] and is associated with reproductive failure in sows, characterized by stillbirth, mummified fetuses, embryonic death, and infertility (SMEDI) [8]. To date, PPV1 is the only parvovirus clearly associated with disease in pigs. While additional PPVs have been identified, Koch's postulates for association of these viruses with disease still need to be fulfilled. Specifically, ungulate tetraparvovirus 3, also known as PPV2, was first discovered in Myanmar in 2001 [9]. In 2008, ungulate tetraparvovirus 2, also known as PPV3, was identified in Hong Kong [10]. In the USA, ungulate copiparvovirus 2, also known as PPV4, was discovered in 2010 [4] as well as the unclassified PPV5 (closely related to PPV4), which was discovered in 2013 [11]. Ungulate copiparvovirus 4, also known as PPV6, was identified in China in 2014 [12]. Finally, the currently unclassified PPV7 (proposed genus "*Chappaparvovirus*") was identified in the USA in 2016 [13].

Since their discovery, the prevalence of different parvoviruses has been investigated by different groups in different countries. The first identification of PPV6 occurred in North America in 2015 [14] and in Poland in 2016 [15], while PPV7 was first observed in Korean and Chinese pigs in 2018 [16, 17]. However, using archived samples from domestic pigs in the USA and Italy, PPV2 [18, 19], PPV3 [18], PPV4 [18, 19] and PPV6 [19] could be traced back to 1998, while PPV5 was identified in samples from 1997 [18]. Furthermore, phylodynamics and phylogeography history studies have suggested that PPV2 has been circulating at least since the 1920s, PPV3 since the1930s, and PPV4 since the1980s [19] and that PPV1 originated approximately 120 years ago [20].

In Vietnam, the SMEDI syndrome caused by ungulate protoparvovirus 1, or PPV1, has been of interest since the early 1990s, and at that time, the disease caused great losses in breeding herds. Currently, it is effectively controlled by inactivated or subunit vaccines. A survey of the seroprevalence of ungulate protoparvovirus 1 in Long An province (southern Vietnam) revealed that this virus is an important factor in the decline of fertility in breeding sows [21]. Until now, no studies on pig parvoviruses concentrating on other genera and their molecular characterization have been reported in Vietnam. In this study, we report the prevalence of ungulate protoparvovirus 1 (PPV1), ungulate tetraparvovirus 2 (PPV3), ungulate tetraparvovirus 3 (PPV2), and ungulate copiparvovirus 2 (PPV4) in pigs sampled from 13 provinces belonging to three main parts of Vietnam. After the first detection of PPV4 from lung lavage of PCV2-infected pigs in the USA [4], the virus has been investigated by several research groups and was also identified in other countries with different prevalence rates: 1.8% (13/705) in China [22], 6.4% (25/392) in Hungary [23], 10% (12/120 in Romania [24], 44% (41/80) in Thailand [25], 33% (40/120) in Japan [26], 2.5% (6/247) in Poland [27], 43.6% (48/110) in South Africa [28], and 20.0% (10/50) in Cameroon [29]. To obtain additional information about PPV4, nearly complete genomic sequences of circulating PPV4 isolates were determined in this study.

Materials and methods

Sample collection

From 2016 to 2019, 231 healthy 4.5- to 5.5-month-old pigs were sampled at abattoirs located in 13 provinces in three regions of Vietnam, including northern, central and southern Vietnam. Lung tissue (from one lung lobe, approximately 2 × 2 cm in size) and in some instances serum samples (3-5 ml blood collected in serum separation tubes) were collected randomly by local veterinarians. The preferred sample was lung tissue, but in some cases lungs could not be collected, and serum samples were collected instead. In each participating abattoir, a maximum of five samples were collected, and the number of abattoirs visited was 2-6 for each province. In total, 136 lung and 95 serum samples were collected. Detailed information on the collected samples, including numbers, sample types, collection years, and collection locations is presented in Table 1. After collection, the samples were stored on ice (4ºC) and immediately shipped to the laboratory. Serum was separated upon arrival, and all samples were frozen at -20ºC until testing.

DNA extraction and PCR analysis

Total viral DNA was extracted from serum or frozen lung tissues using a commercial kit (GeneJET Viral DNA/RNA Purification Kit, Thermo Scientific, Lithuania). The extracted concentration of the DNA was measured using a NanoDrop spectrophotometer and stored at 4°C until testing. All of the samples had a concentration of 92 to 446 ng/μl, making them suitable for further processing.

All DNA samples were tested by conventional PCR assays using previously described primers (Table 2). The PCR assays specific for PPV1 through PPV4 were carried out in a single reaction for each parvovirus using 1.5-2.0 μl of DNA, 2× PCR master mix (Thermo Scientific, Lithuania), and 5 pmol of each primer. The specific annealing temperature (Ta) for each primer pair is shown in Table 2. A Vertiti Thermal Cycler (AB Applied Biosystem) was used. The resulting PCR products were analyzed by electrophoresis on a 2% agarose gel using a 100-bp DNA ladder (Thermo Fisher Scientific). Appropriate positive and negative extraction and PCR controls (obtained from the University of Edinburgh and confirmed by sequencing) were used for each extraction and PCR run. When a faint DNA band was seen on the gel, the PCR was carried out again with double the

Table 1 Information about the samples collected in this study, including location, sample type and year of collection

Region	Province	Number of abattoirs visited	Number of samples collected[1]	Sample type[2]		Collection year
				Lung	Serum	
North	Quang Ninh	4	19	19	0	2019
	Bac Giang	3	10	0	10	2018
	Ha Noi	5	24	19	5	2017-2018
	Hoa Binh	4	20	20	0	2016
Central	Ha Tinh	4	20	10	10	2017
	Quang Tri	5	22	22	0	2019
	Thua Thien Hue	6	28	28	0	2019
	Quang Nam	4	16	10	6	2018-2019
South	Ho Chi Minh	3	13	3	10	2016
	Dong Nai	3	14	5	9	2017
	Can Tho	7	32	0	32	2017
	Ben Tre	2	6	0	6	2017
	Vinh Long	2	7	0	7	2017
Total	14	52	231	136	95	

[1]The number of samples collected in each abbottoir was less than five.

[2]From each animal, only a single sample was collected (either lung or serum).

Table 2 Primers used in this study for genotyping and sequencing. The virus targets were ungulate protoparvovirus 1 (PPV1), ungulate tetraparvovirus 3 (PPV2), ungulate tetraparvovirus 2 (PPV3), and ungulate copiparvovirus 2 (PPV4).

Purpose	Target	Primer sequence (5'-3')	T (°C)	PCR size (bp)	Reference
Genotyping	PPV1	F: GGGAGGGCTTGGTTAGAATCTC[1] R: ACCACACCCCCCATGCGTTAGC	56	196	[34]
	PPV2	F: AGATTCTTGCAGGCCGTAGA R: CCAAGGGTCAGCACCTTTTA	60	222	
	PPV3	F: GCAGTCTGCGCTTAACTT R: CTGCTTCATCCACTGGTC	50	392	[24]
	PPV4	F: GCATTGGTGTGTGTCTGTGTCC R: GTGGCACATTTGTACATGGGAG	54	345	[23]
Sequencing	PPV4-1	F: *TGACGCAGTACAGACCGACGAGA (356-379)* R: *AATGCAAGTGCAAGCCACCTTTT (1175-1197)*	62	842	[23]
	PPV4-2	F: *AGTAATCTGGTAATCGCTGTTCG* (1050-1072) R: *ATGTTAGTCTTTCCTGTTGTGGC* (1920-1942)	62	893	
	PPV4-3	F: *GCTGGTGGATAACAACATCTGCT* (1568-1590) R: *GTTTCTTCTTTCTCGGTGCTTCT* (2531-2553)	60	986	
	PPV4-4	F: *AAGAAGCACCGAGAAAGAAGAAA (2530-2552)* R: *AAATCTAAGGGACAAGGCAAACG* (3391-3413)	60	884	
	PPV4-5	*F: TACTAAGAAAGACAAGGTGGAG (3366-3387)*[2] R: *AATAATAGAAGGTATAGCGTC (4243-4263)*[3]	60	898	
	PPV4-6	F: *ACCTGCTCCTCCATCTTCTCCAC (3918-3940)* R: *GGCCGTCATCATACATTCTGCTC (4895-4897)*	62	980	
	PPV4-7	F: *ACTTACTGTTCTATGATGTCTGGAG (4219-4243)* R: *ATATCATCTGCGGTGTCTGGG (5842-5862)*	62	1,644	

[1]Due to an observed mutation at the 3'end, A was changed to T

[2]Original primer sequence PF3363: 5'-AGATACTAAGAAAGACAAGGTGGAG-3'

[3]Original primer sequence PR4263: 5'-AATAATAGAAGGTATAGCGTCTCCA-3'

volume of DNA. Selected PCR products were purified and sequenced on an ABI 3730xl DNA Analyzer (Thermo Fisher Scientific), and the PPV sequences were confirmed by Basic Local Alignment Search Tool (BLAST) analysis. For PPV1, PPV2, and PPV3, the positive samples in each province were counted. If fewer than 10 samples were positive, one was

selected at random to be sequenced. If more than 10 samples tested positive, two random samples were sequenced. All PPV4-positive samples were sequenced.

PPV4 genome sequencing and sequence analysis

Seven previously described primer pairs [22] were used to amplify portions of the nearly complete genomes of the three PPV4-positive strains (Table 2). Amplification products were purified using a GeneJET PCR Purification Kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions and sequenced by the standard Sanger method on an ABI 3730xl system. The nucleotide sequences of PPVs were identified using BLAST at the National Center for Biotechnology Information [30]. Multiple nucleotide and amino acid sequence alignments were carried out in BioEdit 7.2.5, using published PPV sequences as references [31]. Phylogenetic analysis was conducted using MEGA X [32] and the maximum-likelihood method based on the Tamura-Nei model [33]. Bootstrap analysis (1,000 replicates) was used to assess the robustness of phylogeny.

Table 3 Number of DNA-positive samples (percentage) in this study. The samples were tested for ungulate protoparvovirus 1 (PPV1), ungulate tetraparvovirus 3 (PPV2), ungulate tetraparvovirus 2 (PPV3), and ungulate copiparvovirus 2 (PPV4).

