GENETIC CHARACTERIZATION OF CANINE PARVOVIRUS AND CIRCOVIRUS IN DOGS IN VIETNAM

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ลักษณะทางอณูชีวโมเลกุลและวิวัฒนาการทางพันธุกรรมของเชื้อพาร์โวไวรัสและเซอร์โคไวรัสในสุนัขในสาธารณรัฐสังคมนิยมเวียดนาม

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Thesis Title	GENETIC CHARACTERIZATION OF CANINE PARVOVIRUS AND CIRCOVIRUS IN DOGS IN VIETNAM
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เหงียน มานห์ เตือง : ลักษณะทางอณูชีวโมเลกุลและวิวัฒนาการทางพันธุกรรมของเชื้อพาร์โวไวรัสและเซอร์โคไวรัสในสุนัขในสาธารณรัฐสังคมนิยมเวียดนาม. (GENETIC CHARACTERIZATION OF CANINE PARVOVIRUS AND CIRCOVIRUS IN DOGS IN VIETNAM) อ.ที่ปรึกษาหลัก : สมพร เตชะงามสุวรรณ, อ.ที่ปรึกษาร่วม : อนุเทพ รังสีพิพัฒน์

ไวรัสพาร์โวในสุนัข (Canine parvovirus-2, CPV-2) **เริ่มระบาดและก่อโรคลำไส้อักเสบรุนแรงในสุนัขตั้งแต่ปี ค.ศ**. 1970 **สำหรับสายพันธุ์ล่าสุด** CPV-2c **ที่มีการระบาด ถือเป็นสายพันธุ์ที่มีความรุนแรงและพบการแพร่กระจายทั่วโลก และมีการรายงานการพบ** CPV-2c **ครั้งแรกในประเทศเวียดนามตั้งแต่ปี ค.ศ**. 2002 **แต่ยังพบว่ายังขาดข้อมูลจีโนมของไวรัส ดังนั้น ในการศึกษาครั้งนี้ ทางผู้วิจัยได้ทำการศึกษาข้อมูลจีโนมไวรัสทั้งตัวของ** CPV-2c **ที่แยกได้จากสุนัขป่วยเป็นโรคลำไส้อักเสบในประเทศเวียดนาม และรายงานข้อมูลเป็นครั้งแรกของ** CPV-2c **ที่ได้จากทวีปเอเชีย ผลการวิเคราะห์พบว่า** CPV-2c **ที่แยกได้ในครั้งนี้มีการกลายพันธุ์ในตำแหน่งที่พบได้บ่อยทั้งในส่วนของโปรตีนโครงสร้างและที่ไม่ใช่โครงสร้าง และมีรูปแบบของวิวัฒนาการของไวรัสเหมือนกับสายพันธุ์อื่นที่แยกได้จากประเทศในแถบเอเชีย นอกจากนี้ สายพันธุ์ของประเทศเวียดนามมีการแสดงออกของการกลายพันธุ์ในตำแหน่งใหม่และเมื่อศึกษาทางแผนภูมิวัฒนาการต้นไม้มีการสร้างเคลดใหม่ร่วมกับสายพันธุ์จากประเทศจีน จึงให้ชื่อว่า** “Asia-IV” **ทำการประเมินอัตราการสลับเปลี่ยนของนิวคลีโอไทด์ของไวรัสจากฐานข้อมูลที่มีจำนวนซีเควนท์รวมทั้งสิ้น** 199 **ซีเควนท์ จากทั้งหมด** 42 **ปีตั้งแต่เริ่มมีการะบาดของไวรัส พบว่ามีอัตราการสลับเปลี่ยนสูงซึ่งถือได้ว่า** CPV-2 **เป็นไวรัสที่มีอัตราการกลายพันธุ์ได้รวดเร็วถึงแม้เป็นดีเอ็นเอไวรัสสายเดี่ยว และยังมีการติดเชื้อร่วมกับไวรัสอื่นที่ก่อโรคในระบบทางเดินอาหารสูงอีกด้วย ซึ่งในครั้งนี้ พบว่า ไวรัสเซอร์โคในสุนัข** (Canine circovirus, CanineCV) **เป็นไวรัสที่ก่อโรคร่วมกับไวรัสพาร์โวในสุนัขในเวียดนามสูงถึงร้อยละ** 19.7 **ทำการศึกษาลักษณะทางพันธุกรรมของ** CanineCV **แบบทั้งจีโนม ผลการศึกษาทางแผนภูมิวิวัฒนาการต้นไม้ พบว่า** CanineCV **สามารถจำแนกออกได้ทั้งหมด** 4 **จีโนไทป์** (CanineCV-1, -2, -3 **และ** -4) **ด้วยลักษณะการแทนที่กรดอะมิโนในตำแหน่งที่จำเพาะในโปรตีน** Replicase **และ** Capsid **ผู้วิจัยพบการแพร่กระจายร่วมกันของ** CanineCV 2 **จีโนไทป์ คือ** CanineCV-1 **และ** -4 **ซึ่งมีความคล้ายคลึงของระดับนิวคลีโอไทด์ร้อยละ** 86-87.6 **นอกจากนี้ ยังค้นพบการผสมข้ามสายพันธุ์ของ** CanineCV **จำนวน** 1 **สายพันธุ์** (CanineCV-VN-6) **ที่ตำแหน่งนิวคลีโอไทด์ที่** 420-1020 **โดยมีผสมระหว่างสายพันธุ์จากประเทศอเมริกาและประเทศจีน ในการศึกษาในอนาคตควรเน้นการเฝ้าระวังอย่างต่อเนื่องของไวรัสทั้ง** 2 **ชนิดในเขตอื่นๆ ของประเทศเวียดนาม เพื่อให้ได้ข้อมูลในภาพรวมทางระบาดวิทยา จีโนไทป์ การกลายพันธุ์ และการวิวัฒนาการของไวรัสต่อไป**

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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KEYWORD: canine circovirus canine parvovirus genetic evolution Vietnam

Nguyen Manh Tuong : GENETIC CHARACTERIZATION OF CANINE PARVOVIRUS AND CIRCOVIRUS IN DOGS IN VIETNAM . Advisor: Assoc. Prof. Dr. SOMPORN TECHANGAMSUWAN, DVM. M.Sc., Ph.D., DTBVP. Co-advisor: Assoc. Prof. ANUDEP RUNGSIPIPAT, DVM, PhD, DTBVP

Canine parvovirus (CPV-2) emerged and caused severe enteritis in dogs since the 1970s. The last variant, CPV-2c is considered as the high virulent strain and spread ubiquitous worldwide. This strain was early detected in Vietnam since 2002, but the whole genome is still fragmentary. In this study, we attempted to gain the whole genome of CPV-2c from the Vietnamese infected dogs as the first report of the full-length CPV-2c sequence originated from Asia. The Vietnamese CPV-2c exhibited the common mutations in both structural and nonstructural proteins and shared a typical evolutionary pattern with Asian strains. Besides, Vietnamese sequences presented some new mutations and clustered with current Chinese CPV-2 to create a new Asia-IV clade in the phylogeny. The substitution rate was estimated based on the data set of 199 sequences over the last 42 years of viral emergence, and confirmed that CPV-2 is a rapidly evolving ssDNA virus. Moreover, CPV-2 is highly co-infected with other enteric viruses to cause the disease. Among them, Canine circovirus (CanineCV) is the most prevalent, about 19.7%. Then, we further characterized the full-genome of this virus. Phylogenetic analysis revealed that CanineCV is divided into four different genotypes (CanineCV-1, -2, -3, and -4) with typically replaced amino acids in the *Replicase* and *Capsid* proteins. Both CanineCV-1 and 4 co-circulated in Vietnam and shared 86-87.6% of nucleotide similarity. The recombination event has occurred at nucleotide position 420-1020 in one Vietnamese CanineCV strain (CanineCV-VN-6) with the major and minor parents derived from America and China, respectively. Future study should be emphasized on the continuous surveillance of CPV-2 and CanineCV in other regions in Vietnam to draw the whole information regarding epidemiology, genotype, mutation and evolution of both enteric viruses.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

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Nguyen Manh Tuong

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ABBREVIATIONS

CanineCV	Canine Circovirus
CAdV	Canine adenovirus
CBoV	Canine Bocavirus
CCoV	Canine Coronavirus
CDV	Canine Distemper Virus
CPV	Canine parvovirus
FPLV	Feline panleukopinea
MCMC	Markov Chain Monte Carlo
ML	Maximum Likelihood
MRCA	Most Recent Common Ancestor
NS	Nonstructural Protein
nt	nucleotide
ORF	Open reading frame
PCV	Porcine circovirus
PMX	Paramyxovirus
TfR	Transferrin Receptor
VP	Viral Protein

CHAPTER 1
INTRODUCTION

1.1 The relation between two manuscripts in the thesis

Canine parvovirus type 2 (CPV-2) is a key pathogen in dogs causing fatal hemorrhagic enteritis. Among different variants of CPV, the CPV-2c has become a global distribution and gains attention for parvoviral evolution monitoring. In the first manuscript, we surveyed the CPV-2 variants circulating in Vietnam during 2017-2018 and investigated the characterization of the whole genome of CPV-2c as the first document for full-length sequence of CPV-2c isolated from Asia. The data set including 199 full-genomes of three different CPV-2 variants, isolated from main continents during 42 years since viral emergence, is used for evolution, recombination, and selective pressure analysis. The results confirmed that CPV-2 is a rapidly mutated virus, and the mutations in both structural and nonstructural genes have contributed to the emergence of a new variant. Even though the evidence of genetic recombination of Vietnamese CPV-2c strains was not established, the potential positive selection sites were observed in both structural and nonstructural genes.

The CPV-2-infected dogs initially show lymphopenia and are accompanied by severe immunosuppression, which facilitated for opportunistic pathogen infection. Then, we investigated other enteric viruses co-infected with CPV-2. Surprisingly, Canine circovirus (CanineCV) was the predominant pathogen that was detected among CPV-2c-infected Vietnamese dogs with the prevalence of 19.7%. However, there has been no information of CanineCV in Vietnam. In the second manuscript, we detected and characterized the molecular genetics of CanineCV in Vietnam. Recently, it is known that CanineCV is divided into four different genotypes (CanineCV genotype-1, 2, 3, and 4) and our study found that CanineCV genotype-1 and -4 are co-circulating in Vietnam. In addition, recombination event was found in one genomic sequence of Vietnamese CanineCV (CanineCV-VN-6) strain with an American CanineCV (KC241983) and a Chinese CanineCV (MG279125) as the major and the minor parents,

respectively. Only single dog revealed triple detections of CPV-2c, CanineCV and CAdV (1.2%). This study provides evidence of the co-circulation of two different genotypes of CanineCV and CPV-2c in dogs in Vietnam. The continuous surveillance is necessary to understand the epidemiology and genetic evolution of CanineCV.

These two manuscripts are a part of the fulfillment of the requirement for the degree of Doctor of Philosophy in Veterinary Science and Technology program.

1.2 Importance and Rationale

Hemorrhagic enteritis is a common disease in the dog population; it is caused by several infectious pathogens including viruses, bacteria, and parasites. Of these, CPV-2 is a crucial etiological agent with highly transmissible and causes a severe disease. Although the modified CPV-2 live vaccine has been developed and used to protect animals from this devastating and deadly virus, CPV-2-infection is the leading cause of death for dogs. CPV-induced myocarditis causes disease in all pups in a litter with 100% of death. In older CPV-infected dogs, viruses replicate in lymphoid cells and enterocytes of the small intestine, causing intensive necrosis of the intestinal cryptal epithelial cells; dogs gain a high risk of mortality reaching to 91% in untreated case (Boosinger *et al.*, 1982).

CPV-2 emerged as a new virus in the late 1970s (Peneva-Todorova *et al.*, 1982). It is well-established that the emergence of CPV-2 resulted from the site-specific mutation from feline panleukopenia virus (FPLV) to gain the ability for viral replication in dogs (Chang *et al.*, 1992). CPV-2 is a small DNA virus with four genes coding for two nonstructural (NS) and two structural proteins (VP). The change of a few amino acids at specific VP2 residue can alter relevant viral antigenicity and lead to the emergence of new antigenic variants. Between 1978 and 1981, CPV-2 mutated to CPV-2a and 2b by changing the amino acid at position 426 (asparagine for CPV-2a and isoleucine for CPV-2b), and position 555 (isoleucine to valine) in VP2 protein (Parrish *et al.*, 1985). In 2001, a new CPV variant (CPV-2c) was identified in dogs in

Italy with a novel mutation at residue 426 (glutamate) (Buonavoglia *et al.*, 2001), then, this variant (CPV-2c) has been progressively detected in dog populations worldwide. This mutation related to the viral epitope and might cause an effect on viral antigenicity (Hernández-Blanco and Catala-López, 2015). Therefore, this variant has gained the attention of researchers working in virology, evolutionary, and practitioners.

In Vietnam, the vaccination program has not been widely applied by practitioners to protect puppies from infectious diseases, and the outbreak of CPV-2 frequently occurs. Especially in the Northern area where has a humid subtropical climate, the outbreak of CPV-2-infection with a high rate of morbidity lasts over several months every year. The survey in 2002 revealed that CPV-2a and 2b were circulated in Vietnam, and this report was early identification of CPV-2c in the world (Nakamura *et al.*, 2004b) Notably, CPV-2c has become the most prevalent at 94% in Vietnam in 2017 (Hoang *et al.*, 2019).

The CPV-2-infected disease becomes severity if CPV-2 co-infected with other enteric pathogens. Besides, well-known enteric viruses such as canine coronavirus (CCoV), canine adenovirus (CAdV), an emerging virus named as canine circovirus (CanineCV) has been identified among CPV-2-infected dogs. In this study, we performed molecular and phylodynamic analyses of CPV-2c based on the full-length genome and investigated the evidence of other enteric viruses that co-infected with CPV-2. Then, we characterized the Vietnamese CanineCV circulating in dogs in Vietnam.

1.3 Literature reviews

1.3.1 Canine parvovirus

Virus origin and emergence

In the late 1970s and early 1980s, canine parvovirus type 2 (CPV-2) emerged as an unknown infectious agent causing severe hemorrhagic enteritis in domestic dogs. This virus was firstly identified in the USA and spread to other continents, including Asia, Australia, and Europe (Parrish *et al.*, 1988; Parrish, 1999). However, the studies on phylogenetic analysis and molecular clock estimates indicated that the emergence of CPV-2 was likely before 1978. The serum samples collected in Greece (1974) and Belgium (1976) showed positive antibody titers before detecting positive sera in the USA in 1978 (Koptopoulos *et al.*, 1986; Parrish, 1999). Since that year, parvovirus has been a globally pandemic disease in dog populations.

The origin of CPV-2 was suggested as a variant of feline panleukopenia virus (FPLV); this hypothesis widely accepted these days. The similarity between the nucleotides of CPV-2 and FPLV is 98% in identity, but they have specific host ranges. This is demonstrated that the mutations of CPV-2 likely gain the susceptibility on the canine transferrin receptor and allow CPV-2 to infect and replicate in dogs (Shackelton *et al.*, 2005; Palermo *et al.*, 2006).

Taxonomy

The family *Parvoviridae* contains two sub-families, *Parvovirinae* and *Densovirinae*. The sub-family *Parvovirinae* further divides into eight genera including Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus and Tetraparvovirus (Cotmore *et al.*, 2019). CPV-2 belongs to family *Parvoviridae*, subfamily *Parvovirinae* and genus Protoparvovirus. Parvovirus species have been identified based on a specific infected host such as canine parvovirus, feline parvovirus (feline panleukopenia virus), mink enteritis virus,

minute virus mouse, mouse parvovirus, porcine parvovirus, raccoon parvovirus, rat parvovirus. canine parvovirus is designated as CPV type 2 (CPV-2) to distinguish it from canine parvovirus type 1 (CPV-1) or minute virus that was identified from canine in 1967, but there is no antigenic relationship between them.

Genome and gene products

Canine parvovirus (CPV) is a non-enveloped, icosahedral virus with a single-stranded DNA (Figure 1-1). It is a small virus about 20-26 nanometer (nm) in diameter and approximately 5 kb length. DNA may equally occur as positive- (sense) or negative- (antisense) strand, but the prevalence in CPV-2 population is positive form. DNA genome is composed of two major open reading frames (ORFs); a left-hand ORF encodes the non-structural proteins (NS1 and NS2), and the other encodes for three structural proteins (VP1, VP2 and VP3) (Figure 1-2) (Reed *et al.*, 1988).

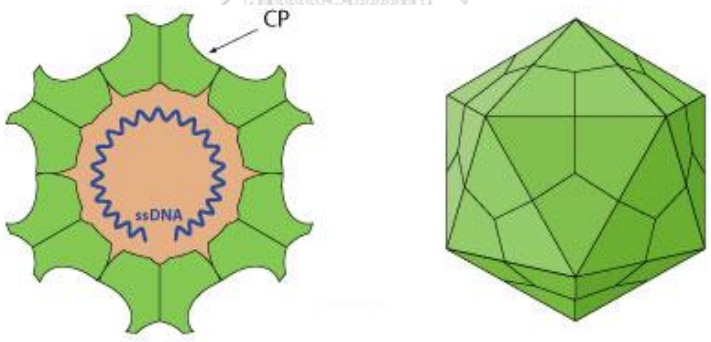

Figure 1-1 The characteristic of CPV genome (Swiss Institute of Bioinformatics, 2009)

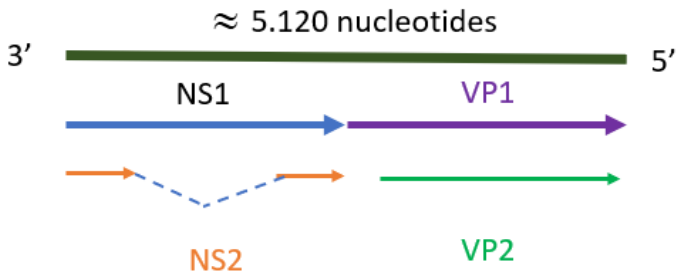

Figure 1-2 Schematic diagram of the canine parvovirus genome (2017)

The non-structural proteins are replicate proteins that control genomic replications. The NS1 is a major non-structural protein with a size of 83 kDa and plays an important role in viral cytotoxicity and pathogenicity. This protein contains several replication-related regions such as DNA and ATP binding regions, and some domains including helicase and transactivation. NS1 binds to the specific sequence of host cells to replicate and maintain the replication fork. Another function of NS1 is related to helicase enzyme activity, which controls the DNA packing into the viral capsid (Nuesch and Rommelaere, 2006). The significant shorter NS2 protein contained a part of NS1 with 87 amino-terminal amino acids and joined to 78 amino acids from an alternative open reading frame. Its function has not yet been described in detail. It is found in the nucleus and cytoplasm of host and it is very likely involved in viral replication too (Wang *et al.*, 1998).

The viral capsid proteins, VP1 and VP2, are the products from the translation of VP1 and VP2 genes but VP3 is a post-transcriptionally proteolytic product of VP2; it does not encode by any particular gene. The size of the VP1 protein is 82.3 kDa and contains 727 amino acids. It has the complete sequence of VP2 plus the nucleotides at its N-terminus (Reed*et al.*, 1988; Wang*et al.*, 1998; Parrish and Kawaoka, 2005). The unique terminal of VP1 contains groups of four amino acids that resemble nuclear localization signals (NLS), and acts as transportation of other proteins into the cell nucleus (Vihinen-Ranta *et al.*, 2002). Therefore, the parvovirus VP1 capsid protein relates to infectivity rather than capsid assembly.

The VP2 protein is the major component in capsid proteins consisting of 584 amino acid residues and has a size of 65 kDa (Reed*et al.*, 1988; Wang*et al.*, 1998; Parrish and Kawaoka, 2005). The function of VP2 protein related to DNA packaging in full capsid particle. The VP2 plays a key role in the determination of the capsid surface, which associated with host invasion and host range. Some residues of VP2 sequence are responsible for targeting and effective interaction with host cells by

binding to the transferrin receptor. The changes of very few structural determinant residues lead to the change of interaction of virus and the host cell transferrin receptor (Tfr) and result in shifting of CPV-2 host range. Therefore, the mutation on VP2 is used to classify the CPV-2 variants (Parrish, 1991; Hueffer and Parrish, 2003)

The apparent size of VP3 protein is 63.5 kDa. The VP3 is derived from VP2 by post-translational proteolytic cleavage of 20 amino acid residues from N-terminus. It is not present in viral empty capsid and considered as the minor protein in capsid component of the complete virion (Tsao *et al.*, 1991; Langeveld *et al.*, 2001; Parrish and Kawaoka, 2005). The VP3 generally appears in the viral genome with completed capsid assembly and packaging. Notably, the study on the adeno-associated viruses suggested that the VP3 was responsible for the capsid assembly and virion stability (Sonntag *et al.*, 2010) but, the function of VP3 in the parvovirus life cycle still is discrete.

