Transition of dominant canine parvovirus type from 2b to 2c in Vietnamese dogs

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Summary
Canine parvovirus (CPV) is one of the most important pathogens causing enteritis in dogs. Although there have been a few reports of CPV in Vietnam, recent information on CPV infection in domestic dogs in Vietnam is limited. Faecal samples collected from 30 diarrheic and 50 healthy dogs were examined by PCR for detection of CPV DNA. The prevalence of CPV in diarrheic dogs (43.3%, 13/30) was significantly higher than in healthy dogs (4.0%, 2/50), indicating that CPV was a major cause of diarrhoea in domestic dogs. Genotyping of 15 CPV strains showed that both CPV-2a and CPV-2c were circulating and that CPV-2c was a dominant CPV variant in Vietnam. Virus isolation was performed from faecal samples using A72/cSLAM cells, and nine CPV strains were successfully isolated. The dominant genotype spreading among Vietnamese dogs has changed from CPV-2b to CPV-2c.

Introduction

Canine parvovirus (CPV) is one of the most important pathogens in dogs with diarrhoea. CPV has a single-stranded DNA genome and is a member of the *Parvoviridae* family, *Protoparvovirus* genus. The CPV genome encodes three structural proteins (VP1, VP2 and VP3) and two non-structural proteins (NS1, NS2). VP2 protein can be cleaved into VP3 by a host protease after virion assembly (Agbandje *et al.* 1995, Decaro *et al.* 2012, Mirand *et al.* 2016). The VP2 protein plays important roles in antigenicity and the determination of host range (Tsao *et al.* 1991, Truyen *et al.* 1995, Phromnoi *et al.* 2010).

CPV-2 was first identified in the late 1970s as an agent of severe haemorrhagic gastroenteritis in dogs. Two antigenic variants emerged in the 1980s and were termed CPV-2a (VP2 87Leu, 101Thr, 300Gly, 305Tyr, 375Asp, 426Asn, 555Ile) and -2b (VP2 87Leu, 101Thr, 300Gly, 305Tyr, 375Asp, 426Asp) (Truyen *et al.* 1996, Decaro *et al.* 2012). CPV-2a and -2b with a mutation at residue 297 from Ser to Ala in VP2 have been classified as new CPV-2a and -2b variants (Martella *et al.* 2005). In 2000, CPV-2c (VP2 426Glu) was recognized in Italy (Buonavoglia *et al.* 2001) and since then CPV-2c has been reported in many countries in Europe, Asia and the United States (Buonavoglia *et al.* 2001, Decaro *et al.* 2007, Hong *et al.* 2007, Decaro and Buonavoglia 2012). In Vietnam, CPV-2c and CPV-2b were reported to have spread among the dog population and CPV-2b was reported as the predominant genotype in 2004 (Ikeda *et al.* 2000, Nakamura *et al.* 2004). In this study, we analyzed the most recent Vietnamese CPV strains circulating in domestic dogs.

Materials and methods

Faecal swab samples

Faecal swabs were collected from 30 diarrheic dogs (dogs showed clinical signs of gastroenteritis and diarrhoea) and 50 healthy dogs in Ho Chi Minh City, Vietnam, from 2013 to 2015. Ages ranged from 2 months to 13 years old. Swab samples were dissolved in 2 ml of phosphate-buffer saline (PBS) and filtered through a 0.22 µm filter (Millipore, Carrigtwohill, Iceland) before being stored at - 80 °C until analysis.

Ethics statement

In the present study, the collection of swab samples from dogs was according to the Animal Ethics Procedures and Guidelines of the Yamaguchi University, Japan.

