Canine parvovirus type 2c in Vietnam continues to form distinct descendants with new mutations restricted to the Vietnamese variants

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Brief Report

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Abstract

The complete sequence for viral protein 2 (VP2) of canine parvovirus (CPV) was obtained from 33 isolates collected from dogs with clinical symptoms and a vaccine in Vietnam from Mar 2017– June 2019. Molecular analysis revealed the descent evolution in CPV-2c-“new”, forming a “new var.” sub-lineage of the substantial 5G, 447M mutations restricted to the Vietnamese isolates in the epitope stretches. These were found besides the common 80R, 87L, 93N, 101T, 103A, 232I, 267Y, 297A, 300G, 305Y, 323N, 324I, 334A, 341P, 370R, 426E, 440T, 447I, 555V, 564S, and 568G typical to the “new” CPV-2c. The novel 447M mutation seemed to emerge in recent years in Vietnam and was present in 38 of 58 CPV-2c strains isolated from December 2016 to June 2019 by our retrospective analysis, counting for 65.5%. With strong nodal support (98%), the topology of the phylogenetic tree revealed the substantially distinct Vietnamese sub-lineage of 2c-“new-var.” (5G/426E/447M) separated from the Vietnamese 2c-“new” (5G/426E/447I), within the 2c-(Asia)/Asia-2c. The Asia-2c strains of Vietnam were grouped with the recent Thai, Chinese, Mongolian, Taiwanese, Korean, and the Asian variants newly imported into Italy, Egypt, and Nigeria from Asia. The changes of amino acids in the epitope stretch in VP2 have effective alteration not only on the continual formation of substantial sub- or new genotypic variants but may be of subjects for antigenicity/immunogenicity and virulence to the CPV-2 strains and vaccine failure worldwide.

Main Text

Canine parvovirus type 2 (CPV-2) and its antigenic variants are of the most highly contagious viral pathogens causing a globally spread infectious disease with acute hemorrhagic gastroenteritis and subacute myocarditis in domestic and wild dogs, raccoons, foxes, mainly puppies of one to 6 months of age [1, 2]. CPV-2 first emerged in the late 1970s and was detected throughout the United States, Asian countries, Australia, and Europe [2, 3–6]. CPV-2 was such a DNA virus that the new antigenic descendants periodically have undergone fast mutations and evolutionary processes to form globally dominant variants, termed CPV2a, CPV2b, and CPV2c [2, 7]. CPV-2 and its new antigenic variants cause enormous economic loss in companion pets and service dogs worldwide due to the high morbidity, severe mortality, and slow recovery from the infection [5]. Strains of the new variants, CPV-2a, CPV-2b, and CPV-2c, have limited antigenic compatibility to each other, although only a few amino acid changes occurred in the epitope sites in VP2, leading to vaccination failure when antigenically different variant vaccines being used [1, 8–10].

The causative agent, CPV-2, is a linear, non-enveloped, single-stranded DNA (ssDNA) virus, and is the species *carnivore protoparvovirus 1*, a member of the *Protoparvovirus* genus. This virus belongs to the family *Parvoviridae* whose genome is about 5.2 kb, consisting of two large open reading frames (ORFs), one encoding the two non-structural proteins (NS1 and NS2) and the other encoding the two capsid proteins (VP1 and VP2), respectively (https://talk.ictvonline.org/ictv-reports/ictv_online_report/ssdna-viruses/w/parvoviridae) [11]. The *carnivore protoparvovirus 1* group also comprises Feline parvovirus (FPV), Mink enteritis virus (MEV), and Raccoon parvovirus (RPV)

[11]. Subjected to a high rate of point mutations and substitutions in NS1 and VP2, the CPV viruses may lead to fast evolution for the emergence of novel antigenic variants interfering with their transmission, epidemiology, vaccination, and control programs [1, 5, 9, 12–15]. Evidence has also been documented for the emerging genetic recombination between CPV field variants and vaccine strains and between CPV and FPV, influencing epidemiology, evolution, and genotypic identification [16, 17]. The fact is that the fast global spread, country-crossing, and intercontinental transmission, and the dominance/co-existence of the new antigenic variants (CPV-2a, new CPV-2a, CPV-2b, new CPV-2b), and particularly, CPV-2c and new CPV-2c have been reported worldwide [2, 18–26].

Viral protein 2 (VP2) is encoded by a large ORF at 3' end of the genomic sequence, accounting for 90% of the viral capsid proteins, and is highly responsible for virus-host cell interactions, tissue tropism, and immunogenicity [2, 27–29]. The VP2 protein of CPV-2 viruses exhibits a large extent of genetic and antigenic diversity, involving particular mutations at several key positions of inducing amino acid changes which are investigated for use for CPV host range, antigenic determination, phylogenetic analysis, and genotyping/ genogrouping of CPV-2 strains [2, 29]. Amino acids at positions 93, 323, 324 in VP2 are responsible for transferrin receptor (TfR), at 87, 101, 297, 299, 300, 305 for host range, and changes of these residues have shifted the adaptivity from feline to canine hosts [27, 28].

