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

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17 **Abstract**

18 *Ungulate protoparvovirus* 1, also known as porcine parvovirus 1 (PPV1), is considered to be
19 one of the major causes of reproductive failure in pig breeding herds. In addition, in pigs, other
20 parvoviruses have also been identified, including *Ungulate tetraparvovirus* 3 or PPV2,
21 *Ungulate tetraparvovirus* 2 or PPV3, and *Ungulate copiparvovirus* 2 or PPV4 but their
22 significance for pigs is unknown. In the present study, the prevalence of PPV1-4 was
23 investigated using a total of 231 lung and serum samples collected from slaughter houses in 13
24 provinces throughout Vietnam. The overall prevalence was 54.5% (126/231) for PPV1, 28.0%
25 (65/231) for PPV2, 17.7% (41/231) for PPV3, and 7.8% (18/231) for PPV4. While PPV1 and
26 PPV2 appeared in 11/13 provinces, PPV4 was detected in only 3/13 provinces. PPV1, PPV2
27 and PPV3 co-circulation was frequently observed with PPV1/PPV2 co-infection,
28 predominating with 20.8% (48/231). All four PPVs were only detected together in one sample
29 from Thua Thien Hue. Three nearly complete PPV4 genomes of 5,453 bp in size were obtained
30 and deposited in GenBank. Genomic alignment and comparison of the three sequences showed
31 high identities at both the nucleotide (99.5-99.6%) and the deduced amino acid levels (99.6-
32 99.9%) of open reading frames 1-3 and also with Vietnamese or Chinese strains (98.9-99.3%
33 and 99.4-99.7%, respectively). Phylogenetic analysis further confirmed a close relationship
34 between Vietnamese and Chinese PPV4 strains. These results are the first to report the
35 prevalence of **PPV1, PPV2, PPV3, and PPV4** and nearly complete genomic sequences of PPV4
36 in pigs from slaughterhouses in Vietnam.

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

39 Introduction

40 Parvoviruses are small, non-enveloped viruses with a linear, single stranded DNA genome of
41 4-6 kb [1]. Their genome is characterized by a hairpin structure at the two 5'-3' ends and two
42 open reading frames (ORF) coding for non-structural protein (NSP) and viral coat and capsid
43 protein (VP) [2, 3]. A small additional ORF3 located between ORF1 and ORF2 has been
44 described for some parvoviruses [4]. Parvoviruses in vertebrates, including pigs, are members
45 of the family *Parvoviridae* and the subfamily *Parvovirinae* [5]. There are eight monophyletic
46 genera in this subfamily, four of which include viruses that infect pigs. Specifically, these
47 genera include *Bocaparvovirus* (*Ungulate bocaparvovirus 2, 3, 4* and 5), *Copiparvovirus*
48 (*Ungulate copiparvovirus 2* and 4), *Protoparvovirus* (*Ungulate protoparvovirus 1* and 2), and
49 *Tetraparvovirus* (*Ungulate tetraparvovirus 2* and 3) [6]. *Ungulate protoparvovirus 1* or
50 porcine parvovirus 1 (PPV1) was first isolated in 1965 in Germany [7] and associated with
51 reproductive failure in sows characterized by stillbirth, mummified fetuses, embryonic death,
52 and infertility (SMEDI) [8]. To date, PPV1 is the only parvovirus clearly associated with
53 disease in pigs. While additional PPVs have been identified, Koch's postulates for disease
54 association of any of these still need to be fulfilled. Specifically, *Ungulate tetraparvovirus 3*,
55 also known as PPV2, was first discovered in Myanmar in 2001 [9]. In 2008, *Ungulate*
56 *tetraparvovirus 2*, also known as PPV3, was identified in Hong Kong [10]. In the USA,
57 *Ungulate copiparvovirus 2*, also known as PPV4, was discovered in 2010 [4] as well as an
58 unclassified PPV5 (closely related to PPV4) which was discovered in 2013 [11]. *Ungulate*
59 *copiparvovirus 4*, also known as PPV6, was identified in China in 2014 [12]. Finally, currently
60 an unclassified PPV7 (proposed genus *Chappaparvovirus*) was identified in the USA in 2016
61 [13].

62 Since their discovery, the prevalence of different parvovirus species has been investigated by
63 different groups in different countries. The first identification of PPV6 occurred in North
64 America in 2015 [14] and in Poland in 2016 [15] while PPV7 was first observed in Korean and

65 Chinese pigs in 2018 [16, 17]. Furthermore, using archived samples from domestic pigs located
66 in the USA or Italy, PPV2 [18,19], PPV3 [18], PPV4 [18,19] and PPV6 [19] could be traced back
67 to 1998, while PPV5 was identified in samples from 1997 [18]. Furthermore, phylodynamics and
68 phylogeography history studies suggested that PPV2-4 were circulating at least since the 1920s
69 (PPV2), 1930s (PPV3), 1980s (PPV4) [19] and PPV1 originated approximately 120 years ago
70 [20].

71
72 In Vietnam, the SMEDI syndrome caused by *Ungulate protoparvovirus 1* or PPV1 has
73 been of interest since the early 1990s and at that time, the disease caused great losses in
74 breeding herds. Currently, it is effectively controlled by inactivated or subunit vaccines. A
75 survey on seroprevalence of *Ungulate protoparvovirus 1* in Long An province (southern
76 Vietnam) revealed that this virus is an important factor impacting breeding sow fertility
77 decline [21]. Until now, no study on pig parvoviruses concentrating on other genera and their
78 molecular characterization has been reported in Vietnam. In this study, we report the
79 prevalence of *Ungulate protoparvovirus 1* (PPV1), *Ungulate tetraparvovirus 2* (PPV3),
80 *Ungulate tetraparvovirus 3* (PPV2), and *Ungulate copiparvovirus 2* (PPV4) in pigs sampled
81 from 13 provinces which belong to three main parts of Vietnam. After the first detection of
82 PPV4 from lung lavage of PCV2-infected pigs in the US [4], the virus has been investigated by
83 several research groups and was also identified in other countries with different prevalence rates:
84 1.8% (13/705) in China [22], 6.4% (25/392) in Hungary [23], 10% (12/120) in Romania [24],
85 44% (41/80) in Thailand [25], 33% (40/120) in Japan [26], 2.5% (6/247) in Poland [27], 43.6%
86 (48/110) in South Africa [28] and 20.0% (10/50) in Cameroon [29]. To further enhance the
87 existing knowledge of PPV4, nearly complete genomic sequences of circulating PPV4 isolates
88 were obtained in this study.

90 **Materials and methods**

91

1 92 **Sample collection**

2
3 93 During 2016 to 2019, a total of 231 individual 4.5 to 5.5 month old healthy pigs were
4
5
6 94 sampled at abattoirs located in 13 provinces in three regions of Vietnam, including northern,
7
8 95 central and southern Vietnam. Lung tissue (from one lung lobe, approximately 2 × 2 cm in
9
10 96 size) and in some instances serum samples (3-5 ml blood collected in serum separation tubes)
11
12
13 97 were randomly collected by local veterinarians. The preferred sample was lung tissue but in
14
15
16 98 some cases lungs could not be collected and serum samples were collected instead. In each
17
18 99 participating abattoir a maximum of five samples were collected and the number of abattoirs
19
20
21 100 visited was 2-6 for each province. Overall 136 lung and 95 serum samples were collected.
22
23 101 Detailed information on the collected samples including numbers, sample types, collection
24
25 102 year and collection location is presented in [Table 1](#). After collection, the samples were stored
26
27
28 103 on ice (4°C) and immediately shipped to the laboratory. Serum was separated upon arrival
29
30 104 and all samples were frozen at -20°C until testing.

