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

Huong Thi Thanh Doan1,2 · Xuyen Thi Kim Le1,2 · Roan Thi Do1,2 · Khue Thi Nguyen1,2 · Thanh Hoa Le1,2

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Abstract
Viral protein 2 (VP2) of canine parvovirus (CPV) exhibits a high degree of genetic and antigenic diversity. We analyzed 88 Vietnamese CPV-VP2 sequences (1755 bp), 34 from this study and 54 from previous studies, and discovered a new sublineage, “new var.”, within the lineage CPV-2c-“new”, characterized by the mutation 5G/447M, which is restricted to the Vietnamese isolates. These new mutants appear to have emerged in recent years, accounting for 65.5% of the total. With strong nodal support (98%), the distinct Vietnamese 2c-“new-var.” sublineage (5G/426E/447M) was found to be separate from the 2c-“new” sublineage (5G/426E/447I) within the 2c-(Asia)/Asia-2c lineage. Amino acid changes in epitopes of VP2 might have led to the generation of subvariants and affected the antigenicity, immunogenicity, or virulence of the virus, resulting in vaccine failure worldwide.

Canine parvovirus type 2 (CPV-2) and its antigenic variants are highly contagious viral pathogens. CPV-2 has a global distribution and causes acute hemorrhagic gastroenteritis and subacute myocarditis in domestic and wild dogs, raccoons, foxes, and puppies 1 to 6 months old [1, 2]. CPV-2 first emerged in the late 1970s and has been detected in the United States, Asian countries, Australia, and Europe [2–6]. CPV-2 is a DNA virus that undergoes rapid mutation. This has led to the emergence of new antigenic variants, termed CPV2a, CPV2b, and CPV2c [2, 7]. CPV-2 and its new antigenic variants pose a danger to companion pets and service dogs worldwide, with high morbidity and mortality, and recovery from infection tends to be slow [5]. A few amino acid changes occurring in antigenic sites of viral protein 2 (VP2) can lead to vaccination failure when different antigenic variants are used in vaccines [1, 8–10].

CPV-2 is a linear, nonenveloped, single-stranded DNA (ssDNA) virus belonging to the species Carnivore protoparvovirus 1 of the genus Protoparvovirus, family Parvoviridae. This virus has a genome of about 5.2 kb, containing two large open reading frames (ORFs), one encoding the two nonstructural proteins (NS1 and NS2) and the other encoding the two capsid proteins (VP1 and VP2) (https://talk.ictvonline.org/ictv-reports/ictv_online_report/ssdna-viruses/w/parvoviridae) [11]. The species Carnivore protoparvovirus I includes feline parvovirus (FPV), mink enteritis virus (MEV), and raccoon parvovirus (RPV) [11]. The high rate of point mutations and substitutions in NS1 and VP2 can lead to rapid evolution and the emergence of novel antigenic variants. These variants interfere with the success of epidemiology, vaccination, and control programs [1, 5, 9, 12–15]. Evidence for genetic recombination between CPV field variants and vaccine strains and between CPV and FPV has been documented. The circulation of recombinant viruses complicates studies of epidemiology and evolution as well as genotypic identification [16, 17]. The new antigenic variants have quickly spread globally, and short-term coexistence of CPV-2a and new CPV-2a, CPV-2b and new CPV-2b, and CPV-2c and new CPV-2c has been reported worldwide [2, 18–26].

VP2 is encoded by a large ORF at the 3’ end of the genomic sequence. It accounts for 90% of the viral capsid
proteins and is important for virus–host cell interactions, tissue tropism, and immunogenicity [2, 27–29]. The VP2 proteins of CPV-2 viruses are genetically and antigenically diverse. Mutations at several key positions can induce amino acid changes that affect the host range, antigenicity, and virulence of the virus and complicate phylogenetic analysis and genotyping/genogrouping of CPV-2 strains [2, 29]. Amino acids at positions 93, 323, and 324 of VP2 are involved in binding to the transferrin receptor, and those at positions 87, 101, 297, 299, 300, and 305 affect the host range of the virus. Changes in some of these residues have been shown to alter the specificity of the virus for feline or canine hosts [27, 28].