The VP2 structurally has two major epitope regions, namely B-cell epitope A (aa 1–23) containing peptide 2L21 (aa 1–21) at the N-terminus of the VP2 protein [30, 31]; and B-cell epitope B (a stretch of aa 38–498) which contained b-strands of eight-stranded, anti-parallel b-barrels, named b_A (aa 40–57), b_B (aa 59–74), b_C (aa 109–112), b_D (aa 136–149), b_E (aa 170–178), b_F (aa 251–257), b_G (aa 259–264), b_H (aa 496–506), and an extra one, b_I (aa 523–537) [32–35]. Between the b-strands there are four conformational threefold “spikes” labeled as Loops 1 to 4, such as Loop 1 [b_{B–C}, aa 84–89], Loop 2 [b_{E–F}, aa 216–235], Loop 3 [b_{G–H}, aa 295–306], and Loop 4 [b_{G–H}, aa 409–444] [32]. Within and between the epitope regions and b-barrels/loops there are 10 antigenic sites that have been identified responsible for eliciting and binding B-cell mediated specific antibodies [32, 33]. Amino acids in these barrels and their central flexible loops, particularly those at the protruding tops, for example, at 87 (M87L) in b_{B–C} loop 1; at 297, 300, 305 (S297A; A300G; D305Y) in b_{G–H} loop 3; at 426 (N/D426E) and at 440 (T440A) in b_{G–H} loop 4 determine conformational B-cell epitopes responsible for the antigenicity of a canine parvovirus strain [1, 2, 4, 5, 36].

Timeline analyses of new antigenic variants indicated that the restrictive mutations in the epitopes and loops in VP2 could lead to the very soon emergence of the new descendants comprising of amino acids distinct from the previous genogroups. CPV-2, characterized by K80R, K93N, V103A, D323N, N564S, and A568D in VP2 emerged from FPV in 1974–1978, and only 2–3 years later, in 1979, it was replaced by CPV-2a (M87L, I101T, A300G, D305Y, V505I); and in 1984 CPV-2a by CPV-2b (N426D and I555V); and then in the late 1990s to early 2000s CPV-2b was replaced by CPV-2c with the unique change of D426E to date [2]. Antigenic mutations in the epitope regions and b-barrels/loops of VP2, interspecific

recombination, incompatible antigenicity, and multi-lineage CPV-2c diversification in CPV strains demonstrate the need for a reappraisal of a new nomenclature for canine parvoviruses.

Within the new antigenic variant CPV-2c, however, strains have dynamically continued to evolve to form different lineages of distinct substitutions, particularly in Vietnam and Asia [12, 13], and tentatively are divided into major lineages of Western (global strains, VT-I, -II, -III) and of Asian origins (Asia-I, -II, -III, -IV), respectively [18, 20, 22, 26]. The substantially mutated Asian CPV-2c strains have been evidenced to appear in the European and African countries since 2018–2019 by any means, one of which, ie. via dog importation [18, 22, 23]. Severe parvoviral outbreaks in vaccinated populations with old/classical live attenuated CPV vaccines were worldwide documented [1, 8, 9].

In Vietnam, the FPV and CPV causative agents were first reported in the late 1990s and early 2000s in cats and dogs in Ha Noi, Da Nang, and Ho Chi Minh City by few studies on clinical epidemiology, serosurvey, and some detections of the viral pathogens were made [12, 36, 37] (**Fig. 1**). Novel CPV-2 variants were revealed to appear in Vietnam in 1997, and at that time the new variant CPV-2c was detected by genetic retrospective analyses [12, 13, 36–38]. In fact, after the early, intensive studies of feline and canine parvoviruses in Vietnam around the 2000s, there were no continual investigations until October 2013 when some CPV-2 sequences deposited in Genbank.

Imported attenuated CPV-2 vaccines have been used with boosted dose mainly for the imported breeds and mixed hybrid puppies between 6 weeks to 6 months of age in Vietnam. The indigenous Vietnamese dogs were rarely vaccinated and hence, they might be one of the most sustainable reservoirs for CPV-2 subsequent transmission and maintaining viruses for mutations. Despite the application of vaccination, severe canine parvoviral infections of novel variants have been reported throughout the country and also frequently been reported elsewhere in many other countries worldwide [1, 8, 9]. The reasons for vaccination failure were evidenced by the interference of maternally derived antibodies (MDA) and the emergence of new lineages with mutations in VP2 in the new antigenic variant CPV-2c. Some descendent mutations occurring in the CPV-2c genotype in recent years in Asian countries and in Vietnam tend to divide the currently circulating CPV-2c variants into “old” and “new” CPV-2c groups, of which many newly mutated strains originally emerged within the Vietnamese CPV2c population [12, 13, 20, 26].

There remains a need for current and retrospective molecular analyses of the detected strains from 1997 to 2019 in Vietnam, including our study conducted from February 2017 to June 2019 for accurate understandings of diversifying CPV-2 and 2c genotypes, particularly from the clinically infected dogs. The timeline molecular analyses in this study provided evidence for descendent evolution in CPV-2c lineages in Vietnam that continually evolve to form distinct CPV-2c sub-genotypes or independent lineages of new variants (termed CPV-2c “new var.”) with novel mutations originally restricted to the Vietnamese CPV-2c isolates.

Thirty-three fecal samples of dogs clinically showing hemorrhagic diarrhea and symptoms of canine parvoviral infection in the period of February 2017 and June 2019 from eight provinces of northern and central Vietnam were collected, and from this source, 33 complete VP2 sequences including 3 isolated in

