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

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. In addition, in pigs, other parvoviruses have also been identified, 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 slaughter houses 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 appeared in 11/13 provinces, PPV4 was detected in only 3/13 provinces. PPV1, PPV2 and PPV3 co-circulation was frequently observed with PPV1/PPV2 co-infection, predominating with 20.8% (48/231). All four PPVs were only detected together in one sample from Thua Thien Hue. Three nearly complete PPV4 genomes of 5,453 bp in size were obtained and deposited in GenBank. Genomic alignment and comparison of the three sequences showed high identities at both the nucleotide (99.5-99.6%) and the deducted amino acid levels (99.6-99.9%) of open reading frames 1-3 and also with Vietnamese or Chinese strains (98.9-99.3% and 99.4-99.7%, respectively). 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.

Keywords: Porcine parvovirus; PPV1, PPV2, PPV3, PPV4, Vietnam; Pigs; Prevalence; Coinfections; PPV4 sequence.

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 two 5'-3' ends and 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 eight monophyletic genera in this subfamily, four of which include viruses that infect pigs. Specifically, these genera include *Bocaparvovirus* (*Ungulate bocaparvovirus 2, 3, 4* and *5*), *Copiparvovirus* (*Ungulate copiparvovirus 2* and *4*), *Protoparvovirus* (*Ungulate protoparvovirus 1* and *2*), and *Tetraparovirus (Ungulate tetraparvovirus 2* and *3)* [6]. *Ungulate protoparvovirus 1* or porcine parvovirus 1 (PPV1) was first isolated in 1965 in Germany [7] and 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 disease association of any of these 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 an 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, currently an unclassified PPV7 (proposed genus *Chappaparvovirus)* was identified in the USA in 2016 [13].

Since their discovery, the prevalence of different parvovirus species 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]. Furthermore, using archived samples from domestic pigs located in the USA or 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 suggested that PPV2-4 were circulating at least since the 1920s (PPV2), 1930s (PPV3), 1980s (PPV4) [19] and 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 on seroprevalence of *Ungulate protoparvovirus 1* in Long An province (southern Vietnam) revealed that this virus is an important factor impacting breeding sow fertility decline [21]. Until now, no study on pig parvoviruses concentrating on other genera and their molecular characterization has 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 which belong to three main parts of Vietnam. After the first detection of PPV4 from lung lavage of PCV2-infected pigs in the US [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 further enhance the existing knowledge of PPV4, nearly complete genomic sequences of circulating PPV4 isolates were obtained in this study.

Materials and methods

Sample collection

During 2016 to 2019, a total of 231 individual 4.5 to 5.5 month old healthy 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 randomly collected 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. Overall 136 lung and 95 serum samples were collected. Detailed information on the collected samples including numbers, sample types, collection year and collection location 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 DNA concentration was measured by Nanodrop and stored at 4°C until testing. All of the samples had a concentration of 92 to 446 ng/ul which was acceptable.

All DNA samples were tested by conventional PCR assays using primers as previously described (Table 2). The PCRs specific for PPV1 through PPV4 were carried out in a single reaction for each parvovirus using 1.5-2.0 µl of the DNA, 2 × PCR master mix (Thermo Scientific, Lithuania), 5 pmol of the respective primers, and annealing temperatures (Ta) specific to each primer pair as represented in Table 2. A Vertiti Thermal Cycler machine (AB Applied Biosystem) was used. The resulting PCR products were analyzed on a 2% agarose

gel for 40 min using DNA 100 bp ladder (ThermoFisher 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 there was a faint DNA band on the gel, the PCR was carried out again with double the volume of DNA. Selected PCR products were purified, sequenced on an ABI 3730 xl DNA Analyzer (ThermoFisher Scientific), and the PPV sequences were confirmed by 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 then sequenced. All PPV4 positive samples were sequenced.

PPV4 genome sequencing and sequence analysis

To amplify the nearly complete genomes of the three PPV4 positive strains, seven previously described primer pairs [22] were utilized (Table 2). Amplification products were purified using GeneJET PCR purification Kit (Thermo Scientific, Lithuania) as per manufacturer's guidance and sequenced with the standard Sanger method on an ABI 3730xl system. The nucleotide sequences of PPVs were identified by the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information [30]. Multiple nucleotide and amino acid alignments were carried out using the published PPV sequences as references by BioEdit 7.2.5 [31]. The phylogenetic analysis was conducted by MEGA X [32] and Maximum Likelihood methods based on Tamura-Nei model [33]. A boot-strap value of 1,000 replicates was applied for the robustness of phylogeny.