Viral DNA extraction

Viral DNA was extracted from faecal swab samples using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

PCR for detection of viral DNA

PCR was carried out using a TaKaRa Ex Taq kit (TaKaRa, Otsu, Japan) with primers 555F (5'- CAG GAA GAT ATC CAG AAG GA-3') and 555R (5'-GGT GCT AGT TGA TAT GTA ATA AAC A-3') (Buonavoglia *et al.* 2001). PCR conditions were as follows: first pre-denaturation step at 94 °C for 2 min followed by 40 cycles of 98 °C for 10 sec, 50 °C for 30 sec and 72 °C for 1 min with a final extension step at 75 °C for 5 min. Amplified products were confirmed as 583 bp by electrophoresis on 2% agarose gels, and then purified using a QIAquick PCR Purification kit (Qiagen) for sequencing.

Virus isolation

For virus isolation, A72 cells expressing canine SLAM, A72/cSLAM (Nakano *et al.* 2009), were maintained in Dulbecco's minimum essential medium (DMEM, Life Technologies, Carlsbad, CA) containing 10% heat-inactivated fetal calf serum (FBS, Sigma-Aldrich, MO, USA), 100 units/ml penicillin and 100 µg/ml streptomycin (Life Technologies). A72/cSLAM cells in 6 well plates were inoculated with fecal swab extracts and then incubated at 37 °C in a 5% CO_2 incubator. Cells were observed daily for the development of cytopathic effect (CPE). If there was no CPE, cells were blind-passaged five times. PCR was used for determination of isolated viruses.

Nucleotide sequencing

The full length VP2 gene (1,755 nt) was amplified by PCR with primers: VP2FLf (forward), 5'-GTGCAGGACAAGTAAAA-3' (Gallo *et al.* 2012) and 555R (reverse), 5'-GGTGCTAGTTGATATGTAATAAACA-3' (Buonavoglia *et al.* 2001). PCR conditions using KOD-Plus Ver.2 kit (TOYOBO, Osaka, Japan) were as follows: a pre-denaturation step at 94 °C for 2 min, 40 cycles of 98 °C for 10 sec, 55 °C for 30 sec and 68 °C for 2 min and a final extension step at 68 °C for 5 min.

PCR was performed for amplification of overlapping fragments of additional genomic sequences of CPV isolates CPV/dog/HCM/7/2013 and CPV/dog/HCM/22/2013 with primers: 210F 5'-AGACCGTTACTGACATTCGC-3' and 3469R 5'-GTGCCACTAGTTCCAGTATGAG-3'. PCR conditions using KOD-Plus Ver.2 kit (TOYOBO) were as follows: a pre-denaturation step at 94 °C for 2 min, 40 cycles of 98 °C for 10 sec, 50 °C for 30 sec and 68 °C for 4 min and a final extension step at 68 °C for 10 min. PCR products were purified using a QIAquick PCR Purification kit (Qiagen) for sequencing.

Statistical analysis

Fisher's exact and Chi-square tests were used for analysis of data. P values of < 0.05 were considered to be statistically significant.

Results

Detection of CPV in diarrheic and healthy dogs

CPV-2 was detected in samples from 13 diarrheic dogs (43.3%, 13/30) and two healthy dogs (4.0%, 2/50). The prevalence of CPV in diarrheic dogs was significantly higher than that in the healthy dogs ($P < 0.05$) (Supplementary Table, Annex 1).

Genotyping of CPV strains in Vietnamese dogs

CPV genotypes were determined from the nucleotide sequences of partial VP2 genes (519 nt) from 15 CPV-positive samples. Fourteen Vietnamese CPV samples were found to have a Glutamic acid at position 426 of the VP2 protein, indicating that they were CPV-2c (Tables I and II). One CPV strain, CPV/dog/HCM/22/2013, was found to belong to genotype 2 or 2a based on the partial VP2 gene sequence (Asparagine at position 426 of VP2 protein). The full length of the VP2 gene (1,755 nt) from this strain was then analysed and CPV/dog/HCM/22/2013 was classified as a new CPV-2a strain (Tables I, II and Figure 1).