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35 106 **DNA extraction and PCR analysis**

36
37 107 Total viral DNA was extracted from serum or frozen lung tissues using a commercial kit
38
39
40 108 (GeneJET Viral DNA/RNA Purification Kit, Thermo Scientific, Lithuania). The extracted
41
42 109 DNA concentration was measured by Nanodrop and stored at 4°C until testing. All of the
43
44
45 110 samples had a concentration of 92 to 446 ng/ul which was acceptable.
46
47 111 All DNA samples were tested by conventional PCR assays using primers as previously
48
49
50 112 described ([Table 2](#)). The PCRs specific for PPV1 through PPV4 were carried out in a single
51
52 113 reaction for each parvovirus using 1.5-2.0 µl of the DNA, 2 × PCR master mix (Thermo
53
54 114 Scientific, Lithuania), 5 pmol of the respective primers, and annealing temperatures (Ta)
55
56
57 115 specific to each primer pair as represented in [Table 2](#). A Vertiti Thermal Cycler machine (AB
58
59 116 Applied Biosystem) was used. The resulting PCR products were analyzed on a 2% agarose

117 gel for 40 min using DNA 100 bp ladder (ThermoFisher Scientific). Appropriate positive and
118 negative extraction and PCR controls (obtained from the University of Edinburgh and
119 confirmed by sequencing) were used for each extraction and PCR run. When there was a faint
120 DNA band on the gel, the PCR was carried out again with double the volume of DNA.
121 Selected PCR products were purified, sequenced on an ABI 3730 xl DNA Analyzer
122 (ThermoFisher Scientific), and the **PPV sequences were** confirmed by BLAST analysis. For
123 PPV1, PPV2 and PPV3 the positive samples in each province were counted. If fewer than 10
124 samples were positive, one was selected at random to be sequenced. If more than 10 samples
125 tested positive, two random samples were then sequenced. All PPV4 positive samples were
126 sequenced.

128 **PPV4 genome sequencing and sequence analysis**

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

140 **Results**

141 **Prevalence of the four PPVs in slaughter age pigs in Vietnam**

142 All four PPVs were detected in the 231 samples tested, with some samples positive for more
143 than one genotype (Table 3). The geographic location of the different PPVs is shown in Fig.
144 1. The overall PPV prevalence rates in pigs at slaughter age across both sample types
145 collected were 53.7% for PPV1, 28.0% for PPV2, 17.7% for PPV3 and 7.8% for PPV4.
146 PPV1 and PPV2 were observed in most of tested provinces (11/13), whereas PPV4 was only
147 detected in pigs from the Quang Ninh, Quang Tri and Thua Thien Hue provinces in northern
148 and central Vietnam. High rates of PPV1 positive samples ranging from 70.0% to 85.6%
149 were observed in Hanoi, Ha Tinh, Quang Tri and Thua Thien Hue provinces. The highest
150 prevalence of PPV2 was 71.4% in Thua Thien Hue and for PPV3 it was 62% in Ha Noi.
151 According to sample type, among 136 lung samples, 67.6% (92/136) were positive for PPV1,
152 32.4% (44/136) were PPV2 positive, 18.4% (25/136) were positive for PPV3 and 7.6%
153 (18/136) were positive for PPV4. Among the 95 serum samples, 33.7% (32/95) were PPV1
154 positive, 22.1% (21/95) were PPV2 positive and 16.8% (16/95) were PPV3 positive. PPV4
155 DNA was not detected in serum samples.
156 Often, pigs were positive for more than one genotype. A summary of co-infection of co-
157 infection with more than one PPV in the study pigs is presented in Table 4. The overall
158 highest co-infection rate was seen in PPV1/PPV2 at 20.8%. The rates of infection of
159 PPV1/PPV3 and PPV2/ PPV3 were lower with 10.0% and 4.3%, respectively. Only 2.6% of
160 the samples were positive for PPV1, PPV2, and PPV3.
161 PPV4 was detected in three provinces (Table 4) and often co-circulated with PPV1, PPV2
162 and PPV3. The infection rates of PPV1 and PPV4 was 23.3%, it was 14.5% for PPV2 and
163 PPV4, 1.5% for PPV3 and PPV4 and also for PPV1, PPV2, PPV3 and PPV4. Only one pig
164 from Thua Thien Hue was infected with all four PPVs.

165 166 **Diversity of PPV4 genome in pigs from slaughterhouses in Vietnam**

167 To investigate the diversity of PPV4 circulating in Vietnam, the nearly complete genome of
168 three PPV4 strains collected from Quang Ninh and Quang Tri were sequenced and analyzed.
169 The entire sequence length of the three isolates was 5,367 bp with no insertion and deletions
170 in the coding regions, the sequences were deposited in Genbank with accession numbers
171 MT434667-MT434669. The similarities at nucleotide (nt) and amino acid (aa) levels among
172 the three PPV4 strains, between Vietnamese strains and PPV4 reference strains from Chinese,
173 US and from wild boar host are summarized in Table 5.
174 The multiple sequence comparison revealed a base substitution at position 124 (G→A)
175 resulting in an amino acid change (42: D→N) in ORF1 and three amino acid changes in
176 ORF2 (455: E→D/Q, 469: I→V, and 531: H→Q). No variation was detected in the deduced
177 aa sequence of ORF3. As seen in Table 5, the three obtained sequences contained two main
178 ORFs, which are 1,797 bp of ORF1 encoding 598 aa and 2,187 bp of ORF2 encoding 728 aa.
179 A small ORF3 with the length of 615 bp encodes 204 aa. The rate of nucleotide sequence
180 similarity among the PPV4 genomes obtained from pigs raised in Vietnam was high, 99.6-
181 99.9% (ORF1); 99.2-99.7% (ORF2) and 99.6-99.8% (ORF3). Considering the full sequence,
182 PPV4 strains share 99.3–99.6% nt identity and 99.6-99.9% aa residues among themselves.
183 High identities at the nucleotide or amino acid level were observed between Vietnamese
184 PPV4 strains and Chinese strains (98.8-99.4% and 99.4-99.7%, respectively). The amino acid
185 sequences of ORF3 showed 100% similarity within Vietnamese strains and between
186 Vietnamese and other reference strains. Data presented in Table 5 also show that
187 ORF1/ORF2 nucleotide and amino acid sequences of Vietnamese PPV4 strains exhibited
188 high identities with Chinese and Romanian strains and less with USA strains. To analyze the
189 genetic relationship between the PPV4 sequences obtained in this study and the reference
190 strains from other geographic locations, a phylogenetic tree was constructed based on nearly
191 full genomic sequences. As seen in Fig. 2, the three Vietnamese PPV4 strains (black dots;
192 **MT434667-MT434669**) group in a clade together with Romanian (JQ868713-JQ868714) and

193 Chinese (GU978964-GU978968; HM031134-HM031135) sequences. The other clade was
194 formed by PPV4 sequences from the USA (black square boxes; GQ387499, GQ387500m
195 BC014665), two sequences from Romania (JQ868715, RJQ868716), and a sequence from
196 China (MG345027). The bootstrap values in all branches were higher than 63% indicating the
197 reliability of phylogenetic tree.

199 Discussion

200 Vietnam is among the highest pork producing countries, therefore, it is not
201 exceptional that parvoviruses are commonly found in the pig population. The previously
202 reported seroprevalence of PPV1 in Long An, a southern province of Vietnam, showed that
203 PPV1 contributed to reproductive failure in sows [21]. The results from this present study
204 confirm the circulation of PPV1-4 genotypes in pigs in slaughterhouses in Vietnam.

