Molecular and phylodynamic analysis of Vietnamese canine parvovirus 2C isolated from dogs reveals a new Asia-IV clade

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Abstract

Canine parvovirus type 2 (CPV-2) is a small, single-stranded DNA virus causing fatal hemorrhagic enteritis in dogs. Currently, CPV-2 has been classified into CPV-2a, CPV-2b, and CPV-2c based on genetic variation in the VP2 gene. The CPV-2c variant has become ubiquitous worldwide and gained attention for monitoring parvoviral evolution. In this study, we characterized the full-length genome sequences of CPV-2c isolates obtained from 59 dogs in Vietnam. Molecular analysis revealed that Vietnamese CPV-2c shared a common evolutionary pattern with the Asian CPV-2 clade, which is marked by genetic signature patterns in the structural and nonstructural proteins. In addition, these Vietnamese CPV-2c strains exhibited unique Thr112Ile and Ile447Met mutations in the VP1 and VP2 sequence, respectively. Interestingly, phylogenetic analysis indicated that the mutations of amino acid residues in both the structural and nonstructural genes have contributed to the emergence of a new clade, designated here as the Asia-IV clade. The substitution rates, estimated from a dataset containing 199 sequences over the last 40 years, confirmed that CPV-2 showed a high rate of nucleotide substitution, at about 2.49 x 10-4 nucleotide substitutions per site per year (nt/s/y), with VP1/2 and NS1/2 estimates of 3.06 x 10-4 and 3.16 x 10-4 nt/s/y, respectively. Even though no evidence of genetic recombination in these Vietnamese CPV-2c strains was established, potential positive selection sites were observed in both the structural and nonstructural genes, suggesting the viral evolutionary process has occurred in both the structural and nonstructural proteins. Genetic and evolutionary analysis of the full-length genome sequence is necessary to gain evolutionary insight of CPV-2. Further studies are needed to elucidate the potential role of these observed mutations in the novel Asia-IV clade.

Original article

Molecular and phylodynamic analysis of Vietnamese canine parvovirus 2C isolated from dogs reveals a new Asia-IV clade

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Running title: CPV-2c variant in Vietnam clustered in Asia-IV

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Summary

Canine parvovirus type 2 (CPV-2) is a small, single-stranded DNA virus causing fatal hemorrhagic enteritis in dogs. Currently, CPV-2 has been classified into CPV-2a, CPV-2b, and CPV-2c based on genetic variation in the VP2 gene. The CPV-2c variant has become ubiquitous worldwide and gained attention for monitoring parvoviral evolution. In this study, we characterized the full-length genome sequences of CPV-2c isolates obtained from 59 dogs in Vietnam. Molecular analysis revealed that Vietnamese CPV-2c shared a common evolutionary pattern with the Asian CPV-2 clade, which is marked by genetic signature patterns in the structural and nonstructural proteins. In addition, these Vietnamese CPV-2c strains exhibited unique Thr112Ile and Ile447Met mutations in the VP1 and VP2 sequence, respectively. Interestingly, phylogenetic analysis indicated that the mutations of amino acid residues in both the structural and nonstructural genes have contributed to the emergence of a new clade, designated here as the Asia-IV clade. The substitution rates, estimated from a dataset containing 199 sequences over the last 40 years, confirmed that CPV-2 showed a high rate of nucleotide substitution, at about 2.49 x 10^{-4} nucleotide substitutions per site per year (nt/s/y), with VP1/2 and NS1/2 estimates of 3.06 x 10^{-4} and 3.16 x 10^{-4}nt/s/y, respectively. Even though no evidence of genetic recombination in these Vietnamese CPV-2c strains was established, potential positive selection sites were observed in both the structural and nonstructural genes, suggesting the viral evolutionary process has occurred in both the structural and nonstructural proteins. Genetic and evolutionary analysis of the full-length genome sequence is necessary to gain evolutionary insight of CPV-2. Further studies are needed to elucidate the potential role of these observed mutations in the novel Asia-IV clade.

