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Original article

Isolation and sequence analysis of the complete VP2 gene of canine parvovirus from Chinese domestic pets and determination of the pathogenesis of these circulating strains in beagles

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Abstract

Canine parvovirus (CPV) causes acute gastroenteritis in domestic dogs, cats, and several wild carnivore species. In this study, the full-length VP2 gene of 36 CPV isolates from dogs and cats infected between 2016 and 2017 in Beijing was sequenced and analyzed. The results showed that, in dogs, the new CPV-2a strain was the predominant variant (n = 18; 50%), followed by the new CPV-2b (n = 6; 16.7%) and CPV-2c (n = 3; 8.3%) strains, whereas, among cats, the predominant strain was still CPV-2 (n = 9; 25%). One new CPV-2a strain, 20170320-BJ-11, and two CPV-2c strains, 20160810-BJ-81 and 20170322-BJ-26, were isolated and used to perform experimental infections. Multiple organs of beagles that died tested PCR positive for CPV, and characteristic histopathological lesions were observed in organs, including the liver, spleen, lungs, kidneys, small intestines, and lymph nodes. Experimental infections showed that the isolates from the epidemic caused high morbidity in beagles, indicating their virulence in animals and suggesting the need to further monitor evolution of CPV in China.

Key words: canine parvovirus, molecular epidemiology, phylogenetic analysis, pathogenesis

Introduction

Canine parvovirus (CPV) is an important pathogen that causes acute hemorrhagic enteritis and myocarditis in dogs and several wild carnivore species (Decaro et al. 2009). CPV-2 infection can result in acute gastroenteritis, characterized by loss of appetite, vomiting, fever, diarrhea (from mucoid to hemorrhagic), and leucopenia, with high mortality in young dogs, especially puppies between 6 weeks and 6 months of age. It was first identified in 1978 in the USA as a virus that infected dogs and other members of the family *Canidae*, but not cats, and it was shown to cause high morbidity and mortality greater than 10% (Zhong et al. 2014). Although it is a DNA virus, its genomic substitution rate is approximately 10^{-4} substitutions per site per year, which is similar to that of RNA viruses (Shackelton et al. 2005). Amino acid substitutions in the VP2 gene have been responsible for changes in genetic and antigenic properties. Between 1979 and 1981, an antigenic variant, CPV-2a, was found. In 1984, a second variant, CPV-2b, was identified (Decaro et al. 2012). Five amino acid differences in the VP2 sequence were found between CPV-2 (Met-87, Ile-101, Ala-300, Asp-305, and Val-555) and CPV-2a (Leu-87, Thr-101, Gly-300, Tyr-305, and Ile-555) (Martella et al. 2005), whereas CPV-2b differed from CPV-2a in the substitution Asn426Asp (Pratelli et al. 2001). Canine parvovirus type 2a/2b strains that have undergone the Ser297Ala substitution are designated “new CPV-2a/2b” (Decaro et al. 2012). Currently, new CPV-2a and new CPV-2b appear to have replaced the prototypical CPV-2a and CPV-2b and become the predominant types; these types are co-circulating in many countries, including China (Decaro et al. 2006, Mukhopadhyay et al. 2008). Another antigenic variant with the amino acid substitution Asp426Glu, designated CPV-2c, was first reported in Italy in 2000 (Dogonyaro et al. 2013) and is now the most common variant in Italy, Germany, Uruguay, and Argentina (Hoelzer et al. 2008, Nandi et al. 2010).

In China, CPV-2 was first reported in 1982 (Zhao et al. 2013); CPV-2a was reported in 1986 and then became predominant (Zhong et al. 2014). CPV-2b emerged in 1997 and co-circulated with CPV-2a. In recent years, new CPV-2a and new CPV-2b have both been circulating in China (Xu et al. 2011). The new CPV-2a virus is distributed throughout all of China, whereas new CPV-2b only circulates in the southern part of China (Zhang et al. 2010, Zhao et al. 2013). CPV-2c was first identified in Jilin province in 2010 (Chiang et al. 2016). Currently, the antigenic variants CPV-2a/2b/2c have completely replaced the original type 2 CPV and are distributed worldwide in the canine population. Most of the commercial

vaccines against CPV are based on the original CPV-2 strain, which may contribute to vaccine failure (Zhao et al. 2013). Therefore, it is important to elucidate the epidemiology and molecularly characterize CPV strains currently circulating in Beijing.