205 PPV1 is currently the most prevalent genotype in pigs in several countries. The
206 overall prevalence of PPV1 observed in this study was 53.7% (124/231). This result is similar
207 to that of commercial pig herds in Thailand (53.0%) [25]. The prevalence rates of PPV1 in
208 Japan (67%) [26] and Germany (61%) [34] were slightly higher with not much difference
209 when compared to that of our study. In contrast, PPV1 circulation in China (5.6%) [35],
210 South Korea (4.6%) [36], North America (8.9%) [37] and Argentina (11.3%) [38] was much
211 lower. This perhaps could be due to difference in PPV1 vaccination programs for breeding
212 herds in these selected regions. The PPV2 prevalence rate in this study was 28.0% (65/231)
213 in pigs across the 13 provinces. Reported PPV2 prevalence rates from North America
214 (36.8%), Poland (19%), and in South Africa (21.8%) indicated little difference [27, 37, 39].
215 In contrast, high PPV2 prevalence rates in swine herds were observed in Hungary (51.0%)
216 [40], Germany (78.0%) [34], Thailand (82.5%) [25] and Japan (58.0%) [41]. The reported
217 circulation of PPV3 in pigs from Asian countries appeared high with 72.5% in Thailand [25],
218 45.1% in China [35] and 39.0% in Japan [26]. In contrast, the detection rate of PPV3 in this

219 study was relatively lower at 17.7%. Meanwhile, PPV3 also appears at a lower rate in
220 Europe, including 21.3% in Hungary [40], 20.0% in Germany [34], 7.7% in Poland [27], and
221 19.1% in Slovakia [42]. PPV3 prevalence in South Africa was also low with 5.5% [28].
222 PPV4 was detected in three of 13 investigated provinces in the northern and central part of
223 Vietnam and only in lung samples. PPV4 infection rates ranged from 21.4%-31.6% (Table 3).
224 This is similar to what has been observed in domestic pigs in other Asian countries, such as
225 33.0% in Japan [26], 21.6% in China [35], and 44.0% in Thailand [25], as well as 43.6% in
226 South Africa [28]. Much lower infection rates of PPV4 were detected in pigs in the US with
227 2.9% positive samples [37] and in European countries, including 6.4% in Hungary [23],
228 10.0% in Romania [19], 7.0% in Germany [34], and 2.5% in Poland [27].

229 In Vietnamese pigs at slaughter age, coinfection of PPV1 and PPV2 was present in
230 20.8% of the investigated animals, the highest detected, and was followed by PPV1 and
231 PPV3 coinfection in 10.0% of the pigs and PPV2 and PPV3 coinfection in 4.3% of the pigs.
232 Triple infection with PPV1, PPV2 and PPCV3 was only found in 2.6% of the investigated
233 pigs. In a previous study from Romania, concurrent infection with PPV2 and PPV3 was
234 dominant and present in 79% (31/39) of domestic pigs and 95% (169/177) of wild boars
235 investigated [19]. When six pig farms in Poland were inspected, PPV2 was most common
236 and detected in 80.2% (65/81) of positive samples for at least one PPV species [27]. In Japan,
237 where 120 pigs aged about 6 months sampled from slaughterhouse were tested, 67% were
238 PPV1 positive, 58% were PPV2 positive, 39% were PPV3 positive, and 33% were PPV4
239 positive [26]. In Thailand, across five genotypes (PPV1-4 and PBo-likeV), over 60% of the
240 pigs carried more than three PPVs, and more than four PPVs were identified in 28% of tonsil
241 samples [25]. Concurrent infection of PPV2, PPV3 and PPV4 and PCV2 was analysed using
242 a biobank of archival pig samples ($n = 695$) [43]. The samples originated from Northern
243 Ireland, the Republic of Ireland, Great Britain and other neighbouring European countries and
244 were collected from 1997 to 2012. Concurrent infection of PPV2 and PPV3 occurred in 3.0%

245 3.0% of the samples (23/ 695), dual infection of PPV2 and PPV4 was identified in 1.2% of all
246 samples (8/695), and PPV3 and PPV4 coinfection occurred 0.6% of the samples (4/695),
247 respectively [43]. As shown in Table 4, in this study only a single sample was concurrently
248 infected with all four investigated PPVs. Considering the sample type investigated in our
249 study, the prevalence of PPVs was higher in lung tissue compared to serum samples in
250 healthy slaughter age pigs. Similarly, previously, the prevalence of PPV 1-5 in tissue samples
251 was also higher than in serum samples [37]. Taken together, results on PPV co-infection
252 indicated that dual, triple, and quadruple infection rate were lower compared to other studies.
253 The reported variations among geographic regions may be caused by differences in sample
254 types investigated, age of collection, test used, health status of the pig at collection, and
255 overall number of samples tested, regardless of individual or coinfection with all four PPVs
256 tested.

257 PPV4 was first identified in the lung lavage of a pig co-infected with porcine
258 circovirus type 2 in the USA in 2010 [4]. Genomic characterization indicated it has a genome
259 size of about 5,9 kb. PPV4 has two major ORFs (which are ORF1 located at the 5'-end
260 encoding for non-structural proteins and ORF2 at the 3'-end encoding for structural proteins)
261 and a small ORF3 located between ORF1 and ORF2 [4]. The initial discovery of PPV4
262 resulted in further investigations and it was quickly confirmed that PPV4 was present on a
263 global basis. In this study, PPV4 was further characterized by genomic sequencing. PPV4
264 was selected over PPV1, PPV2 or PPV3 during the early stages of the investigation and for
265 no particular reason. In hindsight, and after having all PPV prevalence rates available, it
266 would perhaps have been better to chosen another, more prevalent, PPV. However, the results
267 of this study still contribute to the overall knowledge base of PPV4 and are therefore
268 important.

269 The sequence length of the three Vietnamese PPV4 strains analyzed in this study was
270 5,367 bp. Three variations in the deducted amino acid sequence were observed in ORF2 of all

271 three Vietnamese PPV4 strains supporting the suggestion that ORF2 mutates at a faster rate
272 than ORF1 [14]. This may be caused by higher pressure of the host immune system on the
273 viral capsid protein. Comparison of the nucleotide and amino acid sequences among
274 Vietnamese PPV4 strains revealed a high similarity, suggesting a similar origin of these
275 viruses. Sequence comparison and phylogenetic analysis based on nearly complete genome
276 sequence of PPV4 showed that the Vietnamese strains were closely related to Romanian and
277 Chinese PPV4 sequences. They clustered together in a clade, which was separate from the
278 clade formed by PPV4 sequences from USA, China and Romania (Fig. 2). These results are
279 consistent with a previous phylogenetic analysis based on PPV4 genome sequences [22] and
280 further confirm the close genetic relationship among investigated PPV4 strains. The lack of
281 circulating PPV4 in the southern regions of Vietnam in this study, together with the high
282 nucleotide and amino acid identities between Vietnamese and Chinese PPV4 strains, suggests
283 a possible introduction of this genotype into Vietnam via the geographic northern border with
284 China. However, further investigations are needed, with a larger sample size, in order to
285 confirm this hypothesis.