Province	n	PPV1	PPV2	PPV3	PPV4
Quang Ninh	19	15 (78.9)	9 (47.4)	0	6 (31.6)
Bac Giang	10	3 (30)	6 (60)	6 (60)	0
Ha Noi	24	19 (79)	3 (12)	15 (62)	0
Hoa Binh	20	7 (35)	0	3 (15)	0
Ha Tinh	20	14 (70.0)	3 (15.0)	6 (30)	0
Quang Tri	22	18 (81.8)	10 (45.5)	3 (13.6)	6 (27.2)
Thua Thien Hue	28	24 (85.7)	20 (71.4)	3 (10.7)	6 (21.4)
Quang Nam	16	0	6 (37.5)	0	0
Ho Chi Minh	13	2 (15.4)	1 (7.7)	0	0
Dong Nai	14	2 (14.3)	1 (7.1)	3 (21.4)	0
Can Tho	32	18 (56.3)	5 (15.6)	0	0
Ben Tre	6	0	1 (16.7)	2 (33.3)	0
Vinh Long	7	2 (28.6)	0	0	0
Total	231	124 (53.7%)	65 (28.0%)	41 (17.7%)	18 (7.8%)

Results

Prevalence of the four PPVs in slaughter-age pigs in Vietnam

All four PPVs were detected in the 231 samples tested, with some samples testing positive for more than one genotype (Table 3). The geographical location of the different PPVs is shown in Fig. 1. The overall PPV prevalence rates in pigs at slaughter age in both sample types collected were 53.7% for PPV1, 28.0% for PPV2, 17.7% for PPV3, and 7.8% for PPV4. PPV1 and PPV2 were observed in most of the provinces sampled (11/13), whereas PPV4 was only detected in pigs from Quang Ninh, Quang Tri and Thua Thien Hue provinces in northern and central Vietnam. High rates of PPV1 positivity ranging from 70.0% to 85.6% were observed in Hanoi, Ha Tinh, Quang Tri, and Thua Thien Hue provinces. The highest prevalence of PPV2 was 71.4% in Thua Thien Hue and for PPV3 it was 62% in Hanoi.

The distribution according to sample type was as follows: Of the 136 lung samples, 67.6% (92/136) were positive for PPV1, 32/4% (44/136) were positive for PPV2, 18.4% (25/136) were positive for PPV3, and 7.6% (18/136) were positive for PPV4. Of the 95 serum samples, 33.7% (32/95) were positive for PPV1, 22.1% (21/95) were positive for PPV2 and 16.8% (16/95) were positive for PPV3. PPV4 DNA was not detected in any serum samples.

Often, pigs were positive for more than one genotype. A summary of coinfections with more than one PPV is presented in Table 4. The overall highest coinfection rate was seen with PPV1/PPV2, at 20.8%. The rates of infection of PPV1/PPV3 and PPV2/ PPV3 were lower, at 10.0% and 4.3%, respectively. Only 2.6% of the samples were simultaneously positive for PPV1, PPV2, and PPV3.

PPV4 was detected in three provinces (Table 4) and often co-circulated with PPV1, PPV2 and PPV3. The infection rates were 23.3% for PPV1 and PPV4, 14.5% for PPV2 and PPV4, 1.5% for PPV3 and PPV4, and also for PPV1, PPV2, PPV3 and PPV4. Only one pig from Thua Thien Hue was infected with all four PPVs.

Diversity of PPV4 genomes in pigs from slaughterhouses in Vietnam

To investigate the diversity of PPV4 circulating in Vietnam, the nearly complete genome sequences of three PPV4 isolates collected from Quang Ninh and Quang Tri were determined and analyzed. The entire sequence length of the three isolates was 5,367 nt with no insertion and deletions in the coding regions, and the sequences were deposited in the GenBank database with the accession numbers MT434667-MT434669. The nucleotide (nt) and amino acid (aa) sequence identity values for the three PPV4 strains, Vietnamese strains, and PPV4 reference strains from China and the USA, and from wild boar hosts are summarized in Table 5.

A multiple sequence comparison revealed a base substitution at position 124 (G→A) resulting in an amino acid change (42: D→N) in ORF1 and other substitutions resulting in three amino acid changes in ORF2 (455: E→D/Q,

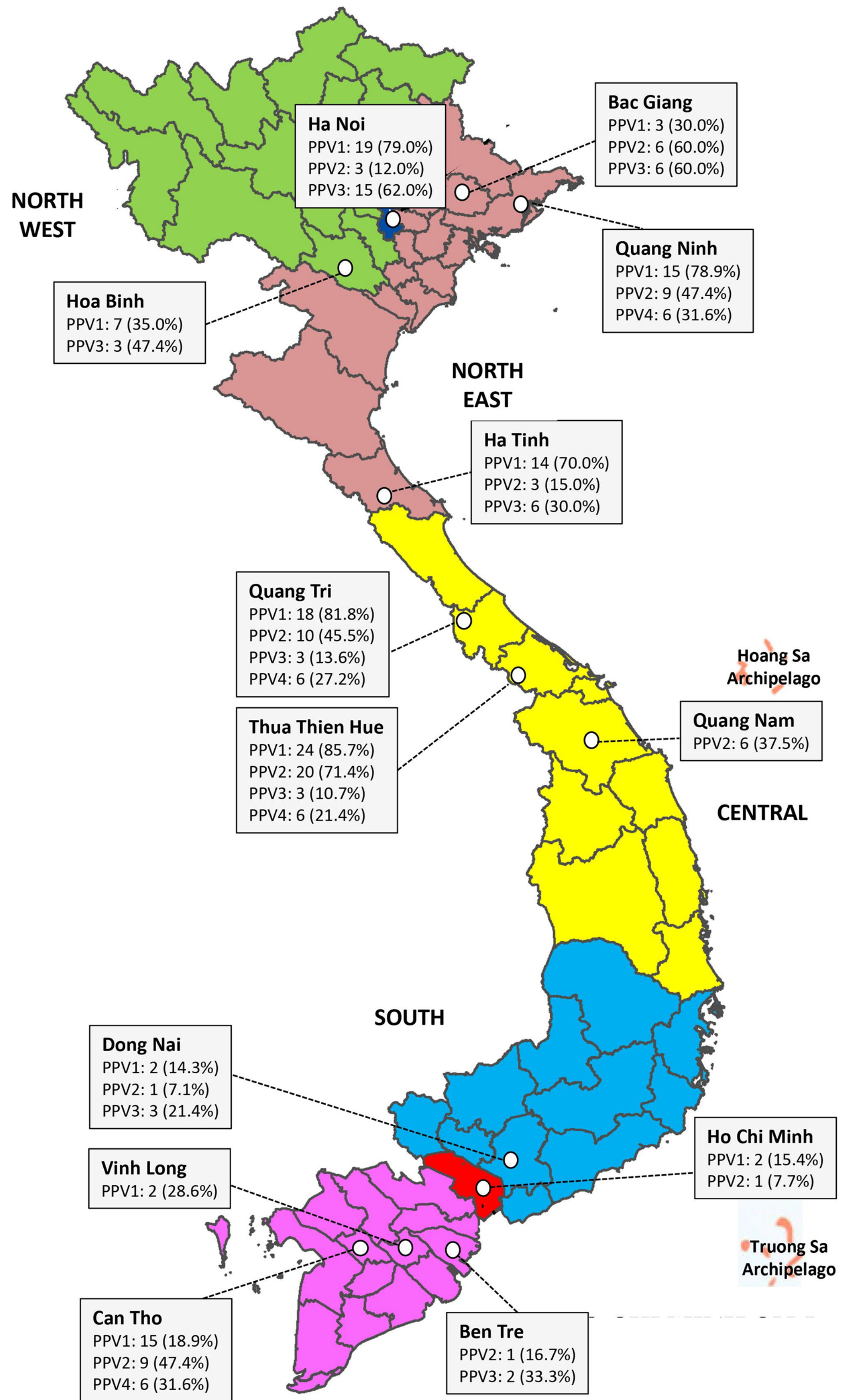

Fig. 1 Map of Vietnam, indicating the different provinces sampled in this study and also the detection rates of parvoviruses in pigs in these areas

Table 4 Rates of coinfection with different parvoviruses in pigs. The samples were tested for ungulate protoparvovirus 1 (PPV1), ungulate tetraparvovirus 3 (PPV2), ungulate tetraparvovirus 2 (PPV3), and ungulate copiparvovirus 2 (PPV4).

Province	n	PPV1+2	PPV2+3	PPV1+3	PPV1+2+3	PPV1+4	PPV2+4	PPV3+4	PPV1+2+3+4
Quang Ninh	19	8 (42.1)	0	0	0	5 (26.3)	1 (5.3)	0	0
Bac Giang	10	2 (20.0)	5 (50.0)	3 (30)	1 (10.0)	0	0	0	0
Ha Noi	24	3 (12.5)	0	11 (45.8)	1 (4.2)	0	0	0	0
Hoa Binh	20	0	0	1 (5.0)	0	0	0	0	0
Ha Tinh	20	3 (15.0)	0	2 (10.0)	0	0	0	0	0
Quang Tri	22	9 (40.9)	2 (9.1)	3 (13.6)	2 (9.1)	5 (22.7)	3 (13.6)	0	0
Thua Thien Hue	28	19 (67.9)	3 (10.7)	2 (7.14)	2 (7.1)	6 (21.4)	6 (21.4)	1 (3.6)	1 (3.6)
Quang Nam	16	0	0	0 (0)	0	0	0	0	0
Ho Chi Minh	13	0	0	0 (0)	0	0	0	0	0
Dong Nai	14	0	0	1 (7.1)	0	0	0	0	0
Can Tho	32	4 (12.5)	0	0	0	0	0	0	0
Ben Tre	6	0	0	0	0	0	0	0	0
Vinh Long	7	0	0	0	0	0	0	0	0
Total	231	48 (20.8%)	10 (4.3%)	23 (10.0%)	6 (2.6%)	16 (23.2%)	10 (14.5%)	1 (1.5%)	1 (1.5%)

Table 5 Percent of nucleotide and amino acid sequence identity for different open reading frames (ORF) of Vietnamese ungulate copiparvovirus 2 (PPV4) sequences investigated in this study (MT434667-MT434669) compared with reference strains from domestic pigs in China (GU978965-GU978967; HM031134; MG345027), the USA (GQ387499-GQ387500; NC014665), and Romania (JQ868713-JQ868716)