Keywords: Asia-IV subclade, canine parvovirus-2c, dog, evolution, phylodynamic, Vietnam

1. Introduction

In the late 1970s, canine parvovirus (CPV) emerged as a highly contagious virus that causes severe hemorrhagic enteritis in dogs (Carman & Povey, 1985; Kelly, 1978). The CPV origin was designated as CPV type 2 (CPV-2) to distinguish it from canine minute virus or CPV type 1. It is well-established that the emergence of CPV-2 resulted from the site-specific mutation of feline panleukopenia virus or FPV-like virus to gain the ability for replication in canine cells (Chang, Sgro, & Parrish, 1992; Truyen & Parrish, 1992). This evidence indicates the role of cross-species transmission. Thus, the emergence of CPV-2 variants has gained wide attention for studying its structure, phylogenomic, and evolutionary dynamics for disease quarantine, monitoring, and prevention.

The CPV-2 is a small, non-enveloped, single-stranded DNA virus with two main open reading frames (ORFs). The first ORF encodes two nonstructural proteins (NS1 and NS2) that are responsible for viral replication, pathogenicity, and cytotoxicity. The second ORF encodes two structural proteins (VP1 and VP2), which are related to the viral tropism and antigenicity (Parker & Parrish, 1997; Reed, Jones, & Miller, 1988; D. Wang, Yuan, Davis, & Parrish, 1998). Among the two structural proteins, VP1 has the most abundant composition in the capsid, while changes in the amino acid sequences of VP2 may alter the antigenic properties of the virus. Thus, most studies have focused on the description of nucleotide mutations in the VP2 gene, while information on the nucleotide substitution and evolution in the nonstructural proteins is limited.

Based on the VP2 sequence, CPV-2 is divided into the three variants, CPV-2a, CPV-2b, and CPV-2c, which have recently co-circulated in dog populations worldwide. The CPV-2a variant differs from the original CPV-2 by five amino acid mutations (Met87Leu, Ile101Thr, Ala300Gly, Asp305Tyr, and Val555Ile), while CPV-2b reveals a new mutation in residue Asn426Asp. Until now, the CPV-2a and CPV-2b variants replaced the

original CPV-2 since the 1980s (Parrish et al., 1991; Parrish et al., 1988), whereas the last variant of CPV-2, CPV-2c, firstly emerged in Italy in 2001 (Buonavoglia et al., 2001). The CPV-2c variant has a specific 426Glu mutation in the VP2 gene. In recent years, CPV-2c has globally spilled over in many geographic regions and currently causes fatal disease in both domestic and wild dog populations (Aldaz et al., 2013; Decaro & Buonavoglia, 2012; Geng et al., 2015; Parthiban, Mukhopadhyay, Antony, & Pillai, 2010).

The CPV-2c variant was first identified in Vietnam in 2002, which was the first report in Asia (Nakamura et al., 2004). Later, this variant was identified in dogs from other Asian countries, including China, India, Taiwan, Indonesia, Laos, and Thailand (Charoenkul et al., 2019; Chiang, Wu, Chiou, Chang, & Lin, 2016; Geng et al., 2015; Nandi, Chidri, Kumar, & Chauhan, 2010; Vannamahaxay et al., 2017). These recent studies have described the molecular characteristics of the VP2 gene in Asian CPV-2c, but the full-length genome characterization of CPV-2c in Asia has not been reported. In this study, we performed molecular and phylodynamic analyses of the full-length genome of CPV-2c variants isolated from domestic dogs in Vietnam.