Structure of virus capsid

CPV-2 nucleocapsid composes 60 capsomer units of VP2 and between 6 to 10 copies VP1. In the infectious particles, VP2 monomers are modified and designated as VP3 units. Like other icosahedral viruses, the core structure of CPV-2 capsids comprises four loops containing eight-stranded antiparallel β-barrel motifs to make up much of the surface of the virus. Loops 1, 3 and 4 are built by three VP2 molecules; these subunits are intertwined together to form a threefold spike, which presents the residues related to the viral antigenic properties. Two major neutralization epitopes of virus have been identified in these loops (Figure 1-3) (Tsao*et al.*, 1991; Wu and Rossmann, 1993; Strassheim *et al.*, 1994).

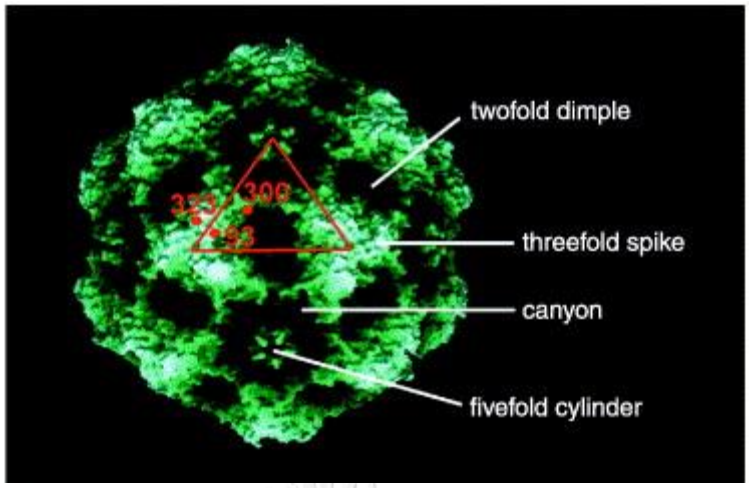

Figure 1-3 The structure of parvovirus (Hueffer and Parrish, 2003)

Virus replication

Initially, the virus binds to the transferrin receptor on the membrane of target cells; then host cells take viral virion into its cytoplasm by the clathrin-mediated pathway. Once inside cells, endocytic vesicle traffics within the cytoplasm through the endosomal pathway including early endosomes and late endosomes toward lysosomes. It is unclear how the virus exists from the endosomal traffic pathway, but the viral VP1 protein may be involved. With a phospholipase A2 (PLA2) enzyme on an N-terminal region, VP1 may modify the endosomal membrane to escape from the lysosome. The nuclear localization signal helps viral DNA entering the nucleus through the nuclear pore complex. The viral DNA begins to replicate by using host DNA replication machinery, and this process requires S-phase of the host cell division cycle. The assembly of viral capsid also takes place in the nucleus (Figure 1-4).

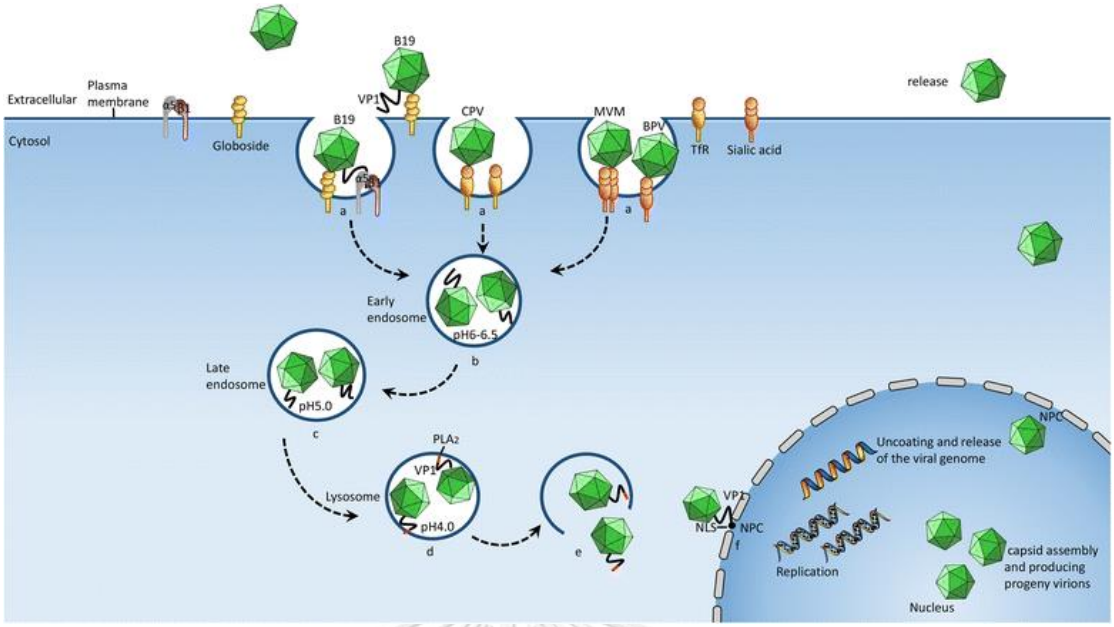

Figure 1-4 A schematic of the parvovirus infection process (Tu *et al.*, 2015)

Viral evolution and variants

The most important evolution of CPV-2 is to gain cross-species transmission. The mutation of some amino acids related to the threefold spike on viral capsid allowed CPV-2 successful transfer and adapt to the canine host cells (Truyen *et al.*, 1995). In specific, the changes of VP2 residues Lys93Asn, 103 Val103Ala and Asp323Asn were sufficient for CPV-2 to acquire the canine host range. However, other mutations at residue Lys80Arg, Asn564Ser and Ala568Gly leaded to loss ability to replicate in feline host cells (Chang*et al.*, 1992; Truyen and Parrish, 1992).

After a short period of the CPV-2 origin detection, the virus continues to undergo mutation to emerge a new variant called CPV-2a in 1979. The mutation at residue Ile101Thr, Ser297Ala and Val555Ile in these new strains leads to be a more strongly epidemiological advantage to completely replace CPV-2 origin worldwide over a few years later (1979-1982). (Parrish *et al.*, 1991; Tsao*et al.*, 1991; Truyen *et al.*, 1996).

The new mutation at residue 426 of VP2 (Asn to Asp) located at the top of the threefold spike and related to the major antigenic region (epitope A) was firstly detected in the USA in 1984 and designated as CPV-2b. Another mutation occurred at residue 426 (Asn to Gly) was detected in Italy in 2000 and designated as newest variant, CPV-2c (Martella *et al.*, 2006; Ohshima *et al.*, 2008; Decaro *et al.*, 2009a). However, a retrospective study showed that CPV-2c strain circulated in Germany for at least 4 years before it was detected in Italy (Decaro *et al.*, 2007a).

Some non-synonymous substitutions presented in VP2 protein have been detected in CPV-2 variants. Note, a mutation of amino acid 297 (Ser to Ala) that located closed to epitope B was observed both in CPV-2a and -2b but the effect of this mutation on viral antigenicity has not been reported. In some studies, these strains considered as new CPV-2a and CPV-2b (Decaro*et al.*, 2009a). Other mutations were detected at VP2 residue 440Ala that located in the GH loop of on the surface of the capsid and residue 324Ile likely affect the host range of CPV-2 have simultaneously detected in the same strains (Hong *et al.*, 2007). These mutations probably gain more advantage to adapt and replicate other host such feline or raccoon (Kaelber *et al.*, 2012).

Epidemiology

The natural susceptible host of CPV-2 is family *Canidae* including domestic dogs, bush dogs, coyotes, wolves, crab-eating foxes, and maned wolves. New CPV-2 variants (CPV-2a, b, c) can replicate in feline cells and infect cats under both experimental and natural circumstances. Among domestic dogs, CPV-2 infects adult dogs, but disease will be more severe in pups, special aged from six weeks to six months. All breed of dogs can be suffered from an acute CPV-2 infectious disease, nevertheless, the risk of infection might increase in some large size breeds of dogs including Alaska, Rottweiler, Doberman (Nandi and Kumar, 2010; Greene, 2011).

The major transmission route of CPV-2 infection is exposure to feces releasing from infected dogs. In addition, humans, insects, rodents, and contaminated equipment play as vectors to carry the infectious agent. CPV-2 can exist on haircoat of infected dogs or in ground-soil up to a year. The incubation period of CPV-2 infection ranges from 7 to 14 days, but in the experimental study, the incubation time is 4 to 6 days (2006). From fecal samples, the enzyme-linked immunosorbent assay (ELISA) can detect antigen from 7 to 10 days post-inoculation but molecular methods such as PCR or Realtime PCR can detect virus for several weeks. From serious infected dogs, antibody titers can be observed from days 3 or 4 after infection and last for at least one year (Greene, 2011).

Geographic distribution of CPV-2 variants

CPV-2 origin was firstly reported in the USA, then spread to other countries including Europe, Asia and Australia. This strain circulated from 1978 to 1981 before replaced by new variant strains. Nowadays, CPV-2 has reported in about 50 countries distributed among five continents. CPV-2a mainly circulates in Australia, most Asian and European countries, especially in New Zealand only this strain has been detected. However, it does not report in Czech, Slovenia, Sweden. The prevalence of CPV-2b is predominant in several countries such as in Ireland, UK, USA. Both CPV-2a and -2b were distributed in an equal portion in some European countries. The last CPV-2 variant, CPV-2c currently widespread distributes in high frequencies in many geographic regions since the first detection in Italy in 2001. This day, CPV-2c mainly circulates in some European countries such as Italy and Germany, Spain and France (Decaro and Buonavoglia, 2012). In Ecuador, CPV-2c was detected at high frequencies about 54.7% (Aldaz *et al.*, 2013) while America and China revealed at 25.9% and 14.7%, respectively (Geng *et al.*, 2015). CPV-2c also have identified in South America including Uruguay, Brazil and Argentina (Perez *et al.*, 2007) including India, China,

Korea, Japan, Taiwan and Thailand. Noteworthy, CPV-2c strain has been circulated at 97% in Vietnam (Hoang*et al.*, 2019)

Pathogenesis

CPV-2 is transmitted to other dogs by oral exposure to contaminated feces from infected dogs. Once virus entry in the oropharynx, viruses begin to replicate in lymphatic tissues such as pharyngeal lymphoid tissue, thymus and mesenteric lymph nodes. Then, the virus gets into the circulation system and the viral concentration reaches a peak in blood after 1-5 days. Subsequent to viremia, CPV-2 continues to spread to other organs such as small intestine, lung, heart, spleen, liver, and kidney. However, the predominant localization of viruses is in the epithelium of tongue, oral and esophageal mucosa, small intestine, lymph nodes, and bone marrow (Greene, 2011). After the period of incubation (about 3-7 days), clinical signs including vomiting, hemorrhagic diarrhea, losses of appetite, depression, fever and dehydration will occur. Before showing clinical signs, virus begins the active excretion on day 3 or day 4 after infection. In small intestine, virus attacks in crypt cells on the villus of the intestinal mucosa make these villi become shorten, which leads to a collapse of the villi and results to maldigestion and malabsorption. Bacteremia, endotoxemia, and disseminated intravascular coagulation are the secondary complications from the damaged intestine. In lymphatic tissue, CPV-2 also destroys the precursors of leukocytes and lymphoid cells in bone marrow and cause neutropenia and lymphopenia. If infected dogs developing systemic inflammatory response syndrome (SIRS) or sepsis, their mortality rate reach to 91%. In neonatal puppies, virus infects myocardiocytes to induce non-suppurative myocarditis and leads to 100% of death in infected puppies in a litter.

Clinical signs

In domestic dogs, CPV-2 infection produces systemic and intestinal diseases but many dogs that become naturally infected will never develop overt clinical signs, especially in pups that get maternal antibody from mothers. CPV-2 infectious virion will rapidly grow if co-infected with other intestinal pathogens such as helminths, protozoa, *Clotridium perfringens*, *Campylobacter* spp. and *Salmonella* spp (Greene and Decaro, 2013). These dogs will display more severe clinical signs and high mortality. CPV-2 infected dogs may progress rapidly and death can occur after the onset of illness during first two days. In early stage, dogs normally show clinical signs such as fever, lethargy, anorexia and weakness. Vomiting and diarrhea are often severe and quickly lead to dehydration. Abdominal tenderness with intestinal tract filled with fluid. In severe cases, gram-negative sepsis can be associated with tachycardia or bradycardia, and poor pulse quality and followed by disseminated intravascular coagulation. Mental obtundation and neurologic signs such as tremors and seizures are uncommonly observed. Puppies under two months can developed myocarditis and increased lung sounds (Greene and Decaro, 2013).

Pathologic findings

The lesion is firstly found in the small intestinal tract, especially in the distal duodenum and jejunum. The extensive lesions can see through all segments of the small intestine in severe cases. The wall of the intestine becomes thicken, and serous membrane is segmentally discolored. The intestinal mucosa is atrophy; the color of content in the lumen ranges from yellowish to reddish or brownish. Microscopically, the necrosis present in the cryptal epithelial cells and the viral inclusion bodies may be observed in the nucleus of epithelial cells. The villi are shortened or obliterated and the lamina propria is collapsed because of the lack of epithelial replacement. CPV-2 also causes lesions in the lymphatic system. Besides the depletion of lymphoid cells, necrosis is always found in many lymphoid tissues

including Peyer's patches, mesenteric lymph nodes, thymus, and spleen. Pulmonary edema or alveolitis are non-specific lesions, which may be seen in cases of death caused by septicemia (Greene, 2011).

The myocardial lesion is characterized by white bands or pale streaks in the myocardium of the ventricle. Histological examination shows that the myocardium is infiltrated with plasma cells and lymphocytes; basophilic intranuclear inclusion bodies are rarely observed in myocardiocytes. Hypercellularity and thickening of the alveolar walls are observed in the lung in case of heart failure (Bastianello, 1981; Greene, 2011).

Diagnosis

The clinical signs such as foul-smelling and bloody diarrhea are similar to other enteric diseases caused by viruses, bacteria or intestinal parasites; those signs are not typical for CPV-2 diagnosis. The method based on antigen-antibody interactions is a suitable technique to detect CPV-2 infection and have currently applied in veterinary clinics. However, the host immune response will be affected their sensitivity and this method is inferior in comparison to molecular assays. In the laboratory, gene amplification-based assays have been developed for the diagnosis of CPV-2. The molecular assays provide greater sensitivity and specificity, but it requires labor-intensive and time-consume. Polymerase chain reaction (PCR) is used as the first molecular method to identify CPV-2 from fecal specimens since 1993 (Mochizuki *et al.*, 1993). Currently, an alternative method, loop-mediated isothermal amplification (LAMP), has been also proposed to detect CPV-2. Based on the Tagman technology, a real-time PCR assay has been developed as a quantitative assay for the detection of CPV-2 DNA. It is a rapid, specific and sensitive method with several advantages over conventional PCR. Real-time PCR allows a large increase in throughput and enables the simultaneous processing of several samples. It was successfully employed to evaluate the distribution of viruses in study of the

pathogenesis of natural infections (Decaro *et al.*, 2007b). Another quantitative assay for detection of CPV-2 is SYBR green-based real-time PCR assay; it is proposed as an alternative method to the Tagman technology. This method displays the same detection limit with Tagman assay (Elia *et al.*, 2007).

1.3.2 Circovirus

Origin and name

Before 2012, the two circoviruses were detected in pigs and named porcine circovirus type 1 (PCV-1) and type 2 (PCV-2); these viruses are known as the only mammalian circoviruses. Two viruses are closely related; however PCV-1 is generally considered to be non-pathogenic (Allan *et al.*, 1995) while PCV-2 has been associated with a variety of pathological conditions in pigs (Kim *et al.*, 2003; Johnstone and Lawton, 2008). In 2012, canine circovirus (CanineCV) was firstly detected in serum samples collected from healthy dogs in the USA (Kapoor *et al.*, 2012a). Because of the "CaCV" notation already used for other viruses such as canary circovirus, canine calicivirus, the name "dog circovirus" was suggested (Li *et al.*, 2013a). To avoid confusion and make species name uniform for all members in whole family *Cirvoviridae*, the International Committee on Taxonomy of Viruses (ICTV) designated canine circovirus as "CanineCV".

Taxonomy

CanineCV is a member of the family *Circoviridae*. In 2016, this family further divided into genera *Circovirus* and *Gyrovirus*. However, the genus *Gyrovirus* was lately removed from *family Circoviridae* and added in *family Anelloviridae* based on viral structure and genome. And the taxon *Cyclovirus* reassigned to belong to the family *Circoviridae*. Most members of the genus Circovirus have been reported in vertebrates (Breitbart *et al.*, 2017).

Psittacine beak and feather disease virus was a first Circovirus described in avian species and, subsequently other circoviruses are discovered in fishes, bats, chimpanzees, humans, mink, and three species of porcine circovirus in pigs (PCV-1, PCV-2, and PCV-3) (Palinski *et al.*, 2017; Phan *et al.*, 2016). Since 2012, the first report revealed CanineCV in dogs (Kapoor*et al.*, 2012a; Phan *et al.*, 2016; Palinski *et al.*, 2017).

Molecular genome

The genome of CanineCV contains a circular single-strand DNA. The complete genome of CanineCV comprised 2,063 nucleotides with two putative open reading frames (ORFs). One of ORFs encodes for Replicase (Rep) protein carrying 303 amino acid (aa) located in complementary strands in the opposite orientation. The other ORF encodes for Capsid (Cap) protein with 270 aa. Besides, the virus has two intergenic noncoding regions, one contains 135 nt, and the other has 203 nt long. Between the two major ORFs is a noncoding region with a thermodynamically stable stem-loop containing 9 nt (TAGTATTAC), and function for initiation of rolling-circle replication (Figure 1-5) (Kapoor*et al.*, 2012a).

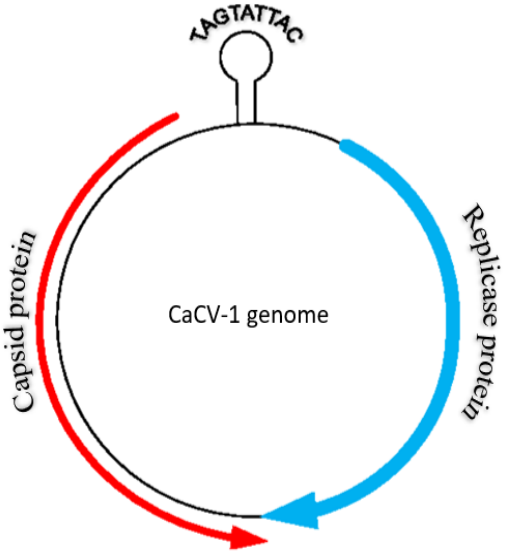

Figure 1-5 Schematic diagram of the genomic organization of canine circovirus

Disease association with CanineCV

CanineCV has been isolated from dogs with different pathological conditions in multi-organs; however, the pathogenic role of this virus has been unknown yet. Some studies reported that this canine pathogen caused hemorrhagic diarrhea (Anderson *et al.*, 2017) and this virus also detected in an outbreak of fatal pups with enteritis in Italy (Decaro *et al.*, 2014). However, another study indicated that the role of CanineCV as a primary cause of gastroenteritis is limited (Dowgier *et al.*, 2017). The detection of CanineCV in healthy dogs also supports this observation (Gentil *et al.*, 2017). Moreover, CanineCV has been detected in dual infection with other pathogens such as CPV and CCoV (Thaiwong *et al.*, 2016), which suggests that CanineCV probably acts in synergism with other pathogens in the development of a disease. The histopathological examination from fatal dogs with enteritis shows that together with the hemorrhage throughout gastrointestinal tracts, necrotizing vasculitis and granulomatous lymphadenitis of the mesenteric lymph nodes also noted (Li*et al.*, 2013a).

Pathogenesis

Currently, there has been no data regarding the pathogenesis of CanineCV infected dogs. Some laboratories attempted to isolate the virus but not yet successful. The detection of CanineCV in different tissues of dogs and red foxes (Li*et al.*, 2013a; De Arcangeli *et al.*, 2019) indicates that viruses naturally infection is systemic. However, the knowledge of viral entry, primary replication, organ distribution is still limited. The detection of CanineCV has been found in the outbreak of pups, which suggests the horizontal transmission by direct contact. It is necessary to conduct more studies to ascertain the potential excretion routes of the virus.

Diagnosis

Currently, the detection of the virus in the laboratory is mainly based on molecular techniques. Several methods, including conventional PCR (Sun *et al.*, 2019), overlap PCR (Kotsias *et al.*, 2019), SYBR Green-based real-time PCR (Thaiwong*et al.*, 2016) and Real-time Tagman PCR (Anderson*et al.*, 2017) have been developed. Additionally, the serological test using recombinant capsid enzyme-linked immunosorbent assay (ELISA) is recently established (Wang *et al*., 2020). The virus is also detected on histological tissues by using the *In situ* hybridization (ISH) technique (Thaiwong*et al.*, 2016); but immunohistochemistry (IHC) method has not performed yet due to lack of the specific antibody, which leads to the study of virus distribution is not completely performed. Other studies of viral isolation, serology, and detection of viral components in tissues are needed.

1.3.3 Other enteritis viruses in dogs

Canine distemper virus

Canine distemper virus (CDV) is the highly contagious agent in domestic dogs; it was the first detection in 1760 and successfully isolated in 1905. Canine distemper has been reported to infect other families such as terrestrial carnivores, peccary, seal, and monkeys. Currently, based on the sequence of hemagglutinin gene, there are 14 distinct genetic lineages of CDV including Asia-1-4, America-1-5, South America-1-2, Africa-1-2 and Arctic-like.