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 positive for more than one genotype (Table 3). The geographic location of the different PPVs is shown in Fig. 1. The overall PPV prevalence rates in pigs at slaughter age across 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 tested provinces (11/13), whereas PPV4 was only detected in pigs from the Quang Ninh, Quang Tri and Thua Thien Hue provinces in northern and central Vietnam. High rates of PPV1 positive samples 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 Ha Noi. According to sample type, among 136 lung samples, 67.6% (92/136) were positive for PPV1, 32/4% (44/136) were PPV2 positive, 18.4% (25/136) were positive for PPV3 and 7.6% (18/136) were positive for PPV4. Among the 95 serum samples, 33.7% (32/95) were PPV1 positive, 22.1% (21/95) were PPV2 positive and 16.8% (16/95) were PPV3 positive. PPV4 DNA was not detected in serum samples.

Often, pigs were positive for more than one genotype. A summary of co-infection of co-infection with more than one PPV in the study pigs is presented in Table 4. The overall highest co-infection rate was seen in PPV1/PPV2 at 20.8%. The rates of infection of PPV1/PPV3 and PPV2/ PPV3 were lower with 10.0% and 4.3%, respectively. Only 2.6% of the samples were 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 of PPV1 and PPV4 was 23.3%, it was 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 genome in pigs from slaughterhouses in Vietnam

To investigate the diversity of PPV4 circulating in Vietnam, the nearly complete genome of three PPV4 strains collected from Quang Ninh and Quang Tri were sequenced and analyzed. The entire sequence length of the three isolates was 5,367 bp with no insertion and deletions in the coding regions, the sequences were deposited in Genbank with accession numbers MT434667-MT434669. The similarities at nucleotide (nt) and amino acid (aa) levels among the three PPV4 strains, between Vietnamese strains and PPV4 reference strains from Chinese, US and from wild boar host are summarized in Table 5.

The multiple sequence comparison revealed a base substitution at position 124 (G→A) resulting in an amino acid change (42: D→N) in ORF1 and three amino acid changes in ORF2 (455: E→D/Q, 469: I→V, and 531: H→Q). No variation was detected in the deducted aa sequence of ORF3. As seen in Table 5, the three obtained sequences contained two main ORFs, which are 1,797 bp of ORF1 encoding 598 aa and 2,187 bp of ORF2 encoding 728 aa. A small ORF3 with the length of 615 bp encodes 204 aa. The rate of nucleotide sequence similarity among the PPV4 genomes obtained from pigs raised in Vietnam was high, 99.6-99.9% (ORF1); 99.2-99.7% (ORF2) and 99.6-99.8% (ORF3). Considering the full sequence, PPV4 strains share 99.3–99.6% nt identity and 99.6-99.9% aa residues among themselves. High identities at the nucleotide or 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 showed 100% similarity within Vietnamese strains and between Vietnamese and other reference strains. Data presented in Table 5 also show that ORF1/ORF2 nucleotide and amino acid sequences of Vietnamese PPV4 strains exhibited high identities with Chinese and Romanian strains and less with USA 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 full 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 in all branches were higher than 63% indicating the reliability of phylogenetic tree.

Discussion

Vietnam is among the highest pork producing countries, therefore, it is not exceptional that parvoviruses are commonly found in the pig population. The previously reported 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 PPV1-4 genotypes in pigs in slaughterhouses in Vietnam.

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 result is similar to that of commercial pig herds in Thailand (53.0%) [25]. The prevalence rates of PPV1 in Japan (67%) [26] and Germany (61%) [34] were slightly higher with not much difference when compared to that of our study. In contrast, PPV1 circulation in China (5.6%) [35], South Korea (4.6%) [36], North America (8.9%) [37] and Argentina (11.3%) [38] was much lower. This perhaps could be due to difference 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 the 13 provinces. Reported PPV2 prevalence rates from North America (36.8%), Poland (19%), and in South Africa (21.8%) indicated little difference [27, 37, 39]. In contrast, high PPV2 prevalence rates in swine herds were 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 from Asian countries appeared 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 lower at 17.7%. Meanwhile, PPV3 also appears 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]. PPV3 prevalence in South Africa was also low with 5.5% [28]. PPV4 was detected in three of 13 investigated provinces in the northern and central part of Vietnam and only in lung samples. PPV4 infection rates ranged from 21.4%-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 US 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 of PPV1 and PPV2 was present in 20.8% of the investigated animals, the highest detected, and was 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 only found in 2.6% of the investigated pigs. In a previous study from Romania, concurrent infection with PPV2 and PPV3 was dominant 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 most common and detected in 80.2% (65/81) of positive samples for at least one PPV species [27]. In Japan, where120 pigs aged about 6 months sampled from 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 of 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 of PPV2 and PPV3 occurred in 3.0%