2. Materials and Methods

2.1 Sample collection and nucleic acid extraction

A total of 59 fecal swabs were collected from dogs in Vietnam residing in Hanoi (n = 19), Da Nang (n = 16), and Ho Chi Minh (n = 24) cities during July 2017 to August 2018. Samples were primarily tested for parvovirus and coronavirus infection using the rapid ELISA testkit (Bionote, South Korea). Essential information of the animals, including sex, age, breed, and vaccination status was also recorded (Supplementary Table S2). The swab was immersed in 0.5 mL sterile phosphate buffer saline pH 7.4 and stored at -80 ^{0}C until used. Viral DNA was extracted using a viral DNA/RNA extraction kit II (Geneaid Biotech, Taiwan) following the manufacturer's recommendations.

2.2 Polymerase chain reaction (PCR) and sequencing analysis

Extracted viral nucleic acids were subjected for parvovirus PCR detection using specific primers targeting the VP2 gene (Supplementary Table S1) as reported (Mochizuki et al., 1996). The PCR reaction contained 3 µL of DNA template, 1 µL of each primer (10 µM; VPF and VPR), 12.5 µL of Gotaq Green Master mix (Promega, USA), and nuclease-free water to 25 µL. The thermal cycling program was comprised of an initial 94 ^{0}C for 10 min, followed by 35 cycles of 94 ^{0}C for 30 s, 55 ^{0}C for 2 min, and 72 ^{0}C for 2 min, and then a final 72^{0}C for 10 min. The PCR products were resolved on a 1.5% (w/v) agarose gel and then visualized under UV illumination. Positive PCR products were purified using a NucleoSpin Extract II (Macherey-Nagel, Germany) and submitted for commercial bi-directional Sanger's sequencing (Macrogen, Korea). Subsequently, the selected CPV-2 PCR positive samples were further subjected for whole genome sequencing using multiple primer sets, as described previously (Perez et al., 2014), under the same thermal cycling condition as mentioned above (Supplementary Table S1).

The generated sequences were aligned by the MAFFT (Multiple Alignment using Fast Fourier Transform program (https://mafft.cbrc.jp) and compared to those CPV-2 sequences available in GenBank. These alignments were then subjected for nucleotide and deduced amino acid sequence analyses as implemented in the BioEdit software package version 7.2 (http://www.mbio.ncsu.edu)

2.3 Phylogenetic and evolutionary analyses

Eleven full-length genome sequences derived from this study were analyzed and compared with the dataset of 188 full-length genome sequences of CPV-2, originally isolated from domestic dogs during 1979 to 2017, available in the GenBank database (Supplementary Table S4). The substitution rate of the full-length genome sequences and each individual structural and nonstructural gene coding region was estimated as the number of nucleotide substitutions per site per year (nt/s/y), implemented in the Bayesian Evolutionary Analysis Sampling Trees (BEAST) version 1.8.4 software (http://tree.bio.ed.ac.uk/) . The rates were calculated based on the two main phylogenetic clades of the CPV-2 strains discovered in Asia (Asian clade) and the CPV-2 strains reported in Europe and America (Western clade). The best-fit model of substitution (HKI + I + G4) was

determined using the model section function in the MEGA 7.0 software (*https://www.megasoftware.net/*). The coalescent Bayesian skyline was applied under exponential relaxed-clock models (Drummond & Rambaut, 2007; Li et al., 2017). The Markov Chain Monte Carlo (MCMC) algorithm was run for 200-million generations and logged every 10,000 states. The convergence of all parameters was checked by observing that the effective sample size (ESS) > 200 with TRACER version 1.6.1. The maximum clade credibility (MCC) tree was obtained from the posterior distribution of trees using TreeAnnotator version 1.8 after discarding 10% of steps as burn-in. The phylogenetic tree with timeline estimated divergences, posterior probability (PP) and 95% highest probability density (HPD) values were generated using FigTree version 1.4.2.

2.4 Recombination analysis

In order to find potential natural genetic recombination events in the evolution of the Vietnamese CPV-2 strains, the dataset of CPV-2 genomes used in the evolutionary analysis (section 2.3) were further subjected to recombination analysis using various statistical methods, including recombination detection program (RDP), GENECONV, BootScan, MaxChi, Chimaera, SiScan, and 3Seq with the default settings in the RDP package version 4.0 (Piewbang et al., 2018). Because many recombination signals would be detected during the test run and the different algorithms might be inconsistent, then the acceptance criteria of any potential breakpoint signal must be revealed by at least four methods with$p$ -values < 0.01 was applied to recognize any potentially positive recombination event.