The VP2 protein has two major epitopes, named B-cell epitope A (aa 1–23), containing the peptide 2L21 (aa 1–21) at the N-terminus [30, 31], and B-cell epitope B (aa 38–498), containing an eight-stranded, antiparallel β-barrel consisting of the β strands βA (aa 40–57), βB (aa 59–74), βC (aa 109–112), βD (aa 136–149), βE (aa 170–178), βF (aa 251–257), βG (aa 259–264), βH (aa 496–506), and an extra one, βI (aa 523–537) [32–35]. Between the β-strands are four conformational threefold “spikes” called loop 1 (βB–C, aa 84–89), loop 2 (βE–F, aa 216–235), loop 3 (βG–H, aa 295–306), and loop 4 (βG–H, aa 409–444) [32]. Within and between the epitope regions and β-barrels/loops, 10 antigenic sites responsible for eliciting and binding B-cell-mediated specific antibodies have been identified [32, 33]. Amino acids in these barrels and their central flexible loops, particularly those at the protruding tops (e.g., at 87 [M87L] in βB–C loop 1; 297, 300, and 305 [S297A, A300G, and D305Y] in βG–H loop 3; and 426 [N/D426E] and 440 [T440A] in βG–H loop 4) determine conformational B-cell epitopes responsible for the antigenicity of a CPV strain [1, 2, 4, 5, 36].

Timeline analysis of new antigenic variants has indicated that restrictive mutations in the epitopes and loops in VP2 can quickly lead to the emergence of new variants that differ from the previous genogroups. CPV-2, characterized by the mutations K80R, K93N, V103A, D323N, N564S, A568D in VP2 emerged from FPV from 1974 to 1978, and only 2 to 3 years later, in 1979, it was replaced by CPV-2a (M87L, I101T, A300G, D305Y, V505I). In 1984, CPV-2a was replaced by CPV-2b (N426D and I555V), and in the late 1990s to early 2000s, CPV-2a was replaced by CPV-2c, which contains the unique mutation D426E [2]. The occurrence of mutations in the epitope regions and β-barrels/loops of VP2, interspecific recombination, changes in antigenicity, and the formation of multiple lineages of the CPV-2c demonstrate the need for a reappraisal of the nomenclature of CPVs.

The new antigenic variant CPV-2c has continued to evolve, giving rise to different lineages with distinct substitutions, particularly in Vietnam and Asia [12, 13]. CPV-2c strains are tentatively divided into two lineages, one of which is of Western origin (global strains, VT-I, VT-II, VT-III), and the other is of Asian origin (Asia-I, Asia-II, Asia-III, Asia-IV) [18, 20, 22, 26]. Mutated forms of Asian CPV-2c strains were found in European and African countries from 2018 to 2019 and could have been introduced by various means (e.g., via importation of dogs) [18, 22, 23]. Severe parvoviral outbreaks in populations that had been vaccinated with classical live attenuated CPV vaccines have been documented in several countries [1, 8, 9].

In Vietnam, FPV and CPV were first reported in the late 1990s and early 2000s in cats and dogs in Ha Noi, Da Nang, and Ho Chi Minh City. There have been only a few studies on the clinical epidemiology and serotype prevalence of these viruses [12, 36, 37] (Supplementary Fig. S1). Novel CPV-2 variants appeared in Vietnam in 1997, but soon the new variant CPV-2c was detected by retrospective genetic analysis [12, 13, 36–38]. After the early, intensive studies of FPV and CPV in Vietnam around 2000, there were no continuing investigations until October 2013, when some CPV-2 sequences were deposited in the GenBank database.

In Vietnam, imported attenuated CPV-2 vaccines have been used mainly in imported breeds and mixed-breed puppies between 6 weeks and 6 months old. Indigenous Vietnamese dogs are rarely vaccinated, and hence, they might be some of the most sustainable reservoirs for CPV-2 transmission. They could also be a source of mutants. Despite the use of vaccines, severe infections with novel canine parvovirus variants have been reported nationwide. Parvovirus outbreaks in vaccinated dogs have also been reported in many other countries [1, 8, 9]. The reasons for vaccination failure include an interference of maternally derived antibodies and the emergence of new lineages of the new antigenic variant CPV-2c with mutations in VP2. The CPV-2c viruses circulating in Vietnam and Asian countries can be divided into “old” and “new” CPV-2c groups, from which many new mutants have emerged [12, 13, 20, 26].