286

287 **Conclusions**

288 This study is the first survey of PPV1, PPV2, PPV3 and PPV4 genotypes in slaughter
289 age pigs in three regions throughout Vietnam. The obtained PPV infection rate within
290 Vietnamese swine herds contributes substantially to the general disease knowledge base and
291 will be useful for future disease management and other pathogen investigations in this region.
292 However, the limitations of this study include sample type (only lung and serum were
293 investigated), disease status of investigated pigs (only healthy pigs at slaughter were tested),
294 and age (only 4-6 month old pigs were tested). The reported results allow a preliminary
295 insight into the prevalence of the major PPVs in healthy pigs in Vietnam. In addition,
296 genomic characterization of PPV4 is also provided. Additional epidemiological studies that

297 also include PPV5 and PPV6 and whole genome sequencing for the other PPVs are necessary
1 298 to further contribute to the overall knowledge of parvoviruses in the Vietnamese pig
2
3 299 population. While the role of PPV1 in reproductive failure in breeding herds is well
4
5 300 recognized, disease association of the other PPVs needs to be established. This will
6
7
8 301 ultimately aid in achieving an improved understanding and control of porcine pathogen
9
10 302 transmission.

13 303

16 304 **Declarations**

18
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33 311 Roslin Institute.

36 312

38 313 **Conflicts of interest/Competing interests** (include appropriate disclosures) The authors
39
40 314 declare that they have no competing interests.

43 315

45 316 **Ethics approval** (include appropriate approvals or waivers) Not applicable

48 317

50 318 **Consent to participate** (include appropriate statements) Not applicable

53 319

55 320 **Consent for publication** (include appropriate statements) The authors agree to publish this
56
57 321 manuscript.

60 322

323 **Availability of data and material** (data transparency) Not applicable

1 324

2
3 325 **Code availability** (software application or custom code) Not applicable

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8 327 **Authors' contributions** *Nguyen Thi Dieu Thuy*: Responsible for conception this study,

9
10 328 laboratory work, and drafting the manuscript); *Nguyen Tran Trung*: Sample collection from

11
12 329 central provinces and assisting with genotyping experiments; *Tran Quoc Dung*: Sample

13
14 330 collection from northern provinces and DNA extraction; *Do Vo Anh Khoa*: Sample collection

15
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17
18 332 Assisting with sequencing. All authors read and approved the final manuscript.

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

3

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19 **Running title: Parvoviruses in slaughterhouse pigs in Vietnam**

20

21 **Abstract**

22 **Ungulate parvovirus 1**, also known as porcine parvovirus 1 (PPV1), is considered to be
23 one of the major causes of reproductive failure in pig breeding herds. Other parvoviruses
24 have also been identified in pigs, including **ungulate tetraparvovirus 3**, or PPV2, **ungulate**
25 **tetraparvovirus 2**, or PPV3, and **ungulate copiparvovirus 2**, or PPV4, but their significance
26 for pigs is unknown. In the present study, the prevalence of PPV1-4 was investigated using a
27 total of 231 lung and serum samples collected from **slaughterhouses** in 13 provinces
28 throughout Vietnam. The overall prevalence was 54.5% (126/231) for PPV1, 28.0% (65/231)
29 for PPV2, 17.7% (41/231) for PPV3, and 7.8% (18/231) for PPV4. While PPV1 and PPV2
30 were found in 11 provinces, PPV4 was detected in only three provinces. Co-circulation of
31 PPV1, PPV2 and PPV3 was frequently observed, with PPV1/PPV2 coinfection
32 predominating, with 20.8% (48/231). All four PPVs were detected together in only one
33 sample from Thua Thien Hue. Three nearly complete PPV4 genome sequences of 5,453 nt
34 were determined and deposited in the GenBank database. Alignment and comparison of the
35 three genome sequences showed 99.5-99.6% nucleotide sequence identity, and the deduced
36 amino acid sequences of open reading frames 1-3 were 99.6-99.9% identical to each other,
37 98.9-99.3% identical to those of other Vietnamese strains and 99.4-99.7% identical to those
38 of Chinese strains). Phylogenetic analysis further confirmed a close relationship between
39 Vietnamese and Chinese PPV4 strains. These results are the first to report the prevalence of
40 PPV1, PPV2, PPV3, and PPV4 and nearly complete genomic sequences of PPV4 in pigs
41 from slaughterhouses in Vietnam.

42
43 **Keywords:** porcine parvovirus; PPV1, PPV2, PPV3, PPV4, Vietnam; pigs; prevalence;
44 coinfections; PPV4 sequence

45 Introduction

46 Parvoviruses are small, non-enveloped viruses with a linear, single-stranded DNA genome of
47 4-6 kb [1]. Their genome is characterized by a hairpin structure at the 5' and 3' ends and
48 contains two open reading frames (ORF) coding for non-structural protein (NSP) and viral
49 coat and capsid protein (VP) [2, 3]. A small additional ORF3 located between ORF1 and
50 ORF2 has been described for some parvoviruses [4]. Parvoviruses in vertebrates, including
51 pigs, are members of the family *Parvoviridae* and the subfamily *Parvovirinae* [5]. There are
52 10 monophyletic genera in this subfamily, four of which include viruses that infect pigs.
53 Specifically, these include *Bocaparvovirus* (species *Ungulate bocaparvovirus 2, 3, 4* and 5),
54 *Copiparvovirus* (species *Ungulate copiparvovirus 2* and 4), *Protoparvovirus* (species
55 *Ungulate protoparvovirus 1* and 2), and *Tetraparvovirus* (species *Ungulate tetraparvovirus 2*
56 and 3) [6]. *Ungulate protoparvovirus 1*, or porcine parvovirus 1 (PPV1), was first isolated in
57 1965 in Germany [7] and is associated with reproductive failure in sows, characterized by
58 stillbirth, mummified fetuses, embryonic death, and infertility (SMEDI) [8]. To date, PPV1 is
59 the only parvovirus clearly associated with disease in pigs. While additional PPVs have been
60 identified, Koch's postulates for association of these viruses with disease still need to be
61 fulfilled. Specifically, *ungulate tetraparvovirus 3*, also known as PPV2, was first discovered
62 in Myanmar in 2001 [9]. In 2008, *ungulate tetraparvovirus 2*, also known as PPV3, was
63 identified in Hong Kong [10]. In the USA, *ungulate copiparvovirus 2*, also known as PPV4,
64 was discovered in 2010 [4] as well as the unclassified PPV5 (closely related to PPV4), which
65 was discovered in 2013 [11]. *Ungulate copiparvovirus 4*, also known as PPV6, was identified
66 in China in 2014 [12]. Finally, the currently unclassified PPV7 (proposed genus
67 "*Chappaparvovirus*") was identified in the USA in 2016 [13].
68 Since their discovery, the prevalence of different parvoviruses has been investigated by different
69 groups in different countries. The first identification of PPV6 occurred in North America in 2015
70 [14] and in Poland in 2016 [15], while PPV7 was first observed in Korean and Chinese pigs in

71 2018 [16, 17]. **However**, using archived samples from domestic pigs **in** the USA **and** Italy, PPV2
72 [18, 19], PPV3 [18], PPV4 [18, 19] and PPV6 [19] could be traced back to 1998, while PPV5
73 was identified in samples from 1997 [18]. Furthermore, phylodynamics and phylogeography
74 history studies **have** suggested that **PPV2 has been** circulating at least since the 1920s, **PPV3**
75 **since the**1930s, **and PPV4 since the**1980s [19] and **that** PPV1 originated approximately 120
76 years ago [20].