2.5 Selective pressure analysis

To determine whether the high rate of nucleotide substitutions might result in the rapid adaptation of the CPV-2, selective pressure tests were assessed on the individual NS and VP genes. Non-neutral selection of nucleotide substitutions was calculated using the ratio between nonsynonymous (dN) and synonymous (dS) substitutions, as assessed by phylogenetic reconstruction using the maximum likelihood (ML) model with general reversible nucleotide substitution, available on the Datamonkey web server (http://www.datamonkey.org). Non-neutral selection was implemented using different models for pervasive individual site measurement, including the single-likelihood ancestor counting (SLAC) and fixed-effects likelihood (FEL) on the HyPhy software package. A p -value of 0.1 was set as significant in all the described methods. The Bayes factor was set at 50 to estimate the rate of dN and dS within each individual codon. Positive selection (adaptive molecular evolution), neutral mutations, and negative selection (purifying selection) were defined as dN/dS > 1, dN/dS $= 1$, and dN/dS < 1, respectively.

3. Results

3.1 High prevalence of the CPV-2c variant in Vietnam

All 59 sampled dogs were found to be CPV-2 PCR positive and confirmed by sequencing a partial fragment of the VP2 gene. There were no breed and sex predilections on the affected dogs, while their ages ranged from 2–9 month-old, with most (86.4%) being 2–5 months-old. Even though 11 (18.6%) dogs had been completely vaccinated against parvovirus, their samples were still positive in the CPV-2 PCR assay. In addition, almost all the dogs (58/59; 98.3%) showed a positive parvovirus infection with the rapid testkit, the single exception being a dog from Hanoi (HN-62) that was positive for both parvovirus and coronavirus (Supplementary Table S2).

Analysis of the deduced amino acid sequence of the VP-2 gene showed that the most of samples (58/59; 98.3%) carried residue 426Glu, and so were classified as CPV-2c variants. However, one sample, derived from a dog in Ho Chi Minh (HCM-16), showed amino acid residue 426Asn, and so was designated as a CPV-2a variant. No CPV-2b variant was detected in this study (Supplementary Table S2).

3.2 Similarity of the full-length genome, nucleotide and amino acid sequence of NS and VP regions of Vietnamese CPV-2c

The complete full-length genetic sequences of various Vietnamese CPV-2, ten CPV-2c (HN04, HN06, HN07, HN08, HN30, DN01, DN02, HCM01, HCM03, and HCM09) and one CPV-2a (HCM16) strains were selected

for sequencing using multiple primer pairs for parvovirus to cover the whole genome. The obtained full-length genome sequences of the Vietnamese CPV-2 variants were submitted to GenBank (accession nos. MT106228–38). Sequence analysis showed that the Vietnamese CPV-2c strains were highly similar among strains, ranging from 99.5–99.8% in nucleotide similarity (Table 1, Supplementary Table S3a). Neither nucleotide differences nor specific amino acid mutations were evident among the Vietnamese CPV-2c strains detected from different geographic regions.

The complete genomes of the Vietnamese CPV-2c strains from this study were found to be closely related to the recently isolated (in 2017) Italian CPV-2c strain (MF510157) and the Chinese CPV-2a strain (KR002800) isolated in 2014, with 99.6–99.8% and 99.3–99.5% nucleotide identity, respectively. Meanwhile, the Vietnamese CPV-2c strains were genetically distant from the previous Italian CPV-2c strain (KU508407) isolated in 2009 and the single Vietnamese CPV-2a variant isolated in this study, with 98.8–98.9% and 98.6–98.7% nucleotide identity, respectively (Table 1, Supplementary Table S3a).