3.0% of the samples (23/ 695), dual infection of PPV2 and PPV4 was identified in 1.2% of all samples (8/695), and PPV3 and PPV4 coinfection occurred 0.6% of the samples (4/695), respectively [43]. As shown in Table 4, in this study only a single sample was concurrently infected with all four investigated PPVs. Considering the sample type investigated in our study, the prevalence of PPVs was higher in lung tissue compared to serum samples in healthy slaughter age pigs. Similarly, previously, the prevalence of PPV 1-5 in tissue samples was also higher than in serum samples [37]. Taken together, results on PPV co-infection indicated that dual, triple, and quadruple infection rate were lower compared to other studies. The reported variations among geographic regions may be caused by differences in sample types investigated, age of collection, test used, health status of the pig at collection, and overall number of samples tested, regardless of individual or coinfection with all four PPVs tested.

PPV4 was first identified in the lung lavage of a pig co-infected with porcine circovirus type 2 in the USA in 2010 [4]. Genomic characterization indicated it has a genome size of about 5,9 kb. PPV4 has two major ORFs (which are ORF1 located at the 5'-end encoding for non-structural proteins and ORF2 at the 3'-end encoding for 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 on a global basis. In this study, PPV4 was further characterized by genomic sequencing. PPV4 was selected over PPV1, PPV2 or 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 chosen another, more prevalent, PPV. 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 bp. Three variations in the deducted amino acid sequence were observed in ORF2 of all

three Vietnamese PPV4 strains supporting the suggestion that ORF2 mutates at a faster rate than ORF1 [14]. This may be caused by higher pressure of the host immune system on the viral capsid protein. Comparison of the nucleotide and amino acid sequences among Vietnamese PPV4 strains revealed a high similarity, suggesting a similar origin of these viruses. Sequence comparison and phylogenetic analysis based on nearly complete genome sequence of PPV4 showed that the Vietnamese strains were closely related to Romanian and Chinese PPV4 sequences. They clustered together in a clade, which was separate from the clade formed by PPV4 sequences from 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 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 identities between Vietnamese and Chinese PPV4 strains, suggests a possible introduction of this genotype into Vietnam via the geographic northern border with China. However, further investigations are needed, with a larger sample size, in order to confirm this hypothesis.

Conclusions

This study is the first survey of PPV1, PPV2, PPV3 and PPV4 genotypes in slaughter age pigs in three regions throughout Vietnam. The obtained PPV infection rate within Vietnamese swine herds contributes substantially to the general disease knowledge base and will be useful for future disease management and other pathogen investigations 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 4-6 month old pigs were tested). The reported 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 further contribute to the overall knowledge of parvoviruses in the Vietnamese pig population. While the role of PPV1 in reproductive failure in breeding herds is well recognized, disease association of the other PPVs needs to be established. This will ultimately aid in achieving an improved understanding and control of porcine pathogen transmission.

Declarations

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Authors' contributions *Nguyen Thi Dieu Thuy*: Responsible for conception this study, laboratory work, and drafting the manuscript); *Nguyen Tran Trung*: Sample collection from central provinces and assisting with genotyping experiments; *Tran Quoc Dung*: Sample collection from northern provinces and DNA extraction; *Do Vo Anh Khoa*: Sample collection from southern provinces; *Tanja Opriessnig*: Manuscript review; *Dinh Thi Ngoc Thuy*: Assisting with sequencing. All authors read and approved the final manuscript.

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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.

Keywords: porcine parvovirus; PPV1, PPV2, PPV3, PPV4, Vietnam; pigs; prevalence; coinfections; PPV4 sequence

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 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 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.

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, 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.

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

Authors' contributions *Nguyen Thi Dieu Thuy*, responsible for conception this study, laboratory work, and drafting the manuscript). *Nguyen Tran Trung*, sample collection from central provinces and assisting with genotyping experiments. *Tran Quoc Dung*, sample collection from northern provinces and DNA extraction. *Do Vo Anh Khoa*, sample collection from southern provinces. *Tanja Opriessnig*, manuscript review; *Dinh Thi Ngoc Thuy*, assisting with sequencing. All authors read and approved the final manuscript.