Virus isolation and nucleotide sequences

Nine CPV strains were successfully isolated from faecal swab samples. Full length VP2 genes from seven isolates including CPV/dog/HCM/2/2013, CPV/dog/HCM/5/2013, CPV/dog/HCM/6/2013, CPV/dog/HCM/8/2013, CPV/dog/HCM/13/2013, CPV/dog/HCM/14/2013, CPV/dog/HCM/20/2013, and the complete coding sequence of two representative CPV genomes, CPV/dog/HCM/7/2013 (CPV-2c) and CPV/dog/HCM/22/2013 (new CPV-2a), were determined.

Table I. *Genotyping of canine parvoviruses (CPV) based on amino acid differences in the VP2 protein gene.*

	Amino acid variations in the VP2 protein of canine parvoviruses							
Position	87	101	297	300	305	375	426	555
CPV-2	Met	Ile	Ser	Ala	Asp	Asn	Asn	Val
CPV-2a	Leu	Thr	Ser	Gly	Tyr	Asp	Asn	Ile
CPV-2b	Leu	Thr	Ser	Gly	Tyr	Asp	Asp	Val
New CPV-2a	Leu	Thr	Ala	Gly	Tyr	Asp	Asn	Val
New CPV-2b	Leu	Thr	Ala	Gly	Tyr	Asp	Asp	Val
CPV-2c	Leu	Thr	Ala	Gly	Tyr	Asp	Glu	Val
Isolated CPV in this study								
CPV/dog/HCM/2/2013 (CPV-2c)	Leu	Thr	Ala	Gly	Tyr	Asp	Glu	Val
CPV/dog/HCM/5/2013 (CPV-2c)	Leu	Thr	Ala	Gly	Tyr	Asp	Glu	Val
CPV/dog/HCM/6/2013 (CPV-2c)	Leu	Thr	Ala	Gly	Tyr	Asp	Glu	Val
CPV/dog/HCM/7/2013 (CPV-2c)	Leu	Thr	Ala	Gly	Tyr	Asp	Glu	Val
CPV/dog/HCM/8/2013 (CPV-2c)	Leu	Thr	Ala	Gly	Tyr	Asp	Glu	Val
CPV/dog/HCM/13/2013 (CPV-2c)	Leu	Thr	Ala	Gly	Tyr	Asp	Glu	Val
CPV/dog/HCM/14/2013 (CPV-2c)	Leu	Thr	Ala	Gly	Tyr	Asp	Glu	Val
CPV/dog/HCM/20/2013 (CPV-2c)	Leu	Thr	Ala	Gly	Tyr	Asp	Glu	Val
CPV/dog/HCM/22/2013 (New CPV-2a)	Leu	Thr	Ala	Gly	Tyr	Asp	Asn	Val

Table II. *Variations between VP2 amino acid sequences in Vietnamese canine parvovirus (CPV) strains.*

		Amino acid variations in the VP2 protein of canine parvoviruses													
	Genotype	5	13	87	101	265	267	297	300	305	324	370	375	426	440
CPV-b (M38245)	CPV-2	A	P	M	I	T	F	S	A	D	Y	Q	N	N	T
In previous study in Vietnam															
HNI-2-13 (AB120724)	New CPV-2b	.	.	L	T	.	Y	A	G	Y	.	.	D	D	.
HNI-3-4 (AB120725)	New CPV-2b	.	.	L	T	.	.	A	G	Y	.	.	D	D	.
HNI-3-1 (AB120726)	New CPV-2b	.	.	L	T	.	.	A	G	Y	.	.	D	D	.
HCM-6 (AB120720)	**New CPV-2b**	**.**	**.**	**L**	**T**	**.**	**.**	**A**	**G**	**Y**	**.**	**.**	**D**	**D**	**.**
HCM-8 (AB120721)	New CPV-2b	.	S	L	T	K	.	A	G	Y	.	.	D	D	.
HCM-18 (AB120722)	New CPV-2b	.	.	L	T	.	Y	A	G	Y	.	.	D	D	.
HCM-23 (AB120723)	New CPV-2b	.	.	L	T	.	.	A	G	Y	.	.	D	D	.
HCM-4-1(AB120727)	CPV-2c	.	.	L	T	.	.	A	G	Y	.	.	D	E	.
In this study															
Full length VP2 (Isolated CPV)															
CPV/dog/HCM/2/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM/5/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM/6/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM/7/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM/8/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM/13/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM/14/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM/20/2013	CPV-2c	G	.	L	T	.	Y	A	G	Y	I	R	D	E	.
CPV/dog/HCM/22/2013	New CPV-2a	.	.	L	T	.	Y	A	G	Y	I	.	D	.	A
Partial -length VP2 (Detected CPV)															
CPV/dog/HCM/1/2013	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.
CPV/dog/HCM/4/2013	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.
CPV/dog/HCM/9/2013	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.
CPV/dog/HCM/18/2013	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.
CPV/dog/HCM/82/2015	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.
CPV/dog/HCM/88/2015	CPV-2c	-	-	-	-	-	-	-	-	-	-	-	-	E	.