In addition, the nucleotide and amino acid sequence of the NS region were similar between these Vietnamese CPV-2c strains, and the Italian CPV-2c (MF510157) and Chinese CPV-2a (KR002800) strains, ranging from 99.7–100% similarity (Table 1, Supplementary Table S3b). Meanwhile, there were different nucleotide and amino acid sequence similarities in the VP region, accounting for 99.4–99.6% and 99.5–99.7%, respectively, compared with MF510157, and 98.9–99.1% and 99.0–99.3%, respectively, compared with KR002800. Whereas the Vietnamese CPV-2c strains showed a lower degree of sequence similarity compared to KU508407 and the single Vietnamese CPV-2a strain from this study (Table 1, Supplementary Table S3c).

3.3 Specific amino acid mutations in the NS and VP proteins of Vietnamese CPV-2c

Analysis of the deduced amino acid sequences of the NS1/NS2 genes revealed that all the Vietnamese CPV-2c strains presented the Ile60Val, Tyr544Phe, Glu545Val, and Leu630Pro mutations that were similar to the residues in the Italian CPV-2c (MF510157) and Chinese CPV-2a (KR002800) strains, but were not observed in the Vietnamese CPV-2a (HCM16) strain (Table 2).

Analysis of the deduced amino acid residues of the structural sequence revealed that all the Vietnamese CPV-2c and CPV-2a strains from this study contained the Arg116Lys, Leu125Ile, and Ala131Thr mutations in the VP1 sequence, and the Ala5Gly, Phe267Tyr, Tyr324Ile, and Gln370Arg mutations in VP2 in all Vietnamese CPV-2c strains, but no changes in Ala5Gly and Gln370Arg were seen in the Vietnamese CPV-2a strain. Interestingly, six Vietnamese CPV-2c variants (HN04, HN08, HN30, HCM01, HCM03, and HCM09) had the Thr112Ile and Ile447Met mutations in VP1 and VP2, respectively (Table 2).

3.4 Distinctive amino acid mutations in Vietnamese CVP-2c creating a new Asia subclade

Phylogenetic analysis of the 199 full-length CPV-2 genome sequences, including the 11 sequences from this study, revealed that the CPV-2 lineages separated into two main clades. The first clade consisted of sequences from Europe and America (Western clade), while the other clade was comprised of most of the strains from Asian countries (Asian clade), where the VP2 residue 324 is 324Ile in the Asian clade and 324Tyr in the Western clade (Table 3).

The most recent common ancestor (MRCA) of the Western clade was estimated to be in 1982, while the MRCA of the Asian clade was estimated to be in 1994 (Figure 1). Later, in 1986, the Western clade was further divided into the Western-I (WT-I; including the CPV-2a/b strains from Brazil, Argentina, Uruguay, and Germany) and Western-II (WT-II; including the CPV-2a/b/c strains from Italy, Germany, Ecuador, Brazil, USA, Canada, Japan, and New Zealand), based on the mutation of NS1 residue Tyr544Phe. By 1996, the Western-III (WT-III) sub-clade, which was composed of only CPV-2c strains from Italy, Argentina, Uruguay, Paraguay, Brazil, Australia, Ecuador, France, and Albania, emerged and was well-defined by the change in the VP2 residue to either Asn426Glu or Asp426Glu.

In the Asian clade, the majority of the CPV-2 isolates expressed Phe267Tyr in VP2, forming an Asia-II subclade in 1998, which included CPV-2a strains from Vietnam (HCM16 in this study), China, and Uruguay, and a few strains that carried Phe 267 but clustered in the Asia-I subclade (CPV-2a isolated in

China, India, and Canada). Thereafter, CPV-2 sequences further separated into Asia-III (CPV-2a/b strains from China and Uruguay) based on the Tyr544Phe and Glu545Val mutations in the NS1 gene in 2002. Interestingly, the most recent mutations of NS1 residues at Ile60Val, Leu630Pro, and Ala5Gly in VP2 in the Vietnamese CPV-2c (10 strains from this study), Chinese CPV-2a (KR002800), and Italian CPV-2c (MF510157) established a new Asian subclade since 2005, named here as Asia-IV (Tables 2 and 3, Figure 1, Supplementary Table S4).