CDV is a negative-sense, single-stranded RNA virus and belongs to the order *Mononegavirales*, family *Paramyxoviridae*, genus *Morbillivirus*. Canine distemper genome contains six genes coding for structural proteins: nucleocapsid (N), matrix (M), fusion (F), hemagglutinin (H), Phospo-(P) and large-(L), and two genes for nonstructural proteins, C and V proteins. The H gene is one of the most variable genes in CDV, which is used to differentiate CDV strains (Carvalho *et al.*, 2012).

CDV contaminates in aerosol droplets. Once virus contacts with the epithelium of the upper respiratory tract of dogs, it will begin to replicate in tissue macrophages, started from tonsils, retropharyngeal and bronchial lymph nodes during first 4 days. The virus continues to replicate in lymphoid follicles, spleen, stomach and small intestine until day 6 post-infection. Lately, virus spreads to all epithelial tissues and the central nervous system. In the case of dogs with poor immune status, virus will spread to other tissues including skin, exocrine and endocrine glands and clinical signs in these dogs are dramatically worse and severe. The severity of clinical signs varies depending on the virulence of virus strains; however, vesicular and pustular dermatitis in puppies, footpad and nasal hyper keratinization, neurologic manifestations and hemorrhagic diarrhea usually are observed (Greene and Decaro, 2006; Carvalho*et al.*, 2012).

Canine coronavirus

Canine coronavirus (CCoV) was first isolated from an epizootic disease in a canine military unit in Germany in 1971. Then, several CCoV outbreaks have been reported worldwide, and it recognized as an essential enteropathogen in dogs. CCoV is a single-stranded, positive-sense RNA virus and belongs to order Nidovirales, family *Coronaviridae*, subfamily *Coronavirinae*, genus Alphacoronavirus, species Alphacoronavirus-1. Currently, CCoV is divided into two genotypes including CCoV-I and II, and CCoV-II is further divided into two subgenotypes, IIa and IIb, based on N-terminal domain of Spike (S) protein (Buonavoglia and Martella, 2007; Le Poder *et al.*, 2013). Coronavirus genome consists of two overlapping ORFs; the ORF located in first two-third part of genome encode non-structural proteins including the viral RNA-dependent RNA polymerase and proteases, and another ORF encodes the major structural (S), envelope (E), membrane (M), and nucleocapsid (N) proteins that located in last one-third part. The S protein has an important role in binding to the host cell receptor and triggers fusion between viral and cellular membranes. In

addition, CCoV encodes small nonstructural proteins: ORFs 3a, 3b, 3c, located between S and E genes and ORFs 7a, 7b, located between the N gene and poly A. The function of these nonstructural proteins still remains unknown (Le Poder*et al.*, 2013).

The virus is released mainly in feces from ill dogs and fecal-oral route is a natural transmission. After dog exposure to CCoV-contaminated feces by oral route, CCoV spread by entering into the mature epithelial cells in the villi of the small intestine. In neonatal puppies, the virus appears to replicate in the villus tips of enterocytes of the small intestine. Virus causes a lytic infection followed by desquamation, which leads to the shortening of the villi. Then, malabsorption and deficiency of digestive enzymes occur, and followed by diarrhea within 18-72 hours post-infection. In some severe cases, diarrhea can become watery, which result in the dehydration and imbalance of electrolyte. However, most of the affected dogs recover spontaneously 8-10 days after the onset of symptoms (Pratelli *et al.*, 2004; Decaro *et al.*, 2009b; Decaro *et al.*, 2010).

Canine bocarvirus

Canine bocavirus (CBoV) is a small, non-enveloped, icosahedral virus with a linear ssDNA genome and it is a member in genera *Bocaparvovirus* of *Parvovirinae* family. The *bocaparvovirus* genus contains bovine parvovirus (BPV), minute virus (MVC) or canine bocavirus 1 (CBoV-1), gorilla bocavirus (GBoV) and four species of human bocaviruses (HBoV1-4), and a new member of canine bocavirus 3 (CBoV-3). CBoV-1 and CBoV-2 shared less than 63%, 62% and 64% amino acid identity in NS, NP and VP proteins, respectively (Kapoor *et al.*, 2012b; Cotmore *et al.*, 2014a). CBoV-3 shared less than 60% amino acid identity with other bocaviruses. CBoV-1 (MVC) is discovered in 1967 from feces of healthy dogs. It can cause severe respiratory infection in newborn puppy but it induces subclinical sign in adult dogs. CBoV-2 was firstly identified in dogs with respiratory disease in the USA in 2012 while CBoV-3

associated with the canine liver diseases. However, CBoV-2 is reported as pathogen related to the lesion of the gastrointestinal tract (Li *et al.*, 2013b; Bodewes *et al.*, 2014; Choi *et al.*, 2015).

Canine adenovirus

Canine adenovirus (CAdV) is a non-enveloped icosahedral double-stranded DNA virus belong to the genus *Mastadenovirus* of the family *Adenoviridae* (Appel, 1987) . CAdV is divided into two distinct serotypes, CAdV-1 and CAdV-2. Genome sizes of CAdV-1 and CAdV-2 are about 30.5 and 31.3 kb, respectively. In dogs, CAdV-1 caused hepatitis, interstitial nephritis and diarrhea whereas, CAdV-2 causes only mild upper respiratory tract infection (Decaro *et al.*, 2008). CAdV-1 has also detected from wild animals including foxes, wolves, bears and coyotes (Woods *et al.*, 2001). Transmission of virus occurs through contact contaminated tools with infectious saliva, feces, urine and respiratory secretion.

1.4 Objectives of the study

(1) To analyze the genetic characteristics and molecular evolution of CPV-2 and CanineCV circulating in infected dogs in Vietnam.

(2) To investigate the evidence of other enteric viruses coinfected with CPV-2 in diarrheic dogs in Vietnam.

1.5 Hypothesis

The CPV-2 highly coinfects with CanineCV in dogs in Vietnam. The molecular characterization of Vietnamese CPV-2 and CanineCV contributes to the diversity of viral genome.

1.6 Conceptual framework

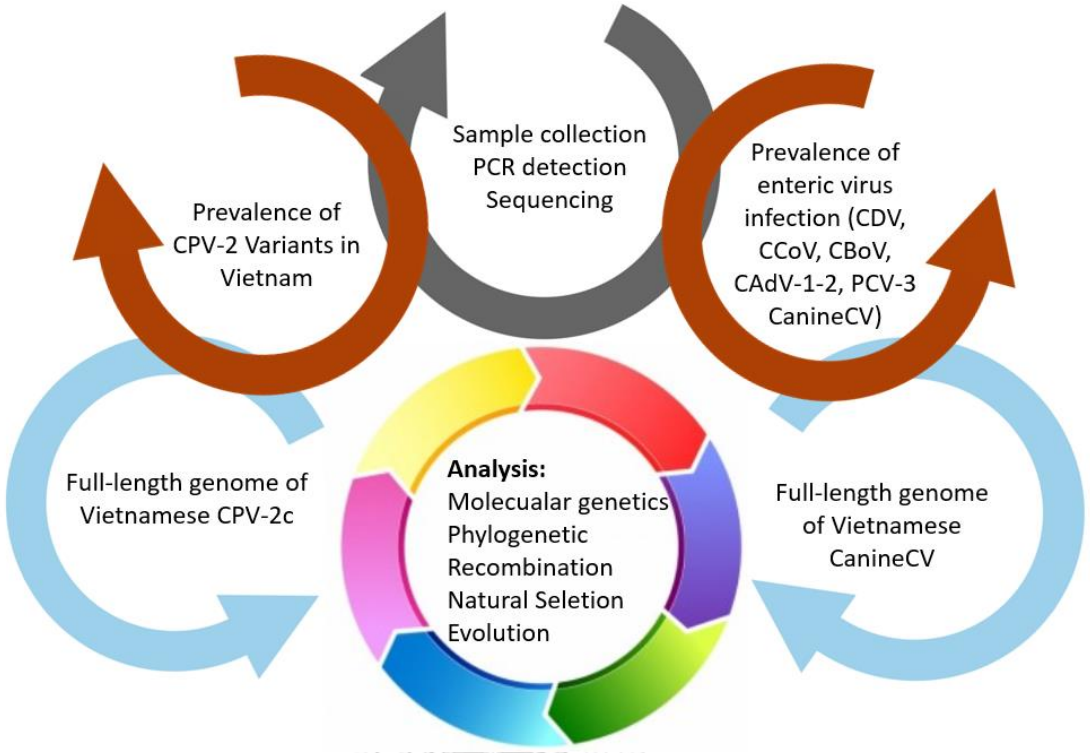

Figure 1-6 Research conceptual framework

1.7 Advantages of the study

(1) Gain information about genetic characterization of CPV-2 and CanineCV circulating in Vietnam.

(2) Gain information of other viruses co-infected with CPV-2 that cause enteritis in dogs in Vietnam.

(3) Understanding the molecular evolution of CPV-2 and CanineCV can provide information about the mechanisms driving virus mutation associated with the host range.

CHAPTER 2

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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2.1 Summary

Canine parvovirus type 2 (CPV-2) is a small, single-stranded DNA virus causing fatal hemorrhagic enteritis in dogs. Currently, CPV-2 is 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 strains 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 42 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.

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

2.2 Introduction

Canine parvovirus (CPV) is a non-enveloped, single-stranded DNA virus and belongs to the genus *Protoparvovirus* within the family *Parvoviridae,* which includes the species carnivore protoparvovirus 1 together with the feline panleukopeniea virus (FPV), mink enteritis virus (MEV), and raccoon parvovirus (RaPV) (Cotmore *et al.*, 2014b). The CPV emerged as a highly contagious virus that causes severe hemorrhagic enteritis in dogs in the late 1970s (Parrish and Kawaoka, 2005). The original CPV 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 FPV or FPV-like virus (Truyen et al., 1995). Later, as a consequence of mutations on the viral capsid surface of CPV, the cross-species transmission of CPV occurs by altering the species-specific binding of viral capsid to the host receptor, transferrin receptor (TfR), with dynamic motion (Lee et al., 2019).

The genome of CPV-2 contains 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 (Reed*et al.*, 1988; Chang*et al.*, 1992; Parker and Parrish, 1997; Wang*et al.*, 1998). Among the two structural proteins, VP2 is the most abundant 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 VP2 gene, while information on evolution in the nonstructural proteins is limited.

Between 1979 and 1980, an antigenic variant was identified in dogs in the USA and termed as CPV-2a that differed from the original CPV-2 by five amino acid mutations in the VP2 (Met87Leu, Ile101Thr, Ala300Gly, Asp305Tyr, and Val555Ile). These amino acid changes lead to the difference in two neutralizing antigenic sites on the surface of viral capsid (Hoelzer and Parrish, 2010; Stucker *et al.*, 2012; Allison *et al.*, 2016). Up-to-date, it is clearly showed that CPV-2a spread worldwide and is the key ancestor for the later variants, designated as CPV-2b, CPV-2c or new CPV-2a,

which are distributed variably (Voorhees *et al*., 2020). Current epidemological surveys show that the CPV-2a variant predominantly circulates in most Asia and in European countries while the CPV-2b variant is a major antigenic variant in Iceland, the UK, the USA, Africa and some Asian countries (Clark *et al.*, 2018). The last variant of CPV-2, CPV-2c, first detected in Italy in 2001 with a specific 426Glu mutation in the VP2 (Buonavoglia*et al.*, 2001). Later, a retrospective study indicated that this strain circulated in Germany at least four years before it was recognized in Italy (Decaro*et al.*, 2007a). This mutation is involved in the interaction of the virion with its cellular receptor and potentially affects the biology and pathogenesis of the CPV (Agbandje *et al.*, 1995; Lee *et al.*, 2019). After its emergence, CPV-2c became widely distributed in other European and Latin American countries (Zhou *et al.*, 2017). In recent years, CPV-2c has spread to many geographic regions and currently causes fatal disease in both domestic and wild dog populations (Parthiban *et al.*, 2010; Decaro and Buonavoglia, 2012; Alda*et al.*, 2013; Geng*et al.*, 2015; Voorhees *et al.*, 2019).

The CPV-2c variant was first identified in Vietnam in 2002, which was the first report in Asia (Nakamura*et al.*, 2004b). Later, this variant was identified in dogs from other Asian countries, including China, India, Taiwan, Indonesia, Laos, and Thailand (Nandi *et al.*, 2010; Geng*et al.*, 2015; Chiang *et al.*, 2016; Vannamahaxay *et al.*, 2017; Charoenkul *et al.*, 2019). 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 phylogenetic analyses of the full-length genome of CPV-2c variants isolated from domestic dogs in Vietnam.

2.3 Materials and Methods

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 (Table 2-2). 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.

Polymerase chain reaction (PCR) and sequencing analysis

Extracted viral nucleic acids were subjected to parvovirus PCR detection using specific primers targeting the VP2 gene (Table 2-1) 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 30s, 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 to whole genome sequencing using multiple primer sets, as described previously (Perez *et al.*, 2014), under the same thermal cycling condition as mentioned above (Table 2-1).

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)

Table 2-1 The set of primers using in this study

Primers	Sequence (5'-3')	Nucleotide position (nt)
VPF[†]	ATGGCACCTGGCAAAGA	2285–2303
VPR[†]	TTTCTAGGTGCTAGTTGAG	4530–4512
FPLV_10[†]	TGTCATAAAGCCATGT	3108–3125
FPLV_51[†]	CCAACTAAAAGAAGTAAACC	2745–2726
FPLV_1[†]	GTACATTTAAATATGCCAGA	3029–3048
FPLV_52[†]	ATTAATGTTCTATCCCATTG	3480–3461
FPLV_5[†]	AGCTATGAGATCTGAGACA	3388–3406
FPLV_9[†]	TCCTGCTGGATATCTTCCT	4060–4042
FPLV_8[†]	AATACAAACTATATTACTGAAG	3785–3806
FPLV_23[†]	CTTTCCTCCAAAAATCTGA	4415–4397
FPLV_41[†]	ATTGTATACCATATAACAAACC	4738–4759
NS-Fext[‡]	GACCGTTACTGCATTCGCTTC	206–227
NS-Rext[‡]	GAAGGGTTAGTTGGTTCTCC	2460-2441
NS-Fint[‡]	GTTGAAACCACAGTGACGACAG	1055–1076
NS-Rint[‡]	CATCATCCAGTCTTCAGGTG	1186–1167
2161F[‡]	TTGGCGTTACTCACAAAGACGTRC	2161–2184
3475R[‡]	GTTGGTGTGCCACTAGTTCCAGTA	3475–3452

[†] (Mochizuki*et al.*, 1996), [‡] (Perez*et al.*, 2014)

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 from 1979 to 2019, available in the GenBank database (Table 2-9). 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 and 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 was generated with timeline estimated divergences, posterior probability (PP) and 95% highest probability density (HPD) values were generated using FigTree version 1.4.2.

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 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.*, 2018b). 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.

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 applied to 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) in 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.

2.4 Results

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 varied breed and sex of 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 (Table 2-2).

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 (Table 2-2).

Table 2-2 Clinical data and genotype of the 59 Vietnamese CPV strains in this study

No	Isolate	Location	Sampling time[†]	Breed	Age (month)[‡]	Sex[§]	Vaccination time[¶]	Testkit result for CPV/CCV[$]	Residue 426	Genotype
1	HN-4	Hanoi	2017/Sep	Mixed	3	F	0	+/-	Glu	CPV-2c
2	HN-6	Hanoi	2017/Sep	Poodle	4	F	2	+/-	Glu	CPV-2c
3	HN-7	Hanoi	2017/Sep	Bulldog	2	M	2	+/-	Glu	CPV-2c
4	HN-8	Hanoi	2017/Sep	Poodle	2	F	1	+/-	Glu	CPV-2c
5	HN-29	Hanoi	2017/Sep	Shih Tzu	5	M	3	+/-	Glu	CPV-2c
6	HN-30	Hanoi	2017/Sep	Mixed	NA	F	NA	+/-	Glu	CPV-2c
7	HN-31	Hanoi	2017/Sep	Mixed	3	F	0	+/-	Glu	CPV-2c
8	HN-39	Hanoi	2017/Sep	Malinois	4	M	0	+/-	Glu	CPV-2c
9	HN-40	Hanoi	2017/Sep	Pug	6	Mc	2	+/-	Glu	CPV-2c
10	HN-45	Hanoi	2017/Sep	Poodle	2	F	2	+/-	Glu	CPV-2c
11	HN-46	Hanoi	2017/Sep	Poodle	4	M	3	+/-	Glu	CPV-2c
12	HN-52	Hanoi	2017/Sep	Pug	4	M	2	+/-	Glu	CPV-2c
13	HN-54	Hanoi	2017/Sep	Pomeranian	5	F	NA	+/-	Glu	CPV-2c
14	HN-61	Hanoi	2017/Sep	Poodle	3	F	2	+/-	Glu	CPV-2c
15	HN-62	Hanoi	2017/Sep	Malinois	2	M	1	+/+	Glu	CPV-2c
16	HN-63	Hanoi	2017/Sep	Bulldog	2	M	0	+/-	Glu	CPV-2c
17	HN-64	Hanoi	2017/Sep	Pug	4	M	2	+/-	Glu	CPV-2c

18	HN-71	Hanoi	2017/Sep	Pug	3	F	2	+/-	Glu	CPV-2c
19	HN-72	Hanoi	2017/Sep	Shih Tzu	3	M	2	+/-	Glu	CPV-2c
20	DN-1	Danang	2017/Nov	Poodle	8	F	3	+/-	Glu	CPV-2c
21	DN-2	Danang	2017/Nov	Chihuahua	2	F	2	+/-	Glu	CPV-2c
22	DN-3	Danang	2017/Nov	Pug	5	M	2	+/-	Glu	CPV-2c
23	DN-4	Danang	2017/Nov	Mixed	2	M	0	+/-	Glu	CPV-2c
24	DN-5	Danang	2017/Nov	Mixed	2	M	0	+/-	Glu	CPV-2c
25	DN-6	Danang	2017/Nov	Poodle	4	M	2	+/-	Glu	CPV-2c
26	DN-8	Danang	2017/Nov	Malinois	3	F	0	+/-	Glu	CPV-2c
27	DN-11	Danang	2017/Nov	Poodle	3	F	3	+/-	Glu	CPV-2c
28	DN-14	Danang	2017/Nov	Pomeranian	4	M	3	+/-	Glu	CPV-2c
29	DN-15	Danang	2017/Nov	Bulldog	2	F	1	+/-	Glu	CPV-2c
30	DN-17	Danang	2017/Nov	Bulldog	2	F	1	+/-	Glu	CPV-2c
31	DN-18	Danang	2017/Nov	Bulldog	2	F	1	+/-	Glu	CPV-2c
32	DN-20	Danang	2017/Nov	Pug	6	Mc	1	+/-	Glu	CPV-2c
33	DN-21	Danang	2017/Nov	Chihuahua	3	M	2	+/-	Glu	CPV-2c
34	DN-22	Danang	2017/Nov	Malinois	4	M	0	+/-	Glu	CPV-2c
35	DN-23	Danang	2017/Nov	Poodle	3	M	2	+/-	Glu	CPV-2c
36	HCM1	HoChiMinh	2017/Dec	Bulldog	3	F	2	+/-	Glu	CPV-2c
37	HCM3	HoChiMinh	2017/Dec	Bulldog	4	F	2	+/-	Glu	CPV-2c

38	HCM4	HoChiMinh	2017/Dec	Pomeranian	4	F	3	+/-	Glu	CPV-2c
39	HCM5	HoChiMinh	2017/Dec	Pomeranian	4	F	3	+/-	Glu	CPV-2c
40	HCM6	HoChiMinh	2017/Dec	Pomeranian	4	M	3	+/-	Glu	CPV-2c
41	HCM8	HoChiMinh	2017/Dec	Dachshund	3	M	3	+/-	Glu	CPV-2c
42	HCM9	HoChiMinh	2017/Dec	Bulldog	3	M	0	+/-	Glu	CPV-2c
43	HCM 10	HoChiMinh	2017/Dec	Bulldog	3	F	0	+/-	Glu	CPV-2c
44	HCM 11	HoChiMinh	2017/Dec	Shih Tzu	2	F	2	+/-	Glu	CPV-2c
45	HCM 12	HoChiMinh	2017/Dec	Poodle	3	F	3	+/-	Glu	CPV-2c
46	HCM 13	HoChiMinh	2017/Dec	Bulldog	4	F	3	+/-	Glu	CPV-2c
47	HCM 14	HoChiMinh	2017/Dec	Mixed	3	M	0	+/-	Glu	CPV-2c
48	HCM 15	HoChiMinh	2017/Dec	Shih Tzu	NA	M	NA	+/-	Glu	CPV-2c
49	HCM 16	HoChiMinh	2017/Dec	Poodle	4	M	3	+/-	Asn	CPV-2a
50	HCM 18	HoChiMinh	2017/Dec	Pomeranian	7	Fs	2	+/-	Glu	CPV-2c
51	HCM 19	HoChiMinh	2017/Dec	Mixed	5	F	1	+/-	Glu	CPV-2c
52	HCM 20	HoChiMinh	2017/Dec	Mixed	5	F	1	+/-	Glu	CPV-2c
53	HCM 21	HoChiMinh	2017/Dec	Bulldog	3	F	3	+/-	Glu	CPV-2c
54	HCM 22	HoChiMinh	2017/Dec	Pug	3	F	3	+/-	Glu	CPV-2c
55	HCM 23	HoChiMinh	2017/Dec	Mixed	2	F	0	+/-	Glu	CPV-2c
56	HCM 24	HoChiMinh	2017/Dec	Poodle	9	Mc	0	+/-	Glu	CPV-2c
57	HCM 25	HoChiMinh	2017/Dec	Poodle	6	F	NA	+/-	Glu	CPV-2c

58	HCM 26	HoChiMinh	2017/Dec	Bulldog	2	M	2	+/-	Glu	CPV-2c
59	HCM 27	HoChiMinh	2017/Dec	Bulldog	5	F	3	+/-	Glu	CPV-2c

[†] Sampling time: year/month; [‡] NA: No data available; [§] M: male, F: female, Mc: castrated male, Fs: sprayed female; [¶] Vaccination time: number of vaccination for parvovirus; [$] +: positive, -: Negative

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) strains and one CPV-2a (HCM16) strain 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 2-3, 2-4). 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 an Italian CPV-2c strain (MF510157) isolated in 2017 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 2-3, 2-5).