February 2017, 6 in March 2017, 3 in August 2017, 9 in March 2018, 2 in May 2018, 4 in April 2019, and 4 in June 2019 (**Table 1**). The clinical samples included 9 from Hanoi ([21°01'42.5"N, 105°51'15.0"E](#)), 8 from Thai Nguyen ([21°35'39.19"N, 105°50'53.41"E](#)), two from Bac Giang ([21°16'23.05"N, 106°11'40.56"E](#)), three from Bac Ninh ([21°10'60.00"N, 106°02' 60.00"E](#)), one from Hung Yen ([20°38'46.93"N, 106°03'4.03"E](#)), and two from Hai Phong ([20°50'59.99"N, 106°40'59.99"E](#)) in northern Vietnam; 4 from Nghe An ([18°40'24.13"N, 105°41'32.35"E](#)) and 4 from Ha Tinh ([18° 20'34.15"N, 105°54'20.48"E](#)) Provinces in central Vietnam (**Fig. 1; Table 1**). Fresh fecal samples from diarrhea dogs were taken by a tampon tool and put into a tube and were stored at -20°C until used for genomic DNA extraction. One commercial combined, multivalent vaccine, widely used in Vietnam, namely *Vanguard®Plus5-CV* (Zoetis, US), comprising an attenuated strain of prototype CPV-2 was also collected for molecular genotyping. The infected dogs were puppies of 6 weeks to 6 months old and the majority were of 3–4 months; including the breeds, ie. Fox, Rottweiler, Pomeranian, Corgi, Border Colli, Pit Bull, Dobermann, Malinois, service Berger (hybrid) or German shepherd, and several indigenous Vietnamese dogs (**Table 1**). The detailed vaccination status was not exactly recorded, however, almost all the imported or mixed breeds (companion pets and service dogs) were vaccinated with the imported vaccines, mainly with the *Vanguard®Plus5-CV* multivalent vaccine.

A tampon of the individual fecal sample or the content of a vaccine vial was homogenized in sterile water and centrifuged at 13,000 rpm in 15 min. Total genomic DNA was extracted from the supernatant of the processed clinical samples or vaccine suspension using the GeneJET™ Genomic DNA Purification Kit (Thermo Scientific Inc., MA, USA) as instructed, eluted in 50 μL , and stored at -20°C until use. Primers for amplification of the entire VP2 gene were designed based on the conserved sequences of the available CPV genomes, including the Forward, CPF1: 5' AGCTAAAAAGGCAATTGCTCC 3' (2346–2366, coding frame numbering) and the Reverse, CPR4: 5' TATAGACAGTATACGAGGCC 3' (4555–4754); and two internal primers, CPF2: 5' ACCAACAGATCCAATTGGAG 3' and CPR3: 5' GCATTACATGAAGTCTTGG 3' for sequencing. The PCR products (2,229 bp) were sequenced directly, or after cloning using pCR2.1-TOPO TA-cloning vector (Invitrogen, USA), by a service company. The complete VP2 sequence of 1,755 nucleotides obtained from each sample in this study (**Table 1**) and reference strains from previous studies and GenBank were used for molecular analysis.

Totally 109 VP2 sequences including 33 Vietnamese isolates and one vaccine strain in this study (Mar 2017–June 2019), and 54 other Vietnamese strains from previous studies by other authors (1997; 2002; October 2013; 2017; and December 2016–January 2018) and 22 reference strains (**Fig. 1; Table 1 and 2**) were aligned for sequence and phylogenetic tree construction using GENEDOC 2.7 (<http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html>) and MEGA X (www.megasoftware.net), with maximum-likelihood method tested by bootstrapping with 1000 replications [40]. The alignment of deduced amino acid sequences was further used for comparative genotypic and antigenic characterization. From the alignment, 10 Vietnamese strains of this and previous studies, and 3 reference strains representing CPV vaccines, CPV-2, 2a, 2b, and 2c variants were used for

drawing a schematic presentation of CPV epitope stretches spanning b-strands and loops in the VP2 protein (**Fig. 2**).

Identical amino acid sequences were obtained from 4 groups of 16, 12, 3, and 2 isolates in each. The amino acid analysis of 34 VP2 sequences in this study (Mar 2017–June 2019) indicated that 33 belonged to the “new” CPV-2c and one was the *Vanguard@Plus5*-CV multivalent vaccine of CPV-2a (from Zoetis, US). No CPV-2a and CPV-2b variants were found. All such residues as 80R, 87L, 93N, 101T, 103A, 232I, 267Y, 297A, 300G, 305Y, 323N, 324I, 334A, 341P, 370Q/R, 426N/D/E, 440T, 555V, 564S, 568G, common to the global and typical to the Asian CPV-2c strains (summarized in [2]) were found, substantially confirming the status of the Asian “new” CPV-2c genogroup. However, the distinctness was revealed in these Vietnamese “new” CPV-2c strains. In these 33 “new” CPV-2c strains there was the presence of the descendants of A5G, P13S, and I447M mutations which are characteristically restricted to the Vietnamese CPV-2c-“new” strains sharply distinct from the global CPV-2c up-to-date known. We suggest naming these Vietnamese “new” CPV-2c strains as “new-var.” CPV-2c viruses with the aim to discriminate them from the “old” or “common” and “new” global and Asian CPV-2c isolates (**Table 1 and 2**). The *Vanguard@Plus5*-CV multivalent vaccine used in Vietnam sequenced in August 2018 revealed to contain the CPV-2a typical residues completely different from those of the common and the “new”/“new var.” CPV-2c in Vietnam (**Table 2**). The mismatching antigenic residues essential for (sub)genotypic immune response reflected well-explained incompatibility in the complete protection of the Vietnamese pets/dogs against the current circulating virulent CPV-2c viruses, and vaccine failure or partial or incomplete protection has been observed in other countries as well [8–10, 39, 41].