ORF	Size[1]	Vietnam	China	USA	Romania
1	1,797 nt	99.6-99.9	99.4-99.6	99.1-99.2	99.1-99.2
	598 aa	99.6-99.8	99.3-99.6	98.3-98.9	99.1-100
2	2,187 nt	99.2-99.7	99.3-99.7	98.6-99.3	99.2-99.7
	728 aa	99.5-100	99.4-99.8	99.4	99.7-99.8
3	615 nt	99.6-99.8	99.1-99.8	99.6-99.8	99.8-100
	204 aa	100	100	100	100
Whole sequence	5,367 nt	99.3-99.6	98.9-99.4	98.8-99.0	99.0-99.5
	1,500 aa	99.6-99.9	99.4-99.7	99.1-99.3	99.4-99.8

[1]nt, nucleotide; aa, amino acid

469: I→V, and 531: H→Q). No variation was detected in the deduced aa sequence of ORF3. As seen in Table 5, the three sequences contained two main ORFs, ORF1 (1797 nt), encoding a 598-aa protein, and ORF2 (2,187 nt), encoding a 728-aa protein, as well as a small ORF (ORF3) with a length of 615 nt, encoding a 204-aa protein. The level of nucleotide sequence similarity among the PPV4 genomes obtained from pigs raised in Vietnam was high: 99.6-99.9% in ORF1, 99.2-99.7% in ORF2 and 99.6-99.8% in ORF3. When full sequences were compared, the PPV4 strains shared 99.3-99.6% nt sequence identity and 99.6-99.9% aa sequence identity. High levels of sequence identity at the nucleotide and amino acid level were observed between Vietnamese PPV4 strains and Chinese strains (98.8-99.4% and 99.4-99.7%, respectively). The amino acid sequences of ORF3 were 100% identical among the Vietnamese strains and between the Vietnamese strains and other reference strains. The data presented in Table 5 also show that ORF1/ORF2 nucleotide and amino acid sequences of Vietnamese PPV4 strains exhibited a high degree of similarity to Chinese and Romanian strains, and less to US strains. To analyze the genetic relationship between the PPV4 sequences obtained in this study and the reference strains from other geographic locations, a phylogenetic tree was constructed based on nearly complete genomic sequences. As seen in Fig. 2, the three Vietnamese PPV4 strains (black dots; MT434667-MT434669) group in a clade together with Romanian (JQ868713-JQ868714) and Chinese (GU978964-GU978968; HM031134-HM031135) sequences. The other clade was formed by PPV4 sequences from the USA (black square boxes; GQ387499, GQ387500m BC014665), two sequences from Romania (JQ868715, RJQ868716), and a sequence from China (MG345027). The bootstrap values for all branches were higher than 63%, indicating the reliability of phylogenetic tree.

Discussion

Vietnam is among the world's major pork-producing countries; therefore, it is not exceptional that parvoviruses are commonly found in the pig population. A previous study on the seroprevalence of PPV1 in Long An, a southern province of Vietnam, showed that PPV1 contributed to reproductive failure in sows [21]. The results from this present study confirm the circulation of PPV genotypes 1-4 in pigs in slaughterhouses in Vietnam.

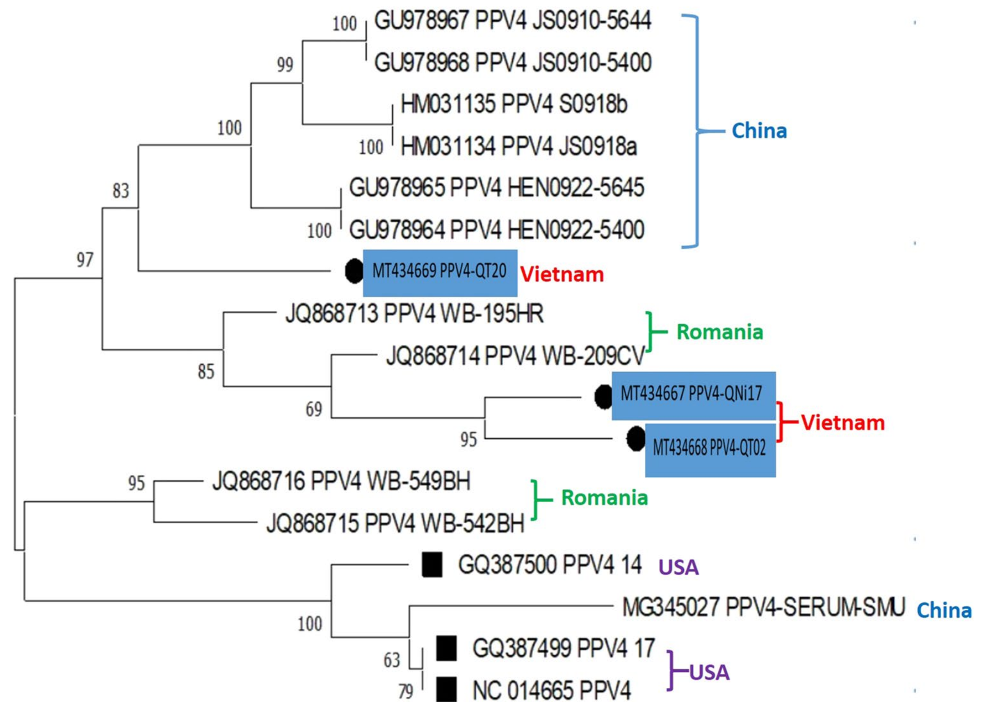

Fig. 2 Phylogenetic tree constructed based on nearly complete genome sequences of PPV4 (5,367 nt) obtained from Vietnam (this study) and reference sequences from the GenBank database. QNi, Quang Ninh province; QT, Quang Tri province. The analysis was conducted in MEGA X [32] with 1,000 replicates. Black square boxes indicate reference PPV4 sequences from the USA [4]; black dots represent the Vietnamese PPV4 sequences (this study).

PPV1 is currently the most prevalent genotype in pigs in several countries. The overall prevalence of PPV1 observed in this study was 53.7% (124/231). This is similar to that in commercial pig herds in Thailand (53.0%) [25]. The prevalence rates of PPV1 reported in Japan (67%) [26] and Germany (61%) [34] were slightly higher. In contrast, the rate of PPV1 circulation in China (5.6%) [35], South Korea (4.6%) [36], North America (8.9%) [37], and Argentina (11.3%) [38] is much lower. This might be due to differences in PPV1 vaccination programs for breeding herds in these selected regions. The PPV2 prevalence rate in this study was 28.0% (65/231) in pigs across 13 provinces. The reported PPV2 prevalence rates in North America (36.8%), Poland (19%), and in South Africa (21.8%) are in a similar range [27, 37, 39]. In contrast, high PPV2 prevalence rates in swine herds have been observed in Hungary (51.0%) [40], Germany (78.0%) [34], Thailand (82.5%) [25], and Japan (58.0%) [41]. The reported circulation of PPV3 in pigs in Asian countries is high, with 72.5% in Thailand [25], 45.1% in China [35], and 39.0% in Japan [26]. In contrast, the detection rate of PPV3 in this study was relatively low, at 17.7%. PPV3 is also present at a lower rate in Europe, including 21.3% in Hungary [40], 20.0% in Germany [34], 7.7% in Poland [27], and 19.1% in Slovakia [42]. The reported prevalence of PPV3 in South Africa is also low, at 5.5% [28]. PPV4 was detected in three of 13 provinces investigated in the northern and central part of Vietnam, and only in lung samples. PPV4 infection rates ranged from 21.4% to 31.6% (Table 3). This is similar to what has been observed in domestic pigs in other Asian countries, such as 33.0% in Japan [26], 21.6% in China [35], and 44.0% in Thailand [25], as well as 43.6% in South Africa [28]. Much lower infection rates of PPV4 were detected in pigs in the USA, with 2.9% positive samples [37], and in European countries, including 6.4% in Hungary [23], 10.0% in Romania [19], 7.0% in Germany [34], and 2.5% in Poland [27].

In Vietnamese pigs at slaughter age, coinfection with PPV1 and PPV2 was detected in 20.8% of the investigated animals, making it the most frequent combination, followed by PPV1 and PPV3 coinfection in 10.0% of the pigs and PPV2 and PPV3 coinfection in 4.3% of the pigs. Triple infection with PPV1, PPV2 and PPCV3 was found in only 2.6% of the investigated pigs. In a previous study from Romania, concurrent infection with PPV2 and PPV3 was frequent and present in 79% (31/39) of domestic pigs and 95% (169/177) of wild boars investigated [19]. When six pig farms in Poland were inspected, PPV2 was the most common and was detected in 80.2% (65/81) of samples that were positive for at least one PPV type [27]. In Japan, where 120 pigs, aged about 6 months, sampled from a slaughterhouse, were tested, 67% were PPV1 positive, 58% were PPV2 positive, 39% were PPV3 positive, and 33% were PPV4 positive [26]. In Thailand, across five genotypes (PPV1-4 and PBo-likeV), over 60% of the pigs carried more than three PPVs, and more than four PPVs were identified in 28% of tonsil samples [25]. Concurrent infection with PPV2, PPV3 and PPV4 and PCV2 was analysed using a biobank of archival pig samples (n = 695) [43]. The samples originated from Northern Ireland, the Republic of Ireland, Great Britain, and other neighbouring European countries and were collected

from 1997 to 2012. Concurrent infection with PPV2 and PPV3 occurred in 3.0% of the samples (23/695), dual infection with PPV2 and PPV4 was identified in 1.2% of the samples (8/695), and PPV3 and PPV4 coinfection occurred in 0.6% of the samples (4/695) [43]. As shown in Table 4, in this study, only a single sample was concurrently infected with all four investigated PPVs. The overall prevalence of PPVs was higher in lung tissue than in serum samples in healthy slaughter-age pigs. This is in agreement with a previous study showing the prevalence of PPV1-5 to be higher in tissue samples was also higher than in serum samples [37]. Taken together, the data on PPV coinfections indicate that the dual, triple, and quadruple infection rates in our study were lower than in other studies. The reported variations among geographic regions may be due to differences in the sample types investigated, the animal's age at sample collection, the test used, the health status of the pig, and the overall number of samples tested.