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, 2-6). 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 2-3, 2-6).

Table 2-3 Sumary of nucleotide and amino acid similarity (%) of Vietnamese CPV-2 and reference strains

Strain	Accession No		Full genome	NS gene	VP gene
Italian CPV-2c	KU508407	nt	98.8–98.9	99.0–99.1	98.8–98.9
2009†		aa		99.2–9.4	98.7–99.0
Italian CPV-2c,	MF510157	nt	99.6–99.8	99.7–100	99.4–99.6
2017†		aa		99.8–100	99.5–99.8
Chinese CPV-2a,	KR002800	nt	99.3–99.5	99.7–100	98.9–99.1
2016†		aa		99.7–100	99.0–99.3
Vietnamese CPV-2c	MT106228-	nt	99.5–99.8	99.6–99.9	99.5–99.9
2017‡	MT106237	aa		99.5–100	99.7–100
Vietnamese CPV-2a	MT106238	nt	98.6–98.7	98.7–98.9	98.5–98.7
2017‡		aa		98.2–98.5	98.7–99.0

† Year isolated virus, ‡ Sequences in this study, nt: nucleotide, aa amino acid

Table 2-4 Full-genome sequence similarity of the Vietnamese CPV-2c and CPV-2a variants along with reference strains[†]

	Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13
1	MF510157[†]													
2	KU508407[†]	0.991												
3	KR002800[†]	0.996	0.991											
4	HN07	0.998	0.989	0.995										
5	HN06	0.996	0.988	0.994	0.997									
6	HN04	0.996	0.988	0.994	0.998	0.996								
7	HN08	0.997	0.988	0.994	0.998	0.996	0.997							
8	HN30	0.997	0.988	0.994	0.998	0.996	0.998	0.998						
9	DN01	0.996	0.988	0.994	0.998	0.996	0.997	0.996	0.996					
10	DN02	0.996	0.989	0.994	0.997	0.997	0.996	0.996	0.996	0.997				
11	HCM01	0.996	0.988	0.993	0.997	0.997	0.997	0.996	0.996	0.996	0.996			
12	HCM03	0.996	0.988	0.993	0.997	0.996	0.996	0.997	0.997	0.996	0.995	0.996		
13	HCM09	0.997	0.988	0.994	0.998	0.997	0.997	0.998	0.998	0.996	0.996	0.997	0.997	
14	HCM16	0.988	0.989	0.991	0.987	0.986	0.986	0.986	0.986	0.986	0.987	0.986	0.985	0.986

[†] *Reference strains*

Table 2-5 VP1/VP2 sequence similarity of the Vietnamese CPV-2c and CPV-2a variants along with three reference strains[†]

	Sequence[‡]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	MF510157[†]		0.991	0.991	0.997	0.997	0.995	0.995	0.995	0.997	0.998	0.995	0.995	0.995	0.988
2	KU508407[†]	0.993		0.991	0.988	0.988	0.987	0.987	0.987	0.988	0.990	0.987	0.987	0.987	0.986
3	KR002800[†]	0.992	0.992		0.991	0.991	0.990	0.990	0.990	0.991	0.993	0.990	0.990	0.990	0.994
4	HN07	0.996	0.989	0.991		0.997	0.998	0.998	0.998	1.000	0.998	0.998	0.998	0.998	0.988
5	HN06	0.995	0.989	0.990	0.997		0.998	0.998	0.998	0.997	0.998	0.998	0.998	0.998	0.988
6	HN04	0.994	0.988	0.989	0.997	0.997		1.000	1.000	0.998	0.997	1.000	1.000	1.000	0.987
7	HN08	0.995	0.989	0.990	0.997	0.997	0.997		1.000	0.998	0.997	1.000	1.000	1.000	0.987
8	HN30	0.995	0.988	0.990	0.997	0.996	0.999	0.997		0.998	0.997	1.000	1.000	1.000	0.987
9	DN01	0.994	0.988	0.989	0.997	0.996	0.996	0.996	0.995		0.998	0.998	0.998	0.998	0.988
10	DN02	0.996	0.989	0.991	0.998	0.997	0.996	0.996	0.996	0.998		0.997	0.997	0.997	0.990
11	HCM01	0.994	0.989	0.989	0.997	0.998	0.997	0.997	0.996	0.996	0.996		1.000	1.000	0.987
12	HCM03	0.995	0.989	0.990	0.997	0.996	0.996	0.997	0.997	0.996	0.996	0.997		1.000	0.987
13	HCM09	0.995	0.988	0.990	0.997	0.997	0.996	0.997	0.997	0.995	0.996	0.997	0.997		0.987
14	HCM16	0.988	0.987	0.994	0.987	0.986	0.985	0.986	0.986	0.985	0.987	0.985	0.986	0.986	

[†] Reference strains; [‡] Upper half shows amino acid similarity; lower half shows nucleotide similarity

Table 2-6 NS1/NS2 sequence similarity of the Vietnamese CPV-2c and CPV-2a variants along with three reference strains[†]

	Sequence[‡]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	MF510157[†]		0.994	1.000	1.000	0.998	0.998	0.998	1.000	1.000	0.997	1.000	0.998	1.000	0.985
2	KU508407[†]	0.991		0.994	0.994	0.992	0.992	0.992	0.994	0.994	0.991	0.994	0.992	0.994	0.991
3	KR002800[†]	1.000	0.991		1.000	0.998	0.998	0.998	1.000	1.000	0.997	1.000	0.998	1.000	0.985
4	HN07	1.000	0.991	1.000		0.998	0.998	0.998	1.000	1.000	0.997	1.000	0.998	1.000	0.985
5	HN06	0.998	0.990	0.998	0.998		0.997	0.997	0.998	0.998	0.995	0.998	0.997	0.998	0.983
6	HN04	0.999	0.991	0.999	0.999	0.997		0.997	0.998	0.998	0.995	0.998	0.997	0.998	0.983
7	HN08	0.998	0.990	0.998	0.998	0.997	0.997		0.998	0.998	0.995	0.998	0.997	0.998	0.983
8	HN30	0.999	0.991	0.999	0.999	0.998	0.998	0.999		1.000	0.997	1.000	0.998	1.000	0.985
9	DN01	0.999	0.991	0.999	0.999	0.997	0.998	0.997	0.998		0.997	1.000	0.998	1.000	0.985
10	DN02	0.999	0.990	0.999	0.999	0.997	0.998	0.997	0.998	0.998		0.997	0.995	0.997	0.982
11	HCM01	0.999	0.991	0.999	0.999	0.997	0.999	0.997	0.998	0.998	0.998		0.998	1.000	0.985
12	HCM03	0.997	0.990	0.997	0.997	0.996	0.996	0.997	0.998	0.996	0.996	0.996		0.998	0.983
13	HCM09	0.999	0.990	0.999	0.999	0.997	0.998	0.998	0.999	0.998	0.998	0.998	0.997		0.985
14	HCM16	0.989	0.992	0.989	0.989	0.988	0.989	0.988	0.989	0.989	0.988	0.989	0.987	0.988	

[†] Reference strains; [‡] Upper half shows amino acid similarity; lower half shows nucleotide similarity

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 2-7).

Analysis of the deduced amino acid residues of the structural protein sequences 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 2-7).

Table 2-7 Non-synonymous nucleotide and amino acid variations in the NS1/NS2 and VP1/VP2 genes

Strain	Genome type	Nucleotide/amino acid position												
		NS1/NS2				VP1				VP2				
		178	1631	1633	1889	335	347	373	391	14	800	971	1109	1341 (nt)
		60	544	545	630	112	116	125	131	5	267	324	370	447 (aa)
KU508407†	CPV-2c	A/Ile	A/Tyr	A/Glu	T/Leu	C/Thr	G/Arg	C/Leu	G/Ala	C/Ala	T/Phe	T/Tyr	A/Gln	A/Ile
MF510157†	CPV-2c	G/Val	T/Phe	T/Val	C/Pro	C/Thr	A/Lys	C/Leu	A/Thr	G/Gly	A/Tyr	A/Ile	G/Arg	A/Ile
KR002800†	CPV-2a	G/Val	T/Phe	T/Val	C/Pro	C/Thr	A/Lys	T/Ile	G/Ala	C/Ala	A/Tyr	A/Ile	A/Gln	A/Ile
HN04	CPV-2c	G/Val	T/Phe	T /Val	C/Pro	T/Ile	A/Lys	T/Ile	A/Thr	G/Gly	A/Tyr	A/Ile	G/Arg	G/Met
HN06	CPV-2c	G/Val	T/Phe	T/Val	C/Pro	C/Thr	A/Lys	T/Ile	A/Thr	G/Gly	A/Tyr	A/Ile	G/Arg	G/Met
HN07	CPV-2c	G/Val	T/Phe	T/Val	C/Pro	T/Ile	A/Lys	T/Ile	A/Thr	G/Gly	A/Tyr	A/Ile	G/Arg	A/Ile
HN08	CPV-2c	G/Val	T/Phe	T/Val	C/Pro	T/Ile	A/Lys	T/Ile	A/Thr	G/Gly	A/Tyr	A/Ile	G/Arg	G/Met
HN30	CPV-2c	G/Val	T/Phe	T/Val	C/Pro	T/Ile	A/Lys	T/Ile	A/Thr	G/Gly	A/Tyr	A/Ile	G/Arg	G/Met
DN01	CPV-2c	G/Val	T/Phe	T/Val	C/Pro	T/Ile	A/Lys	T/Ile	A/Thr	G/Gly	A/Tyr	A/Ile	G/Arg	A/Ile
DN02	CPV-2c	G/Val	T/Phe	T/Val	C/Pro	C/Thr	A/Lys	T/Ile	A/Thr	G/Gly	A/Tyr	A/Ile	G/Arg	A/Ile
HCM01	CPV-2c	G/Val	T/Phe	T/Val	C/Pro	T/Ile	A/Lys	T/Ile	A/Thr	G/Gly	A/Tyr	A/Ile	G/Arg	G/Met
HCM03	CPV-2c	G/Val	T/Phe	T/Val	C/Pro	T/Ile	A/Lys	T/ Ile	A/Thr	G/Gly	A/Tyr	A/Ile	G/Arg	G/Met
HCM09	CPV-2c	G/Val	T/Phe	T/Val	C/Pro	T/Ile	A/Lys	T/ Ile	A/Thr	G/Gly	A/Tyr	A/Ile	G/Arg	G/Met
HCM16	CPV-2a	A/Ile	A/Tyr	A/Glu	T/Leu	C/Thr	A/Lys	T/Ile	A/Thr	C/Ala	A/Tyr	A/Ile	A/Gln	A/Ile

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 2-8).

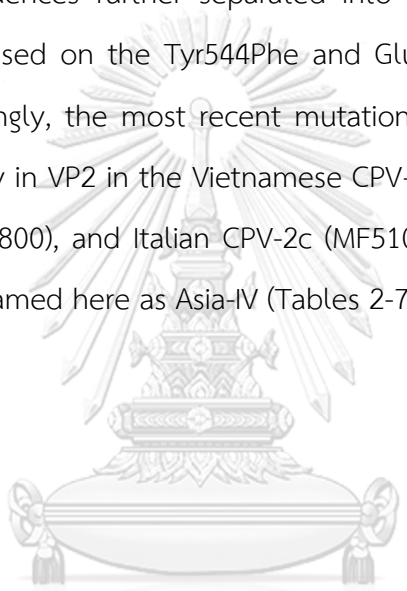
Table 2-8 Amino acid signatures of the NS1 and VP2 proteins for evolutionary analysis

Clade	Subclade	NS1				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

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 in 1986 (Figure 2-1). 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 2-7, 2-8, 2-9 and Figure 2-1).



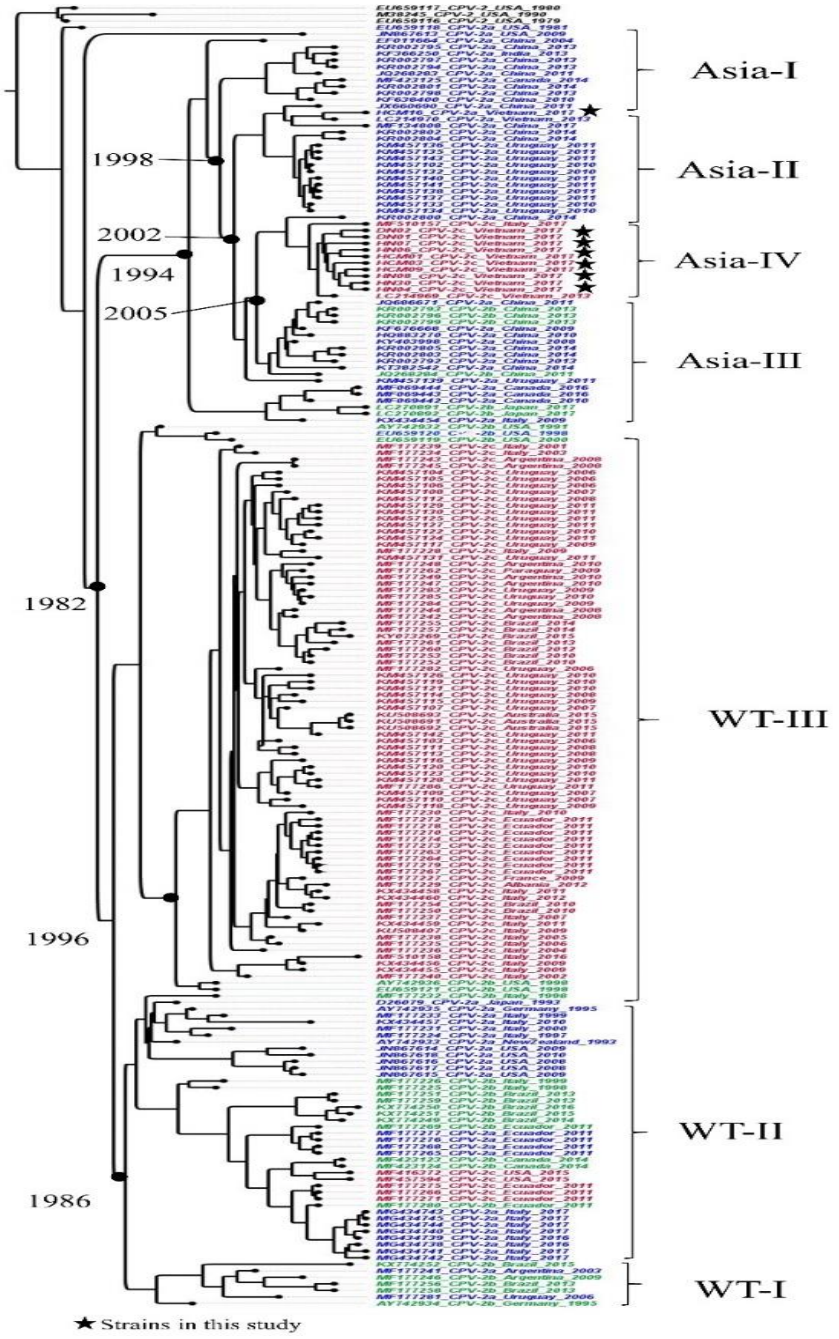


Figure 2-1 Phylogenetic tree (ML) with timeline estimated divergences based on 199 full-length genomes of CPV-2 isolated from 1978 to 2019. Evolutionary clades are indicated as Asia and Western (WT) with their subclades as Asia-I to -IV, and WT-I to -III. The strains are colored according to the CPV genotype. * Represents the CPV strains from this study.

Table 2-9 Amino acid signature of the NS1 and VP2 protein sequence in each evolutionary group (totally 199 sequences)

No	Strain[1]	Clade	NS1[2]				VP2[2]		
			60	544	545	630	267	324	426
1	EU659116_CPV-2_USA_1979	CPV-2	I	Y	E	L	F	Y	N
2	EU659117_CPV-2_USA_1980	CPV-2	I	Y	E	L	F	Y	N
1	EF011664_CPV-2a_China_2004	Asia-I	I	Y	E	L	F	I	N
2	KF638400_CPV-2a_China_2010	Asia-I	I	Y	E	L	F	I	N
3	JQ268283_CPV-2a_China_2011	Asia-I	I	Y	E	L	F	I	N
4	KR002795_CPV-2a_China_2013	Asia-I	I	Y	E	L	F	I	N
5	KF366250_CPV-2a_India_2013	Asia-I	I	Y	E	L	F	Y	N
6	KR002794_CPV-2a_China_2013	Asia-I	I	Y	E	L	F	I	N
7	KR002797_CPV-2a_China_2013	Asia-I	I	Y	E	L	F	I	N
8	KR002798_CPV-2a_China_2013	Asia-I	I	Y	E	L	F	I	N
9	KR002801_CPV-2a_China_2014	Asia-I	I	Y	E	L	F	I	N
10	MF423125_CPV-2a_Canada_2014	Asia-I	I	Y	E	L	F	I	N
1	KM457102_CPV-2a_Uruguay_2010	Asia-II	I	Y	E	L	Y	I	N
2	KM457132_CPV-2a_Uruguay_2010	Asia-II	I	Y	E	L	Y	I	N
3	KM457133_CPV-2a_Uruguay_2010	Asia-II	I	Y	E	L	Y	I	N
4	KM457134_CPV-2a_Uruguay_2010	Asia-II	I	Y	E	L	Y	I	N
5	KM457135_CPV-2a_Uruguay_2011	Asia-II	I	Y	E	L	Y	I	N
6	KM457136_CPV-2a_Uruguay_2011	Asia-II	I	Y	E	L	Y	I	N
7	KM457137_CPV-2a_Uruguay_2011	Asia-II	I	Y	E	L	Y	I	N
8	KM457138_CPV-2a_Uruguay_2011	Asia-II	I	Y	E	L	Y	I	N
9	KM457140_CPV-2a_Uruguay_2011	Asia-II	I	Y	E	L	Y	I	N
10	KM457141_CPV-2a_Uruguay_2011	Asia-II	I	Y	E	L	Y	I	N
11	KM457143_CPV-2a_Uruguay_2011	Asia-II	I	Y	E	L	Y	I	N
12	JX660690_CPV-2a_China_2011	Asia-II	I	Y	E	L	Y	I	N
13	KR002802_CPV-2a_China_2014	Asia-II	I	Y	E	L	Y	I	N
14	KR002804_CPV-2a_China_2014	Asia-II	I	Y	E	L	Y	I	N
15	MF134808_CPV-2a_China_2017	Asia-II	I	Y	E	L	Y	I	N
16	LC214970_CPV-2a_Vietnam_2013	Asia-II	I	Y	E	L	Y	I	N