To unravel the historical mutations that occurred in the derivative CPV-2 populations in Vietnam we have successfully collected 88 Vietnamese CPV-2a, b, c sequences up-to-date available and used for molecular analysis. These comprised 18 CPV-2a, b, and 70 CPV-2c according to the authors’ report including 33 in this and 38 from previous studies [12, 13, 20, 26] and from GenBank. However, the amino acid analysis revealed some misidentified antigenic variants among the three variants, and in fact, there were 7 CPV-2a strains (having 426N), 14 CPV-2b (426D), and 67 CPV-2c (426E) [2]. Involving 4 strains in [12] and [13], LCPV-V139/1997 (GenBank: AB054222), LCPV-V140/1997 (AB054223) and LCPV-203/1997 (AB054224) were revealed CPV-2a, and HNI-41/2002 (AB120727) was CPV-2b variants, respectively, instead of 2c strains (**Table 2**).

All the 19 Vietnamese CPV-2a, b strains before 2002 had 426N/D and 5A, 13P and 447I [12, 13], while 66 CPV-2c strains investigated from October 2013 to June 2019 had the 5G, 13P/S, 38V/E, 447I/M residues. The P13S changes occurred only in 4 strains of 2016–2017 and V38E in 2 strains of March 2018 isolation whilst the novel, substantial 447M mutations were present in 38 strains isolated from December 2016 to June 2019 (**Table 2 and 3**). These 447M descendants seemed to emerge in recent years from the continual evolution of the intra-geographically formed CPV-2c derivatives in Vietnam. The 5G/447M mutants of CPV-2c “new var.” strains were found present at the average rate of 65.5% in the CPV-2c population in Vietnam by our random statistic analysis (**Table 3**). These mutations were found in the epitope regions and restricted to the CPV-2c of Vietnam although many of the studied strains were

from different geographical localities throughout the country. We suggest tentatively terming these novel 5G/447M-CPV-2c as “2c-new-var.” and keeping the 5G/447I-CPV-2c viruses as usual “2c-new” group (**Table 1; Fig. 1**). They are the representative antigenic variants of Vietnam completely distinct from the rest of the global CPV-2c population.

Phylogenetic analysis and tree construction were performed based on the alignment of 109 complete VP2 sequences of CPV-2a, b, c, and some vaccines of CPV-2 developed in the 1980s (as an outgroup) (a majority are listed in **Tables 1 and 2**). Eighty-eight Vietnamese CPV-2 sequences were phylogenetically clustered into two major complexes, one is the “mixed” of CPV-2a and b; and another is the “Asia-2c” (2c-Asia). Within the “Asia-2c” of the Vietnamese strains, obviously, the “new-var.” CPV-2c (5G/426E/447M) formed a majority separated from the “new” CPV-2c ((5G/426E/447I). All the Vietnamese specimens of the 1997 and 2002 isolations consisting of CPV-2a, b, and mis-genotyped CPV-(2c?)/2a, b, were placed in one, while all others isolated in 2013 and between 2016–2019 comprising of “new” and “new var.” CPV-2c isolates formed another cluster (**Table 2; Fig. 3**).

In Vietnam, a strain of “mixed” CPV-2b and 2c antigenic variants was historically discovered in 2002 (ie., HNI-4-1, GenBank AB120727) which had residues of 5A/267F/324Y/370Q/426E/447I, and lacked such 5G/267Y/324I/370R typically to define the Asian and the Vietnamese CPV-2c [13, 42]. This strain was not included in the phylogenetic tree with the Asia-2c majority clade (**Fig. 3**). However, this strain might be speculated as one of the transforming variants, being a representative for those experiencing genetic transition between the global CPV-2b and the Asian CPV-2c strains. The topology of the phylogenetic tree showed that other CPV-2c strains of Vietnam belonged to the “old” or “classical” CPV-2c which indicated the early introduction into Vietnam from the originally formed global lineage. They might play the role of an ancestral cluster for initiating the generation of the substantial “Asia-2c” and the Vietnamese “new” and “new-var.” descendants during the timeline evolution (**Fig. 3**).

Currently, there are many reports regarding the circulation of the CPV-2c strains in many countries in Europe, Asia, North America, and South America, and tentatively divided into two global lineages of, ie. either the world/global (VT) and the Asian CPV-2c mutant strains (Asia-2c) [19, 21, 25, 26]. The latter variants were evidenced to be originated in Asian countries and spread back to the European and African countries, ie., Italy, Egypt, Nigeria, respectively [19, 22, 23, 43]. The Asian CPV-2c strains were reported circulating in India, China, Mongolia, Laos, Taiwan, Thailand, Korea, and Vietnam since 2010 [20, 24, 25, 42, 44, 45]. These Asian CPV-2c strains geographically emerged in Asia, including those of the Vietnamese strains which were statistically grouped into a clade termed as Asia-IV originated from 2005, sharply distinguishable from the global VT-III CPV-2c strains [26]. Our latest data supports the two existing lineages of CPV-2 viruses in the world and the formation of the distinct Vietnamese sub-lineage of CPV-2c that continues to evolve to form novel, descendent mutations within the CPV-2c population in Vietnam. The changes of amino acids in the epitope stretch in VP2 have effective alteration not only on the continual formation of substantial sub- or new genogroups but may be of subjects for antigenicity/immunogenicity and virulence to the CPV-2 strains worldwide.

The universal naming system proposed and used in [26] such as VT-I; VT-II and VT-III for global, and Asia-I, Asia-II, Asia-III, and Asia-IV have not yet been fully transparent by sequence analyses. However, new and separate two major lineages appeared on tree topology by the maximum-likelihood method with 1000 bootstrap resamplings and typical characteristics of the Asian and Vietnamese CPV-2c-“new” and “new-var.” have proven to be reasonably reappraisal [20, 26, 42]. The Vietnamese clinical CPV-2c “new” and “new var.” isolates of the period 2016–2019 of this and previous studies formed a well-defined lineage, distinct from the typical global and previous Asian classes, indicating the continual evolution of multiple sub-lineages within the Asia-2c. The CPV-2c populations, particularly the “new” and “new var.” strains that have been circulating in Vietnam since whatsoever time of their formation for two decades (1997–2019) appear to belong to a unique lineage, indicating the possibility that this evolved from a common ancestral source.