There remains a need for current and retrospective molecular analyses of the strains circulating in Vietnam from 1997 to 2019. This study was conducted from February 2017 to June 2019 to investigate the diversity of CPV-2 and CPV-2c genotypes, particularly in dogs with clinical illness. This study provides information about the evolution of CPV-2c in Vietnam, which has resulted in distinct CPV-2c sublineages or independent lineages of new variants. The term “CPV-2c new var.” refers to Vietnamese CPV-2c isolates containing novel mutations.

In this study, 33 fecal samples were collected from February 2017 to June 2019 from dogs from eight provinces of northern and central Vietnam with hemorrhagic diarrhea and canine parvoviral infection symptoms. From these samples, complete VP2 sequences were obtained from different isolates, including three from February 2017, six from March 2017, three from August 2017, nine from March 2018, two
from May 2018, four from April 2019, and four from June 2019 (Table 1). The clinical samples included nine from Hanoi (21°01′42.5″N, 105°51′15.0″E), eight 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 and four from Nghe An (18°40′24.13″N, 105°41′32.35″E) and four from Ha Tinh (18°20′34.15″N, 105°54′20.48″E) in central Vietnam (Table 1; Supplementary Fig. S1). Fresh fecal samples from dogs with diarrhea were collected and stored at –20°C until use for genomic DNA extraction. The commercial multivalent vaccine Vanguard® Plus 5-CV (Zoetis, USA), which is widely used in Vietnam, was also collected for molecular genotyping. The infected dogs were puppies 6 weeks to 6 months old, and the majority were 3 to 4 months old. They included the following breeds, fox

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

| No. | Province         | Strain name | Year- month of isolation | Dog breed      | Age (m) | GenBank accession no. | Amino acid position | Genotype          |
|-----|------------------|-------------|--------------------------|----------------|---------|-----------------------|---------------------|-------------------|
| 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 terrier   | 2       | MW239578              | G P E I             | 2c-new           |
| 3   |                  | CPV6p-HN-VN | 2017-Mar                 | Fox terrier   | 3.5     | MW239579              | G P E M             | “2c-new-var.”   |
| 4   |                  | CPV7p-HN-VN | 2017-Mar                 | Fox terrier   | 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 terrier   | 3       | MW239582              | G P E I             | 2c-new           |
| 7   |                  | CPV10p-HN-VN| 2018-Mar                 | Fox terrier   | 4       | MW239583              | G P E I             | 2c-new           |
| 8   |                  | CPV20-HN-VN | 2019-Apr                 | Fox terrier   | 3.5     | MW239584              | G P E M             | “2c-new-var.”   |
| 9   |                  | CPV22-HN-VN | 2019-Apr                 | Fox terrier   | 3       | MW239585              | G P E M             | “2c-new-var.”   |
| 10  | 2. Thai Nguyen   | CPV14-TN-VN | 2017-Mar                 | Indigenous    | 5       | MW239586              | G P E I             | 2c-new           |
| 11  |                  | CPV15p-TN-VN| 2017-Mar                 | Fox terrier   | 4       | MW239587              | G P E M             | “2c-new-var.”   |
| 12  |                  | CPV16n-TN-VN| 2017-Mar                 | Poodle        | 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  | 3. Hung Yen      | ParHN51-HY-VN| 2017-Feb                 | Rottweiler    | NA      | MW239599              | G P E I             | 2c-new           |
| 19  |                  | ParHN11-HP-VN| 2017-Feb                 | Corgi         | 2       | MW239600              | G S E I             | 2c-new           |
| 20  |                  | ParHN31-HP-VN| 2017-Feb                 | Fox terrier   | NA      | MW239601              | G P E I             | 2c-new           |
| 21  | 4. Hai Phong     | PCV24-BN-VN | 2018-Mar                 | Pitt 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 terrier   | 3       | MW239598              | G P E M             | “2c-new-var.”   |
| 24  | 5. Bac Ninh      | PCV01-BG-VN | 2018-May                 | Border collie | 6       | MW239594              | G S E I             | 2c-new           |
| 25  |                  | PCV02-BG-VN | 2018-May                 | Fox terrier   | 2.5     | MW239595              | G S E I             | 2c-new           |
| 26  | 6. Bac Giang     | 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  | 8. Ha Tinh       | 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 breed   | 4       | MW239608              | G P E M             | “2c-new-var.”   |
| 33  |                  | PCV32-HT-VN | 2019-Jun                 | Mixed breed   | 4.5     | MW239609              | G P E M             | “2c-new-var.”   |
| 34  | *Vaccine         | Vanguard® Plus5 | 2018-Aug              |               |         | MW239610              | 2a                  |                  |