77 In Vietnam, the SMEDI syndrome caused by **ungulate protoparvovirus 1**, or PPV1, has
78 been of interest since the early 1990s, and at that time, the disease caused great losses in
79 breeding herds. Currently, it is effectively controlled by inactivated or subunit vaccines. A
80 survey **of the** seroprevalence of **ungulate protoparvovirus 1** in Long An province (southern
81 Vietnam) revealed that this virus is an important factor **in the decline of fertility in** breeding
82 sows [21]. Until now, no **studies** on pig parvoviruses concentrating on other genera and their
83 molecular characterization **have** been reported in Vietnam. In this study, we report the
84 prevalence of **ungulate protoparvovirus 1** (PPV1), **ungulate tetraparvovirus 2** (PPV3),
85 **ungulate tetraparvovirus 3** (PPV2), and **ungulate copiparvovirus 2** (PPV4) in pigs sampled
86 from 13 **provinces** belonging to three main parts of Vietnam. After the first detection of PPV4
87 from lung lavage of PCV2-infected pigs in the USA [4], the virus has been investigated by
88 several research groups and was also identified in other countries with different prevalence rates:
89 1.8% (13/705) in China [22], 6.4% (25/392) in Hungary [23], 10% (12/120) in Romania [24],
90 44% (41/80) in Thailand [25], 33% (40/120) in Japan [26], 2.5% (6/247) in Poland [27], 43.6%
91 (48/110) in South Africa [28], and 20.0% (10/50) in Cameroon [29]. To **obtain additional**
92 **information about** PPV4, nearly complete genomic sequences of circulating PPV4 isolates
93 were **determined** in this study.

94 **Materials and methods**

95 **Sample collection**

97 From 2016 to 2019, 231 healthy 4.5- to 5.5-month-old pigs were sampled at abattoirs located
1 98 in 13 provinces in three regions of Vietnam, including northern, central and southern
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3 99 Vietnam. Lung tissue (from one lung lobe, approximately 2 × 2 cm in size) and in some
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5 instances serum samples (3-5 ml blood collected in serum separation tubes) were collected
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8 101 randomly by local veterinarians. The preferred sample was lung tissue, but in some cases
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10 lungs could not be collected, and serum samples were collected instead. In each participating
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12 abattoir, a maximum of five samples were collected, and the number of abattoirs visited was
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14 2-6 for each province. In total, 136 lung and 95 serum samples were collected. Detailed
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16 information on the collected samples, including numbers, sample types, collection years, and
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18 collection locations is presented in Table 1. After collection, the samples were stored on ice
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20 (4°C) and immediately shipped to the laboratory. Serum was separated upon arrival, and all
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22 samples were frozen at -20°C until testing.
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30 110 DNA extraction and PCR analysis

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32 111 Total viral DNA was extracted from serum or frozen lung tissues using a commercial kit
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34 (GeneJET Viral DNA/RNA Purification Kit, Thermo Scientific, Lithuania). The extracted
35 113
36 concentration of the DNA was measured using a NanoDrop spectrophotometer and stored at
37 114
38 4°C until testing. All of the samples had a concentration of 92 to 446 ng/μl, making them
39 115
40 suitable for further processing.
41
42 116 All DNA samples were tested by conventional PCR assays using previously described
43 117
44 primers (Table 2). The PCR assays specific for PPV1 through PPV4 were carried out in a
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46 single reaction for each parvovirus using 1.5-2.0 μl of DNA, 2× PCR master mix (Thermo
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48 Scientific, Lithuania), and 5 pmol of each primer. The specific annealing temperature (Ta) for
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50 each primer pair is shown in Table 2. A Veriti Thermal Cycler (AB Applied Biosystem) was
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52 used. The resulting PCR products were analyzed by electrophoresis on a 2% agarose gel
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55 using a 100-bp DNA ladder (Thermo Fisher Scientific). Appropriate positive and negative
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123 extraction and PCR controls (obtained from the University of Edinburgh and confirmed by
124 sequencing) were used for each extraction and PCR run. When a faint DNA band was seen
125 on the gel, the PCR was carried out again with double the volume of DNA. Selected PCR
126 products were purified and sequenced on an ABI 3730xl DNA Analyzer (Thermo Fisher
127 Scientific), and the PPV sequences were confirmed by Basic Local Alignment Search Tool
128 (BLAST) analysis. For PPV1, PPV2, and PPV3, the positive samples in each province were
129 counted. If fewer than 10 samples were positive, one was selected at random to be sequenced.
130 If more than 10 samples tested positive, two random samples were sequenced. All PPV4-
131 positive samples were sequenced.

132

133 **PPV4 genome sequencing and sequence analysis**

134 Seven previously described primer pairs [22] were used to amplify portions of the nearly
135 complete genomes of the three PPV4-positive strains (Table 2). Amplification products were
136 purified using a GeneJET PCR Purification Kit (Thermo Scientific, Lithuania) according to
137 the manufacturer's instructions and sequenced by the standard Sanger method on an ABI
138 3730xl system. The nucleotide sequences of PPVs were identified using BLAST at the
139 National Center for Biotechnology Information [30]. Multiple nucleotide and amino acid
140 sequence alignments were carried out in BioEdit 7.2.5, using published PPV sequences as
141 references [31]. Phylogenetic analysis was conducted using MEGA X [32] and the
142 maximum-likelihood method based on the Tamura-Nei model [33]. Bootstrap analysis (1,000
143 replicates) was used to assess the robustness of phylogeny.

144

145 **Results**

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

147 All four PPVs were detected in the 231 samples tested, with some samples testing positive for
148 more than one genotype (Table 3). The geographical location of the different PPVs is shown

149 in Fig. 1. The overall PPV prevalence rates in pigs at slaughter age in both sample types
150 collected were 53.7% for PPV1, 28.0% for PPV2, 17.7% for PPV3, and 7.8% for PPV4.
151 PPV1 and PPV2 were observed in most of the provinces sampled (11/13), whereas PPV4 was
152 only detected in pigs from Quang Ninh, Quang Tri and Thua Thien Hue provinces in northern
153 and central Vietnam. High rates of PPV1 positivity ranging from 70.0% to 85.6% were
154 observed in Hanoi, Ha Tinh, Quang Tri, and Thua Thien Hue provinces. The highest
155 prevalence of PPV2 was 71.4% in Thua Thien Hue and for PPV3 it was 62% in Hanoi.
156 The distribution according to sample type was as follows: Of the 136 lung samples, 67.6%
157 (92/136) were positive for PPV1, 32.4% (44/136) were positive for PPV2, 18.4% (25/136)
158 were positive for PPV3, and 7.6% (18/136) were positive for PPV4. Of the 95 serum
159 samples, 33.7% (32/95) were positive for PPV1, 22.1% (21/95) were positive for PPV2 and
160 16.8% (16/95) were positive for PPV3. PPV4 DNA was not detected in any serum samples.
161 Often, pigs were positive for more than one genotype. A summary of coinfections with more
162 than one PPV is presented in Table 4. The overall highest coinfection rate was seen with
163 PPV1/PPV2, at 20.8%. The rates of infection of PPV1/PPV3 and PPV2/PPV3 were lower, at
164 10.0% and 4.3%, respectively. Only 2.6% of the samples were simultaneously positive for
165 PPV1, PPV2, and PPV3.
166 PPV4 was detected in three provinces (Table 4) and often co-circulated with PPV1, PPV2
167 and PPV3. The infection rates were 23.3% for PPV1 and PPV4, 14.5% for PPV2 and PPV4,
168 1.5% for PPV3 and PPV4, and also for PPV1, PPV2, PPV3 and PPV4. Only one pig from
169 Thua Thien Hue was infected with all four PPVs.