Nucleotide sequences of these CPV strains were deposited into the DNA database of Japan (DDBJ) (Accession numbers: LC216904-LC216910, LC214969 and LC214970). When compared to CPV-2 genotype (CPV-b strain) (Parrish 1991) Vietnamese CPV-2c CPV/dog/HCM/7/2013 had two mutations in NS1, I60V (Ile to Val) and L630P (Leu to Pro), and 11 mutations in VP2, A5G (Ala to Gly), M87L (Met to Leu), I101T (Ile to Thr), F267Y (Phe to Tyr), S297A (Ser to Ala), A300G (Ala to Gly), D305V (Asp to Val), Y324I (Tyr to Ile), Q370R (Gln to Arg), N375D (Asn to Asp) and N426E (Asn to Glu). Vietnamese CPV-2a, CPV/dog/HCM/22/2013, had 3 mutations in the NS1 protein, A13G (Ala to Gly), V115I (Val to Ile) and N624K (Asn to Lys) and 9 mutations in VP2, including M87L, I101T, F267Y, S297A, A300G, D305V, Y324I, N375D and T440A (Thr to Ala) (Table II).

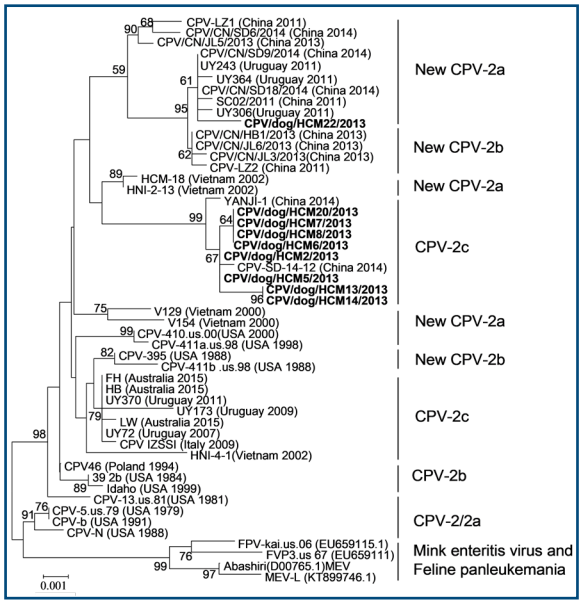

Figure 1. *Phylogenetic tree based on the nucleotide sequences of full length VP2 of canine parvoviruses (CPV).* The bootstrap percentages (supported by at least 50% of the 1,000 replicates) are shown above the nodes. The scale bar indicates the number of nucleotide substitutions per site. Strains identified in Vietnam are shown in bold. GeneBank accession numbers of reference strains are as follows, CPV 2/2a: CPV-b (M38241.1), CPV-N (M19296.1), CPV-5.us.81 (EU659118.1) and CPV-13.us.81 (EU659118.1). CPV new 2a: V129 (AB054216), V154 (AB054217), UY306 (KM457135.1), UY243 (KM457102.1), UY364 (KM457143.1), CPV/CN/SD9/2014 (KR002802.1), CPV/CN/SD18/2014 (KR002804.1), SC02/2011 (JX660690.1), CPV-LZ1 (JQ268283.1), CPV/CN/JL5/2013 (KR002798.1) and CPV/CN/SD6/2014 (KR002801.1). CPV-2b: 39 (M74849.1), 46 (Z46651.1) and Idaho (U22896.1). CPV new 2b: HCM-18 (AB120722), HNI-2-13 (AB120724), CPV-395 (AY742936.1), CPV-411a.us.98 (EU659120.1), CPV-411b.us.98 (EU659121.1), CPV-410.us.00 (EU659119.1); CPV/CN/HB1/2013 (KR002793.1), CPV/CN/JL3/2013 (KR002796.1), CPV/CN/JL6/2013 (KR002799.1) and CPV-LZ2 (JQ268284.1). CPV-2c: CPV-SD-14-12 (KR611522.1), YANJI-1 (KP749854.1), HNI-4-1 (AB120727), HB (KU508691.1), FH (KU508692.1), LW (KU508693.1), UY370c (KM457142.1), UY72 (KM457107.1), UY173 (KM457115.1) and CPV IZSSI (KU508407.1). Mink enteritis virus: MEV-L (KT899746.1) and Abashiri (D00765.1). Feline panleukopenia virus: FVP-3.us 67 (EU659111.1) and FPV-kai.us.06 (EU659115.1).