*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 subgenotype new variant CPV-2c, which contains an amino acid change from isoleucine (I) to methionine (M) at position 447 in VP2 (I447M).
terrier, Rottweiler, Pomeranian, corgi, Border collie, pit bull, dobermann, Malinois, service berger (hybrid), and German shepherd, as well as several indigenous Vietnamese breeds (Table 1). Although there was no detailed record of their vaccination status, almost all of the imported or mixed breeds (companion pets and service dogs) had been vaccinated with imported vaccines, usually with the Vanguard®Plus 5-CV multivalent vaccine.

Fecal samples or the contents of a vaccine vial were homogenized in sterile water and centrifuged at 13,000 rpm for 15 min. Total genomic DNA was extracted from the supernatants of the processed clinical samples or vaccine suspension using a 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 conserved sequences in the available CPV genome sequences. These included the forward primer CPF1 (5’-AGCTAA AAAGCCAATTGCTCC-3’; nt 2346–2366, coding frame numbering, the reverse primer CPR4 (5’-TATAGACGTAT ACGAGGCC-3’; nt 4555–4754), and two internal primers for sequencing, CPF2 (5’-ACAAACAGATCAATTGG AG-3’) and CPR3 (5’-GCATTACATGAACTTGG-3’). The PCR products (2229 bp) were sequenced directly or after cloning into the pCR2.1-TOPO TA-cloning vector (Invitrogen, USA) by a service company. The complete VP2 sequence of 1755 nucleotides obtained from each sample in this study (Table 1) and reference sequences from previous studies and from the GenBank database were used for molecular analysis.

An alignment of 109 complete VP2 nucleotide sequences was performed for sequence comparisons and phylogenetic analysis. This alignment included 34 sequences from this study (March 2017 to June 2019), 54 from previous studies (obtained in 1997, 2002, October 2013, 2017, and December 2016 to January 2018) and 22 reference strains (Table 1; Supplementary Table S1). The GENESE 2.7 program was used for alignment (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html), and MEGA X (www.megasoftware.net) was used for phylogenetic reconstruction using the maximum-likelihood (ML) method with 1000 replications of bootstrapping [39]. An alignment of the deduced amino acid sequences was used for genotypic and antigenic comparisons. From the alignment, 10 Vietnamese strains and three reference strains representing CPV vaccines, CPV-2, CPV-2a, CPV-2b, and CPV-2c variants were analyzed. A schematic representation of CPV epitopes involving β-strands and loops of the VP2 protein is shown in Fig. 1.

Identical amino acid sequences were obtained from four groups, consisting of 16, 12, three, and two isolates, respectively. Analysis of 34 VP2 amino acid sequences from this study indicated that 33 belonged to the “new” CPV-2c and one was the Vanguard®Plus 5-CV vaccine strain of CPV-2a (Zoetis). No CPV-2a and CPV-2b variants were found. The residues 80R, 87L, 93N, 101T, 103A, 232I, 267Y, 297A, 300G, 305Y, 323N, 324I, 334A, 341P, 370Q/R, 426D/E, 440T, 555V, 564S, and 568G, which are typical of Asian CPV-2c strains [reviewed in reference 2] were found in all of the isolates. These key residues substantially confirmed the status of the Asian “new” CPV-2c genogroup. However, the Vietnamese “new” CPV-2c strains also contained the mutations 5G, P13S, and I447M, which distinguishes them from previously described CPV-2c isolates. We suggest naming this new Vietnamese lineage “new-var. CPV-2c viruses” to distinguish these isolates from the “old” or “common” and “new” global and Asian CPV-2c isolates (Table 1; Supplementary Table S1). The Vanguard®Plus 5-CV multivalent vaccine used in Vietnam, sequenced in August 2018, was revealed to contain variations that distinguish them from the common and the “new/var.” CPV-2c strains in Vietnam (Supplementary Table S1). These variations within antigenic sites are likely to be responsible for the incomplete protection of Vietnamese dogs against the currently circulating virulent CPV-2c viruses by the vaccine. Vaccine failure or partial or incomplete protection has been observed in other countries as well [8–10, 40, 41].