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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.

Fig. 2. Phylogenetic tree constructed based on nearly complete genome sequences of PPV4 (5,367 bp) obtained from Vietnam (this study) and reference sequences from GenBank. 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).

Figure 1

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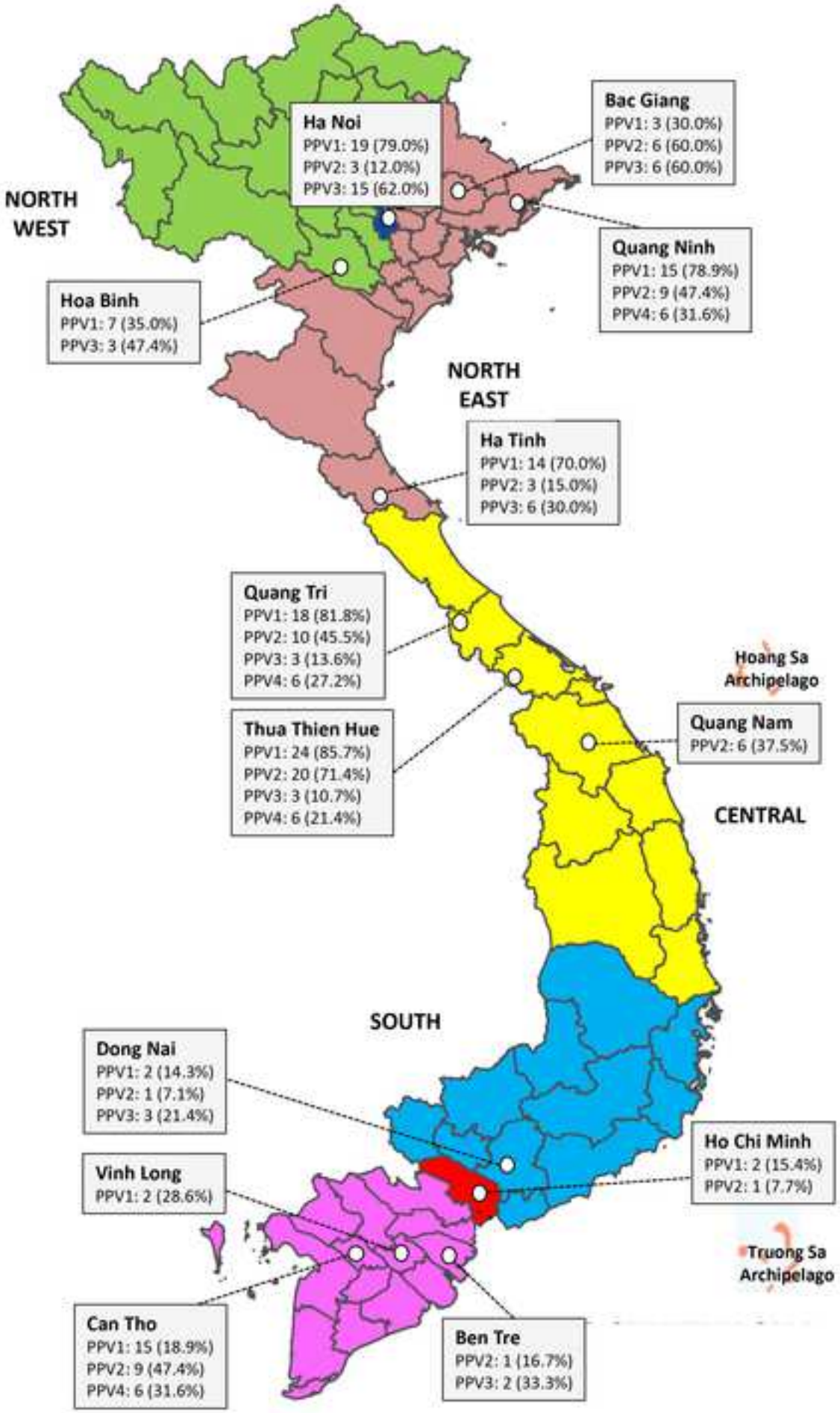