PPV4 was first identified in the lung lavage of a pig that was coinfected with porcine circovirus type 2 in the USA in 2010 [4]. It was found to have a genome size of about 5.9 kb. PPV4 has two major ORFs (ORF1, located at the 5'end, encoding non-structural proteins, and ORF2, at the 3'end, encoding structural proteins) and a small ORF3 located between ORF1 and ORF2 [4]. The initial discovery of PPV4 resulted in further investigations, and it was quickly confirmed that PPV4 was present globally. In this study, PPV4 was further characterized by genomic sequencing. PPV4 was selected over PPV1, PPV2, and PPV3 during the early stages of the investigation and for no particular reason. In hindsight, and after having all PPV prevalence rates available, it would perhaps have been better to have chosen another, more prevalent, PPV type. However, the results of this study still contribute to the overall knowledge base of PPV4 and are therefore important.

The sequence length of the three Vietnamese PPV4 strains analyzed in this study was 5,367 nt. Three variations in the deduced amino acid sequence of the protein encoded by ORF2 were observed in all three Vietnamese PPV4 strains, supporting the suggestion that ORF2 mutates at a faster rate than ORF1 [14]. This may be due to higher selection pressure by the host immune system on the viral capsid protein. Comparison of the nucleotide and amino acid sequences among Vietnamese PPV4 strains revealed high similarity, suggesting a common origin of these viruses. Sequence comparisons and phylogenetic analysis based on nearly complete genome sequences of PPV4 showed that the Vietnamese isolates were closely related to Romanian and Chinese PPV4 strains. They clustered together in a clade, which was separate from the clade formed by PPV4 sequences from the USA, China and Romania (Fig. 2). These results are consistent with a previous phylogenetic analysis based on PPV4 genome sequences [22] and further confirm the close genetic relationship among the investigated PPV4 strains. The lack of circulating PPV4 in the southern regions of Vietnam in this study, together with the high nucleotide and amino acid sequence similarity between Vietnamese and Chinese PPV4 strains, suggests a possible introduction of this genotype into Vietnam via the northern border with China. However, further investigations are needed, with a larger sample size, in order to test this hypothesis.

Conclusions

This study is the first survey of PPV1, PPV2, PPV3, and PPV4 genotypes in slaughter-age pigs in three regions of Vietnam. The information obtained about the PPV infection rate within Vietnamese swine herds contributes substantially to the general disease knowledge base and will be useful for future disease management and investigations of other pathogens in this region. However, the limitations of this study include sample type (only lung and serum were investigated), disease status of investigated pigs (only healthy pigs at slaughter were tested), and age (only 4- to 6-month-old pigs were tested). The results allow a preliminary insight into the prevalence of the major PPVs in healthy pigs in Vietnam. In addition, genomic characterization of PPV4 is also provided. Additional epidemiological studies that also include PPV5 and PPV6 and whole genome sequencing for the other PPVs are necessary to gain an overview of the prevalence of parvoviruses in the Vietnamese pig population. While the role of PPV1 in reproductive failure in breeding herds is well recognized, the association of the other PPVs with disease still needs to be established. This will ultimately aid in achieving an improved understanding and control of porcine pathogen transmission.

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Author contributions NTDT, responsible for conception this study, laboratory work, and drafting the manuscript). NTT, sample collection from central provinces and assisting with genotyping experiments. TQD, sample collection from northern provinces and DNA

extraction. DVAK, sample collection from southern provinces. TO, manuscript review; DTNT, assisting with sequencing. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no competing interests.

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Prevalence and structural protein encoding gene sequence (VP) of porcine parvovirus 2 (PPV2) in slaughtered pigs in Central provinces of Vietnam

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Abstract. Porcine parvovirus (PPV) is a DNA virus and causative agent of several reproductive problems in sows. This study was conducted for the prevalence and analyzing the DNA sequence of structural protein encoding gene (VP) of PPV2 genotype in pig. A total of 146 samples (lung and blood samples) was collected from slaughtered pigs of seven provinces in central of Vietnam during 2018-2019. The overall prevalence of PPV2 was 56.2% (82/146). PPV2 positive rate in each province ranged from 37.5% for Quang Nam to 100% for Quang Binh, with the exception of Da Nang, where no PPV2 positive samples were detected. Nearly complete PPV2-VP gene sequences of three strains were identified with the length of 2,493 nucleotides and deposited in GenBank with accession number of OL913365-OL913367. Four nucleotide substitutions were detected in Vietnamese PPV2 isolates and were not observed in PPV2 reference strains. Multiple alignment and comparison of nucleotide and deducted amino acid sequences showed the high similarity within Vietnamese PPV2 strains (95.6-96.5% and 94.7-96.9%, respectively). The PPV2 strains from this study clustered together with the "primitive" PPV2 strains from Myanmar, and strains from China in a main clade in the phylogenetic tree (Cluster A). This is the first report on the prevalence of PPV2 genotype and its VP gene sequence in pigs in Vietnam. This also provides the value information of the molecular evolution of locally circulating PPV2 and contributes to the control of PPV-induced SMEDI syndrome in sows, especially in Central provinces of Vietnam.

Keywords: Porcine parvovirus 2, prevalence, pigs, VP gene nucleotide sequence, phylogenetic tree

1 Introduction

To date, the clinical significance of porcine parvovirus (PPV) has been well-described with the predominant effect being impaired fertility in sows [1]. PPV are small, non-enveloped, isometric, 18-26 nm in diameter viruses, with a genome structure that resembles a linear, non-segmented molecule of ssDNA which is approximately 4-6.3 kb in size [2] that causes porcine reproductive failure in swine [3]. PPV genome is characterized by a hairpin structure at two 5′-3′ ends [4, 5] and two open reading frames (ORF) coding for non-structural protein (NSP) and viral coat and capsid protein (VP). PPV is stable to environmental factors, living in a pH range of 3.0-8.0 and is heat resistant for hours at 80°C [6].

PPV is a member of the family Parvoviridae [7]. *Ungulate protoparvovirus 1*, or Porcine parvovirus 1 (PPV1- known as “classical” PPV) was first isolated in 1965 in Germany [8] and is

the causative agent of reproductive dysfunction in sows, characterized by stillbirth, mummified fetuses, embryonic death, and infertility (SMEDI) [3]. In the following years, other genotypes of PPV were also confirmed, including: Porcine parvovirus 2 (PPV2) (formally name is *Ungulate tetraparvovirus 3),* belongs to the *Tetraparvovirus* genus, was discovered in 2001 during a survey for Hepatitis E virus (HEV) in swine sera collected in Myanmar [9]; PPV3 (*Ungulate tetraparvovirus 2*) was discovered in Hong Kong in 2008; PPV4 (*Ungulate copiparvovirus 2*) and PPV5 (unclassifed) were identified in the USA in 2010 and 2013, respectively; PPV6 (*Ungulate copiparvovirus 4*) was discovered in China in 2014 and most recently, PPV7 (proposed genus *Chappaparvovirus*) was identified in the USA in 2016 [10, 11, 12, 13, 14, 15, 16].

Analysis of the genetic relationship between PPV1 and PPV2 revealed that PPV2 is "distantly related" to PPV1 with 32.2-34.5% genomic identity and amino acid identity 20.2-21.5 % for ORF1 and 16.5-16.9% for ORF2 [17]. The efforts to propagate the PPV2 *in vitro* have so far been unsuccessful, so studies on the pathogenicity of PPV2 have not been possible [18]. However, there is recent evidence shows that PPV2 may be a co-factor or triggering agent associated with PCVAD [17] or 'high fever disease' [19].

Further epidemiological studies of PPV2 have reported differently worldwide [20]. About a decade after it was first detected, PPV2 was identified in 8.8% of serum samples obtained from commercial pig farms in Southeastern China [19] Subsequently, PPV2 has been discovered in other samples including hearts, blood, faeces and lungs in many other countries including Hungary, USA, Germany, Japan, and Thailand with prevalence of 6.4%, 20.7%, 55%, 58% and 83% respectively [18, 21, 22, 23, 24]. Meanwhile, using tonsil samples in PPV2 screening has reported a higher prevalence, ranging from 78% (Germany) to 100% (Japan), leading to a hypothesis that viral prevalence may depend on the organ type examined, although the routes of infection and viral loci are yet to be elucidated experimentally [22, 23, 24].

In Vietnam, SMEDI syndrome in sows caused by PPV is nationwide and has been of concern since the early 1990s. Previous studies have investigated the prevalence of PPVs using serological testing and PCR methods. Objective of this study is to analyze the genotypic prevalence of PPVs and molecular characterization of VP gene of PPV2 from slaughtered pigs in central Vietnam.

2 Materials and methods

2.1 Sample collection

Lung tissue and blood samples of grow-finish healthy pigs (18-25 weeks) were sampled at abattoirs located in seven provinces in Central of Vietnam. In each participating abattoir, a maximum of five samples were collected, and the number of abattoirs visited was 3-6 for each province. Only one blood or lung tissue sample was collected from each individual pig to avoid duplication. In total, 136 lung and 29 blood samples were collected. Detailed information on the collected sample, including number, sample type, collection year, and collection location is presented in Table 1. After collection, the samples were stored on ice (4°C) and immediately shipped to the laboratory and all samples were frozen at -20°C until testing.

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Table 1. Sample information collected in this study

Province	Number of abattoirs visited[1]	Number of samples collected[2]	Sample type		Collection year
			Lung	Blood	
Quang Binh	4	17	0	17	2019
Quang Tri	5	22	22	0	2019
Thua Thien Hue	6	28	28	0	2019
Da Nang	3	14	8	6	2018
Quang Nam	4	16	10	6	2018-2019
Quang Ngai	5	24	24	0	2018-2019
Binh Dinh	5	25	25	0	2018
Total	32	146	117	29	

[1]The number of samples collected in each abattoir was less than five.

[2]From each pig, only a single sample was collected (either lung or blood).

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2.2 DNA extraction and PCR analysis

Total DNA was extracted and purified according to the methods of Sambrook and Russell [25] with small modifications: small modifications: blood samples were washed several times with PBS buffer until they become white (try to remove all the hemoglobin) before using for DNA extraction. DNA was diluted in TE buffer, and stored at 4°C for analysis.

To detect PPV2, specific PCR assays were carried out in a single reaction with 50-100 ng of DNA template 2× PCR master mix (Thermo Scientifc, Lithuania), and 5 pmol of each primer. The PPV2 primers for detection were followed Streck et al. [22]. Primers for the PPV2 sequencing (Table 2) were designed using the program Primer3 based on reference sequences listed below (Table 3). Information on primer sequence, annealing temperature (Ta) and product size are shown in Table 2. The PCR consisted of an initial activation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec., annealing at 56/60/50/54 °C for 30 sec., extension at 72°C for 30 sec., final synthesis at 72°C for 5 minutes and holding at 14°C.