17	HCM16_CVP-2a_Vietnam_2017	Asia-II	I	Y	E	L	Y	I	N
1	KY403998_CPV-2a_China_2008	Asia-III	I	F	V	L	Y	I	N
2	KF676668_CPV-2a_China_2009	Asia-III	I	F	V	L	Y	I	N
3	HQ883270_CPV-2a_China_2010	Asia-III	I	F	V	L	Y	I	N
4	JQ686671_CPV-2a_China_2011	Asia-III	I	F	V	L	Y	I	N
5	KR002792_CPV-2a_China_2013	Asia-III	I	F	V	L	Y	I	N
6	KR002805_CPV-2a_China_2014	Asia-III	I	F	V	L	Y	I	N
7	KR002803_CPV-2a_China_2014	Asia-III	I	Y	E	L	Y	I	N
8	KT382542_CPV-2a_China_2014	Asia-III	I	Y	E	L	Y	I	N
9	KM457139_CPV-2a_Uruguay_2011	Asia-III	I	Y	E	L	Y	I	N
10	JQ268284_CPV-2b_China_2011	Asia-III	I	Y	V	L	Y	I	D
11	KR002793_CPV-2b_China_2013	Asia-III	I	F	V	L	Y	I	D
12	KR002796_CPV-2b_China_2013	Asia-III	I	F	V	L	Y	I	D
13	KR002799_CPV-2b_China_2013	Asia-III	I	F	V	L	Y	I	D
1	LC214969_CPV-2c_Vietnam_2013	Asia-IV	V	Y	E	P	Y	I	E
2	KR002800_CPV-2a_China_2014	Asia-IV	V	F	V	P	Y	I	N
3	MF510157_CPV-2c_Italy_2017	Asia-IV	V	F	V	P	Y	I	E
4	HN04_CVP-2c_Vietnam_2017	Asia-IV	V	F	V	P	Y	I	E
5	HN06_CVP-2c_Vietnam_2017	Asia-IV	V	F	V	P	Y	I	E
6	HN07_CVP-2c_Vietnam_2017	Asia-IV	V	F	V	P	Y	I	E
7	HN08_CVP-2c_Vietnam_2017	Asia-IV	V	F	V	P	Y	I	E
8	HN30_CVP-2c_Vietnam_2017	Asia-IV	V	F	V	P	Y	I	E
9	DN01_CVP-2c_Vietnam_2017	Asia-IV	V	F	V	P	Y	I	E
10	DN02_CVP-2c_Vietnam_2017	Asia-IV	V	F	V	P	Y	I	E
11	HCM01_CVP-2c_Vietnam_2017	Asia-IV	V	F	V	P	Y	I	E
12	HCM03_CVP-2c_Vietnam_2017	Asia-IV	V	F	V	P	Y	I	E
13	HCM09_CVP-2c_Vietnam_2017	Asia-IV	V	F	V	P	Y	I	E
1	MF177241_CPV-2a_Argentina_2003	WT-I	I	Y	E	L	F	Y	N
2	MF177281_CPV-2a_Uruguay_2006	WT-I	I	Y	E	L	F	Y	N
3	AY742934_CPV-2b_Germany_1995	WT-I	I	Y	E	L	F	Y	D
4	MF177246_CPV-2b_Argentina_2009	WT-I	I	Y	E	L	F	Y	D
5	MF177256_CPV-2b_Brazil_2013	WT-I	I	Y	E	L	F	L	D

6	MF177258_CPV-2b_Brazil_2013	WT-I	I	Y	E	L	F	L	D
7	KX774252_CPV-2b_Brazil_2015	WT-I	I	Y	E	L	F	L	D
1	D26079_CPV-2a_Japan_1993	WT-II	I	F	E	L	F	Y	N
2	AY742933_CPV-2a_N.Zealand_1993	WT-II	I	F	E	L	F	Y	N
3	AY742935_CPV-2a_Germany_1995	WT-II	I	F	E	L	F	Y	N
4	MF177224_CPV-2a_Italy_1997	WT-II	I	F	E	L	F	Y	N
5	MF177233_CPV-2a_Italy_1999	WT-II	I	F	E	L	F	Y	N
6	MF177231_CPV-2a_Italy_2000	WT-II	I	F	E	L	F	Y	N
7	KX434457_CPV-2a_Italy_2010	WT-II	I	F	E	L	F	Y	N
8	JN867616_CPV-2a_USA_2008	WT-II	I	F	E	L	F	Y	N
9	JN867617_CPV-2a_USA_2008	WT-II	I	F	E	L	F	Y	N
10	JN867614_CPV-2a_USA_2009	WT-II	I	F	E	L	F	Y	N
11	JN867615_CPV-2a_USA_2009	WT-II	I	F	E	L	F	Y	N
12	JN867618_CPV-2a_USA_2010	WT-II	I	F	E	L	F	Y	N
13	MF177225_CPV-2b_Italy_1998	WT-II	I	F	E	L	F	Y	D
14	MF177232_CPV-2b_Italy_1998	WT-II	I	F	E	L	F	Y	D
15	MF177226_CPV-2b_Italy_1999	WT-II	I	F	E	L	F	Y	D
16	MF177251_CPV-2b_Brazil_2013	WT-II	I	F	E	L	F	Y	D
17	MF177259_CPV-2b_Brazil_2013	WT-II	I	F	E	L	F	Y	D
18	KX774249_CPV-2b_Brazil_2014	WT-II	I	F	E	L	F	L	D
19	KX774251_CPV-2b_Brazil_2015	WT-II	I	F	E	L	F	L	D
20	KX774250_CPV-2b_Brazil_2016	WT-II	I	F	E	L	F	L	D
21	MF177265_CPV-2a_Ecuador_2011	WT-II	I	F	E	L	F	Y	N
22	MF177268_CPV-2a_Ecuador_2011	WT-II	I	F	E	L	F	Y	N
23	MF177269_CPV-2b_Ecuador_2011	WT-II	I	F	E	L	F	Y	D
24	MF177276_CPV-2a_Ecuador_2011	WT-II	I	F	E	L	F	Y	N
25	MF177277_CPV-2a_Ecuador_2011	WT-II	I	F	E	L	F	Y	N
26	MG434738_CPV-2a_Italy_2016	WT-II	I	F	E	L	F	L	N
27	MG434739_CPV-2a_Italy_2016	WT-II	I	F	E	L	F	L	N
28	MG434740_CPV-2a_Italy_2017	WT-II	I	F	E	L	F	L	N
29	MG434741_CPV-2a_Italy_2017	WT-II	I	F	E	L	F	L	N
30	MG434742_CPV-2a_Italy_2017	WT-II	I	F	E	L	F	L	N

31	MG434743_CPV-2a_Italy_2017	WT-II	I	F	E	L	F	L	N
32	MG434744_CPV-2a_Italy_2017	WT-II	I	F	E	L	F	L	N
33	MG434745_CPV-2a_Italy_2017	WT-II	I	F	E	L	F	L	N
34	MF423123_CPV-2b_Canada_2014	WT-II	I	F	E	L	F	Y	D
35	MF423124_CPV-2b_Canada_2014	WT-II	I	F	E	L	F	Y	D
36	MF177280_CPV-2b_Ecuador_2011	WT-II	I	F	E	L	F	Y	D
37	MF177275_CPV-2c_Ecuador_2011	WT-II	I	F	E	L	F	Y	E
38	MF177266_CPV-2c_Ecuador_2011	WT-II	I	F	E	L	F	Y	E
39	MF177271_CPV-2c_Ecuador_2011	WT-II	I	F	E	L	F	Y	E
40	MF416372_CPV-2c_USA_2015	WT-II	I	Y	E	L	F	Y	E
41	MF457594_CPV-2c_USA_2015	WT-II	I	Y	E	L	F	Y	E
1	MF177239_CPV-2c_Italy_2001	WT-III	I	Y	E	L	F	Y	E
2	MF177240_CPV-2c_Italy_2002	WT-III	I	Y	E	L	F	Y	E
3	MF177234_CPV-2c_Italy_2003	WT-III	I	Y	E	L	F	Y	E
4	MF177236_CPV-2c_Italy_2004	WT-III	I	Y	E	L	F	Y	E
5	MF177238_CPV-2c_Italy_2005	WT-III	I	Y	E	L	F	Y	E
6	MF177235_CPV-2c_Italy_2006	WT-III	I	Y	E	L	F	Y	E
7	MF177237_CPV-2c_Italy_2007	WT-III	I	Y	E	L	F	Y	E
8	MF177228_CPV-2c_Italy_2009	WT-III	I	Y	E	L	F	Y	E
9	KX434455_CPV-2c_Italy_2009	WT-III	I	Y	E	L	F	Y	E
10	KX434456_CPV-2c_Italy_2009	WT-III	I	Y	E	L	F	Y	E
11	KU508407_CPV-2c_Italy_2009	WT-III	I	Y	E	L	F	Y	E
12	MF177230_CPV-2c_Italy_2010	WT-III	I	Y	E	L	F	Y	E
13	KX434458_CPV-2c_Italy_2011	WT-III	I	Y	E	L	F	Y	E
14	KX434459_CPV-2c_Italy_2011	WT-III	I	Y	E	L	F	Y	E
15	KX434460_CPV-2c_Italy_2012	WT-III	I	Y	E	L	F	Y	E
16	MF510158_CPV-2c_Italy_2016	WT-III	I	Y	E	L	F	Y	E
17	MF177242_CPV-2c_Argentina_2008	WT-III	I	Y	E	L	F	Y	E
18	MF177243_CPV-2c_Argentina_2008	WT-III	I	Y	E	L	F	Y	E
19	MF177244_CPV-2c_Argentina_2008	WT-III	I	Y	E	L	F	Y	E
20	MF177245_CPV-2c_Argentina_2008	WT-III	I	Y	E	L	F	Y	E
21	MF177247_CPV-2c_Argentina_2010	WT-III	I	Y	E	L	F	Y	E

22	MF177248_CPV-2c_Argentina_2010	WT-III	I	Y	E	L	F	Y	E
23	MF177249_CPV-2c_Argentina_2010	WT-III	I	Y	E	L	F	Y	E
24	KM457103_CPV-2c_Uruguay_2006	WT-III	I	Y	E	L	F	Y	E
25	KM457104_CPV-2c_Uruguay_2006	WT-III	I	Y	E	L	F	Y	E
26	KM457105_CPV-2c_Uruguay_2006	WT-III	I	Y	E	L	F	Y	E
27	KM457106_CPV-2c_Uruguay_2006	WT-III	I	Y	E	L	F	Y	E
28	MF177282_CPV-2c_Uruguay_2006	WT-III	I	Y	E	L	F	Y	E
29	KM457107_CPV-2c_Uruguay_2007	WT-III	I	Y	E	L	F	Y	E
30	KM457108_CPV-2c_Uruguay_2007	WT-III	I	Y	E	L	F	Y	E
31	KM457109_CPV-2c_Uruguay_2007	WT-III	I	Y	E	L	F	Y	E
32	KM457110_CPV-2c_Uruguay_2007	WT-III	I	Y	E	L	F	Y	E
33	KM457111_CPV-2c_Uruguay_2008	WT-III	I	Y	E	L	F	Y	E
34	KM457112_CPV-2c_Uruguay_2008	WT-III	I	Y	E	L	F	Y	E
35	KM457113_CPV-2c_Uruguay_2008	WT-III	I	Y	E	L	F	Y	E
36	KM457114_CPV-2c_Uruguay_2008	WT-III	I	Y	E	L	F	Y	E
37	KM457115_CPV-2c_Uruguay_2009	WT-III	I	Y	E	L	F	Y	E
38	KM457116_CPV-2c_Uruguay_2009	WT-III	I	Y	E	L	F	Y	E
39	KM457117_CPV-2c_Uruguay_2009	WT-III	I	Y	E	L	F	Y	E
40	KM457118_CPV-2c_Uruguay_2009	WT-III	I	Y	E	L	F	Y	E
41	MF177283_CPV-2c_Uruguay_2009	WT-III	I	Y	E	L	F	Y	E
42	MF177284_CPV-2c_Uruguay_2009	WT-III	I	Y	E	L	F	Y	E
43	MF177285_CPV-2c_Uruguay_2010	WT-III	I	Y	E	L	F	Y	E
44	KM457119_CPV-2c_Uruguay_2010	WT-III	I	Y	E	L	F	Y	E
45	KM457120_CPV-2c_Uruguay_2010	WT-III	I	Y	E	L	F	Y	E
46	KM457121_CPV-2c_Uruguay_2010	WT-III	I	Y	E	L	F	Y	E
47	KM457122_CPV-2c_Uruguay_2010	WT-III	I	Y	E	L	F	Y	E
48	KM457123_CPV-2c_Uruguay_2010	WT-III	I	Y	E	L	F	Y	E
49	KM457126_CPV-2c_Uruguay_2010	WT-III	I	Y	E	L	F	Y	E
50	KM457124_CPV-2c_Uruguay_2011	WT-III	I	Y	E	L	F	Y	E
51	KM457125_CPV-2c_Uruguay_2011	WT-III	I	Y	E	L	F	Y	E
52	KM457127_CPV-2c_Uruguay_2011	WT-III	I	Y	E	L	F	Y	E
53	KM457128_CPV-2c_Uruguay_2011	WT-III	I	Y	E	L	F	Y	E

54	KM457129_CPV-2c_Uruguay_2011	WT-III	I	Y	E	L	F	Y	E
55	KM457130_CPV-2c_Uruguay_2011	WT-III	I	Y	E	L	F	Y	E
56	KM457131_CPV-2c_Uruguay_2011	WT-III	I	Y	E	L	F	Y	E
57	KM457142_CPV-2c_Uruguay_2011	WT-III	I	Y	E	L	F	Y	E
58	MF177286_CPV-2c_Uruguay_2011	WT-III	I	Y	E	L	F	Y	E
59	MF177262_CPV-2c_Paraguay_2009	WT-III	I	Y	E	L	F	Y	E
60	MF177250_CPV-2c_Brazil_2010	WT-III	I	Y	E	L	F	Y	E
61	MF177252_CPV-2c_Brazil_2010	WT-III	I	Y	E	L	F	Y	E
62	MF177253_CPV-2c_Brazil_2010	WT-III	I	Y	E	L	F	Y	E
63	MF177254_CPV-2c_Brazil_2010	WT-III	I	Y	E	L	F	Y	E
64	MF177260_CPV-2c_Brazil_2012	WT-III	I	Y	E	L	F	Y	E
65	MF177261_CPV-2c_Brazil_2013	WT-III	I	Y	E	L	F	Y	E
66	MF177255_CPV-2c_Brazil_2014	WT-III	I	Y	E	L	F	Y	E
67	MF177257_CPV-2c_Brazil_2014	WT-III	I	Y	E	L	F	Y	E
68	KY073269_CPV-2c_Brazil_2015	WT-III	I	Y	E	L	F	Y	E
69	KU508691_CPV-2c_Australia_2015	WT-III	I	Y	E	L	F	Y	E
70	KU508692_CPV-2c_Australia_2015	WT-III	I	Y	E	L	F	Y	E
71	KU508693_CPV-2c_Australia_2015	WT-III	I	Y	E	L	F	Y	E
72	MF177270_CPV-2c_Ecuador_2011	WT-III	I	Y	E	L	F	Y	E
73	MF177263_CPV-2c_Ecuador_2011	WT-III	I	Y	E	L	F	Y	E
74	MF177264_CPV-2c_Ecuador_2011	WT-III	I	Y	E	L	F	Y	E
75	MF177267_CPV-2c_Ecuador_2011	WT-III	I	Y	E	L	F	Y	E
76	MF177272_CPV-2c_Ecuador_2011	WT-III	I	Y	E	L	F	Y	E
77	MF177273_CPV-2c_Ecuador_2011	WT-III	I	Y	E	L	F	Y	E
78	MF177274_CPV-2c_Ecuador_2011	WT-III	I	Y	E	L	F	Y	E
79	MF177278_CPV-2c_Ecuador_2011	WT-III	I	Y	E	L	F	Y	E
80	MF177279_CPV-2c_Ecuador_2011	WT-III	I	Y	E	L	F	Y	E
81	MF177227_CPV-2c_France_2009	WT-III	I	Y	E	L	F	Y	E
82	MF177229_CPV-2c_Albania_2012	WT-III	I	Y	E	L	F	Y	E

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 2-10).

Table 2-10 Summary statistics of the nucleotide substitution rates

Data and ML estimates	Full-length	NS1/NS2	VP1/VP2	Asian Clade	European 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)[1]	2.49E-04	3.16E-04	3.06E-04	2.12E-04	1.69E-04
Stderr of mean	2.23E-06	2.12E-06	2.06E-06	2.53E-06	1.52E-06
Stdev	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
Effective Sample Size (ESS)	220.2191	462.9358	425.3558	509.4852	311.6939

[†] Nucleotide/site/year

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.

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 (Table 2-11).

Table 2-11 Evidence for positive and negative selection using various detection methods

CPV genome	Selection pressure	SLAC[†]	FEL[‡]
NS gene	Positive selection	2	5
	Negative selection	45	112
	Overall dN/dS	0.116	0.100
VP gene	Positive selection	2	3
	Negative selection	37	155
	Overall dN/dS	0.090	0.096

Diversifying selection sites

[†] SLAC: positive selection for NS at sites 366 and572; for VP at sites 23 and 416

[‡] FEL: positive selection for NS at sites 572, 582, 583, 597, and 626; for VP at sites 131, 467, and 583.

2.5 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 (Kang *et al.*, 2008; Ohshima*et al.*, 2008; Geng*et al.*, 2015; Hoang*et al.*, 2019). 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 strains obtained from 59 Vietnamese dogs.

In this study, Vietnamese CPV-2c strains 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. 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. 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; Wang *et al.*, 2016; Wu *et al.*, 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.

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 (Kang*et al.*, 2008; Phromnoi *et al.*, 2010; Soma *et al.*, 2013; Zhao *et al.*, 2013; Mukhopadhyay *et al.*, 2014; Geng*et al.*, 2015; Zhao *et al.*, 2017), 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 characteristics. 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.

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*et al.*, 2005). More retrieved sequences could lead to a greater precision of 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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2.7 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).

2.8 Conflict of interests

The authors declare no conflict of interests.

CHAPTER 3

Detection and molecular characterization of two canine circovirus genotypes co-circulating in Vietnam

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3.1 Abstract

Canine circovirus (CanineCV), a relatively new virus in dogs, is currently detectable in many countries including USA, Italy, Germany, Thailand, Taiwan, Argentina and China. CanineCV was detected in sera of healthy dogs and in feces/organs of dogs with diarrhea, hemorrhagic gastroenteritis, vasculitis; and frequently coinfected with canine parvovirus-2 (CPV-2). In this study, we examined the prevalence of CanineCV in 81 fecal CPV-2-positive samples collected in Vietnam during 2017-2018. Moreover, other canine viruses including canine bocavirus (CBoV), canine adenovirus (CAdV), paramyxovirus, canine coronavirus and porcine circovirus-3 were also tested. A total of sixteen samples (19.7%) were found to be positive for CanineCV. Eight selected Vietnamese CanineCV (CanineCV-VN) were sequenced, analyzed and phylogram construction. Interestingly, it revealed that those CanineCV-VN strains were clustered into 2 different genotypes which were CanineCV genotype-1 (CanineCV-VN-3, 4, 5, 8 strains) and CanineCV genotype-4 (CanineCV-VN-1, 2, 6, 7 strains), with nucleotide similarity at 86-87.2%. The nucleotide discrepancy among both genotypes altered the deduced amino acid sequence in 14 and 10 residues of Replicase and Capsid proteins, respectively. Recombination event was found in genomic sequence of CanineCV-VN-6 with American CanineCV (KC241983) and Chinese CanineCV (MG279125) as the major and the minor parents, respectively. Only single dog revealed triple detections of CPV-2c, CanineCV and CAdV (1.2%). This study provides evidence of the co-circulation of two different genotypes of CanineCV and CPV-2c in dogs in Vietnam. The continuous surveillance of CanineCV in samples derived from healthy dogs and *in situ* investigation for virus distribution are necessary for further understanding the epidemiology and pathogenesis of CanineCV.

Keywords Canine circovirus, Capsid, Replicase, Vietnam

3.2 Introduction

Canine circovirus (CanineCV) is a member of the genus *Circovirus*, family *Circoviridae*, and belongs together with other mammal and avian circoviruses found in humans, pigs, ducks, gooses, pigeons (ICTV, 2019). In non-human mammals, the

porcine circovirus (PCV) infection in pigs causes immunosuppressive condition, particularly the PCV type 2 (PCV-2), and results in devastating syndromes in swine production economy (Segales *et al.*, 2005). Besides, the CanineCV was firstly detected in serum samples collected from dogs in the USA in 2012 (Kapoor*et al.*, 2012a). After that, the virus has been reported in dogs in Italy, Germany, Thailand, Taiwan, Argentina and China (Decaro et al., 2014; Hsu et al., 2016; Li et al., 2013; Piewbang et al., 2018; Kotsias et al., 2019; Sun et al., 2019), and it is considered as a second non-human mammalian circovirus. Recently, the CanineCV has been divided into four genotypes (CanineCV-1, -2, -3 and -4). The CanineCV-1 genotype is the most detectable prevalence which circulating in the USA, Europe and Latin America, while other CanineCV geotypes are reported in China (Niu *et al*., 2020).

CanineCV is an icohedral, non-enveloped virus containing a small monomeric single circular strand DNA with 2,063 nucleotides in length. Its genome comprises two major putative open reading frames (ORFs) which encode for the replicase (Rep) and the capsid (Cap) protein. The Rep protein contains 303 amino acids that is essential for viral replication (Cheung, 2003); while the Cap protein possesses 270 amino acids which play role as a structural protein (Kapoor*et al.*, 2012a; Li*et al.*, 2013a). Between these two major ORFs, there is a non-coding region containing 9 nucleotides (TAGTATTAC), which is a thermodynamically stable stem-loop with the function for initiation of rolling-circle replication (Kapoor*et al.*, 2012a). Recently, an additional ORF, ORF-3, was identified in the antisense of ORF1 of CanineCV Thai's strain which its function has yet been elucidated (Piewbang *et al*., 2018b). .