171 Diversity of PPV4 genomes in pigs from slaughterhouses in Vietnam

172 To investigate the diversity of PPV4 circulating in Vietnam, the nearly complete genome
173 sequences of three PPV4 isolates collected from Quang Ninh and Quang Tri were determined
174 and analyzed. The entire sequence length of the three isolates was 5,367 nt with no insertion

175 and deletions in the coding regions, and the sequences were deposited in the GenBank
176 database with the accession numbers MT434667-MT434669. The nucleotide (nt) and amino
177 acid (aa) sequence identity values for the three PPV4 strains, Vietnamese strains, and PPV4
178 reference strains from China and the USA, and from wild boar hosts are summarized in Table
179 5.

180 A multiple sequence comparison revealed a base substitution at position 124 (G→A)
181 resulting in an amino acid change (42: D→N) in ORF1 and other substitutions resulting in
182 three amino acid changes in ORF2 (455: E→D/Q, 469: I→V, and 531: H→Q). No variation
183 was detected in the deduced aa sequence of ORF3. As seen in Table 5, the three sequences
184 contained two main ORFs, ORF1 (1797 nt), encoding a 598-aa protein, and ORF2 (2,187 nt),
185 encoding a 728-aa protein, as well as a small ORF (ORF3) with a length of 615 nt, encoding
186 a 204-aa protein. The level of nucleotide sequence similarity among the PPV4 genomes
187 obtained from pigs raised in Vietnam was high: 99.6-99.9% in ORF1, 99.2-99.7% in ORF2
188 and 99.6-99.8% in ORF3. When full sequences were compared, the PPV4 strains shared
189 99.3-99.6% nt sequence identity and 99.6-99.9% aa sequence identity. High levels of
190 sequence identity at the nucleotide and amino acid level were observed between Vietnamese
191 PPV4 strains and Chinese strains (98.8-99.4% and 99.4-99.7%, respectively). The amino acid
192 sequences of ORF3 were 100% identical among the Vietnamese strains and between the
193 Vietnamese strains and other reference strains. The data presented in Table 5 also show that
194 ORF1/ORF2 nucleotide and amino acid sequences of Vietnamese PPV4 strains exhibited a
195 high degree of similarity to Chinese and Romanian strains, and less to US strains. To analyze
196 the genetic relationship between the PPV4 sequences obtained in this study and the reference
197 strains from other geographic locations, a phylogenetic tree was constructed based on nearly
198 complete genomic sequences. As seen in Fig. 2, the three Vietnamese PPV4 strains (black
199 dots; MT434667-MT434669) group in a clade together with Romanian (JQ868713-
200 JQ868714) and Chinese (GU978964-GU978968; HM031134-HM031135) sequences. The

201 other clade was formed by PPV4 sequences from the USA (black square boxes; GQ387499,
202 GQ387500m BC014665), two sequences from Romania (JQ868715, RJQ868716), and a
203 sequence from China (MG345027). The bootstrap values for all branches were higher than
204 63%, indicating the reliability of phylogenetic tree.

206 Discussion

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

212 PPV1 is currently the most prevalent genotype in pigs in several countries. The
213 overall prevalence of PPV1 observed in this study was 53.7% (124/231). This is similar to
214 that in commercial pig herds in Thailand (53.0%) [25]. The prevalence rates of PPV1
215 reported in Japan (67%) [26] and Germany (61%) [34] were slightly higher. In contrast, the
216 rate of PPV1 circulation in China (5.6%) [35], South Korea (4.6%) [36], North America
217 (8.9%) [37], and Argentina (11.3%) [38] is much lower. This might be due to differences in
218 PPV1 vaccination programs for breeding herds in these selected regions. The PPV2
219 prevalence rate in this study was 28.0% (65/231) in pigs across 13 provinces. The reported
220 PPV2 prevalence rates in North America (36.8%), Poland (19%), and in South Africa
221 (21.8%) are in a similar range [27, 37, 39]. In contrast, high PPV2 prevalence rates in swine
222 herds have been observed in Hungary (51.0%) [40], Germany (78.0%) [34], Thailand
223 (82.5%) [25], and Japan (58.0%) [41]. The reported circulation of PPV3 in pigs in Asian
224 countries is high, with 72.5% in Thailand [25], 45.1% in China [35], and 39.0% in Japan
225 [26]. In contrast, the detection rate of PPV3 in this study was relatively low, at 17.7%. PPV3
226 is also present at a lower rate in Europe, including 21.3% in Hungary [40], 20.0% in

227 Germany [34], 7.7% in Poland [27], and 19.1% in Slovakia [42]. The reported prevalence of
1 228 PPV3 in South Africa is also low, at 5.5% [28]. PPV4 was detected in three of 13 provinces
2
3 229 investigated in the northern and central part of Vietnam, and only in lung samples. PPV4
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5 230 infection rates ranged from 21.4% to 31.6% (Table 3). This is similar to what has been
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8 231 observed in domestic pigs in other Asian countries, such as 33.0% in Japan [26], 21.6% in
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10 232 China [35], and 44.0% in Thailand [25], as well as 43.6% in South Africa [28]. Much lower
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12 233 infection rates of PPV4 were detected in pigs in the USA, with 2.9% positive samples [37],
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14 234 and in European countries, including 6.4% in Hungary [23], 10.0% in Romania [19], 7.0% in
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16 235 Germany [34], and 2.5% in Poland [27].
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20 236 In Vietnamese pigs at slaughter age, coinfection with PPV1 and PPV2 was detected in
21
22 237 20.8% of the investigated animals, making it the most frequent combination, followed by
23
24 238 PPV1 and PPV3 coinfection in 10.0% of the pigs and PPV2 and PPV3 coinfection in 4.3% of
25
26 239 the pigs. Triple infection with PPV1, PPV2 and PPCV3 was found in only 2.6% of the
27
28 240 investigated pigs. In a previous study from Romania, concurrent infection with PPV2 and
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30 241 PPV3 was frequent and present in 79% (31/39) of domestic pigs and 95% (169/177) of wild
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32 242 boars investigated [19]. When six pig farms in Poland were inspected, PPV2 was the most
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34 243 common and was detected in 80.2% (65/81) of samples that were positive for at least one
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36 244 PPV type [27]. In Japan, where 120 pigs, aged about 6 months, sampled from a
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38 245 slaughterhouse, were tested, 67% were PPV1 positive, 58% were PPV2 positive, 39% were
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40 246 PPV3 positive, and 33% were PPV4 positive [26]. In Thailand, across five genotypes (PPV1-
41
42 247 4 and PBo-likeV), over 60% of the pigs carried more than three PPVs, and more than four
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44 248 PPVs were identified in 28% of tonsil samples [25]. Concurrent infection with PPV2, PPV3
45
46 249 and PPV4 and PCV2 was analysed using a biobank of archival pig samples ($n = 695$) [43].
47
48 250 The samples originated from Northern Ireland, the Republic of Ireland, Great Britain, and
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50 251 other neighbouring European countries and were collected from 1997 to 2012. Concurrent
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52 252 infection with PPV2 and PPV3 occurred in 3.0% of the samples (23/695), dual infection with
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253 PPV2 and PPV4 was identified in 1.2% of the samples (8/695), and PPV3 and PPV4
1 254 coinfection occurred in 0.6% of the samples (4/695) [43]. As shown in Table 4, in this study,
2
3 255 only a single sample was concurrently infected with all four investigated PPVs. The overall
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5 256 prevalence of PPVs was higher in lung tissue than in serum samples in healthy slaughter-age
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8 257 pigs. This is in agreement with a previous study showing the prevalence of PPV1-5 to be
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11 258 higher in tissue samples was also higher than in serum samples [37]. Taken together, the data
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13 259 on PPV coinfections indicate that the dual, triple, and quadruple infection rates in our study
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15 260 were lower than in other studies. The reported variations among geographic regions may be
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18 261 due to differences in the sample types investigated, the animal's age at sample collection, the
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21 262 test used, the health status of the pig, and the overall number of samples tested.