3.5 High substitution rates for CPV-2 without evidence of recombination

We investigated the substitution rate of CPV-2 based on the 199 full-length genome sequences. The mean substitution rate was found to be 2.49 x 10^{-4} nt/s/y, and similar rates were estimated for individual genes coding for the nonstructural and structural proteins at 3.16 x 10^{-4} and 3.06 x 10^{-4} nt/s/y, respectively. Since the phylogenetic tree divided the CPV-2 into Asian and Western clades, the substitution rate was estimated for each clade, suggesting the Asian CPV-2 evolutionary rate at 2.12 x 10^{-4} nt/s/y was faster than that in the Western clade at 1.69 x 10^{-4} nt/s/y (Table 4).

To further explore possible recombination in Vietnamese CPV-2 evolution, we investigated the recombination analysis in all detected Vietnamese CPV-2 genomes compared to the other CPV-2 strains in GenBank using the RDP method. However, no recombination breakpoints were found in any of the Vietnamese CPV-2 strains.

3.6 CPV-2 undergoes negative selective pressure on its evolution

The dN/dS ratio was determined from the alignment of the individual NS1/NS2 and VP1/VP2 genes from the available CPV-2 genomes. Both SLAC and FEL analyses indicated that overall the CPV-2 gene has undergone negative selective pressure (dN/dS < 1), but potentially positive selection sites were also evident in both the NS1/NS2 and VP1/VP2 genes (Supplementary Table S5).

4. Discussion

CPV-2c strains have been reported at a high prevalence in dogs in many geographic regions in Europe, America, and Asia, and specifically in Vietnam (Geng et al., 2015; Hoang et al., 2019; Kang et al., 2008; Ohshima et al., 2008). In this study, we identified CPV-2c infections at a high incidence (98.3%), which is concordant with a previous study of CPV in Vietnam (Hoang et al., 2019). Previous genetic characterization and analysis has been based on the individual VP genes, whereas this study attempted to characterize the whole genome of CPV-2c isolates obtained from 59 Vietnamese dogs.

In this study, Vietnamese CPV-2c presented the unique non-synonymous Thr112Ile and Ile447Met mutations in the VP1 and VP2 genes, respectively. These two novel mutations are seemingly currently restricted to Vietnam. Thus, further studies are needed to monitor these mutations and elucidate their impact on the pathogenicity and virulence of CPV-2c. In addition, the common mutations that have been widely reported in other Asian CPV-2 strains, such as Phe267Tyr, Tyr324Ile, and Gln370Arg, and the recent Ala5Gly mutation, were observed in the VP2 sequence of all Vietnamese CPV-2c strains. Interestingly, these mutations were not observed in the previous CPV-2c HNI-4-1strain (AB120727), which was firstly isolated in Vietnam in 2002 (Nakamura et al., 2004). Moreover, these CPV-2c isolates exhibit the Ile60Val, Tyr544Phe, Glu545Val, and Leu630Pro mutations in the NS1 gene and the Arg116Lys, Leu125Ile, and Ala131Thr in the VP1 genes, which are similar to those described in Chinese and Taiwanese CPV-2 isolates (Chiang et al., 2016; J. Wang et al., 2016; Wu, Li, Wang, Liu, & Tian, 2018). These findings indicated that Vietnamese CPV-2c strains likely shared a common evolutionary pattern in both their nonstructural and structural proteins with other CVP-2 variants, including the Italian CPV-2c (MF510157) that was isolated from a puppy imported from Thailand to Italy in 2017.