To investigate the history of mutations that occurred in CPV-2 populations in Vietnam, we analyzed 88 currently available Vietnamese CPV-2a, CPV-b, and CPV-c sequences. These included 18 CPV-2a and CPV-b sequences and 70 CPV-2c sequences based on the authors’ reports, including 33 from this study and 38 from previous studies [12, 13, 20, 26] and from the GenBank database. However, amino acid analysis revealed some misidentified antigenic variants among these three variants. There were actually seven CPV-2a strains (having 426N), 14 CPV-2b strains (426D), and 67 CPV-2c strains (426E) [2], including four strains from references 12 and 13. Three (LCPV-V139/1997 [AB054222], LCPV-V140/1997 [AB054223], and LCPV-V203/1997 [AB054224]) were found to be CPV-2a, and the HNI-41/2002 strain (AB120727) was found to be CPV-2b variant instead of a 2c strain (Supplementary Table S1).

All 19 Vietnamese CPV-2a and CPV-b strains isolated before 2002 had 426N/D and 5A, 13P, and 447I [12, 13], whereas 66 CPV-2c strains from October 2013 to June 2019 had 5G, 13P/S, 38V/E, and 447I/M. The P13S mutation was found in only four strains from 2016 to 2017, and V38E was found in two strains from March 2018, whereas the novel 447M mutation was present in 38 strains isolated from December 2016 to June 2019 (Supplementary Tables S1 and S2). These 447M variants appear to have emerged in recent years through the continual evolution of CPV-2c derivatives from Vietnam. The 5G/447M mutants of CPV-2c “new var.” strains were found present at an average rate of 65.5% in the “new” CPV-2c population in Vietnam by our random
Fig. 1 Amino acid positions of the major epitope regions, β-barrels, loops, and epitope sites in the VP2 structural protein of field/vaccine reference strains and representative Vietnamese CPV strains. The month/year of isolation and antigenic group designations is shown to the right of each sequence. The top line is the amino acid sequence of the prototype strain 6us80-US of CPV-2 (GenBank no. EU659117). Identical residues in aligned sequences are indicated by dots, and differences are indicated by single letters. The B-cell epitope A region (aa 1–23) containing the peptide 2L21 (aa 1–21) [31] and the B-cell epitope B region (aa 38–498) containing β-barrels, βA, βB, βC, βD, βE, with their amino acids underlined and four loops, loop 1 (βA-C), loop 2 (βE-F), loop 3 (βG-H), and loop 4 (βG-H) [32, 34, 35] are marked on the top (see text for more details). The amino acid positions of 10 antigenic sites are underlined and labeled as E1 to E10 [33], and the core residues of the epitopes are shaded. The key variable amino acids determining different antigenic variants are indicated by arrows at the top. Positions where frequent and distinct amino acid changes occur and originating in and restricted to Vietnamese CPV isolates are boxed, and the position of the latest Vietnamese mutation I447M is indicated by a double arrow.
Fig. 2 A phylogenetic tree based on the analysis of 109 complete nucleotide sequences of the VP2 gene (1755 nucleotides) of CPV-2. The topology shows the relationships of 33 clinical isolates and a vaccine strain from this study (diamond symbol) and 53 other Vietnamese strains and 22 reference CPV strains of known or well-identified genotypes. In the Asia-CPV-2c cluster, 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 sublineage of Vietnamese CPV-2c strains. CPV-2c strains are identified by the presence of the 426E variation in VP2. The major groups are indicated. The phylogenetic tree was constructed in MEGA X using ML analysis based on the general time-reversible model with 1000 bootstrap replicates [39]. Strain designations include the name of the country where the virus was isolated, the year and month (if available) of isolation (in brackets), 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.

A phylogenetic analysis was performed based on the alignment of 109 complete VP2 sequences of CPV-2a, CPV-b, and CPV-c isolates and some vaccine strains of prototype CPV-2 developed in the 1980s (as an outgroup; the majority are listed in Table 1 and Supplementary Table S1). Eighty-eight Vietnamese CPV-2 sequences were phylogenetically clustered into two major complexes, called “mixed” (CPV-2a and CPV-b); and “Asia-2c” (2c-Asia). Within the “Asia-2c” complex, the majority of the isolates were Vietnamese “new-var.” CPV-2c (5G/426E/447M) strains, which were separate from the “new” CPV-2c (5G/426E/447I) strains. All Vietnamese isolates from 1997 and 2002 that comprised the CPV-2a, CPV-2b, the misidentified CPV-(2c?)/2a, and CPV-2b strains were placed in one cluster. All of the other “new” and “new-var.” CPV-2c isolates from 2013 and from 2016-2019 formed another cluster (Fig. 2; Supplementary Table S1).