Currently, the CanineCV has been detected from dogs associated with different pathological conditions including vasculitis, gastroenteritis and infectious respiratory disease complex (Li*et al.*, 2013a; Decaro*et al.*, 2014; Anderson*et al.*, 2017; Piewbang*et al.*, 2018b). Since the attempt of viral isolation *in vitro* has not yet been successful, the fundamental knowledge regarding the pathogenesis of CanineCV infection in dogs remains poorly understood. However, the CanineCV has been considered as cause of enteritis and is frequently identified in a dual infection with canine parvovirus-2 (CPV-2) (Hsu *et al.*, 2016; Thaiwong*et al.*, 2016) leading to an increasing mortality rate (Anderson*et al.*, 2017). Therefore, it is suggested that

CanineCV probably acts in synergism with other infectious agents in the development of a disease.

In Vietnam, the outbreak of CPV-2 frequently occurs and causes a massive death in dogs which might be caused by the humid subtropical climate and poor vaccination program. So far, there is a lack of CanineCV study in Vietnam; meanwhile numbers of diarrheic dogs is increasing without any supporting evidence for its etiology. We, therefore, investigated the prevalence of CanineCV infection among CPV-2-infected dogs and characterized the molecular genetics of Vietnamese CanineCV strains through its recombination analysis.

3.3 Materials and Methods

Samples, viral nucleic acid extraction and CPV-2 detection

Eighty-one fecal swabs were collected from dogs residing in Hanoi (n=41), Da Nang (n=16) and Ho Chi Minh (n=24) from Vietnam during September-December 2017, immersed in sterile phosphate buffer saline pH 7.4, and preserved in -80 ^{0}C until assayed. All procedures were approved by the Chulalongkorn University Animal Care and Use Committee (No. 2031005). Samples were subjected to viral nucleic acid extraction using a commercial extraction kit (Geneaid Biotech, Taiwan) and further assayed by polymerase chain reaction (PCR) to confirm the CPV-2 infection as previously described (Nguyen Manh *et al.*, 2020). Essential data of the animals, including sex, age, and breed was also noted (Table 2-14).

PCR amplification and full-length genome sequencing of CanineCV

A set of primers, including CanineCV-605F and -1041R, CanineCV-1022F and -1538R retrieving from previous study (Piewbang*et al.*, 2018b) and CanineCV-1448F and -110R, CanineCV-2014F and -776R designing from the alignment of available CanineCV sequences from database, was used to detect and amplify the complete genome of obtained CanineCV strains (Table 3-1). PCR assay was performed using Gotaq Green Master mix (Promega, USA) and amplified the target amplicons

according to the specific primer pairs. The cycler condition consisted of initial denaturation at 94^{0}C for 7 min, 35 cycles of 94^{0}C for 30 sec, 55^{0}C for 5 min and 72^{0}C for 1 min, and final extension at 72^{0}C for 7 min. The PCR products were resolved on 1.0% (w/v) agarose gel containing 0.5% (v/v) ethidium bromide in-gel staining and visualized under a UV transilluminator. All PCR products were purified using NucleoSpin Extract II (Macherey-Nagel, Germany) and submitted for bi-directional Sanger's sequencing (Macrogen, Korea) to confirm the specificity. Selected strains were further sequenced to gain full-length genome for subsequent analysis.

Other canine pathogens including canine bocavirus (CBoV), canine adenovirus (CAdV), paramyxovirus (PMX), CCoV and PCV-3 were also identified using specific primers and PCR programs as mentioned elsewhere in previous studies (Posuwan *et al.*, 2010; Piewbang *et al.*, 2017; Piewbang *et al.*, 2018a; Zhang *et al.*, 2018)

Table 3-1 The set of primers for canine circovirus (CanineCV) detection and sequencing using in this study

Primers	Sequence (5'-3')	Nucleotide position	References
CanineCV-605F	AATGGTGGGAYGGYTACGATGG	605-626	Piewbang *et al.*, 2018
CanineCV-1041R	AAGGGGGGTGAACAGGTAAAC	1041-1021	Piewbang *et al.*, 2018
CanineCV-1022F	TTTACCTGTTCACCCCCCTTCGA	1022-1044	Piewbang *et al.*, 2018
CanineCV-1538R	GGAAGAGGYAATGCTACAAGATCA	1538-1515	Piewbang *et al.*, 2018
CanineCV-1448F	TGAAYGGAGCCTTRTTDGGATC	1448-1469	This study
CanineCV-110R	TCCGGCGCRAGGTTCTTCA	120-110	This study
CanineCV-2014F	GTATTACCCGGCACCTCGTC	2014-2033	This study
CanineCV-776R	CATCAYTATACCAYTCATGAGGC	798-776	This study

Genetic characterization and phylogenetic analysis

The derived full-length sequences were aligned by the MAFFT (Multiple Alignment using Fast Fourier Transform program version 7 (https://mafft.cbrc.jp) and compared to the reference CanineCV sequence (NC_020904) and those CanineCV

sequences available in GenBank. These alignments were further used for nucleotide and deduced amino acid sequence analyses using the BioEdit software package version 7.2 (http://www.mbio.ncsu.edu). The full-length sequences and individual *Rep* and *Cap* genes of Vietnamese CanineCV strains were used to construct a maximum likelihood phylogenetic trees with the dataset of 86 full-genome CanineCV sequences, originally derived from domestic dogs from 2012-2020, available in GenBank. The best-fit model of substitution (TN93 + I + G) was determined using the program implemented in the MEGA 7.0 software (https://www.megasoftware.net/). Phylogenies were constructed by bootstrapping 1,000 replicates with value > 70%.

Recombination analysis

The recombination analysis was run on two independent programs including Recombination Detection Program (RDP) and Simplot, to define the potential natural genetic recombination events in the evolution of the Vietnamese CanineCV strains. Initially, the possibility of genetic recombination in Vietnamese CanineCV was analyzed using RDP package version 4.0 (http://web.cbio.uct.ac.za/) including GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq with the default setting for all parameters. The potential positive recombination was recorded when at least four over six methods showed a breakpoint signal with *p*-values < 0.01. The potential recombination was confirmed by BootScan and similarity plot implanted on Simplot software package (Piewbang*et al.*, 2018b).

3.4 Results

Prevalence of the co-infection of CanineCV in CPV-2c positive Vietnamese dogs

All 81 CVP-2-infected samples were subjected for CanineCV detection using specific primers (CanineCV-605F and -1041R). Sixteen samples (19.8%) revealed the target amplicons at 437 bp suggesting the positive detection of CanineCV, and later confirmed by sequencing. All CanineCV-positive dogs were co-infected with CPV-2c genotype which were the majority of CPV-2 infected genotype (98.8%, 80/81), with

an exception of one CPV-2a infection (No. 71, Supplementary Table S1). The positive rate of CanineCV detection was highest in Hanoi (24.39%, 10/41), then in Ho Chi Minh City (16.67%, 4/24), and Da Nang (12.5%, 2/16), respectively. Other canine pathogens were simultaneously detected including CBoV (6.2%, 5/81), CAdV (3.7%, 3/81), and PMX (2.5%, 2/81); however, there were no evidence of CCoV and PCV-3 positive sample in all dogs. To be noted that only one dog (No.2) from Hanoi revealed triple detections of CPV-2c, CanineCV and CAdV (1.2%) (Table 2-13, 2-14).

Dogs in this study, showing a double infection with CanineCV and CPV-2c, were purebred (5 Poodles, 3 Pomeranians, 2 Pugs, 2 Chihuahuas, 2 Bulldogs, 1 Malinois, and 1 Shih Tzu). Age ranged from 2-7-month-old with the highest prevalence at 3 month-old (37.5%, 6/16) (Table 2-14).

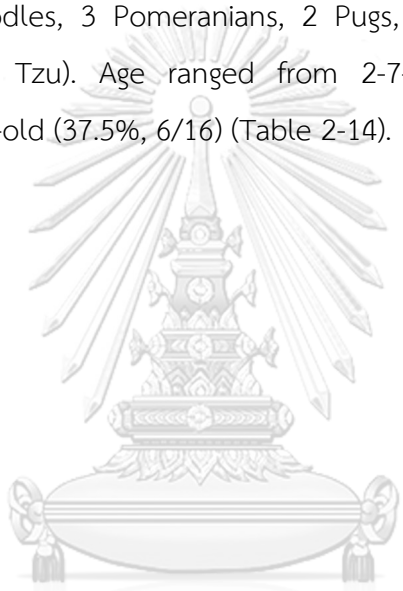

Table 3-2 The detection of other enteric virus coinfection with canine parvovirus-2 (CPV-2) in 81 dogs

Type of infection	Viruses[1]	Positive Samples	Percentage
Dual infection	CanineCV	16[2]	19.8
	CBoV	5	6.2
	CAdV	3	3.7
	PMX	2	2.5
	CCoV	0	0
	PCV-3	0	0
Triple infection	CanineCV and CAdV	1	1.2

[1] CanineCV: Canine circovirus, CBoV: Canine bocavirus, CAdV: Canine adenovirus, PMX: Paramyxovirus, CCoV: Canine coronavirus, PCV-3: Porcine circovirus-3

[2] Number of positive samples: Hanoi (n=10), Ho Chi Minh (n=4), Da Nang (n=2)

Table 3-3 The detection of canine circovirus and other pathogens from canine parvovirus-2 infected dogs in Vietnam

No[1]	Location	Sampling time[2]	Breed	Age (month)[3]	Sex[4]	Viruses[5]						
						CPV-2 Genotype	CanineCV	CBoV	CAdV	PMX	CCoV	PCV-3
1	Hanoi	2017/Sep	Mixed	3	F	2c	-	-	-	-	-	-
2++	Hanoi	2017/Sep	Poodle	4	F	2c	+	-	+	-	-	-
3	Hanoi	2017/Sep	Bulldog	2	M	2c	-	-	-	-	-	-
4	Hanoi	2017/Sep	Poodle	2	F	2c	+	-	-	-	-	-
5	Hanoi	2017/Sep	Shih Tzu	5	M	2c	-	-	-	-	-	-
6	Hanoi	2017/Sep	Mixed	NA	F	2c	-	-	-	-	-	-
7	Hanoi	2017/Sep	Mixed	3	F	2c	-	-	-	-	-	-
8	Hanoi	2017/Sep	Malinois	4	M	2c	-	+	-	-	-	-
9	Hanoi	2017/Sep	Pug	6	Mc	2c	+	-	-	-	-	-
10	Hanoi	2017/Sep	Poodle	2	F	2c	+	-	-	-	-	-
11	Hanoi	2017/Sep	Poodle	4	M	2c	-	-	-	_	-	-
12	Hanoi	2017/Sep	Pug	4	M	2c	+	-	-	-	-	-
13	Hanoi	2017/Sep	Pomeranian	5	F	2c	-	-	-	-	-	-
14++	Hanoi	2017/Sep	Poodle	3	F	2c	+	-	-	-	-	-
15	Hanoi	2017/Sep	Malinois	2	M	2c	-	-	-	-	-	-
16	Hanoi	2017/Sep	Bulldog	2	M	2c	-	-	-	-	-	-
17	Hanoi	2017/Sep	Pug	4	M	2c	-	-	+	-	-	-

18	Hanoi	2017/Sep	Pug	3	F	2c	-	-	-	-	-	-
19	Hanoi	2017/Sep	Shih Tzu	3	M	2c	+	-	-	-	-	-
20	Hanoi	2017/Nov	Poodle	8	F	2c	-	-	-	-	-	-
21	Hanoi	2017/Nov	Chihuahua	2	F	2c	-	+	-	-	-	-
22	Hanoi	2017/Nov	Pug	5	M	2c	-	-	-	-	-	-
23	Hanoi	2017/Nov	Mixed	2	M	2c	-	-	-	+	-	-
24	Hanoi	2017/Nov	Mixed	2	M	2c	-	-	-	-	-	-
25	Hanoi	2017/Nov	Poodle	2	M	2c	-	-	-	-	-	-
26	Hanoi	2017/Nov	Malinois	6	F	2c	-	-	-	-	-	-
27	Hanoi	2017/Nov	Poodle	3	F	2c	-	-	-	-	-	-
28[++]	Hanoi	2017/Nov	Pomeranian	4	M	2c	+	-	-	-	-	-
29	Hanoi	2017/Nov	Poodle	3	F	2c	-	-	-	-	-	-
30	Hanoi	2017/Nov	Pomeranian	3	F	2c	-	-	-	-	-	-
31	Hanoi	2017/Nov	Dachshund	4	F	2c	-	-	-	+	-	-
32	Hanoi	2017/Nov	Poodle	6	Mc	2c	-	-	-	-	-	-
33	Hanoi	2017/Nov	Chihuahua	3	M	2c	+	-	-	-	-	-
34	Hanoi	2017/Nov	Malinois	2	M	2c	-	-	-	-	-	-
35	Hanoi	2017/Nov	Poodle	6	M	2c	-	-	-	-	-	-
36	Hanoi	2017/Dec	Poodle	3	F	2c	+	-	-	-	-	-
37	Hanoi	2017/Dec	Bulldog	4	F	2c	-	-	-	-	-	-
38	Hanoi	2017/Dec	Mixed	3	F	2c	-	-	-	-	-	-

39	Hanoi	2017/Dec	Poodle	3	F	2c	-	-	-	-	-	-
40	Hanoi	2017/Dec	Malinois	4	M	2c	-	-	+	-	-	-
41	Hanoi	2017/Dec	Dachshund	3	M	2c	-	-	-	-	-	-
42	Da Nang	2017/Nov	Poodle	8	F	2c	-	-	-	-	-	-
43	Da Nang	2017/Nov	Chihuahua	2	F	2c	-	+	-	-	-	-
44	Da Nang	2017/Nov	Pug	5	M	2c	-	-	-	-	-	-
45	Da Nang	2017/Nov	Mixed	2	M	2c	-	-	-	-	-	-
46	Da Nang	2017/Nov	Mixed	2	M	2c	-	+	-	-	-	-
47	Da Nang	2017/Nov	Poodle	4	M	2c	-	-	-	-	-	-
48	Da Nang	2017/Nov	Malinois	3	F	2c	-	-	-	-	-	-
49	Da Nang	2017/Nov	Poodle	3	F	2c	-	-	-	-	-	-
50++	Da Nang	2017/Nov	Pomeranian	4	M	2c	+	-	-	-	-	-
51	Da Nang	2017/Nov	Bulldog	2	F	2c	-	-	-	-	-	-
52	Da Nang	2017/Nov	Bulldog	2	F	2c	-	-	-	-	-	-
53	Da Nang	2017/Nov	Bulldog	2	F	2c	-	-	-	-	-	-
54	Da Nang	2017/Nov	Pug	6	Mc	2c	-	-	-	-	-	-
55++	Da Nang	2017/Nov	Chihuahua	3	M	2c	+	-	-	-	-	-
56	Da Nang	2017/Nov	Malinois	4	M	2c	-	-	-	-	-	-
57	Da Nang	2017/Nov	Poodle	3	M	2c	-	-	-	-	-	-
58	Ho Chi Minh	2017/Dec	Bulldog	3	F	2c	-	-	-	-	-	-
59	Ho Chi Minh	2017/Dec	Bulldog	4	F	2c	-	-	-	-	-	-

60	Ho Chi Minh	2017/Dec	Pomeranian	4	F	2c	-	-	-	-	-	-
61	Ho Chi Minh	2017/Dec	Pomeranian	4	F	2c	-	-	-	-	-	-
62	Ho Chi Minh	2017/Dec	Pomeranian	4	M	2c	-	-	-	-	-	-
63	Ho Chi Minh	2017/Dec	Dachshund	3	M	2c	-	-	-	-	-	-
64	Ho Chi Minh	2017/Dec	Bulldog	3	M	2c	-	-	-	-	-	-
65++	Ho Chi Minh	2017/Dec	Bulldog	3	F	2c	+	-	-	-	-	-
66	Ho Chi Minh	2017/Dec	Shih Tzu	2	F	2c	-	-	-	-	-	-
67	Ho Chi Minh	2017/Dec	Poodle	3	F	2c	+	-	-	-	-	-
68	Ho Chi Minh	2017/Dec	Bulldog	4	F	2c	-	-	-	-	-	-
69	Ho Chi Minh	2017/Dec	Mixed	3	M	2c	-	-	-	-	-	-
70	Ho Chi Minh	2017/Dec	Shih Tzu	NA	M	2c	-	-	-	-	-	-
71	Ho Chi Minh	2017/Dec	Poodle	4	M	2a	-	-	-	-	-	-
72++	Ho Chi Minh	2017/Dec	Pomeranian	7	Fs	2c	+	-	-	-	-	-
73	Ho Chi Minh	2017/Dec	Mixed	5	F	2c	-	-	-	-	-	-
74	Ho Chi Minh	2017/Dec	Mixed	5	F	2c	-	+	-	-	-	-
75	Ho Chi Minh	2017/Dec	Bulldog	3	F	2c	-	-	-	-	-	-
76	Ho Chi Minh	2017/Dec	Pug	3	F	2c	-	-	-	-	-	-
77	Ho Chi Minh	2017/Dec	Mixed	2	F	2c	-	-	-	-	-	-
78	Ho Chi Minh	2017/Dec	Poodle	9	Mc	2c	-	-	-	-	-	-
79	Ho Chi Minh	2017/Dec	Poodle	6	F	2c	-	-	-	-	-	-
80++	Ho Chi Minh	2017/Dec	Bulldog	2	M	2c	+	-	-	-	-	-

81	Ho Chi Minh	2017/Dec	Bulldog	5	F	2c	-	-	-	-	-	-

[1] ++: Samples submitting for CanineCV sequencing

[2] Sampling time: year/month

[3] NA: No data available

[4] M: male, F: female, Mc: castrated male, Fs: sprayed female

[5] CPV-2: Canine parvovirus type-2, CanineCV: Canine circovirus, CBoV: Canine bocavirus, CAdV: Canine adenovirus, PMX: Paramyxovirus, CCoV: Canine coronavirus, PCV-3: Porcine circovirus-3

6 +: positive, -: Negative

Genome organization and phylogenetic analysis of Vietnamese CanineCV

Eight out of 16 CanineCV-positive samples were further sequenced with a set of primers to gain the full-length genome of CanineCV. The obtained genome sequences of the Vietnamese CanineCV strains were submitted to GenBank (MT740194-MT740201). Sequence analysis of Vietnamese CanineCV strains revealed the mean values of nucleotide composition of GC% (52.14 ± 0.26) and AT% (47.86 ± 0.25) that were similar to the nucleotide composition of the reference CanineCV UCD1-1698 strain (NC_020904) from USA, 52% and 48%, respectively (Table 2-15). The *Rep* gene located at nt 1-912 and encoded 304 amino acids, while the *Cap* gene placed in antisense of nucleotide sequence at nt 1,116-1,928 and encoded 271 amino acids. The thermodynamical stem-loop representing TAGTATTAC sequence located at nt 2,012-2,020.

Table 3-4 The nucleotide composition of Vietnamese Canine circovirus (CanineCV-VN) strains

Strains	Accession No	Nucleotide composition (2,063 nt)	
		G + C (%)	A + T (%)
UCD1-1698[1]	NC_020904	52.00	48.00
CanineCV-VN-1	MT740194	52.35	47.65
CanineCV-VN-2	MT740195	52.21	47.79
CanineCV-VN-3	MT740198	52.21	47.79
CanineCV-VN-4	MT740201	52.16	47.84
CanineCV-VN-5	MT740199	51.91	48.07
CanineCV-VN-6	MT740196	52.40	47.60
CanineCV-VN-7	MT740197	51.62	48.38
CanineCV-VN-8	MT740200	52.25	47.75

[1] Reference strain of CanineCV

The phylogenetic analysis was constructed based on eight full-genome sequences derived from this study and 86 known sequences retrieved from GenBank during 2012-2020. Total 94 CanineCV strains were segregated into 4 clades corresponding with genotype-1, -2, -3 and -4. Vietnamese CanineCV (CanineCV-VN) strain 3, 4, 5 and 8 were clustered together with CanineCV genotype-1 (CanineCV-1) strains from Italy, Germany, Argentina, USA and China; while CanineCV-VN strain 1, 2, 6 and 7 were grouped in the clade of CanineCV genotype-4 (CanineCV-4) along with other strains from China (KY388494-388496, MF797786), USA (KC241983), and UK (KP260925-260927) (Figure 2-2). Accordingly, phylogenetic analyses based on individual *Rep* and *Cap* genes showed similar results with the phylogeny from the full genomes (Figure 2-3, 2-4).