The phylogenetic analysis showed that the mutation of 324Ile in the VP2 gene, which has been frequently observed in the most recent CPV-2 isolates in Asia (Geng et al., 2015; Kang et al., 2008; Mukhopadhyay et al., 2014; Phromnoi, Sirinarumitr, & Sirinarumitr, 2010; Soma, Taharaguchi, Ohinata, Ishii, & Hara, 2013; H. Zhao et al., 2017; Y. Zhao, Lin, Zeng, Lu, & Hou, 2013), plays a role as the hallmark amino acid to

separate the Asian CPV-2 clade from the Western counterpart. In addition, the prevalence of CPV-2 strains carrying 267Tyr is increasing in Asia (Chiang et al., 2016) as the next step of the evolution process in the CPV-2 subclade. Thus, the 267Tyr and 324Ile mutations of VP2 may serve at present as genetic markers for the Asian CPV-2 strains.

Apart from the amino acid changes in VP1/VP2, mutations in NS1/NS2 might represent the emergence of a subclade in the phylogeny by sharing common characterizations. In the WT-II group, all strains had the Tyr544Phe genotype in NS1, similar to that in a previous study (Grecco et al., 2018). In addition, the Tyr544Phe and Glu545Val mutations in the NS1/NS2 were present in the Asia-III and -IV subclades, and residues Ile60Val and Leu630Pro were in the Asia-IV subclade. These findings suggest that these mutations in NS1/NS2 might play a role in the emergence of new variants. Future studies on genomic analysis, including the NS1/NS2 genes, should be conducted to be better understanding the viral evolution.

Although CPV-2 is a DNA virus, it has been reported (and observed in this study) to have a high nucleotide substitution rate, perhaps as rapid as that found in RNA viruses (Shackelton, Parrish, Truyen, & Holmes, 2005). More retrieved sequences could lead to a greater precision on the substitution rate estimation. The substitution analysis in this study suggested that the CPV-2 genome had a high background mutation rate of 2.49 x 10^{-4} nt/s/y. Notably, the individual NS and VP gene analysis showed a similar substitution rate, suggesting that viral evolution of CPV-2 may not only be observed in the structural proteins, which are associated with immune escape and cellular tropism, but also in the nonstructural proteins. In addition, we found that the evolutionary rate of the Asian clade was higher than that for the Western clade. Further studies are needed to verify this observation and search for the factors that alter the evolutionary rate in the Asian group. Interestingly, the selective pressure analysis in this study revealed that even though most of the CPV-2 has undergone negative selection, there were potential positive selection sites located in both the NS and VP genes. Thus, the function of mutation(s) in the nonstructural protein needs to be verified and might be interesting for further study of CPV evolution.

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Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received by the Chulalongkorn University Animal Care and Use Committee (No. 2031005).

Conflict of interests

The authors declare no conflict of interests.

Data availability statement

The data that support the findings of this study are openly available online at doi:

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Tables

Table 1 Nucleotide and amino acid similarity (%) of Vietnamese CPV-2 and reference strains

Strain	**Accession No**		**Full genome**	**NS gene**	**VP gene**
Italian CPV-2c, 2009^{+}	KU508407	Nucleotide	98.8–98.9	99.0–99.1	98.8–98.9
		Amino acid		99.2–9.4	98.7–99.0
Italian CPV-2c, 2017^{+}	MF510157	Nucleotide	99.6–99.8	99.7–100	99.4–99.6
		Amino acid		99.8–100	99.5–99.8
Chinese CPV-2a, 2016^{+}	KR002800	Nucleotide	99.3–99.5	99.7–100	98.9–99.1
		Amino acid		99.7–100	99.0–99.3
Vietnamese CPV-2c, 2017^{++}	MT106228-MT106237	Nucleotide	99.5–99.8	99.6–99.9	99.5–99.9
		Amino acid		99.5–100	99.7–100
Vietnamese CPV-2a, 2017^{++}	MT106238	Nucleotide	98.6–98.7	98.7–98.9	98.5–98.7
		Amino acid		98.2–98.5	98.7–99.0