In conclusion, VP2 sequence and phylogenetic analyses, mutations of antigenic stretch, and topology of the phylogenetic tree revealed the two majorities of virulent “new” and “new-var.” CPV-2c strains in Vietnam evolving and circulating during February 2017 and June 2019 in this and 2013–2018 by previous studies belonged to the distinct, independent Vietnamese sub-lineage within the well-defined Asian CPV-2c population. The 5G/447M novel CPV-2c mutants presenting their dominance in the recent 2016–2019 strains were investigated and seemed to proceed to replace the co-existing 5G/447I strains. The antigenic amino acid changes at the epitope sites and altering genetic characteristics in the newly formed Asia-2c, the “new” and the “new-var.” CPV-2c may be of the causes of vaccination failure in dogs in Vietnam and worldwide.

Declarations

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. The samples were taken under legal informed consent with the owners.

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Tables

Table 1 List and details of 33 canine parvovirus samples collected in dogs in northern Vietnam between February 2017 – June 2019 and one commercial vaccine used in this study for VP2 sequence characterization and phylogenetic analysis

No	Provinces	Code of strains	Year- Month of isolation	Dog breed	Age (m)	GenBank accession No	Amino acid position				Identified Genotype
							5	13	426	447	
1	1. Ha Noi city	CPV1p-HN-VN	2017-Mar	Berger (hyb)	NA	MW239577	G	P	E	M	"2c-new-var."
2		CPV4p-HN-VN	2017-Mar	Fox	2	MW239578	G	P	E	I	2c-new
3		CPV6p-HN-VN	2017-Mar	Fox	3.5	MW239578	G	P	E	M	"2c-new-var."
4		CPV7p-HN-VN	2017-Mar	Fox	3	MW239580	G	P	E	M	"2c-new-var."
5		CPV8p-HN-VN	2017-Mar	Indigenous	NA	MW239581	G	P	E	M	"2c-new-var."
6		CPV9p-HN-VN	2018-Mar	Fox	3	MW239582	G	P	E	I	2c-new
7		CPV10p-HN-VN	2018-Mar	Fox	4	MW239583	G	P	E	I	2c-new
8		CPV20-HN-VN	2019-Apr	Fox	3.5	MW239584	G	P	E	M	"2c-new-var."
9		CPV22-HN-VN	2019-Apr	Fox	3	MW239585	G	P	E	M	"2c-new-var."
10		CPV14-TN-VN	2017-Mar	Indigenous	5	MW239586	G	P	E	I	2c-new
11		CPV15p-TN-VN	2017-Mar	Fox	4	MW239587	G	P	E	M	"2c-new-var."
12		CPV16n-TN-VN	2017-Mar	Foodle	NA	MW239588	G	P	E	I	2c-new
13		CPV19-TN-VN	2017-Aug	Indigenous	NA	MW239589	G	P	E	M	2c-new var
14		CPV20-TN-VN	2017-Aug	Pomeranian	4	MW239590	G	P	E	I	2c-new
15		CPV21n-TN-VN	2017-Aug	Corgi	NA	MW239591	G	P	E	I	2c-new
16		CPV30-TN-VN	2019-Apr	Indigenous	NA	MW239592	G	P	E	M	"2c-new-var."
17		CPV35-TN-VN	2019-Apr	Corgi	3.5	MW239593	G	P	E	I	2c-new
18		ParHN5l-HY-VN	2017-Feb	<i>Rottweiler</i>	NA	MW239599	G	P	E	I	2c-new
19		ParHN1l-HP-VN	2017-Feb	Corgi	2	MW239600	G	S	E	I	2c-new
20		ParHN3l-HP-VN	2017-Feb	Fox	NA	MW239601	G	P	E	I	2c-new
21	5. Bac Ninh	PCV24-BN-VN	2018-Mar	Pit Bull	3	MW239596	G	P	E	M	"2c-new-var."
22		PCV25b-BN-VN	2018-Mar	Dobermann	2.5	MW239597	G	P	E	I	2c-new
23		PCV26-BN-VN	2018-Mar	Fox	3	MW239598	G	P	E	M	"2c-new-var."
24	6. Bac Giang	CPV01-BG-VN	2018-May	Border Colli	6	MW239594	G	S	E	I	2c-new
25		CPV02-BG-VN	2018-May	Fox	2.5	MW239595	G	S	E	I	2c-new
26	7. Nghe An	PCV27-NA-VN	2018-Mar	Malinois	2.5	MW239602	G	P	E	M	"2c-new-var."
27		PCV28-NA-VN	2018-Mar	Berger (hyb)	1.5	MW239603	G	P	E	M	"2c-new-var."
28		Par25p-NA-VN	2019-Jun	Malinois	4	MW239604	G	P	E	I	2c-new
29		Par27p-NA-VN	2019-Jun	Berger (hyb)	3	MW239605	G	P	E	M	"2c-new-var."
30		PCV29-HT-VN	2018-Mar	Malinois	4	MW239606	G	P	E	M	"2c-new-var."
31		PCV30-HT-VN	2018-Mar	Malinois	1.5	MW239607	G	P	E	M	"2c-new-var."
32		PCV31-HT-VN	2019-Jun	Mixed Bred	4	MW239608	G	P	E	M	"2c-new-var."
33		PCV32-HT-VN	2019-Jun	Mixed Bred	4.5	MW239609	G	P	E	M	"2c-new-var."