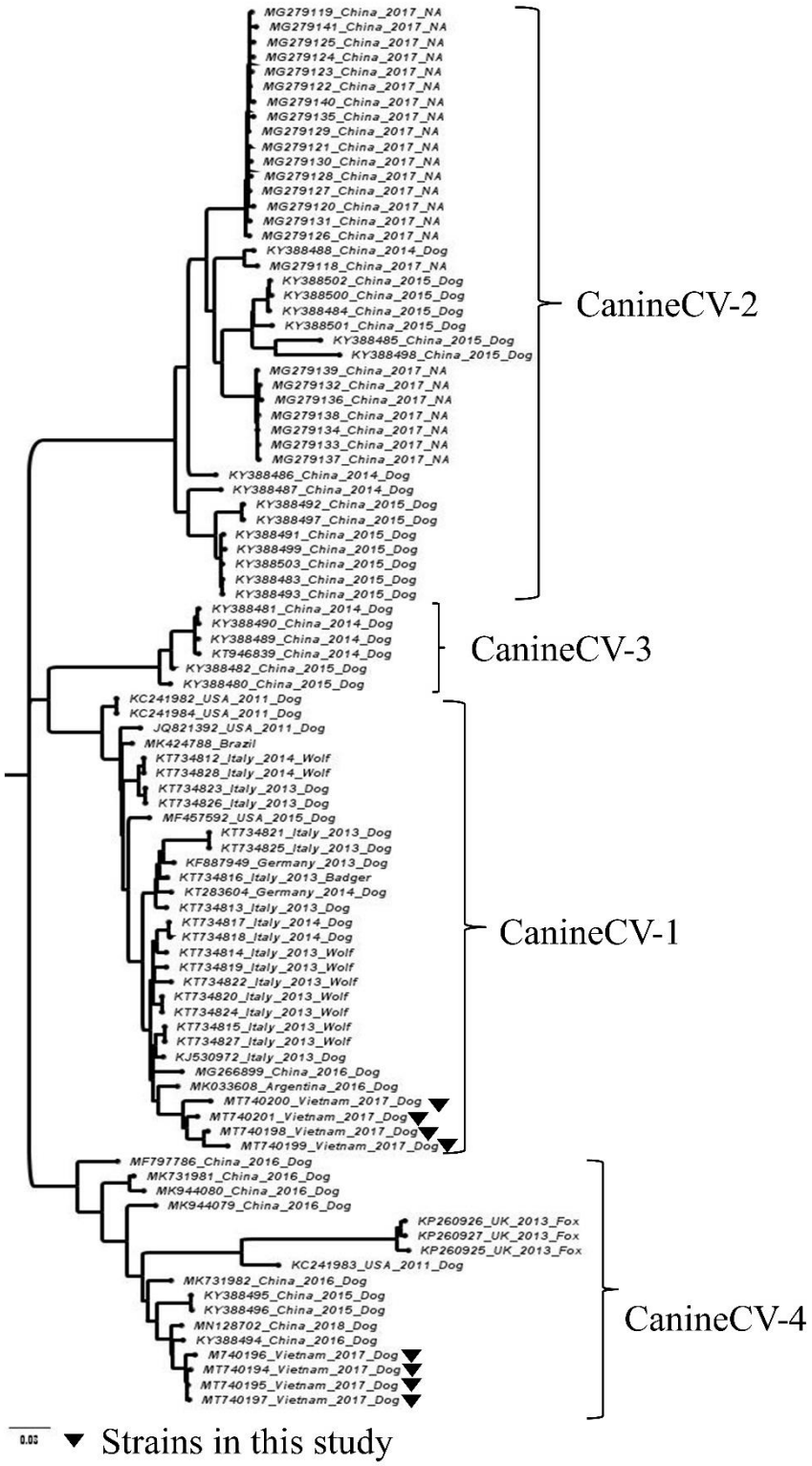

Figure 3-1 Phylogenetic tree with time based on 94 sequences of Replicase gene of Canine circovirus (CanineCV) during 2012 to 2020 (▼ indicated the Vietnamese CanineCV strains in this study)

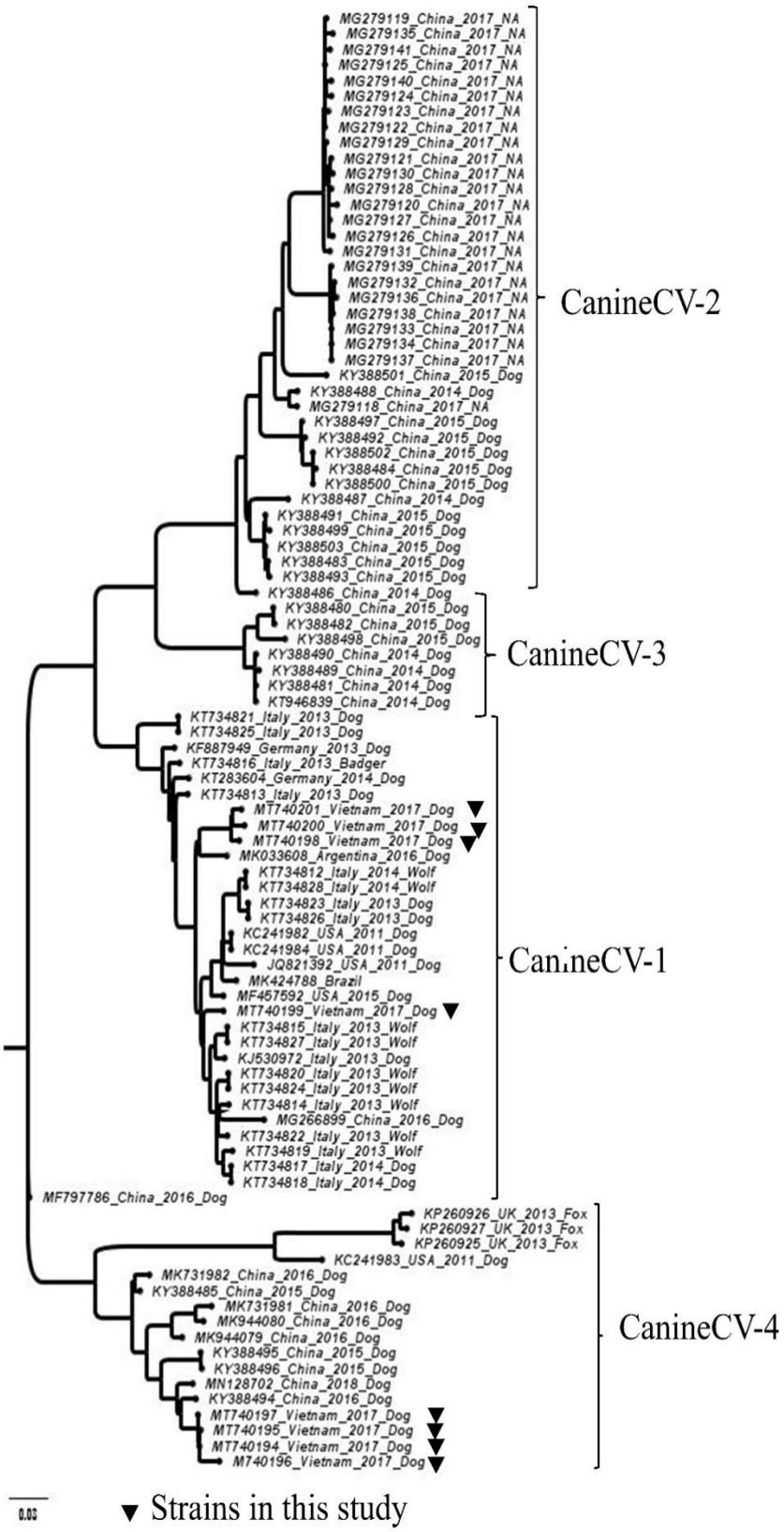

Figure 3-2 Phylogenetic tree with time based on 91 Rep gene of canine circovirus (CaCV) during 2012 to 2019 (▼ Vietnamese CaCV strains in this study).

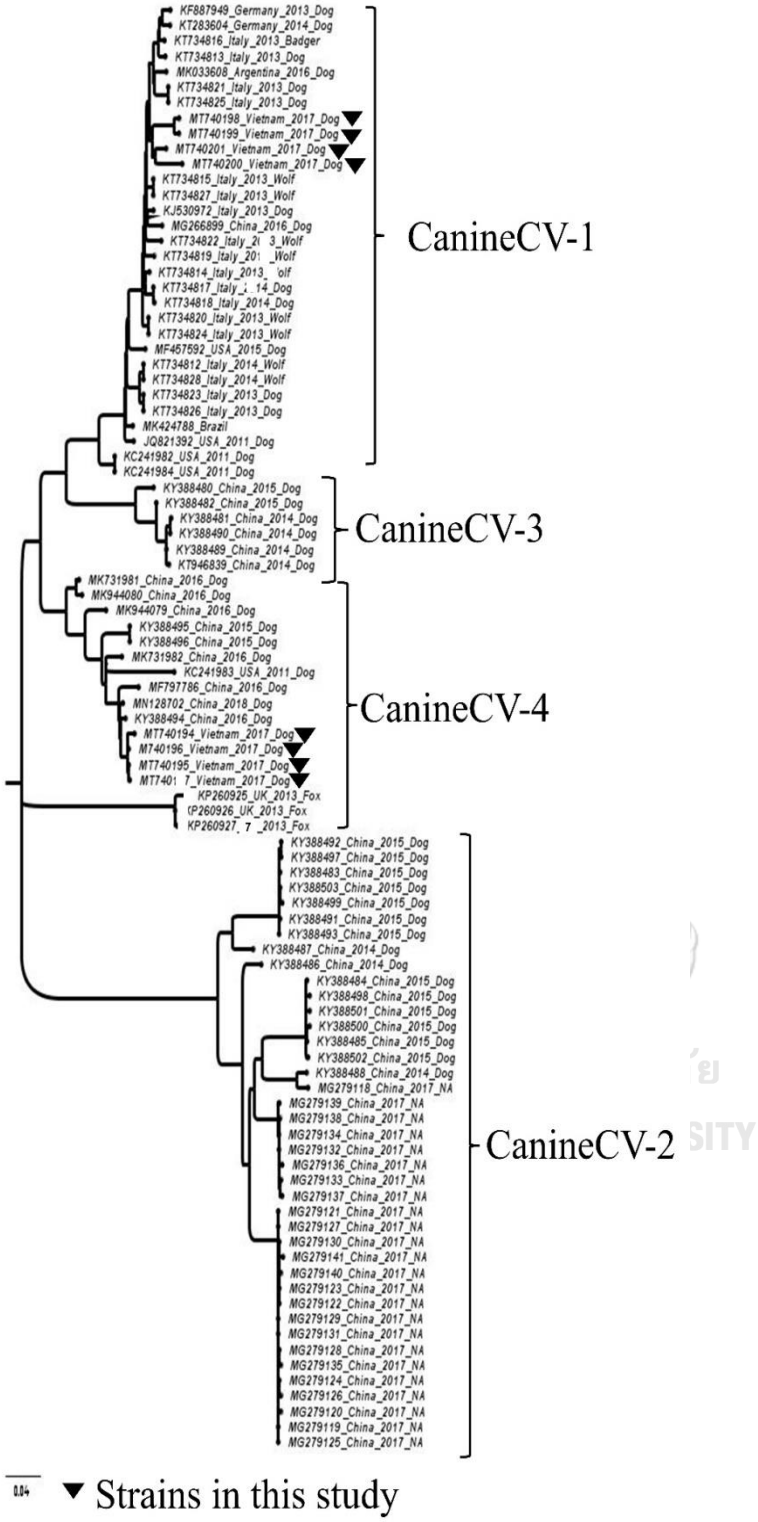

Figure 3-3 Phylogenetic tree with time based on 94 sequences of Capsid gene of Canine circovirus (CanineCV) during 2012 to 2020 (▼ indicated Vietnamese CanineCV strains in this study).

Genetic and amino acid characterizations of different CanineCV genotypes in Vietnam

Genetic analysis of eight full-length genomes revealed that CanineCV-VN strains shared 86-99.8% of similarity and showed the nucleotide similarity among strains in CanineCV genotype-1 (CanineCV-VN-3, -4, -5, -8), and CanineCV genotype-4 (CaCV-VN-1, -2, -6, -7) were 95.2-98% and 98.9-99.8% , respectively. However, the comparison between strains belonging to CanineCV-VN genotype-1 and -4 was 86-87.2% (Table 2-16, 2-17). In addition, similarity analysis of individual *Rep* and *Cap* genes between genotypes, the similarities were 83.8-85.9% for *Rep* gene and 85.3-86.8% for *Cap* gene. For the similarity comparison of deduced amino acid counterpart between two genotypes were 92.7-97.3% for Rep protein and 92.9-97.7% for Cap protein (Table 2-16, 2-18, 2-19).

Among CanineCV-VN genotype-1 and -4, the amino acid variations were obviously observed in 14 and 10 amino acids of Rep and Cap proteins, respectively (Table 2-20, 2-21). Among these positions, almost amino acid sequences of Rep and Cap proteins of CanineCV-VN genotype 1, except for amino acid at 269 (Rep) and 74 (Cap), were identical to the reference UCD1-1698 strain (NC_020904). Further amino acid analysis for each genotype, the results illustrated the unique amino acid variations in both Rep and Cap proteins as shown in Table 2-22, 2-23.

Table 3-5 Similarity percentage of nucleotides and deduced amino acids of Vietnamese Canine circovirus (CanineCV-VN) and reference strains

Genotypes	Strains	Sequences	Similarity (%)		
			Full genome	Replicase	Capsid
	UCD1-1698[1] & CanineCV-VN-3, 4, 5, 8	Nucleotide	93.6-94.4	94.7-96.7	91.3-92.9
		Amino acid		95.7-97.3	97.0-97.7
	UCD1-1698[1] & CanineCV-VN-1, 2, 6, 7	Nucleotide	88.3-88.5	85.7-86.1	89.1-89.6
		Amino acid		93.7-94.3	98.8-95.9
CanineCV-1	CanineCV-VN-3, 4, 5, 8	Nucleotide	95.2-98.0	95.3-98.6	94.2-99.0
		Amino acid		96.0-99.3	97.0-99.6
CanineCV-4	CanineCV-VN-1, 2, 6, 7	Nucleotide	98.9-99.8	98.4-99.8	99.0-99.8
		Amino acid		93.7-100.0	98.5-100.0
CanineCV-1 & CanineCV-4[2]		Nucleotide	86.0-87.2	83.8-85.9	85.3-86.8
		Amino acid		92.7-97.3	92.9-97.7

[1] Reference strain of CanineCV (Accession no. NC_020904); Similarities were compared between UCD1-1698 strains and CanineCV-VN strains.

[2] Similarities were compared between Vietnamese CanineCV-1 and Canine-CV-4 genotypes.

Table 3-6 Similarity percentage of full-genome sequences of Vietnamese circovirus (CanineCV) and reference strain

		CanineCV-4				CanineCV-1			
	NC_020904	VN-1	VN-2	VN-6	VN-7	VN-3	VN-5	VN-8	VN-4
NC_020904									
VN-1	0.883								
VN-2	0.885	0.996							
VN-6	0.883	0.989	0.992						
VN-7	0.884	0.995	0.998	0.990					
VN-3	0.936	0.862	0.864	0.863	0.862				
VN-5	0.944	0.870	0.872	0.870	0.871	0.980			
VN-8	0.941	0.861	0.863	0.860	0.862	0.964	0.952		
VN-4	0.940	0.867	0.869	0.867	0.868	0.979	0.968	0.972	

Table 3-7 Similarity percentage of Replicase sequences of Vietnamese circovirus (CanineCV) and reference strain

		CanineCV-4				CanineCV-1			
	NC_020904	VN-1	VN-2	VN-6	VN-7	VN-3	VN-5	VN-8	VN-4
NC_020904		0.943	0.943	0.937	0.943	0.960	0.973	0.960	0.957
VN-1	0.861		1.000	0.986	1.000	0.933	0.933	0.933	0.933
VN-2	0.860	0.998		0.986	1.000	0.933	0.933	0.933	0.933
VN-6	0.857	0.985	0.985		0.986	0.927	0.927	0.927	0.927
VN-7	0.860	0.998	0.997	0.984		0.933	0.933	0.933	0.933
VN-3	0.951	0.845	0.846	0.845	0.844		0.970	0.993	0.990
VN-5	0.967	0.859	0.858	0.856	0.858	0.966		0.963	0.960
VN-8	0.947	0.844	0.843	0.838	0.843	0.983	0.953		0.990
VN-4	0.949	0.848	0.847	0.845	0.847	0.986	0.957	0.984	

*upper half showed amino acid similarity; lower half showed nucleotide similarity

Table 3-8 Similarity percentage of Capsid sequences of Vietnamese circovirus (CanineCV) and reference strain

		CanineCV-4				CanineCV-1			
	NC_020904	VN-1	VN-2	VN-6	VN-7	VN-3	VN-5	VN-8	VN-4
NC_020904		0.948	0.959	0.959	0.955	0.974	0.970	0.977	0.974
VN-1	0.891		0.988	0.988	0.985	0.929	0.929	0.940	0.940
VN-2	0.896	0.991		1.000	0.996	0.940	0.940	0.951	0.951
VN-6	0.896	0.991	0.997		0.996	0.940	0.940	0.951	0.951
VN-7	0.895	0.990	0.998	0.996		0.937	0.937	0.948	0.948
VN-3	0.913	0.853	0.858	0.858	0.857		0.996	0.974	0.977
VN-5	0.913	0.858	0.863	0.863	0.862	0.990		0.970	0.981
VN-8	0.929	0.857	0.862	0.862	0.861	0.942	0.942		0.981
VN-4	0.926	0.863	0.868	0.868	0.867	0.961	0.969	0.963	

*upper half showed amino acid similarity; lower half showed nucleotide similarity

Table 3-9 Amino acid[1] variations in the Replicase (Rep) proteins of Vietnamese Canine circovirus (CanineCV-VN) strains

	Amino acid sequence of Rep protein[2]																						
	10	**56**	**69**	**71**	**78**	**97**	**106**	**110**	**113**	**115**	**126**	**131**	**140**	**149**	**168**	**174**	**194**	**248**	**252**	**265**	**268**	**269**	**288 (aa)**
NC_020904[3]	G	N	R	T	A	R	L	S	P	S	V	T	S	Y	N	E	E	L	I	S	I	G	N
CanineCV-VN-1	V	S	K	C	A	K	I	A	V	R	V	T	A	F	T	E	S	V	V	N	F	G	S
CanineCV-VN-2	V	S	K	C	A	K	I	A	V	R	V	T	A	F	T	E	S	V	V	N	F	G	S
CanineCV-VN-6	V	S	K	C	A	K	I	A	V	R	V	T	A	F	T	E	S	V	V	N	F	G	S
CanineCV-VN-7	V	S	K	C	A	K	I	A	V	R	V	T	A	F	T	E	S	V	V	N	F	G	S
CanineCV-VN-3	V	N	R	T	G	R	I	S	P	R	I	S	S	Y	T	Q	E	L	I	S	V	P	N
CanineCV-VN-4	V	N	R	T	G	R	I	S	P	R	I	S	S	Y	T	Q	E	L	I	S	V	P	N
CanineCV-VN-5	G	N	R	T	A	R	I	S	P	S	V	T	S	Y	K	E	D	L	I	S	V	P	N
CanineCV-VN-8	V	N	R	T	G	R	I	S	P	R	I	S	S	Y	T	Q	E	L	I	S	V	P	N

[1] Amino acid symbol: A (Alanine), R (Arginine), N (Asparagine); D (Aspartic acid), Q (Glutamine), G (Glycine), I (Isoleucine), L (Leucine), K (Lysine), M (Methionine), F (Phenylalanine), P (Proline), S (Serine), T (Threonine)

[2] Bold numbers indicate the distinct amino acid variations between CanineCV-VN genotype 1 and -4.

[3] Reference strain

Table 3-10 Amino acid[1] variations in the Capsid (Cap) proteins of Vietnamese Canine circovirus (CanineCV-VN) strains

	Amino acid sequence of Cap protein[2]													
	13	14	**16**	**29**	**58**	**74**	**101**	**111**	148	**149**	195	211	**240**	**242(aa)**
NC_020904[3]	S	Y	T	R	T	T	Y	K	R	L	A	V	D	S
CanineCV-VN-1	R	F	A	N	Q	T	F	R	I	G	T	V	E	T
CanineCV-VN-2	R	F	A	N	Q	T	F	R	I	G	T	V	E	T
CanineCV-VN-6	R	F	A	N	Q	T	F	R	I	G	T	V	E	T
CanineCV-VN-7	R	F	A	N	Q	T	F	R	I	G	T	V	E	T
CanineCV-VN-3	S	Y	T	R	T	S	Y	K	R	L	T	I	D	S
CanineCV-VN-4	S	F	T	R	T	S	Y	K	T	L	T	I	D	S
CanineCV-VN-5	S	Y	T	R	T	S	Y	K	T	L	T	I	D	S
CanineCV-VN-8	S	F	T	R	T	S	Y	K	R	L	T	V	D	S

[1] Amino acid symbol: A (Alanine), R (Arginine), N (Asparagine); D (Aspartic acid), Q (Glutamine), G (Glycine), I (Isoleucine), L (Leucine), K (Lysine), M (Methionine), F (Phenylalanine), P (Proline), S (Serine), T (Threonine)

[2] Bold numbers indicate the distinct amino acid variations between CanineCV-VN genotype 1 and -4.