$^{+}$ Year that the virus was isolated

$^{++}$ Sequences in this study

Table 2 Non-synonymous nucleotide and amino acid variations in the NS1/NS2 and VP1/VP2 genes

Nucleotide/amino acid position	**Nucleotide/amino acid position**	**Nucleotide/amino acid position**	**Nucleo**
Strain	**Genome type**	**NS1/NS2**	**NS1/N**
		178	**1631**
		60	**544**
KU508407^{+}	CPV-2c	A/Ile	A/Tyr
MF510157^{+}	CPV-2c	G/Val	T/Phe
KR002800^{+}	CPV-2a	G/Val	T/Phe
HN04	CPV-2c	G/Val	T/Phe
HN06	CPV-2c	G/Val	T/Phe
HN07	CPV-2c	G/Val	T/Phe
HN08	CPV-2c	G/Val	T/Phe
HN30	CPV-2c	G/Val	T/Phe
DN01	CPV-2c	G/Val	T/Phe
DN02	CPV-2c	G/Val	T/Phe
HCM01	CPV-2c	G/Val	T/Phe
HCM03	CPV-2c	G/Val	T/Phe
HCM09	CPV-2c	G/Val	T/Phe
HCM16	CPV-2a	A/Ile	A/Tyr

$^{+}$ Reference strains

Table 3 Amino acid signatures of the NS1 and VP2 proteins for evolutionary analysis

Clade	Subclade	NS1	NS1	NS1	NS1	VP2	VP2	VP2
		60	544	545	630	267	324	426
CPV-2 origin	CPV-2	Ile	Tyr	Glu	Leu	Phe	Tyr	Asn
Western	WT-I	Ile	Tyr	Glu	Leu	Phe	Tyr/Leu	Asp/Asn
	WT-II	Ile	Phe	Glu	Leu	Phe	Tyr/Leu	Asn/Asp/Glu
	WT-III	Ile	Tyr	Glu	Leu	Phe	Tyr	Glu
Asian	Asia-I	Ile	Tyr	Glu	Leu	Phe	Ile/Tyr	Asn
	Asia-II	Ile	Tyr	Glu	Leu	Tyr	Ile	Asn
	Asia-III	Ile	Phe/Tyr	Val/Glu	Leu	Tyr	Ile	Asn/Asp
	Asia-IV	Val	Phe/Tyr	Val/Glu	Pro	Tyr	Ile	Glu/Asn

Table 4 Summary statistics of the nucleotide substitution rates

Data and ML estimates	Full-length	NS1/NS2	VP1/VP2	Asian clade	Western clade
Number of sequences	199	199	199	59	135
Year of collected sequences	1978–2017	1978–2017	1978–2017	2004–2017	1991–2017
Length of sequence (bp)	4269	2007	2184	4269	4269
Mean (nt/s/y)$^{+}$	2.49E-04	3.16E-04	3.06E-04	2.12E-04	1.69E-04
SEM	2.23E-06	2.12E-06	2.06E-06	2.53E-06	1.52E-06
SD	3.31E-05	4.57E-05	4.25E-05	5.70E-05	2.68E-05
Variance	1.10E-09	2.08E-09	1.80E-09	3.25E-09	7.16E-10
Median	2.47E-04	3.13E-04	3.03E-04	2.07E-04	1.68E-04
95% HPD Min	1.87E-04	2.32E-04	2.27E-04	1.06E-04	1.20E-04
95% HPD Max	3.12E-04	4.10E-04	3.90E-04	3.25E-04	2.24E-04
ESS	220.2191	462.9358	425.3558	509.4852	311.6939

$^{+}$Nucleotide/site/year

Figure legends

Figure 1. Phylogenetic tree (ML) with timeline estimated divergences based on 199 full-length genomes of CPV-2 isolated from 1978 to 2017. Evolutionary clades are indicated as Asia and Western (WT) with their subclades as Asia-I to -IV, and WT-I to -III. The strains are colored according to the CPV genotype. * Represents the CPV strains from this study.