23 263 PPV4 was first identified in the lung lavage of a pig that was coinfecting with porcine
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25 264 circovirus type 2 in the USA in 2010 [4]. It was found to have a genome size of about 5.9 kb.
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27 265 PPV4 has two major ORFs (ORF1, located at the 5' end, encoding non-structural proteins,
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29 266 and ORF2, at the 3' end, encoding structural proteins) and a small ORF3 located between
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31 267 ORF1 and ORF2 [4]. The initial discovery of PPV4 resulted in further investigations, and it
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33 268 was quickly confirmed that PPV4 was present globally. In this study, PPV4 was further
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35 269 characterized by genomic sequencing. PPV4 was selected over PPV1, PPV2, and PPV3
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37 270 during the early stages of the investigation and for no particular reason. In hindsight, and
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39 271 after having all PPV prevalence rates available, it would perhaps have been better to have
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42 272 chosen another, more prevalent, PPV type. However, the results of this study still contribute
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45 273 to the overall knowledge base of PPV4 and are therefore important.

49 274 The sequence length of the three Vietnamese PPV4 strains analyzed in this study was
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51 275 5,367 nt. Three variations in the deduced amino acid sequence of the protein encoded by
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53 276 ORF2 were observed in all three Vietnamese PPV4 strains, supporting the suggestion that
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55 277 ORF2 mutates at a faster rate than ORF1 [14]. This may be due to higher selection pressure
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57 278 by the host immune system on the viral capsid protein. Comparison of the nucleotide and
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279 amino acid sequences among Vietnamese PPV4 strains revealed **high** similarity, suggesting a
280 **common** origin of these viruses. Sequence comparisons and phylogenetic analysis based on
281 nearly complete genome sequences of PPV4 showed that the Vietnamese **isolates** were
282 closely related to Romanian and Chinese PPV4 **strains**. They clustered together in a clade,
283 which was separate from the clade formed by PPV4 sequences from **the** USA, China and
284 Romania (**Fig. 2**). These results are consistent with a previous phylogenetic analysis based on
285 PPV4 genome sequences [22] and further confirm the close genetic relationship among **the**
286 investigated PPV4 strains. The lack of circulating PPV4 in the southern regions of Vietnam
287 in this study, together with the high nucleotide and amino acid **sequence similarity** between
288 Vietnamese and Chinese PPV4 strains, suggests a possible introduction of this genotype into
289 Vietnam via **the** northern border with China. However, further investigations are needed, with
290 a larger sample size, in order to **test** this hypothesis.

291

292 **Conclusions**

293 This study is the first survey of PPV1, PPV2, PPV3, and PPV4 genotypes in
294 slaughter-age pigs in three regions of Vietnam. The **information** obtained **about the** PPV
295 infection rate within Vietnamese swine herds contributes substantially to the general disease
296 knowledge base and will be useful for future disease management and investigations of **other**
297 **pathogens** in this region. However, the limitations of this study include sample type (only
298 lung and serum were investigated), disease status of investigated pigs (only healthy pigs at
299 slaughter were tested), and age (only 4- to 6-month-old pigs were tested). The **results** allow a
300 preliminary insight into the prevalence of the major PPVs in healthy pigs in Vietnam. In
301 addition, genomic characterization of PPV4 is also provided. Additional epidemiological
302 studies that also include PPV5 and PPV6 and whole genome sequencing for the other PPVs
303 are necessary to **gain an overview** of **the prevalence of** parvoviruses in the Vietnamese pig
304 population. While the role of PPV1 in reproductive failure in breeding herds is well

305 recognized, **the** association of the other PPVs **with disease still** needs to be established. This
1 306 will ultimately aid in achieving an improved understanding and control of porcine pathogen
2
3 307 transmission.
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7
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30 318 **Conflicts of interest** The authors declare that they have no competing interests.
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35 320 **Authors' contributions** *Nguyen Thi Dieu Thuy*, responsible for conception this study,
36
37 321 laboratory work, and drafting the manuscript). *Nguyen Tran Trung*, sample collection from
38
39 322 central provinces and assisting with genotyping experiments. *Tran Quoc Dung*, sample
40
41 323 collection from northern provinces and DNA extraction. *Do Vo Anh Khoa*, sample collection
42
43 324 from southern provinces. *Tanja Opriessnig*, manuscript review; *Dinh Thi Ngoc Thuy*,
44
45 325 assisting with sequencing. All authors read and approved the final manuscript.
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51 327 **References**

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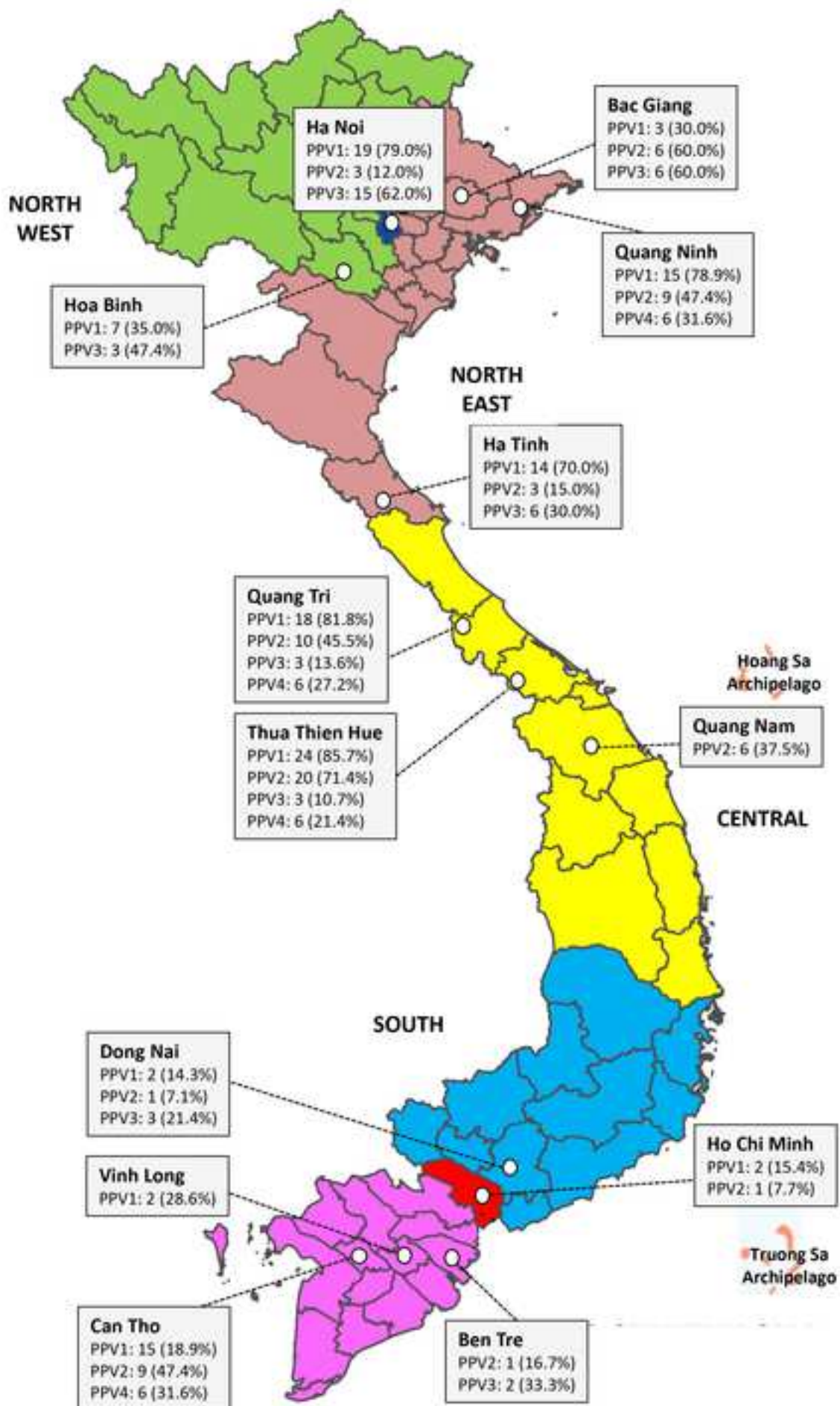
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Fig. 1. Map of Vietnam indicating the different provinces sampled in this study and also the detection rates of parvoviruses in pigs in these areas.