The resulting PCR products were analyzed by electrophoresis on a 2% agarose gel using a 100 bp DNA ladder (Thermo Fisher Scientific). Appropriate positive and negative extraction and PCR controls (obtained from the University of Edinburgh and confirmed by sequencing) were used for each extraction and PCR run.

2.3 PPV2-VP2 gene sequencing and sequence analysis

Five primer pairs designed based on PPV2 gene sequence published on GenBank were used to amplify structural protein encoding genes (VP genes) of three PPV2 positive strains (Table 2).

The PCR products were sent to Macrogen Inc., South Korea for sequencing using an ABI-3100 Avant Genetic Analyzer automated sequencer according to the standard Sanger method [26].

Multinucleotide and amino acid sequence alignments were performed in BioEdit 7.0.9.0 [27]. The published PPV2 sequence information used as a reference is presented in Table 3. Obtained nucleotide sequences were identified with the

Basic Local Alignment Search Tool (BLAST, NCBI) [28].

The phylogenetic tree was constructed with Mega X solfware using the neighbor-joining (NJ) method [29] and computed with the Kimura 2-parameter method [30]. Bootstrap values were calculated using 1.000 replicates of the alignment.

Table 2. Primer information used in this study for genotyping and sequencing. The virus target was *U. tetraparvovirus 3* (PPV2)

Purpose	Target	Primer sequence (5′-3′)	Ta (°C)	PCR size (bp)	Reference
Genotyping	PPV2	F: AGATTCTTGCAGGCCGTAGA R: CCAAGGGTCAGCACCTTTTA	60	222	Streck et al., 2013
Sequencing	PPV2-1	F: CAGCTTCATGGCTTACGGGCA R: ATCCCTCTCCGCCCGCCAGA	50	721	This study
	PPV2-2	F: CATGAGCGCTGCCGACGCGT R: TCCATCAGACCCTGGGCCA	54	730	
	PPV2-3	F: GACCCAGAGATAGATAGCGT R: CCACCAATGAGATCCGCTA	53	773	
	PPV2-4	F: TGGACAAAGTCTTACCGCC R: AGGGTTGTACCACTGAATACT	50	743	
	PPV2-5	F: CATTGGCGCGCAGGCACC R: ATAAACCCCTGAGGAAATA	50	776	

Table 3. List of PPV2 strains used in this study

Strain	Location	Year	Size (bp)	Reference
AB076669	Myamar	2001	5118	Hijikata et al., 2001
KP245940	China	2014	3099	Sun et al., 2015
KP245943		2014	3099	
KP245944		2014	3099	
MK092387		2018	5205	Ren et al., 2020
MK092408		2018	3172	
MN326142		2019	5119	Sun et al., 2021
MN326185		2019	5119	
MZ577029		2021	5426	Li et al., 2021
MG345016		2017	5427	Qin et al., 2018
OL913365	Vietnam	2019	2493	This study
OL913366		2019	2493	This study
OL913367		2019	2493	This study
KY586144	Brazil	2017	5316	Cerva et al., 2017
KX517759	Hungary	2016	5533	Novosel et al., 2017
KC701296		2013	3099	Cadar et al., 2013
KC687100	Cromania	2013	3099	
JQ860238		2012	3096	

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Strain	Location	Year	Size (bp)	Reference
JQ860240	Romania	2012	3096	
JQ860243		2012	3099	
JQ860248		2012	3099	
JX101461	US	2012	5486	Xiao et al., 2013
KF725662		2013	3259	Opriessnig et al., 2014

3 Results and discussion

3.1 Detection PPV2 genotype

The quality and quantity of total extracted DNA were sufficient for analysis with the ratio of OD 260/280 (1.84 to 2.16) and DNA concentration (92 to 446 ng/μl). The PCR products for genotyping of PPV2 are shown in Fig 1. In Fig 1, single clear band was observed in wells 2 and 4-11, corresponding to the size of positive control (+). That means those samples were positive of PPV2 genotype, but this is neccesary to confirm by sequencing. No DNA band appeared at negative control (-) and negative samples (well 1 and 3).

The expected bands represented for PPV2 genotypes were purified and sequenced. Obtained nucleotide sequences of 222 bp in length were identified as PPV2 by BioEdit software, respectively. As shown in Fig 2 the BLAST analyses also revealed the high nucleotide identities (98%) between PPV2 sequence and reference PPV2 strain (MG345019).

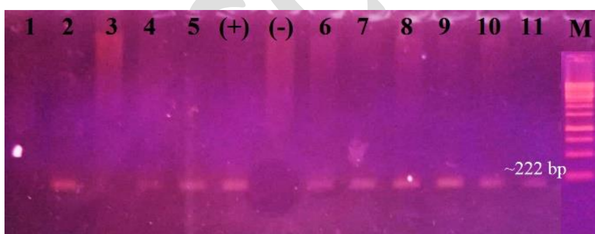

Fig. 1. Agarose gel electrophoresis of PPV2 genotyping PCR product (~222 bp)

M: 1 kb DNA ladder (Thermo Fisher Scientific); *1, 3: negative samples; 2, 4-11: positive samples; (-): negative control; (+): positive control (~222 bp)*

Fig. 2. BLAST result revealed the positive sample with 98% nucleotide identity compared with reference PPV2 strain (MG345019).

In the present study, among the 146 lung and blood samples from 32 different abattoirs in seven provinces of Central Vietnam, PPV2 DNA was detected with 56.2% (82/146). PPV2 prevalence rates for each province ranged from 37.5% for Quang Nam to 100% for Quang Binh, with the exception of Da Nang, where no PPV2-positive samples were detected (Table 4).

Table 4. Number of DNA-positive samples (percentage) in this study

Province	n	PPV2 (%)
Quang Binh	17	17 (100)
Quang Tri	22	10 (45.5)
Thua Thien Hue	28	20 (71.4)
Da Nang	14	0
Quang Nam	16	6 (37.5)
Quang Ngai	24	18 (75)
Binh Dinh	25	11 (44)
Total	146	82 (56.2)

Of the 117 lung and 29 blood samples, the positive PPV2 was detected at 50.4% (59/117) and 79.3% (23/29), respectively (data not shown). However, this result did not reflect the effect of sample types because only a single sample was

collected from each pig (either lung tissue or blood).

Recently, studies using diagnostic methods based on target amplification PCR have detected PPV in commercial pigs in many countries around the world. With a PPV2 prevalence of 56.2%, our study results were in a similar range to the PPV2 prevalence in China (45.1%), Hungary (51.0%) and Japan (58.0%) [31, 32, 23]. A lower prevalence of PPV2 infection was reported in North America (36.8%), Romania (25%), South Africa (21.8%) and Poland (19%) [15, 33, 1, 34]. In contrast, high prevalence of PPV2 in pig herds was observed in Germany (78.0%) and Thailand (82%) [22, 24].

In Vietnam, PPV-induced fertility decline syndrome has been of concern since the early 90s of the last century and has caused considerable damage to the pig industry; Therefore, the detection of PPVs genotypes in pigs, including PPV2, is not an exception. In a previous survey of sows in Long An province by serological testing, Nguyen and Tran [35] reported that the positive rate for PPV was quite high at 69%. In addition, analysis of 52 stillbirth samples detected PPV virus and PPV antibodies simultaneously. Recently, surveys in some localities have initially confirmed the prevalence of PPV2 genotype in pig farms. In a study on pig lung samples collected from abattoirs in four northern provinces of Vietnam, Cuong [36] reported a PPV2 infection rate of 17.6%. Most recently, Thuy et al. [37] found PPV2 prevalence up to 28% in finishing pig of 13 provinces in three regions of Vietnam, including North, Central and South Vietnam.

Taken together, the above data have shown that our results on PPV2 prevalence are in the average range compared with other previous studies. It could be explained by the type of sample collected (mainly lung sample) and status of investigated animal (slaughtered pig) in our study. This result supports the hypothesis of Saekhow and Ikeda [24]: viral prevalence may depend on the type of sample collected and screened, although the routes of infection and the virus tissue tropism are yet to be elucidated.

3.2 Analysis of PPV2-VP gene

The VP gene sequences encoding structural protein of three PPV2 strains collected from Quang Binh, Hue and Quang Ngai were identified and analyzed. The length of partial of VP gene of three PPV2 strains isolates in this study is 2,493 nucleotides, including VP1 and VP2 sequences, without insertions and deletions in the coding regions. The sequences were deposited in the GenBank database with the accession numbers OL913365-OL913367.

Comparing many reference strains found that there are four base substitutions (444: T→A, 734: C→T, 820: A→C, 1974: T→A) in PPV2 QN03-VN strain, completely different compared with the reference strains, two of them (734: C→T, 820: A→C) resulted in amino acid changes in the VP gene sequence (245: S→F, 274: K→Q). No variation was detected in the deduced amino acid sequence of the two strains PPV2 HU10-VN and PPV2 QB05-VN. For the PPV1 genotype, nucleotide changes leading to amino acid changes at several potential sites on the VP2 gene and responsible for antigenicity were observed in previous study [4, 38, 39, 40, 41]. The studies on molecular genetic variability of PPV2 is still limited and therefore, the analysis of molecular changes related to antigenicity, viruslence, immune response, neutralization activity and pathogenesis is necessary.

Comparison results of the VP nucleotide and amino acid sequence (2,493 nucleotides and 832 amino acid) between the PPV2 strains isolated in Vietnam in this study and strains isolated in Myamar, China and Europe are presented in Table 5.

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Table 5. Nucleotide and amino acid sequence identity (%) within Vietnamese *U. tetraparvovirus 3* (PPV2) strains and compared with reference strains

Strain		Molecular size[1]	
		Nucleotide (2,493 nt)	**Amino acid (832 aa)**
Vietnam		95.6-96.8	94.7-96.9
Myanma		96.1-97.9	95.4-98.1
China	Cluster A	95.2-99.5	94.5-99.0
	Cluster B	93.5-94.6	93.2-95.4
Europe		94.9-100	93.3-100

[1]nt, nucleotide; aa, amino acid

Comparison between three strains of PPV2 isolated from pigs raised in central Vietnam showed that the nucleotide similarity between them was quite high, at 95.6-96.5%. Compared with the "original" PPV2 strain first identified from Myanmar in 2001, the PPV2 strains isolated in Vietnam showed nucleotide and amino acid similarity of 96.1-97.9% and 95.4-98.1%, respectively. A high level of sequence identity at the nucleotide and amino acid levels was observed between the Vietnamese PPV2 strains and the European strains (94.9-100% and 93.3-100%, respectively). The data presented in Table 5 also show that: the nucleotide and amino acid sequences of the PPV2 strains in Vietnam have a high degree of similarity with the Chinese strains isolated from 2018-2021 and less with the Chinese strains isolated in 2014.