Discussion

CPV infection in dogs with diarrhoea (prevalence of 40.0%-84.0%) has been reported in many countries (Chollom *et al.* 2013, Soma *et al.* 2013, Yi *et al.* 2016, Csagola *et al.* 2014), Chile (78.0%) (Jamett *et al.* 2015), India (88.0%) (Belsare *et al.* 2014), Zimbabwe (84.0%) (McRee *et al.* 2014), Portugal (71.6%) (Castanheira *et al.* 2014) and Korea (93.8%) (Yang *et al.* 2010). In this study, we found that the prevalence of CPV in diarrheic dogs (43.3%, 13/30) was significantly higher than in healthy dogs (4.0%, 2/50), indicating that CPV was a major cause of diarrhoea in domestic dogs.

CPV-2b was reported as a major CPV variant in leopards and domestic cats in Vietnam in 2000 (Ikeda *et al.* 2000). In 2004, seven CPV-2b and one CPV-2c strains were detected (Nakamura *et al.* 2004). CPV-2a and CPV-2b were reported to be the predominant CPV variant in Asia and Australia (Meer *et al.* 2007, Lin *et al.* 2014) and CPV-2c was reported as a minor CPV variant. Recently, CPV-2c was detected as the predominant genotype in many European countries including Italy, Germany, and Spain (Buonavoglia *et al.* 2001, Decaro *et al.* 2012, Gallo *et al.* 2012, Cságola *et al.* 2014). However, 14 CPV-2c and one CPV-2a strains were detected in Vietnam between 2013-2015. These data suggest that the predominant CPV variant in Vietnam has changed from CPV-2b to CPV-2c.

When compared with the major CPV types globally, Vietnamese CPV-2c strains have four unique mutations at residue 5 (Ala to Gly), 267 (Phe to Tyr), 324 (Tyr to Ile), 370 (Gln to Arg), and Vietnamese CPV-2a has three unique mutations at residue 267 (Phe to Tyr), 324 (Tyr to Ile) and 440 (Thr to Ala) (Table II). On the other hand, the previous CPV-2a and -2b genotypes in Vietnam had single mutations at residue 5 (Ala to Gly) in cats, 267 (Phe to Tyr) in dogs, and 440 (Thr to Ala) in leopard cats (Ikeda *et al.* 2000, Nakamura *et al.* 2004). The mutation at residue 5 (Ala to Gly) was also reported in dogs in China (Wang *et al.* 2016). These data indicate that there might be evolution of the original CPV strains in Vietnam. The mutations in Vietnamese CPV-2c might affect the antigenicity and pathogenicity of the virus and this should be further investigated *in vivo*.