Figure 2

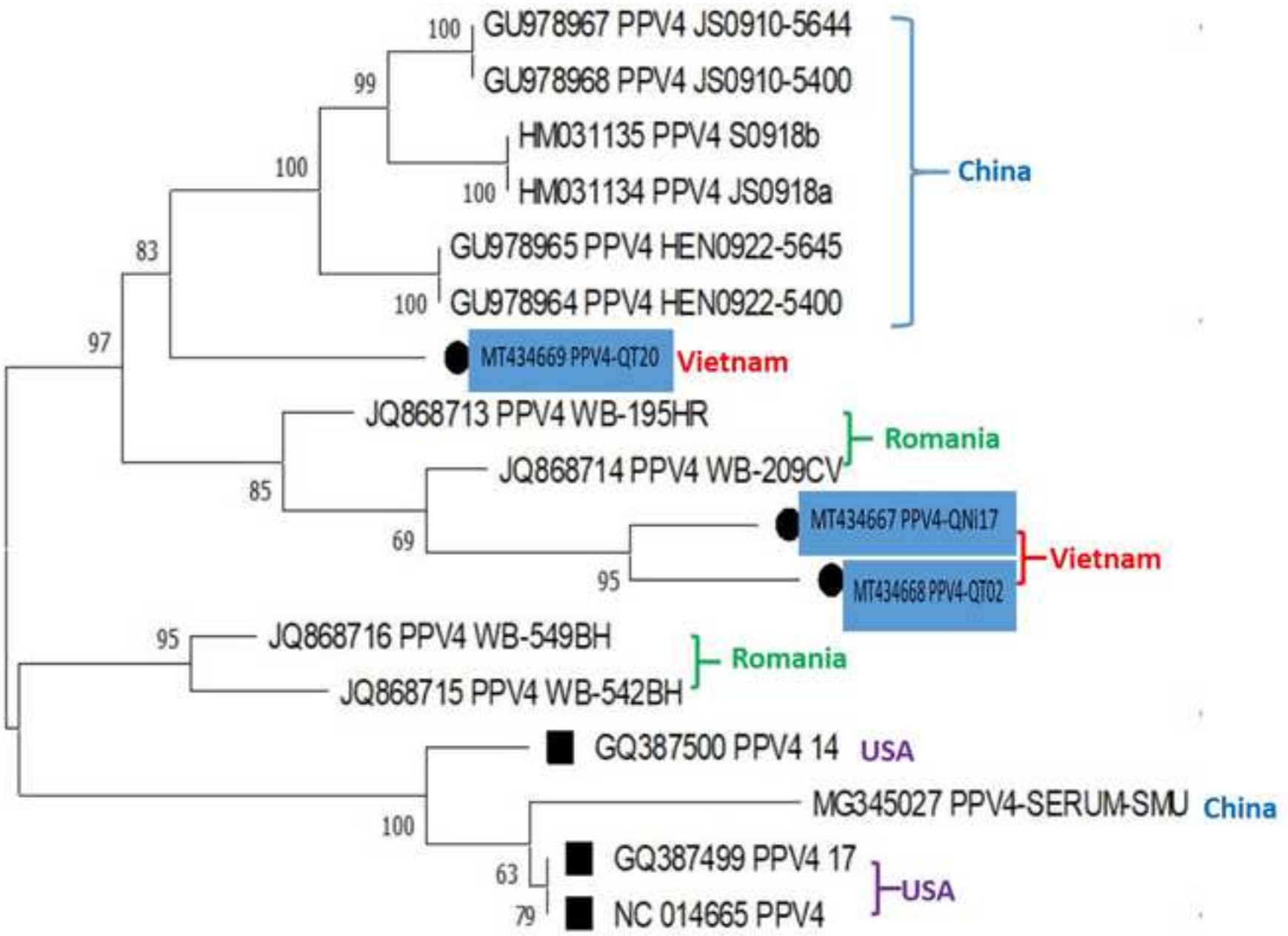

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Table

Table 1. Information on 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 any 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)	References
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

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

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%)***

Table 4. Prevalence rates of co-infection of 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. Percentage of nucleotide and amino acid identities for different open reading frames (ORF) within Vietnamese *Ungulate copiparvovirus 2* (PPV4) sequences investigated in this study (MT434667-MT434669), and compared with other reference strains from domestic pigs in China (GU978965-GU978967; HM031134; MG345027), the USA (GQ387499-GQ387500; NC014665) and from 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	5,367 nt	99.3-99.6	98.9-99.4	98.8-99.0	99.0-99.5
sequence	1.500 aa	99.6-99.9	99.4-99.7	99.1-99.3	99.4-99.8

[1]Abbreviations used: nt, nucleotide; aa, amino acid.

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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
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