To analyze the genetic relationship between the PPV2 sequence obtained in this study and the reference strains from other geographical locations, a phylogenetic tree was constructed based on the sequence of the partial of PPV2-VP gene. As seen in Fig 3, the PPV2 phylogenetic tree splits into two main branches (Cluster A and B). PPV2 strains isolated in Vietnam (black dots), "primitive" PPV2 strains discovered for the first time in Myanmar, strains isolated in China during 2018-2021 together with isolates in Europe gathered together to form Cluster A. Cluster B includes PPV2 strains isolated in China in 2014 and PPV2 strains originating from the USA. The bootstrap value for all branches reached 99%, indicating the reliability of the phylogenetic tree. These results are consistent with a previous phylogenetic analysis based on PPV2 genome sequencing [22, 31] and showed the genetic relationship between PPV2 strains circulating in Vietnam and reference strains.

4 Conclusions

This is the first study on the circulation of PPV2 in commercial pigs in seven Central provinces of Vietnam and the characterization of their VP gene sequence. The partial VP gene sequences of three PPV2 strains (2,493 nucleotides) deposited in the GenBank database showed four nucleotide substitution mutations that were completely different from the reference strains. The PPV2 strains from this study clustered with the "primitive" PPV2 strains from Myanmar, and strains from China to form a large clade in the phylogenetic tree (Cluster A). The obtained result provides the value information of the molecular evolution of locally circulating PPV2 and contributes to the control of PPV-induced SMEDI syndrome in sows, especially in Central provinces of Vietnam.

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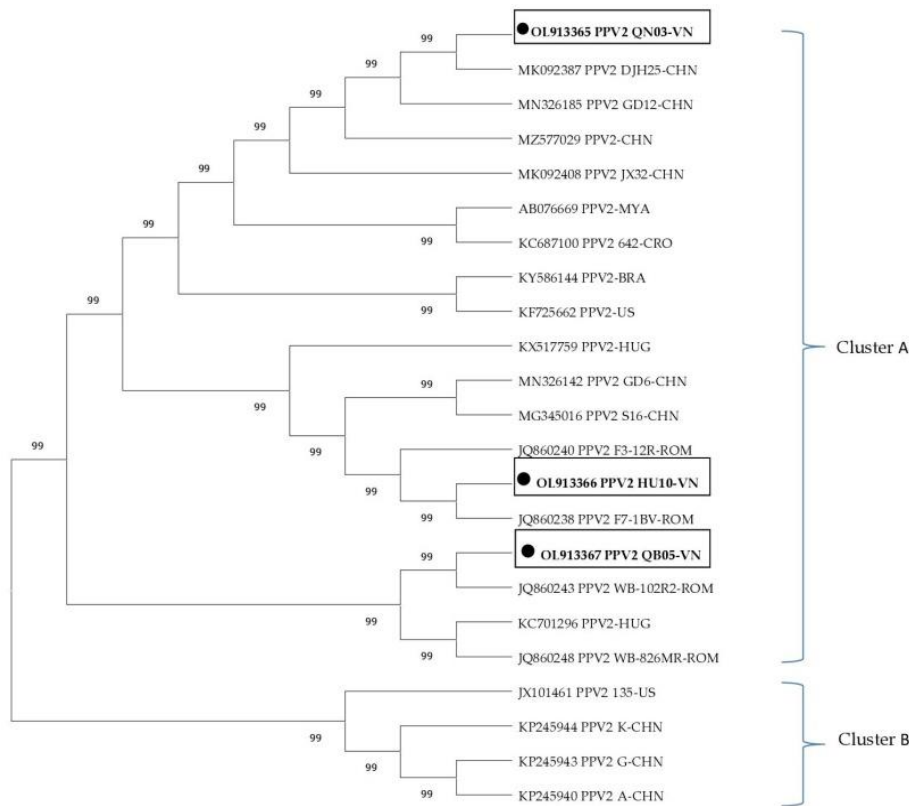

Fig. 3. Phylogenetic tree based on VP nucleotide sequence of PPV2 (2,493 nucleotides) obtained from three provinces of Vietnam (in this study) and reference sequences from GenBank database. The black square box indicates the Vietnamese PPV2 sequence. The number indicated the bootstrap value 1.000 replicates.

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PHÂN TÍCH TRÌNH TỰ VÙNG GEN NS1/VP1 CỦA VIRUS PARVO (PPV3) TRÊN LỢN NUÔI TẠI QUẢNG TRỊ VÀ QUẢNG NGÃI

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Ngày bài báo được chấp nhận đăng: 30/8/2023

TÓM TẮT

Virus Parvo trên lợn (*Porcine Parvovirus* - PPV) là một trong những tác nhân chính gây nên hội chứng suy giảm khả năng sinh sản ở lợn nái. Nghiên cứu này tiến hành phân tích trình tự nucleotide và các biến đổi di truyền vùng gen thuộc gen mã hóa protein không cấu trúc (*Nonstructural protein* - NS1) và gen mã hoá protein vỏ capsid (*Viral coat and capsid protein*-VP) của PPV3 trên lợn nuôi thu tại 2 tỉnh Quảng Ngãi và Quảng Trị. Trình tự nucleotide có kích thước phân tử 995bp (thuộc gen *NS1* và *VP1*) đã được xác định từ 2 chủng PPV3-QN16 và PPV3-QT4. Trình tự gen NS1/VP1 (995bp) của 2 chủng PPV3 bộc lộ 6 vị trí thay thế, tương ứng với mức tương đồng nucleotide là 99,1%. 4/6 biến đổi nucleotide này là hoàn toàn khác biệt với các chủng PPV3 tham chiếu. Cây phát sinh chủng loại xây dựng dựa trên trình tự đoạn gen *VP*1 (305bp) phân thành 2 nhánh với giá trị bootstrap tin cậy. Trong đó, hai chủng PPV3 phân lập từ miền Trung Việt Nam thuộc cùng một nhánh với các chủng PPV3 có nguồn gốc từ Trung Quốc (2017-2019) và Mỹ. Nhánh thứ hai bao gồm các chủng PPV3 có nguồn gốc từ Trung Quốc, Hàn Quốc, châu Âu và châu Mỹ. Nghiên cứu này cung cấp thông tin về biến đổi di truyền trong trình tự nucleotide của PPV3 lưu hành trên đàn lợn thương phẩm nuôi tại Miền Trung.

Từ khoá: *Trình tự nucleotide, Virus Parvo 3, lợn, cây phát sinh chủng loại, Quảng Ngãi, Quảng Trị.*

ABSTRACT

The nucleotide sequence of NS1/VP1 gene of Porcine Parvovirus 3 (PPV3) in finishing pigs raised in Quang Ngai and Quang Tri provinces

Porcine Parvovirus (PPV) is one of the main causes of reproductive failure syndrome in sows. In this study, the nucleotide sequence and genetic variation of nonstructural and capside protein encoding region (NS1/VP1) of the PPV3 genotype in pigs raised in the two provinces of Quang Ngai and Quang Tri were analysed. The nucleotide sequence of NS1/VP1 with a molecular size of 995bp, was determined from two strains PPV3-QN16 and PPV3-QT4. Six nucleotide substitutions were detected in two analyzed PPV3 strains, which is correspondent to 99,1% of nucleotide identity. Out of six nucleotide subtitutions, four were completely different from PPV3 reference strains. The phylogenetic tree based on the VP1 gene sequence (305bp) showed two identical clades with reliable bootstrap values. In which, the two Vietnamese PPV3 strains grouped in a clade together with PPV3 strains originating from China (2017-2019) and the US. The second clade was formed by the PPV3 strains from China, Korea, Europe and America. The results of this study provide information about the genetic variation of PPV3 strains circulating in finishing pigs raised in the central provinces of Vietnam.

Keywords: *Nucleotide sequence, Porcine Parvovirus 3, pigs, phylogenetic tree, Quang Ngai, Quang Tri.*

1. ĐẶT VẤN ĐỀ

Ở các nước có ngành chăn nuôi lợn phát triển, đàn lợn nái thường gặp các vấn đề liên quan đến suy giảm khả năng sinh sản. Porcine Parvovirus (PPV) được xem là nguyên nhân gây nên hội chứng suy giảm khả năng sinh sản ở lợn nái, biểu hiện ở việc lợn nái nhiễm PPV sinh con bị chết non, ướp xác, chết phôi và vô sinh (Stillbirth, Mummification, Embryonic Death and Infertility-SMEDI). PPV là virus không vỏ, hình dạng tròn, có kích thước nhỏ, đường kính virion khoảng 18-26nm, cấu

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*Tác giả liên hệ: TS. Nguyễn Thị Diệu Thúy, Viện Công nghệ sinh học, Viện Hàn lâm Khoa học và Công nghệ Việt Nam. Điện thoại: 0916541273; Email: ntdthuy@ibt.ac.vn

trúc đối xứng 20 mặt (Bern và Hauswirth, 1983). Vật liệu di truyền của PPV là DNA sợi đơn, kích thước phân tử dao động từ 4-6,3kb (Molitor và ctv, 1983). Hệ gen PPV đặc trưng bởi cấu trúc kẹp tóc ở hai đầu 5′-3′, hai khung đọc mở (ORF) mã hoá cho các protein không cấu trúc (nonstructural protein) và vỏ capsid của chúng (capside protein) (Simpson và ctv, 2002). Hiện tại, theo phân loại của Ủy ban Quốc tế về phân loại virus (International Committee on Taxonomy of Viruses-ICTV), 7 kiểu gen PPV đã được xác nhận: PPV1 (*U. protoparvovirus* 1); PPV2 (*U. tetraparvovirus* 3); PPV3 (*U. tetraparvovirus* 2); PPV4 (*U. copiparvovirus* 2); PPV5 (chưa có tên loài); PPV6 (*U. copiparvovirus* 4) và PPV7 (*U. chaphamaparvovirus*) (Walker và ctv, 2020). Gần đây, kiểu gen PPV8 được phát hiện bởi Guo và ctv (2022) trên đàn lợn nuôi tại Trung Quốc và chưa được chính thức phân loại.