Vietnamese CPV strains have two mutations at residues 324 and 267. Because residue 323 is associated with binding to the canine transferrin receptor, the change at residue 324 might also influence infection in cells. In addition, it was reported that the mutation at residue 267 might play an important role in transmission and infection

(Chang *et al.* 1992, Xu *et al.* 2012). Therefore, these mutations might result in changes in the antigenicity of CPV in domestic dogs in Vietnam. The previous reports showed that viruses with a mutation at 324 (Tyr to Ile) were detected in naturally infected dogs for at least 63 days (Lin *et al.* 2014).

In addition, all Vietnamese CPV-2c strains possessed an Arginine at position 370, and this mutation was also observed in some Chinese CPV-2a (Guo *et al.* 2013) and CPV-2c strains (Zhao *et al.* 2015). This residue is close to residues 375 and 377 which are associated with the ability of CPV to agglutinate red blood cells. Residue 370 is also close to residues 379 and 384 which affect binding to the viral receptor and host range (Guo *et al.* 2013). Further experiments are required to clarify the role of mutations at residue 370.

Recently, some mutations (267Y, 297A, 324I and 370R) in VP2 observed in Vietnamese CPV strains have been also reported in Italian and Nigerian CPV-2c strains of Asian origin (Mira *et al.* 2019, Ogbu *et al.* 2020). This indicated that Asian CPV-2c mutation have been spread worldwide.

In this study, we observed a high prevalence of CPV in diarrheic dogs in Vietnam, and CPV was confirmed to be a major pathogen causing enteritis in dogs. In addition, the transition of the predominant genotype from CPV-2b to CPV-2c with some novel mutations was observed. The efficiency of protection generated by recent vaccines against current field isolates should be evaluated.

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Annex 1

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Supplementary Table. *Epidemiological information on canine parvovirus (CPV) infection of domestic dogs in Ho Chi Minh city, Vietnam.*

Dog.No	Sex	Age	Clinical sign	Collection date (month/year)	Breed	PCR detection	Virus isolation
CPV/dog/HCM1/2013	Male	8 months	Diarrhea, cough, nasal discharge	10/2013	Vietnamese	2c	
CPV/dog/HCM2/2013	Female	12 months	Diarrhea, ocular discharge	10/2013	Japanese	2c	CPV-2c
CPV/dog/HCM4/2013	Female	3 months	Diarrhea, cough	10/2013	Vietnamese	2c	
CPV/dog/HCM5/2013	Male	4 months	Diarrhea	10/2013	Japanese	2c	CPV-2c
CPV/dog/HCM6/2013	Male	6 months	Diarrhea, ocular discharge	10/2013	Vietnamese	2c	CPV-2c
CPV/dog/HCM7/2013	Female	4 months	Diarrhea	10/2013	Mixed	2c	CPV-2c
CPV/dog/HCM8/2013	Male	2 months	Diarrhea, cough	10/2013	Vietnamese	2c	CPV-2c
CPV/dog/HCM9/2013	Female	2 months	Diarrhea, nasal discharge	10/2013	Vietnamese	2c	
CPV/dog/HCM13/2013	Female	3 months	Diarrhea, cough, nasal discharge	11/2013	Vietnamese	2c	CPV-2c
CPV/dog/HCM14/2013	Male	3 months	Diarrhea, cough	11/2013	Vietnamese	2c	CPV-2c
CPV/dog/HCM18/2013	Female	8 months	Diarrhea, cough	11/2013	Japanese	2c	
CPV/dog/HCM20/2013	Male	4 months	Diarrhea	11/2013	Japanese	2c	CPV-2c
CPV/dog/HCM22/2013	Female	2 months	Diarrhea	12/2013	Vietnamese	2a	New CPV-2a
CPV/dog/HCM82/2015	Male	3 years old	Healthy	6/2015	Berger	2c	
CPV/dog/HCM88/2015	Male	2 years old	Healthy	6/2015	Vietnamese	2c	