*Commercial attenuated vaccine purchased in 2018 from a veterinary vaccine company in Hanoi; (m): month; (hyb): hybrid; N/A: not available; 2c: Asian genotype CPV-2c; “2c-new-var.”: Asian sub-genotype new variant CPV-2c, which contains amino acid change from Ile (I) to Methionine (M) at position 447 in VP2 (I447M) in this study.

Table 2 Summary of the reference prototype (the United States) and studied Vietnamese strains in this and previous studies providing information on the strain designation, CPV type, GenBank accession number, year/month of isolation, and key amino acid positions of the CPV-2, 2a, 2b, and 2c (“new” and “new-var.”) of the VP2 (excluding 80R; 93N; 103A; 232I; 323N; 334A; 341P; 440T; 555V; 564S; 568G residues present in all the strains) used in the sequence and phylogenetic analyses in this study.

*Strains	CPV types	GenBank	Year/ Month	5 A G	13 P S	38 V E	87 M L	101 I T	267 F Y	297 S A	300 A G	305 D Y	324 Y I	370 Q R	426 N D E	447 I M	
Prototype strains																	
1	5us79-US	2	EU659116	1979	A	P	V	M	I	F	S	A	D	Y	Q	N	I
2	6us80-US	2	EU659117	1980	A	P	V	M	I	F	S	A	D	Y	Q	N	I
3	13us81-US	2	EU659118	1981	A	P	V	L	T	F	S	G	Y	Y	Q	N	I
4	VanguardPlus	2a (vac)	FJ197847	2007	A	P	V	M	I	F	S	A	D	Y	Q	N	I
5	FortDodge	2b (vac)	FJ222822		A	P	V	L	T	F	A	G	Y	Y	Q	D	I
Previous studies (54 strains)																	
6	V120	2a	AB054215	1997	A	P	V	L	T	F	A	G	Y	Y	Q	N	I
7	V123	2b	AB054218	1997	A	P	V	L	T	F	A	G	Y	Y	Q	D	I
8	V129	2a	AB054216	1997	A	P	V	L	T	F	A	G	Y	Y	Q	N	I
9	V154	2a	AB054217	1997	G	P	V	L	T	F	A	G	Y	Y	Q	N	I
10	V209	2b	AB054219	1997	A	P	V	L	T	F	A	G	Y	Y	Q	D	I
11	V217	2b	AB054220	1997	A	P	V	L	T	F	A	G	Y	Y	Q	D	I
12	LCPV-V204	2b	AB054221	1997	A	P	V	L	T	F	A	G	Y	Y	Q	D	I
13	LCPV-V139	2c?/2a	AB054222	1997	A	P	V	L	T	F	A	D	Y	Y	Q	N	I
14	LCPV-V140	2c?/2a	AB054223	1997	A	P	V	L	T	F	A	D	Y	Y	Q	N	I
15	LCPV-V203	2c?/2b	AB054224	1997	A	P	V	L	T	F	A	D	Y	Y	Q	D	I
16	HCM-6	2b	AB120720	2002	A	P	V	L	T	F	A	G	Y	Y	Q	D	I
17	HCM-8	2b	AB120721	2002	A	S	V	L	T	F	A	G	Y	Y	Q	D	I
18	HCM-18	2b	AB120722	2002	A	P	V	L	T	Y	A	G	Y	Y	Q	D	I
19	HCM-23	2b	AB120723	2002	A	P	V	L	T	F	A	G	Y	Y	Q	D	I
20	HNI-1-18	2b	AB120728	2002	A	P	V	L	T	F	A	G	Y	Y	Q	D	I
21	HNI-2-13	2b	AB120724	2002	A	P	V	L	T	Y	A	G	Y	Y	Q	D	I
22	HNI-3-4	2b	AB120725	2002	A	P	V	L	T	F	A	G	Y	Y	Q	D	I
23	HNI-3-11	2b	AB120726	2002	A	P	V	L	T	F	A	G	Y	Y	Q	D	I
24	HNI-4-1	2c?/2b	AB120727	2002	A	P	V	L	T	F	A	G	Y	Y	Q	E	I
25	dog-HCM-2	2c-new	LC216904	2013/Oct	G	P	V	L	T	Y	A	G	Y	I	R	E	I
26	dog-HCM-5	2c-new	LC216905	2013/Oct	G	P	V	L	T	Y	A	G	Y	I	R	E	I
27	dog-HCM-6	2c-new	LC214969	2013/Oct	G	P	V	L	T	Y	A	G	Y	I	R	E	I
28	dog-HCM-7	2c-new	LC216906	2013/Oct	G	P	V	L	T	Y	A	G	Y	I	R	E	I
29	dog-HCM-8	2c-new	LC216907	2013/Oct	G	P	V	L	T	Y	A	G	Y	I	R	E	I
30	dog-HCM-13	2c-new	LC216908	2013/Oct	G	P	V	L	T	Y	A	G	Y	I	R	E	I
31	dog-HCM-14	2c-new	LC216907	2013/Oct	G	P	V	L	T	Y	A	G	Y	I	R	E	I
32	dog-HCM-20	2c-new	LC216910	2013/Oct	G	P	V	L	T	Y	A	G	Y	I	R	E	I
33	DN8	2c-new var.	MK357721	2016/Dec	G	S	V	L	T	Y	A	G	Y	I	R	E	M
34	DN18	2c-new var.	MK357722	2016/Dec	G	P	V	L	T	Y	A	G	Y	I	R	E	M
35	DN26	2c-new var.	MK357723	2016/Dec	G	S	V	L	T	Y	A	G	Y	I	R	E	M
36	DN33 (vac)	2a (vac)	MK357724	2017/Jan	A	P	V	M	I	F	S	A	D	Y	Q	N	I
37	HCM4	2c-new var.	MK357725	2017/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	M
38	HCM16	2c-new var.	MK357726	2017/May	G	P	V	L	T	Y	A	G	Y	I	R	E	M
39	HCM22	2c-new	MK357727	2017/Jun	G	P	V	L	T	Y	A	G	Y	I	R	E	I
40	HCM25	2c-new var.	MK357728	2017/Jun	G	P	V	L	T	Y	A	G	Y	I	R	E	M
41	HCM27	2c-new var.	MK357729	2017/Jun	G	P	V	L	T	Y	A	G	Y	I	R	E	M
42	HCM32	2c-new var.	MK357730	2017/Jul	G	P	V	L	T	Y	A	G	Y	I	R	E	M
43	HCM48	2c-new var.	MK357731	2017/Jul	G	P	V	L	T	Y	A	G	Y	I	R	E	M
44	HCM50	2c-new var.	MK357732	2017/Jul	G	P	V	L	T	Y	A	G	Y	I	R	E	M
45	HN4AA	2c-new var.	MK357735	2018/Jan	G	P	V	L	T	Y	A	G	Y	I	R	E	M
46	HN7AA	2c-new	MK357734	2017/Dec	G	P	V	L	T	Y	A	G	Y	I	R	E	I
47	HN11AA	2c-new var	MK357736	2018/Jan	G	P	V	L	T	Y	A	G	Y	I	R	E	M
48	HN37AA	2c-new var	MK357737	2018/Jan	G	P	V	L	T	Y	A	G	Y	I	R	E	M
49	DN01	2c-new	MT106233	2017	G	P	V	L	T	Y	A	G	Y	I	R	E	I
50	DN02	2c-new	MT106236	2017	G	P	V	L	T	Y	A	G	Y	I	R	E	I
51	HCM01	2c-new var	MT106234	2017	G	P	V	L	T	Y	A	G	Y	I	R	E	M