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



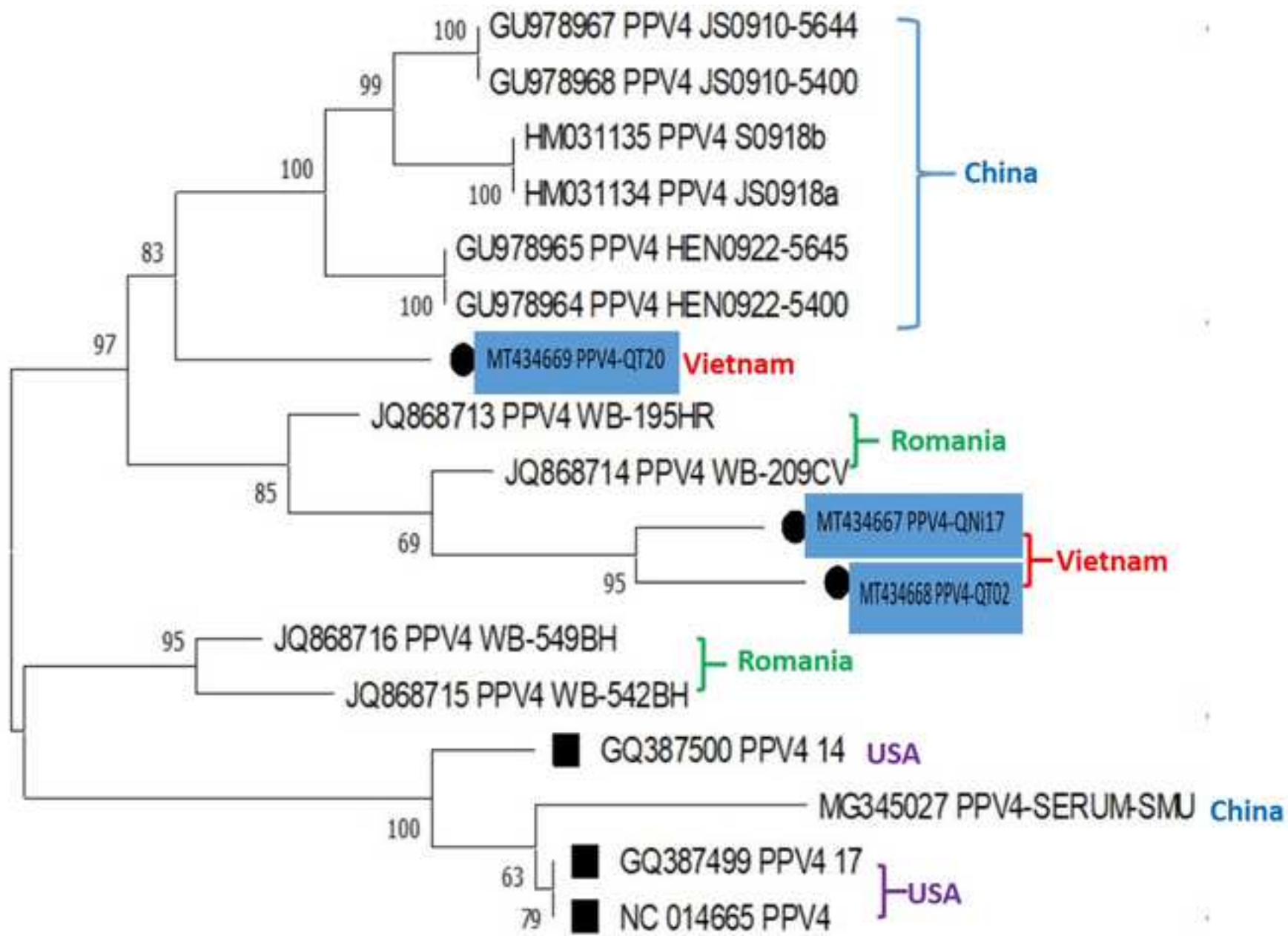


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Table 1. Information on the samples collected in this study including location, sample type and year of collection.

Region	Province	Number of abattoirs visited	Number of samples collected ¹	Sample type ²		Collection year
				<i>Lung</i>	<i>Serum</i>	
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 abattoir 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: AGTAATCTGGTAATCGCTGTTTCG (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: GGCCGTCATCATACTGCTC (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'

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	Size¹	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

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

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: AGTAATCTGGTAATCGCTGTTTCG (1050-1072) R: ATGTTAGTCTTTCTGTTGTGGC (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: GGCCGTCATCATACTTCTGCTC (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'

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%)

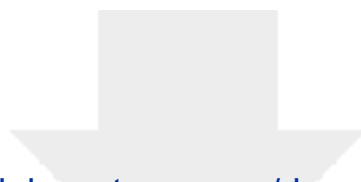
Table 4 Rates of coinfection with different parvoviruses in pigs. The samples were tested for ungulate protoparvovirus 1 (PPV1), ungulate tetraparvovirus 3 (PPV2), ungulate tetraparvovirus 2 (PPV3), and ungulate copiparvovirus 2 (PPV4).

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)

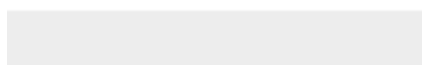
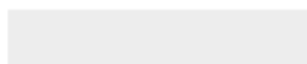
ORF	Size ¹	Vietnam	China	USA	Romania
1	1,797 nt	99.6-99.9	99.4-99.6	99.1-99.2	99.1-99.2
	598 aa	99.6-99.8	99.3-99.6	98.3-98.9	99.1-100
2	2,187 nt	99.2-99.7	99.3-99.7	98.6-99.3	99.2-99.7
	728 aa	99.5-100	99.4-99.8	99.4	99.7-99.8
3	615 nt	99.6-99.8	99.1-99.8	99.6-99.8	99.8-100
	204 aa	100	100	100	100
Whole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

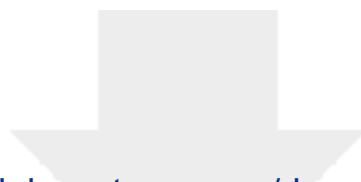
¹ nt, nucleotide; aa, amino acid



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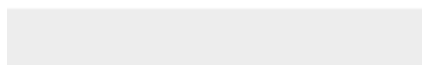
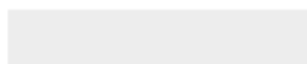
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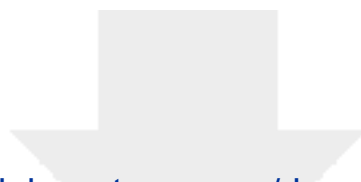




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PPV4 genomic seq 5368bp_QT02.txt





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PPV4 genomic seq 5368bp_QT20.txt