In Vietnam, “mixed” CPV-2b and CPV-2c antigenic variants were discovered in 2002 (HNI-4-1; GenBank no. AB120727; [13]), which had the constellation 5A/267F/324Y/370Q/426E/447I and lacked the 5G/267Y/324I/370R pattern typically found in Asian and Vietnamese CPV-2c [42]. This strain was not included in the phylogenetic tree with the Asia-2c majority clade (Fig. 2), but it might be speculated to be one of the transforming variants representing viruses experiencing a genetic transition between the global CPV-2b and the Asian CPV-2c genotype.

This suggests that there was an early introduction of CPV-2a and CPV-b into Vietnam from the original global lineage, which might have served as an ancestral cluster for the generation of the subsequent “Asia-2c” and Vietnamese “new” and “new-var.” lineages (Fig. 2).

There have been many reports of circulating CPV-2c strains in various countries in Europe, Asia, North America, and South America [19, 21, 25]. CPV-2c strains are believed to belong to two major global lineages: world/global (VT) or Asian CPV-2c (Asia-2c) [26, 42]. The latter variants were shown to have originated from Asian countries and spread to European and African countries, i.e., Italy, Egypt, and Nigeria [19, 22, 23, 43]. The Asian CPV-2c strains were reported to have been circulating in India, China, Mongolia, Laos, Taiwan, Thailand, Korea, and Vietnam since 2010 [20, 24, 25, 42, 44, 45]. These Asian CPV-2c strains emerged in Asia, including the Vietnamese isolates that were grouped into the Asia-IV clade, which originated in 2005 and is clearly distinguishable from the global VT-III CPV-2c strains [26]. These latest data support the existence of two lineages of CPV-2 viruses worldwide and the formation of the distinct Vietnamese sublineage of CPV-2c, which continues to evolve and give rise to new variants within the CPV-2c population in Vietnam. Amino acid changes at antigenically important sites in VP2 not only lead to new sublineages or genogroups but may also affect the antigenicity, immunogenicity, and virulence of CPV-2 strains.

The universal naming system proposed by Nguyen et al. [26], using the groups VT-I, VT-II and VT-III for global strains and Asia-I, Asia-II, Asia-III, and Asia-IV for Asian CPV-2c strains, has not yet been fully transparent by sequence analysis. Two new major lineages with many subclades have appeared in the tree topology, thus requiring a reappraisal of the CPV-2 and CPV-2c groupings. The genotypic characteristics of the Asian and Vietnamese CPV-2c-“new” and “new-var.” isolates analyzed in various studies strongly support these groupings [20, 26, 42]. Clinical isolates from Vietnam during the period of 2016 to 2019 described in this study and previous studies formed a well-defined lineage, distinct from the established global and Asian classes, demonstrating the continuous evolution of multiple sublineages within the Asia-2c lineage. The CPV-2c populations, particularly the “new” and “new-var.” strains that have been circulating in Vietnam since their emergence in 1997–2019, appear to belong to a unique lineage, indicating the possibility that they evolved from a common ancestor.

In conclusion, VP2 sequence comparisons and phylogenetic analysis revealed the presence of two large groups of virulent CPV-2c strains in Vietnam: “new” and “new-var.”. They belong to distinct, independent Vietnamese sublineages within the well-defined Asian CPV-2c population. The dominance of the novel 5G/447M CPV-2c mutants was
observed from 2016 to 2019, when they seemed to replace the 5G/447I strains. Amino acid changes in antigenic sites that alter the genetic characteristics of the members of the newly formed Asia-2c, and "new" and "new-var." CPV-2c groups might also cause vaccination failure in dogs in Vietnam and other countries.

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Availability of data and materials  The datasets generated and/or analyzed in the current study are available in the GenBank database, and the online Supplementary materials and can also be made available by the corresponding author upon reasonable request.

Declarations  

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 collected with legal informed consent of the owners.

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