PPV3 lần đầu tiên được phân lập tại Hồng Kông với tên gọi là PhoV (Porcine Hokovirus) năm 2008. Trình tự hệ gen của PhoV thể hiện mức độ tương đồng cao với Parvovirus 4 ở người và Hokovirus ở bò, tạo thành nhánh riêng trong họ Parvovirus (Lau và ctv, 2008). PPV3 được phát hiện với tỷ lệ 9,7% ở lợn nuôi ở Hungari (Csagola và ctv, 2012), tỷ lệ dương tính PPV3 trên lợn rừng ở Đức (Adlhoch và ctv, 2010), Romania (Cadar và ctv, 2011) và miền Nam nước Ý (Amoroso và ctv, 2019) cao hơn lần lượt là 32,7 và 35%. Trên đối tượng lợn nuôi tại châu Á, PPV3 được phát hiện tại Trung Quốc (Sun và ctv, 2015), Thái Lan (Saekhow và Ikeda, 2015) và Nhật Bản (Saekhow và ctv, 2015) với tỷ lệ cao hơn hẳn là 45,1; 73 và 39%. Gần đây, PPV3 cũng đã được phát hiện tại Nam Phi và Congo với tỷ lệ lần lượt là 5,5 và 17,5% (Afolabi và ctv, 2019 ; Bisimwa và ctv, 2021). Thông tin về khả năng gây bệnh trên lợn của PPV3 cho đến nay vẫn còn hạn chế, tuy nhiên, đã có minh chứng cho thấy PPV3 làm suy giảm khả năng miễn dịch ở lợn (Amoroso và ctv, 2019), đóng vai trò là yếu tố kích hoạt liên quan đến bệnh do PCV2 trên lợn (Patrick và ctv, 2014). Đồng thời, gần đây PPV3 được xác nhận có liên quan đáng kể đến hiện tượng viêm phúc mạc, viêm loét dạ dày, đại tràng và manh tràng, bệnh viêm da và hội chứng bệnh thận ở lợn (Porcine dermatitis and nephropathy syndrome) (Tregakis và ctv, 2020).

Ở Việt Nam, các nghiên cứu gần đây đã xác nhận sự lưu hành của PPV1-4 trên đàn lợn ở một số tỉnh thành trong phạm vi cả nước (Nguyen Tran Trung và ctv, 2019 ; Thuy và ctv, 2021). Trong đó, kiểu gen PPV3 đã được phát hiện trên đàn lợn nuôi ở một số tỉnh miền Bắc, miền Nam với tỷ lệ lần lượt là 43,2; 6,9% (Thuy và ctv, 2021). Nghiên cứu của Giáp và ctv (2022) đã khảo sát tỷ lệ lợn dương tính với PPV3 là 46,5% (năm 2017) và 27,3% (2021). Trình tự hệ gen PPV4, đặc điểm phân tử gen mã hoá của PPV2 phân lập trên lợn nuôi tại một số địa phương đã được phân tích (Thuy và ctv, 2021; Nguyen Tran Trung và ctv, 2023). Trong nghiên cứu này, vùng gen NS1/VP1 (kích thước phân tử 995bp) của chủng PPV3 phân lập tại 2 tỉnh miền Trung (Quảng Trị và Quảng Ngãi) đã được giải trình tự và phân tích biến đổi di truyền.

2. VẬT LIỆU VÀ PHƯƠNG PHÁP NGHIÊN CỨU

Mẫu phổi dương tính với PPV3 thu từ lợn thương phẩm nuôi tại Quảng Ngãi và Quảng Trị lần lượt vào năm 2018 và 2019 (Nguyen Tran Trung và ctv, 2019). Mẫu được giữ ở 4°C và chuyển về phòng thí nghiệm Viện Công nghệ sinh học, Viện Hàn lâm Khoa học và Công nghệ Việt Nam bảo quản ở -20°C cho đến khi sử dụng để tách chiết DNA tổng số.

DNA tổng số được tách chiết theo phương pháp của Sambrook và Russell (2001): sử dụng proteinase K; phenol/phloroform. DNA tổng số được pha loãng trong đệm TE và bảo quản ở 4°C trước khi tiến hành thí nghiệm.

Để khuếch đại vùng gen NS1/VP1 của PPV3, phản ứng PCR được thực hiện với 50-100ng DNA tổng số, hỗn hợp PCR master mix 2X (Thermo Scientifc, Lithuania), 5pmol mỗi mồi xuôi (F: TTGGAGGTACCGGCAGA) và mồi ngược (R: TCATCGTACCGTTCATCG với kích thước sản phẩm khuếch đại theo tính toán lý thuyết là 995bp. Chu trình PCR được

thực hiện với 3 bước: (i) Biến tính ban đầu ở 94°C trong 5 phút; (ii) 35 chu kỳ bao gồm: biến tính ở 94°C trong 30 giây, gắn mồi ở 58/53°C trong 30 giây và kéo dài mạch ở 72°C trong 30 giây; (iii) Kết thúc tổng hợp ở 72°C trong 5 phút và giữ mẫu ở 14°C. Sản phẩm PCR được kiểm tra bằng điện di trên gel agarose 2% sử dụng Marker DNA 100bp (Thermo Fisher Scientific). Sản phẩm PCR được sử dụng để giải trình tự gen trực tiếp 2 chiều theo nguyên lý Sanger bằng thiết bị giải trình tự tự động ABI-3100 Avant Genetic Analyzer (Macrogen, Hàn Quốc).

Các trình tự gen thu được được xử lý bằng phần mềm BioEdit v.7.0.9.0 (Hall, 1999) và tìm kiếm trình tự tương đồng bằng công cụ BLAST trên cơ sở dữ liệu NCBI (Altschul và ctv, 1990). Danh sách các trình tự gen PPV3 tham chiếu được trình bày ở Bảng 1.

Bảng 1. Trình tự nucleotide các chủng PPV3

Mã số GenBank	Quốc gia	Phân lập năm	Tác giả
KJ842609	Trung Quốc	2014	He và ctv
MK378232		2019	He và Tu
MK378244			
MK378240			
KX827776		2017	Sun và ctv
MG345026		2018	Qin và ctv
JQ177085		2013	Pan và ctv
MW355754	Congo	2021	Bisimwa và ctv
EU200671	Hồng Kông	2008	Lau và ctv
OP377042	Hàn Quốc	2023	Park và ctv
OP377043			
KC296751	Đức	2013	Streck và ctv
KC701326	Hungary	2013	Cadar và ctv
KP768509	Slovakia	2015	Sliz và ctv
JQ700072	Brazil	2016	Souza và ctv
KT726332	Bồ Đào Nha	2016	Miranda và ctv
JF738351	Romania	2011	Cadar và ctv
JF738350			
KF561854	Mỹ	2014	Opriessnig và ctv
QN16	Việt Nam	2018	Trung và ctv
QT4		2019	

Cây phát sinh chủng loại được xây dựng bằng phần mềm MEGA X (Tamura, 2013) với các tham số đầu vào bao gồm: Phương pháp suy diễn cây phát sinh chủng loại Neighbor Joining; mô phỏng sự thay đổi nucleotide giữa các trình tự gen dựa vào mô hình Tajima-Nei; mức tin cậy ở các nhánh của cây phát sinh chủng loại được ước tính bằng phép thử bootstrap lặp lại 1.000 lần.

3. KẾT QUẢ VÀ THẢO LUẬN

PPVs được xem là một trong những tác nhân chính gây ra hội chứng suy giảm khả năng sinh sản ở lợn nái và hội chứng này đã gây thiệt hại đáng kể cho ngành chăn nuôi lợn; do đó, việc phát hiện các kiểu gen PPVs ở lợn sử dụng phương pháp PCR khuếch đại gen mục tiêu đã được ứng dụng trong kiểm soát PPVs trên lợn thương phẩm ở nhiều nước trên thế giới.

Nhóm nghiên cứu của chúng tôi đã phát hiện PPV3 lưu hành trên đàn lợn nuôi tại 7 tỉnh khu vực miền Trung với tỷ lệ 5,5% (8/146). Cụ thể, PPV3 đã được phát hiện 3/7 tỉnh thành gồm Quảng Trị, Thừa Thiên Huế và Quảng Ngãi với tỷ lệ lần lượt là 13,6; 10,7 và 8,3% (Nguyen Tran Trung và ctv, 2019). Gần đây, Thuy và ctv (2021) đã phát hiện PPV3 với tỷ lệ nhiễm 17,7% trên lợn trong độ tuổi xuất chuồng tại 13 tỉnh thuộc 3 miền của Việt Nam. Với tỷ lệ phát hiện PPV3 ở bảy tỉnh miền Trung là 5,5%, kết quả nghiên cứu của chúng tôi tương đương với kết quả phát hiện PPV3 trên đàn lợn tại Anh và một số nước châu Âu (5,8%) cũng như tại Nam Phi (5,5%) (Tregakis và ctv, 2020; Afolabi và ctv, 2019). Tỷ lệ phát hiện PPV3 cao hơn đã được báo cáo ở Trung Quốc (45,11%), Thái Lan (73%), Nhật Bản (39%), Đức (20%), Slovakia (11%), Bắc Mỹ (19,2%) và Congo (17,5%) (Streck và ctv, 2013; Opriessnig và ctv 2014; Sliz và ctv, 2015; Sun và ctv, 2015; Saekhow và Ikeda, 2015; Saekhow và ctv, 2016; Bisimwa và ctv 2021).