[3] Reference strain

Table 3-11 Relevant amino acid[1] changes of the Replicase (Rep) and Capsid (Cap) protein sequence in each Canine circovirus (CanineCV) genotypes

Genotypes	Rep protein							Cap protein													
	32	35	69	115	140	149	231	13	29	57	94	95	102	149	150	193	195	211	239	240	242 (aa)
CanineCV-1	E	D	R	R	R/S	Y	C	S	R	Q	F	Y	Y	L	E	D	T	I/V	P	D	S
CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	F	N/S	Q	E	Q	I	A	E	A
CanineCV-3	E	E	N	K	A	Y	L	R	N	Q	Y	Y	Y	M	E	D	T	I	P	D	S
CanineCV-4	E	D	K	R	A	F	C	R	N	Q	F	H	H	G	E	D	T	V	P	E	T

[1] Amino acid symbol: A (Alanine), R (Arginine), N (Asparagine); D (Aspartic acid), C (Cysteine), E (Glutamic acid), Q (Glutamine), G (Glycine), I (Isoleucine), L (Leucine), K (Lysine), M (Methionine), F (Phenylalanine), P (Proline), S (Serine), T (Threonine), Y (Tyrosine), V (Valine)

Table 3-12 Relevant amino acid[1] changes of the Replicase (Rep) and Capsid (Cap) protein sequences in each Canine circovirus (CanineCV) genotypes

Accession number_CanineCV genotypes	Rep Protein (aa)							Cap Protein (aa)													
	32	35	69	115	140	149	231	13	29	57	94	95	102	149	150	193	195	211	239	240	242
KT734812_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	V	L	E	D	T	V	P	D	S
KT734828_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	V	L	E	D	T	V	P	D	S
KT734823_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	V	L	E	D	T	V	P	D	S
KT734826_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	V	L	E	D	T	V	P	D	S
MK424788_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
JQ821392_CanineCV-1	E	D	R	R	S	Y	C	N	R	Q	F	Y	I	L	E	D	T	V	P	D	S
MF457592_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KC241982_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	A	V	P	D	S
KC241984_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	A	V	P	D	S
KT734813_CanineCV-1	E	D	Q	R	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KT734816_CanineCV-1	E	D	N	R	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KF887949_CanineCV-1	E	D	Q	R	S	Y	C	S	R	Q	Y	Y	V	L	E	D	T	V	P	D	S
KT283604_CanineCV-1	E	D	Q	R	S	Y	T	S	R	Q	F	Y	V	L	E	D	T	V	P	D	S
KT734814_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S

KT734820_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KT734824_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KT734817_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KT734818_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KT734819_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KT734815_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KT734827_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KJ530972_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KT734822_CanineCV-1	E	D	R	S	S	F	C	S	R	Q	F	Y	I	L	E	N	T	V	P	D	A
MK033608_CanineCV-1	E	D	Q	R	S	Y	C	S	R	Q	F	F	I	L	E	D	T	V	P	D	S
MG266899_CanineCV-1	E	D	R	S	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	I	P	D	S
MT740198_CanineCV-1[2]	E	D	R	R	S	Y	C	S	R	Q	F	Y	V	L	E	D	T	I	P	D	S
MT740201_CanineCV-1[2]	E	D	R	R	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	I	P	D	S
MT740199_CanineCV-1[2]	E	D	R	S	S	Y	C	S	R	Q	F	Y	V	L	E	D	T	I	P	D	S
MT740200_CanineCV-1[2]	E	D	R	R	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KT734821_CanineCV-1	E	D	K	T	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KT734825_CanineCV-1	E	D	K	T	S	Y	C	S	R	Q	F	Y	I	L	E	D	T	V	P	D	S
KY388483_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	N	Q	E	Q	I	A	E	A
KY388493_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	N	Q	E	Q	I	A	E	A

KY388491_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	N	Q	E	Q	I	A	E	A
KY388503_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	N	Q	E	Q	I	A	E	A
KY388499_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	N	Q	E	Q	I	A	E	A
KY388492_CanineCV-2	A	D	N	K	S	F	L	R	N	T	Y	F	V	N	Q	E	Q	I	A	E	A
KY388497_CanineCV-2	A	D	N	K	S	F	L	R	N	T	Y	F	V	N	Q	E	Q	I	A	E	A
KY388487_CanineCV-2	A	D	N	K	S	F	L	R	N	T	Y	F	V	N	Q	E	Q	I	A	E	A
KY388486_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279119_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279121_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279127_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279130_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279122_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279123_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279129_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279131_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279128_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279140_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279124_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279120_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A

MG279125_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279141_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279135_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279126_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	T
KY388484_CanineCV-2	A	D	N	K	S	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
KY388502_CanineCV-2	A	D	N	K	S	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
KY388500_CanineCV-2	A	D	N	K	S	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
KY388501_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279132_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	T
MG279138_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	T
MG279134_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	T
MG279133_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	T
MG279137_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
MG279139_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	T
MG279136_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
KY388488_CanineCV-2	A	D	N	K	A	F	L	R	N	Q	Y	F	V	S	Q	E	Q	I	A	E	S
MG279118_CanineCV-2	A	D	N	K	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	S
KY388485_CanineCV-2	E	D	K	R	A	F	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A
KY388498_CanineCV-2	E	E	N	K	A	Y	L	R	N	T	Y	F	V	S	Q	E	Q	I	A	E	A

KY388480_CanineCV-3	E	E	N	K	A	Y	L	R	N	Q	F	Y	V	M	E	D	T	I	P	D	S
KY388482_CanineCV-3	E	E	N	K	A	Y	L	R	N	Q	Y	Y	V	M	E	D	T	I	P	D	S
KY388481_CanineCV-3	E	E	N	K	A	Y	L	R	N	Q	Y	Y	V	M	E	D	T	I	P	D	S
KY388490_CanineCV-3	E	E	N	K	A	Y	L	R	N	Q	Y	Y	V	M	E	D	T	I	P	D	S
KY388489_CanineCV-3	E	E	N	K	A	Y	L	R	N	Q	Y	Y	V	M	E	D	T	I	P	D	S
KT946839_CanineCV-3	E	E	N	K	A	Y	L	R	N	Q	Y	Y	V	M	E	D	T	I	P	D	S
KY388494_CanineCV-4	E	D	K	R	A	F	L	R	N	Q	F	Y	I	G	E	D	T	V	P	E	T
MN128702_CanineCV-4	E	D	K	R	A	F	L	R	T	Q	F	Y	I	G	E	D	T	V	P	E	T
MT740194_CanineCV-4[2]	E	D	K	R	A	F	C	R	N	Q	F	H	I	G	E	D	T	V	P	E	T
MT740195_CanineCV-4[2]	E	D	K	R	A	F	C	R	N	Q	F	Y	I	G	E	D	T	V	P	E	T
MT740197_CanineCV-4[2]	E	D	K	R	A	F	C	R	N	Q	F	Y	I	G	E	D	T	V	P	E	T
MT740196_CanineCV-4[2]	E	D	K	R	A	F	C	R	N	Q	F	Y	I	G	E	D	T	V	P	E	T
MK731982_CanineCV-4	E	D	K	K	A	F	L	R	N	Q	F	Y	I	G	E	D	T	V	P	E	T
KY388495_CanineCV-4	E	D	K	R	A	F	L	R	N	Q	F	F	I	H	E	D	T	V	P	E	T
KY388496_CanineCV-4	E	D	K	R	A	F	L	R	N	Q	F	F	I	H	E	D	T	V	P	E	T
MK731981_CanineCV-4	E	E	K	R	A	F	C	R	N	Q	F	Y	I	G	E	D	T	V	P	E	S
MK944080_CanineCV-4	E	D	K	R	A	F	L	R	N	Q	F	Y	I	G	E	D	T	V	P	E	S
MK944079_CanineCV-4	E	D	K	R	A	F	L	S	N	Q	Y	Y	I	G	E	D	T	V	P	E	S
MF797786_CanineCV-4	E	D	K	R	A	F	C	S	N	Q	F	Y	I	S	E	D	T	V	P	E	T

KC241983_CanineCV-4	E	D	K	K	A	F	C	R	N	Q	F	Y	V	G	E	D	T	V	P	D	S
KP260925_CanineCV-4	E	D	K	K	A	F	C	R	N	Q	Y	F	V	T	E	E	S	V	P	E	S
KP260926_CanineCV-4	E	D	K	K	A	F	C	R	N	Q	Y	F	V	T	E	E	S	V	P	E	S
KP260927_CanineCV-4	E	D	K	K	A	F	C	R	N	Q	Y	F	V	T	E	D	S	V	P	E	S

[1] Amino acid symbol: A (Alanine), R (Arginine), N (Asparagine); D (Aspartic acid), C (Cysteine), E (Glutamic acid), Q (Glutamine), G (Glycine), H (Histidine), I (Isoleucine), L (Leucine), K (Lysine), M (Methionine), F (Phenylalanine), P (Proline), S (Serine), T (Threonine), Y (Tyrosine), V (Valine)

[2] Vietnamese strains from this study

Recombination analysis

The recombination analysis applied for all CanineCV-VN strains using the RDP4 software including RDP, GENECONV, Bootscan, Maxchi, Chimaera, SiScan, 3Seg and LARD. The potential recombination breakpoint was found in CanineCV-VN genotype 4 (CanineCV-VN-3, 4, 5, 8) at least 4 positive recombination algorithms. Moreover, these four strains were further analyzed by similarity plot on Simplot software package. The potential recombination signal was confirmed in CanineCV-VN-6 at nucleotide position 420-1020 which had American CanineCV KC241983 and Chinese CanineCV MG279125 as its major and minor parents, respectively (Figure 2-5).

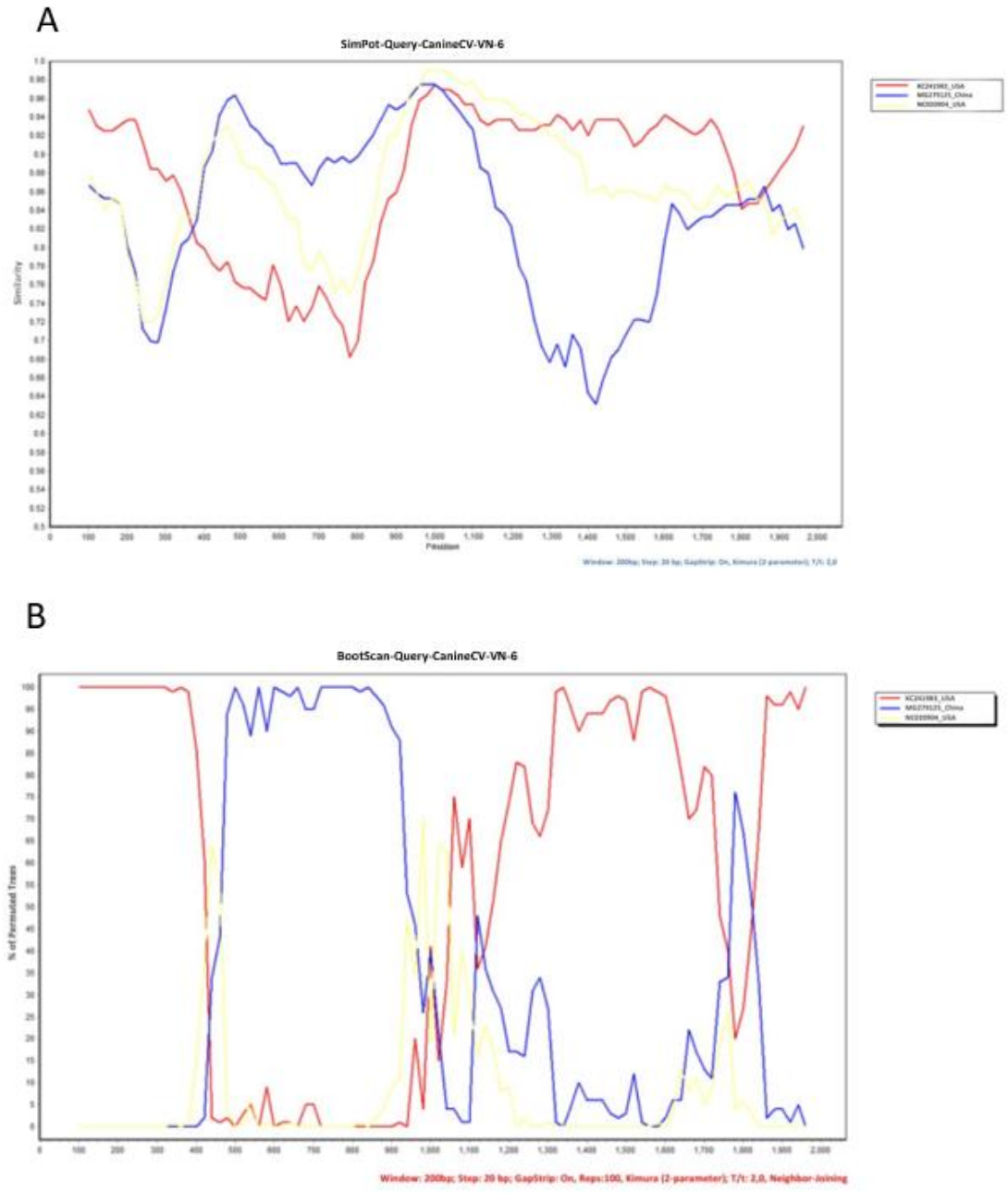

Figure 3-4 The potential recombination events of Vietnamese Canine circovirus (CanineCV-VN-6 as query) strains compared to published CanineCV strains; (A) Similarity plot, (B) Bootscan analysis

3.5 Discussion

This study was the first identification of CanineCV in Vietnam as evidence for the circulation of CanineCV among Vietnamese dogs. The CanineCV was detected from CPV-2c-infected samples with a prevalence of 19.8% which is higher than that was reported in Gemany, about 12.7% (Anderson*et al.*, 2017). This discrepancy might have been due to the differences in latitude, topography and climate trend. The difference in the prevalence of CanineCV also was observed in different regions throughout Vietnam, where dividing into three regions with a different climate. The fecal samples were collected in Northern area (Hanoi, humid subtropical) is detected the highest rate of CanineCV, about 24.39% while in central (Da Nang, tropical moonsoon) and Southern (Ho Chi Minh City, tropical savanna) areas were about 12.5 and 16.67%, respectively. In addition, CanineCV is also reported in healthy and non-CPV-2 infected dogs (Hsu*et al.*, 2016; Niu *et al.*, 2020). Further investigation, including healthy and diarrheic dogs in Vietnam, is required to understand the epidemiology of CanineCV.

The pathogenesis of CanineCV induced disease and possible synergistic function of the virus with other pathogens have not been clarified. There was a study indicated the CanineCV playing role as a primary cause of acute hemorrhagic diarrheic disease in dogs (Anderson*et al.*, 2017). However, some other reports indicated that the CanineCV might act in synergism with other infectious agents in the development of a disease (Zaccaria *et al.*, 2016; Kotsias*et al.*, 2019). In this study, CanineCV is co-detected with CPV-2c in enteritis-suffering dogs, which was in agreement with the previous findings (Kotsias *et al.*, 2019). The exact role of CanineCV should be explored in the future.

The present study described the characteristics of full-length genomes and deduced amino acids of CanineCV in Vietnam to add information on the geographical extent of the virus. The viral genome is encompassed two ORFs encoding for Rep

protein (304 amino acids) and Cap protein (271 amino acids) with a high variability of amino acids. The knowledge related to the mutation in CanineCV is limited. Although selective pressure was demonstrated as the factor to drive the mutations in the Cap gene of PCV-2 for escaping immune defense caused by vaccination in pigs (Franzo *et al.*, 2016), it could not explain in the case of CanineCV since the vaccine for CanineCV has not been available at this moment. Therefore, further study is necessary to understand the importance and evolution of CanineCV.

The phylogenetic analysis based on full-length genome and individual Rep and Cap genes was divided into four clades corresponding to four genotypes with typical changes of amino acids. Notable, CanineCV genotype-1 was mainly detected in Europe (Italy and Germany) and the America (USA, Brazil and Argentina), exception for only one sequence (MG266899) that was detected in China. CanineCV genotype-2 and -3 were distributed in Southwestern China while CanineCV genotype-4 was detected in the UK, the USA, and Northern China (Niu*et al.*, 2020). This study provided more evidence of CanineCV genotype-1 distribution in Asia.

The recombination events have been identified in CanineCV in Rep gene and other parts of the genome (Piewbang*et al.*, 2018b; Sun*et al.*, 2019). In this study, the recombination event occurred in CanineCV-VN-6 strain at nucleotide position 420-1020 with a major parent sequence derived from America strain (KC241983; CanineCV genotype-4) and a minor parent sequence derived from Chinese strain (MG279125; CanineCV genotype-2). To be noted that only CanineCV KC241983 has been previously detected in Vietnam. These findings suggested the various genotypes of CanineCV have been prevalent in Vietnam. The recombinant event should be strictly monitored since the different genotype of CanineCV has simultaneously circulated and co-detected with other pathogens in same the host.

In conclusion, this is the first detection and characterization of full-length genome of CanineCV in Vietnam. This study highlights the role of CanineCV as a co-

infectious agent for developing enteritis in dogs. Phylogenetic analysis revealed that CanineCV was classified into four genotypes marked by typical mutations of amino acid and specific geographic distribution. Further studies are vital to understanding of subtypes and genotypes of CanineCV.

3.6 Acknowledgments

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3.7 Conflict of interests

The authors declare no conflict of interest.

CHAPTER 4

4.1 General discussion and conclusion

Enteritis is one of the major health problems of dogs in Vietnam. Among enteric agents, CPV-2 is one of the most critical pathogenic viruses and responsible for the leading cause of death in Vietnamese dogs. Moreover, this virus coinfected with other enteric pathogens, including CAdV, CanineCV, CBoV and PMX. Notably, CanineCV is the most prevalent co-infectious agent, accounted for 19.7%, while other viruses such as PMX, CBoV, and CAdV were detected at 2.5%, 6.2%, and 3.7%, respectively. Since the coinfection of CPV-2 and CanineCV leads to more severe clinical disease (Thaiwong*et al.*, 2016; Kotsias*et al.*, 2019), the result of this study should be noted for the future investigation regarding diagnosis and treatment of CPV-2 infection.

CPV-2c is the dominant CPV-2 variant, about 98.7%, and a few CPV-2a strain co-circulated in Vietnam. Notably, CPV-2b was not found in the current study even though it was previously reported as the most prevalent variant in Vietnam in 2003 (Nakamura *et al.*, 2004a). The nucleotide sequences of CPV-2 are high homology (99.5-99.8% of similarity) between samples collected from different regions (Northern, Central, and Southern Vietnam). However, the prevalence of CanineCV is different among samples in different regions. The most prevalent was detected in Hanoi, about 24%, whereas the percentages in Da Nang and Ho Chi Minh are 12.5 and 17%, respectively.

The full-length genome of CPV-2c strain in Vietnam presented typical signatures in both NS and VP proteins for Asian CPV-2 strains and shared an evolutionary pattern with other Asian strains. Besides, Vietnamese strains exhibited new mutations that have been detected in the most current CPV-2 strains in China and Taiwan, and these strains clustered together in a new clade.

The evolutionary analysis based on the data set containing the 199 global full-length sequences of three different CPV-2 variants isolated from main continents

during 42 years of viral emergence confirmed that CPV-2 is a rapidly mutated virus. No evidence of genetic recombination event was established for CPV-2, but the potential positive selection sites might contribute to driving the evolutionary process.

CanineCV is an emerging virus that was reported related to enteritis in dogs; however, the pathogenesis of CanineCV induced disease and the possible synergistic effect with other pathogens have not been understood. In the present study, CanineCV was highly prevalent in CPV-2 infected dogs, which highlight the function of CanineCV as a synergism agent in the development of a disease.

Phylogenic analysis based on full-genome of 94 global sequences of CanineCV revealed that CanineCV is classified into four genotypes with typical amino acid characteristics in Rep and Cap proteins. Notably, CanineCV genotype-1, which mostly distributes in Europe and America, is co-circulating with CanineCV genotype-4 among CPV-2c-infected dogs in Vietnam, and the two genotypes shared 86-87.2% of similarity. Compare to CanineCV genotype-1, CanineCV-4 presented 14 and 10 replacement amino acids in Rep and Cap proteins, respectively. Interestingly, most of these replacement amino acids are not observed in the CanineCV reference strain (UCD1-1698).

The recombination events were identified in the Vietnamese CanineCV strain (MT740196) with the major and minor parents are an American CanineCV (KC241983) and a Chinese CanineCV (MG279125), respectively. The recombination event occurred in position 420-1020 nt. Therefore, the recombinant event should be strictly monitored since the different genotypes of CanineCV have simultaneously circulated and co-infected with other pathogens in the same host.

4.2 Limitation of the study

1. The samples in this study were collected from Hanoi, Da Nang, and Ho Chi Minh City where are crowded dog population during 2017-2018. It is considered quite subjective for the study.

2. Only positive-CPV2 samples were used to detect CanineCV, which leads to miss data of CanineCV in healthy and negative-CPV2 diarrheic dogs.

4.3 Suggestions from the study

1. Continuous surveillance of CPV-2 subtypes and genotypes is vital in order to monitor the novel mutation, which may evolve to evade the currently available immunization at any time.

2. The current focus on the evolution on the VP2 gene, which encodes the major viral capsid protein and related to immune evasion and host cell binding, is gained attention, however, whole genome sequence analysis is also necessary to understand the evolutionary insight of CPV-2.

3. CanineCV genotype-1 and -4 were identified in this study as evidence for the circulation of CanineCV in Vietnamese dogs; continued survey including healthy and negative-CPV-2 diarrheic dogs is necessary in order to understand the epidemiology of CanineCV in Vietnam.

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