52	HCM03	2c-new var	MT106237	2017	G	P	V	L	T	Y	A	G	Y	I	R	E	M
53	HCM09	2c-new var	MT106232	2017	G	P	V	L	T	Y	A	G	Y	I	R	E	M
54	HCM16	2a	MT106238	2017	A	P	V	L	T	Y	A	G	Y	I	Q	N	I
55	HN04	2c-new var	MT106229	2017	G	P	V	L	T	Y	A	G	Y	I	R	E	M
56	HN06	2c-new var	MT106235	2017	G	P	V	L	T	Y	A	G	Y	I	R	E	M
57	HN07	2c-new	MT106228	2017	G	P	V	L	T	Y	A	G	Y	I	R	E	I
58	HN08	2c-new var	MT106230	2017	G	P	V	L	T	Y	A	G	Y	I	R	E	M
59	HN30	2c-new var	MT106231	2017	G	P	V	L	T	Y	A	G	Y	I	R	E	M
This study (34 strains)																	
60	CPV1p-HN	2c-new var	MW239577	2017/Mar	G	S	V	L	T	Y	A	G	Y	I	R	E	M
61	CPV4p-HN	2c-new	MW239578	2017/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	I
62	CPV6p-HN	2c-new var	MW239578	2017/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	M
63	CPV7p-HN	2c-new var	MW239580	2017/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	M
64	CPV8p-HN	2c-new var	MW239581	2017/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	M
65	CPV9p-HN	2c-new	MW239582	2018/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	I
66	CPV10p-HN	2c-new	MW239583	2018/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	I
67	CPV20-HN	2c-new var	MW239584	2019/Apr	G	P	V	L	T	Y	A	G	Y	I	R	E	M
68	CPV22-HN	2c-new var	MW239585	2019/Apr	G	P	V	L	T	Y	A	G	Y	I	R	E	M
69	CPV14-TN	2c-new	MW239586	2017/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	I
70	CPV15p-TN	2c-new var	MW239587	2017/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	M
71	CPV16n-TN	2c-new	MW239588	2017/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	I
72	CPV19-TN	2c-new var	MW239589	2017/Aug	G	P	V	L	T	Y	A	G	Y	I	R	E	M
73	CPV20-TN	2c-new	MW239590	2017/Aug	G	P	V	L	T	Y	A	G	Y	I	R	E	I
74	CPV21n-TN	2c-new	MW239591	2017/Aug	G	P	V	L	T	Y	A	G	Y	I	R	E	I
75	CPV30-TN	2c-new var	MW239592	2019/Apr	G	P	V	L	T	Y	A	G	Y	I	R	E	M
76	CPV35-TN	2c-new	MW239593	2019/Apr	G	P	V	L	T	Y	A	G	Y	I	R	E	I
77	ParHN5l-HY	2c-new	MW239599	2017/Feb	G	P	V	L	T	Y	A	G	Y	I	R	E	I
78	ParHN1l-HP	2c-new	MW239600	2017/Feb	G	S	V	L	T	Y	A	G	Y	I	R	E	I
79	ParHN3l-HP	2c-new	MW239601	2017/Feb	G	P	V	L	T	Y	A	G	Y	I	R	E	I
80	PCV24-BN	2c-new var	MW239596	2018/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	M
81	PCV25b-BN	2c-new	MW239597	2018/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	I
82	PCV26-BN	2c-new var	MW239598	2018/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	M
83	CPV01-BG	2c-new	MW239594	2018/May	G	S	V	L	T	Y	A	G	Y	I	R	E	I
84	CPV02-BG	2c-new	MW239595	2018/May	G	S	V	L	T	Y	A	G	Y	I	R	E	I
85	PCV27-NA	2c-new var	MW239602	2018/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	M
86	PCV28-NA	2c-new var	MW239603	2018/Mar	G	P	V	L	T	Y	A	G	Y	I	R	E	M
87	Par25p-NA	2c-new	MW239604	2019/Jun	G	P	V	L	T	Y	A	G	Y	I	R	E	I
88	Par27p-NA	2c-new var	MW239605	2019/Jun	G	P	V	L	T	Y	A	G	Y	I	R	E	M
89	PCV29-HT	2c-new var	MW239606	2018/Mar	G	P	E	L	T	Y	A	G	Y	I	R	E	M
90	PCV30-HT	2c-new var	MW239607	2018/Mar	G	P	E	L	T	Y	A	G	Y	I	R	E	M
91	PCV31-HT	2c-new var	MW239608	2019/Jun	G	P	V	L	T	Y	A	G	Y	I	R	E	M
92	PCV32-HT	2c-new var	MW239609	2019/Jun	G	P	V	L	T	Y	A	G	Y	I	R	E	M
93	VanguardPlusvac	2a	MW239610	2018Aug	A	P	V	M	I	F	S	A	D	Y	R	N	I