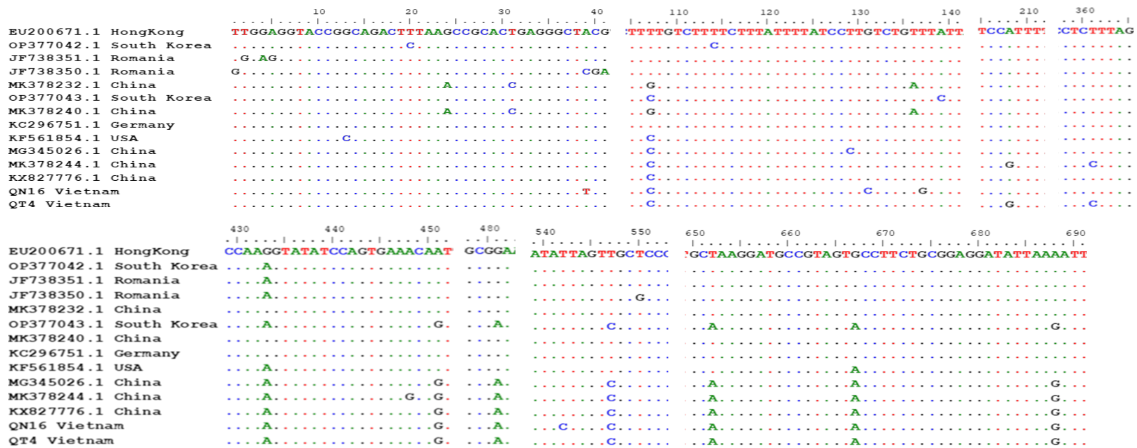
Sự khác biệt về tỷ lệ phát hiện PPV3 trên đàn lợn tại các địa phương khác nhau có thể được giải thích bằng việc thu thập những loại mẫu khác nhau, lứa tuổi, tình trạng lợn, cũng như điều kiện chăn nuôi lợn. Theo Saekhow và Ikeda (2015), tỷ lệ lưu hành của PPVs có thể phụ thuộc vào loại mẫu được thu thập và sàng

lọc. Đồng thời, sự khác biệt về kết quả phát hiện PPV3 ở mỗi địa phương có thể được lý giải bởi sự khác biệt trong quy mô chăn nuôi cũng như hoạt động quản lý vệ sinh dịch tễ thú y ở từng trang trại/địa phương.

3.1. Phân tích trình tự vùng gen NS1/VP1 của PPV3

Trình tự vùng gen NS1/VP1 của hai chủng PPV3-QN16 và PPV3-QT4 lưu hành trên lợn ở Quảng Ngãi và Quảng Trị đã được xác định và phân tích. Kết quả giải trình tự thu được đoạn gen có kích thước phân tử 995bp; trong đó không xuất hiện các đột biến chèn/xoá (Hình 1).

Trong trình tự gen kích thước 995bp, 2 chủng PPV3 bộc lộ 6 vị trí nucleotide khác biệt, cụ thể 39: A→T, 131: G→C, 137: T→G, 208: A→G, 361: T→C và 542: T→C (Hình 1, Bảng 2). Trong số 6 vị trí biến đổi đó, 4 vị trí thay thế nucleotide khác biệt hoàn toàn so với các chủng tham chiếu, bao gồm các vị trí 39: A→T, 131: G→C, 137 T→G và 542: T→C (Bảng 2). Bên cạnh đó, so sánh các vị trí thay thế nucleotide tương tự với các chủng tham chiếu cũng cho thấy hai chủng PPV3 phân lập tại miền Trung một số thay thế nucleotide tương đồng với chủng PPV3 phân lập tại Trung Quốc (2017-2019), Hàn Quốc và Mỹ (107: T→C, 433: G→A, 667: G→A và 709: A→C) (Bảng 2).



Hình 1. Các vị trí khác biệt nucleotide trong vùng gen NS1/VP1 (995bp) của các chủng PPV3 trong nghiên cứu này và các chủng PPV3 tham chiếu

Bảng 2. Vị trí thay thế nucleotide trong vùng gen NS1/VP1 của 2 chủng PPV3-QN16, PPV3-QT4 và các chủng PPV3 tham chiếu

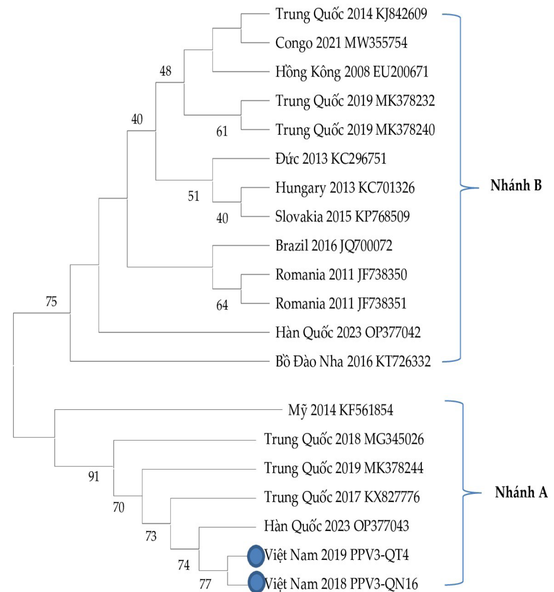
Chủng tham chiếu	Vị trí thay thế nucleotide															
	39	107	131	137	208	361	433	451	481	542	547	652	667	688	709	745
EU200671 HongKong	A	T	G	T	A	T	G	A	G	T	T	T	G	A	A	G
OP377042 South Korea	A	T	G	T	A	T	A	A	G	T	T	T	G	A	C	A
JF738351 Romania	A	T	G	T	A	T	A	A	G	T	T	T	G	A	C	A
JF738350 Romania	C	T	G	T	A	T	A	A	G	T	T	T	G	A	C	A
MK378232 China	A	G	G	T	A	T	G	A	G	T	T	T	G	A	A	A
OP377043 South Korea	A	C	G	T	A	T	A	G	A	T	C	A	A	G	C	A
MK378240 China	A	G	G	T	A	T	G	A	G	T	T	T	G	A	A	A
KC296751 Germany	A	T	G	T	A	T	G	A	G	T	T	T	G	A	A	A
KF561854 USA	A	C	G	T	A	T	A	A	G	T	T	T	A	A	C	A
MG345026 China	A	C	G	T	A	T	A	G	A	T	C	A	A	G	C	A
MK378244 China	A	C	G	T	G	C	A	G	A	T	C	A	A	G	C	A
KX827776 China	A	C	G	T	A	T	A	G	A	T	C	A	A	G	C	A
QN16 Vietnam	**T**	C	**C**	**G**	A	T	A	G	A	**C**	C	A	A	G	C	A
QT4 Vietnam	A	C	G	T	G	C	A	G	A	T	C	A	A	G	C	A

Kết quả so sánh trình tự nucleotide cho thấy 2 chủng PPV3 phân lập tại Quảng Trị và Quảng Ngãi có sự khác biệt ở 6 vị trí nucleotide, tỷ lệ tương đồng giữa hai chủng PPV3 phân lập ở Quảng Ngãi và Quảng Trị là 99,1%. Tỷ lệ tương đồng của hai chủng PPV3 trong nghiên cứu của chúng tôi với các chủng Trung Quốc được phân lập trong cùng thời điểm (2017-2019) dao động 99,2-99,5%, cao hơn tỷ lệ tương đồng nucleotide giữa 2 chủng PPV3 nghiên cứu (99,1%); và cao hơn hẳn khi so sánh với các chủng Trung Quốc được phân lập trong giai đoạn 2014-2019 (97,8-97,9%). So với chủng PPV3 có nguồn gốc từ Mỹ, các chủng PPV3 phân lập tại Quảng Ngãi và Quảng Trị có độ tương đồng nucleotide 98,3-98,4%. Tỷ lệ tương đồng nucleotide này thấp hơn, dao động trong khoảng 97-98,4% khi so sánh với các chủng PPV3 có nguồn gốc từ Châu Âu.

3.2. Cây phả hệ di truyền các chủng PPV3 trên lợn nuôi tại Quảng Ngãi và Quảng Trị

Nghiên cứu dịch tễ học phân tử dựa trên việc phân tích cây phát sinh chủng loại là một vấn đề quan trọng nhằm đánh giá sự biến đổi di truyền của các chủng virus ngoài thực địa, từ đó đánh giá sự biến chủng của virus theo thời gian. Cây phát sinh chủng loại của các chủng PPV3 phân lập trong nghiên cứu này và các chủng tham chiếu có nguồn gốc từ 3 trung tâm chăn nuôi lợn lớn trên thế giới là châu Âu, châu Mỹ và châu Á (Bảng 1) được xây dựng dựa trên trình tự vùng gen mã hoá protein cấu trúc *VP1* của PPV3 với kích thước phân tử 305bp (Hình 2).

Cây phát sinh chủng loại PPV3 dựa trên trình tự vùng gen mã hoá protein cấu trúc VP1 (305bp) phân các chủng PPV3 phân tích thành hai nhánh chính. Hai chủng PPV3 được phân lập vào năm 2018-2019 ở miền Trung Việt Nam tập hợp cùng với các chủng PPV3 được phân lập tại Trung Quốc trong giai đoạn 2017-2019 và chủng PPV3 có nguồn gốc từ Mỹ trong một phân nhánh. Nhánh thứ 2 bao gồm các chủng PPV3 có nguồn gốc từ Trung Quốc, Hàn Quốc, châu Âu, Congo và Brazil. Kết quả nghiên cứu này phù hợp với các phân tích phát sinh chủng loại dựa trên trình tự gen VP của PPV3 được thực hiện bởi các nghiên cứu trước đây (Sun và ctv, 2015; Bisimwa và ctv, 2021).



Hình 2. Cây phát sinh chủng loại được xây dựng dựa trên trình tự vùng gen *VP1* (305bp) của 2 chủng PPV3-QN16 và PPV3-QT4 (chấm tròn) và các chủng PPV3 tham chiếu bằng phần mềm MEGA X (Tamura, 2013).

Các chữ số trên các nhánh là giá trị bootstrap với 1.000 lần lặp lại.

4. KẾT LUẬN

Trình tự vùng gen NS1/VP1 (995bp) của hai chủng PPV3-QN16 và PPV3-QT4 trên lợn nuôi tại 2 tỉnh Quảng Ngãi và Quảng Trị đã được xác định và phân tích. Kết quả bộc lộ 6 vị trí nucleotide khác biệt giữa 2 chủng PPV3 nghiên cứu, trong đó, 4 vị trí thay thế nucleotide khác biệt so với các chủng PPV3 tham chiếu. Hai chủng PPV3 phân lập được xếp cùng nhóm di truyền với các chủng PPV3 có nguồn gốc từ Trung Quốc và Mỹ. Kết quả từ nghiên cứu này cung cấp thông tin về biến đổi di truyền trong trình tự vùng gen NS1/VP1 của PPV3 lưu hành trên lợn nuôi tại 2 tỉnh miền Trung, đóng góp vào cơ sở dữ liệu về dịch tễ phân tử PPVs ở Việt Nam.

LỜI CÁM ƠN

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