***Strains:** No 1–5: prototype including vaccine (vac) strains [5]; No 6–15: strains reported by [12]; No 16–24: strains reported by [13]; No 25–32: taken from GenBank; No 33–48: strains reported by [20]; No 49–59: strains reported by [26]; No 60–93: strains reported by this study.

Table 3 A statistic summary of the random presence of the 5G/447M mutants in the CPV-2c VP2 sequences of the samples studied in 2016–2019 in Vietnam (by authors and by years)

Studies	Period of study	2016	2017	2018	2019	No of Mutants/ Total	Percentage
Hoang et al. (2019)	2016-2018	3/3 (100%)	7/9 (77.8%)	3/3 (100%)		13/15	86.7%
Nguyen et al. (2020)	2017		7/10 (70%)			7/10	70.0%
This study	2017-2019		6/14 (42.9%)	6/11 (54.6%)	6/8 (75%)	18/33	54.6%
Total/year	2016-2019	3/3 (100%)	20/33 (60.6%)	9/14 (64.3%)	6/8 (75%)	38/58	65.5%

Figures

Figure 1

Schematic geographical map of Vietnam indicating the ten provinces/cities where clinical samples were collected from dog population between March 2017 and June 2019 in this study (marked as a solid circle) including (1) Ha Noi; (2) Thai Nguyen; (3) Bac Giang; (4) Bac Ninh; (5) Hung Yen; (6) Hai Phong; (7) Nghe An; (8) Ha Tinh; and in previous studies (marked as a solid square): (1) Ha Noi; (9) Da Nang; (10) Ho Chi Minh City.

Figure 2

Amino acid positions of the major epitope regions, β -barrels, loops, and epitope sites in the VP2 structural protein of field/vaccine reference and representative Vietnamese CPV strains (months/years of isolation and antigenic variants at the end of each sequence). The top line is the amino acid sequence of the prototype strain 6us80-US of CPV-2 (GenBank: EU659117). Identical residues in aligned sequences are indicated by dots (.) and differences by single letters. B-cell epitope A region (aa 1–23) containing peptide

2L21 (aa 1–21) [31] and B-cell epitope B region (a stretch of aa 38–498) containing β -barrels: β A, β B, β C, β D, β E, β F, β G, β H, β I with their amino acids underlined and four Loops: Loop 1 [β B–C], Loop 2 [β E–F], Loop 3 [β G–H], and Loop 4 [β G–H] [32, 34, 35] are marked on the top (more details, see text). Amino acid positions of ten antigenic sites are underlined and marked by designated E1 to E10 [33]; and core residues of the epitopes are shaded. Key variable amino acids determining different antigenic variants are indicated by the arrow on top. Major peaks containing frequent and distinct amino acid changes and those originally formed and restricted to the Vietnamese CPV isolates are boxed, and the latest Vietnamese I447M position is shown by a double arrow.

Figure 3

A phylogenetic tree based on the analysis of 109 complete nucleotide sequences of the VP2 gene (1755 nucleotides) of CPV-2. Topology is shown for relationships of 33 clinical isolates and a vaccine in this study (marked by a diamond symbol) and other 53 Vietnamese strains and 22 reference CPV strains of known or well-identified genotypes. In the Asia-CPV-2c topology, in total, 38 Vietnamese CPV-2c-"new-var." mutants (5G/447M) formed a distinct clade with 20 Vietnamese CPV-2c-"new" sequences (5G/447I),

representing an independent sub-lineage of the descendants within the Vietnamese CPV-2c strains. CPV-2c strains are indicated by the 426E uniquely identified in the VP2. The majorities are shown by notes and arrows. Phylogenetic tree reconstruction was performed by MEGA X using a maximum-likelihood (ML) analysis based on the general time-reversible model; supported for each node by 1000 bootstrap resamplings [40]. Following strain designation is the full name of country (where the virus was isolated), year and month (if available) of isolation (in brackets), then the accession numbers, and the identified genotype/subgenotype (in brackets) at the end of each sequence. The scale bar represents the number of substitutions per site.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GenBank34strForARVI20121.rar](#)