In the present study, we investigated the epidemiology of CPV in domestic dogs of Beijing and isolated three epidemic CPV strains. The objectives of the study were to identify and molecularly characterize the epidemic CPV strains and to determine whether experimental oral gavage infection of beagles resulted in clinical signs.

Materials and Methods

Sample collection

A total of 89 samples from suspected CPV cases with symptoms of vomiting and hemorrhagic diarrhea were collected from GuanZhong Animal Hospital in Beijing from January 2016 to May 2017. The diagnostic criteria used for CPV-2 infection included clinical signs, examination of blood, and a positive result by CPV colloidal gold test strip (Bionote, Inc., Hwaseong, Gyeonggi Province, South Korea) used according to the product protocol. Samples were collected and stored at -80°C by a single skilled veterinarian.

Detection of the CPV genome

All samples were diluted 1:5 in distilled phosphate-buffered saline (PBS), thawed on ice, and centrifuged at $12,000 \times g$ for 10 min at 4°C , and the supernatants were then passed through $0.22\text{-}\mu\text{m}$ filters (Millipore, Billerica, MA, USA) and used for PCR amplification (Xu et al. 2011). Viral DNA was extracted from an aliquot (200 μl) of each dilution by boiling at 100°C for 10 min, chilling immediately on crushed ice for 5 min (Decaro et al. 2006), then extraction using an Axyprep body fluid viral DNA/RNA miniprep kit (Corning, USA) according to the manufacturer's instructions. The extracted DNA samples were frozen at -80°C until used for PCR amplification. A pair of primers pair (VP2-1F/VP2-1R) (shown in Table 1) to detect the CPV genome was designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA), based on the conserved sequence of a previously published CPV genome sequence (GenBank accession no.: JQ268284.1).

Table 1. Primers for detection of the CPV genome and the complete VP2 gene

Name	Sequence (5'-3')	Position (VP2)	Product (bp)	GenBank accession no.
VP2-1F	TTAAAGACTGTTTCAGAATCTGC	3226-3248	746	JQ268284.1
VP2-1R	AATCTCTCAGGTGTTTCTCCTGTT	3948-3971		
VP2-F	ATGAGTGATGGAGCAGTTCAACCAGACGG	2779-2807	1775	JQ268284.1
VP2-R	TTAGT ATAAT TTTCTA GGTGC TAGTT GAGAT	4503-4533		

Amplification and sequencing of the complete VP2 gene

For genotyping, the full-length VP2 gene was amplified as follows. Amplifications were performed with primers VP2-F/VP2-R using Pfu polymerase (TaKaRa Biotechnology Co., Dalian, China). PCR products containing the full-length VP2 gene were cloned into pEASY-Blunt Zero Cloning Vector (TransGen Biotech Company, Beijing, China), and the presence of the desired insert (1775 bp) in the recombinant plasmid DNA was confirmed by PCR amplification (primer pair M13 F/R). After confirmation, five recombinant plasmids of each DNA fragment were sequenced by Invitrogen™ of Shanghai Biotechnology Co., Ltd. (Shanghai, China) (Gallo et al. 2012).

Sequence analysis

The specificity of the sequences was determined using BLAST (Basic Local Alignment Search Tool, <http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi>), and phylogenetic analyses of CPV based on the full-length VP2 nucleotide sequences were performed using the maximum likelihood method in MEGA 5.0. The reliability of the tree topology obtained for the VP2 region was then evaluated by bootstrap testing with 1000 replicates. Multiple alignments of the complete VP2 gene nucleotide sequences and deduced amino acid sequences were obtained using the MegAlign program of DNASTAR (DNASTAR Inc., Madison, WI, USA). The VP2 nucleotide sequences were submitted to GenBank.

Virus isolation

The cat kidney F81 cell line, maintained in DMEM medium (Gibco, USA), was used to isolate CPV from clinical samples that tested positive for CPV by PCR. The samples were filtered through a 0.22-µm Millipore filter (Merck Millipore, Germany) and used to inoculate a confluent F81 monolayer. The supernatant and cells were harvested 72 h post infection (p.i.) [with or without cytopathic effect (CPE)] via three alternating freeze-thaw cycles and then were clarified at 6,000 × g for 15 min in a refrigerated centrifuge. The third and fifth passages of the virus were screened for the presence

of virus by PCR using the same primer pair (VP2-1F and VP2-1R) and an indirect immunofluorescence assay (IFA) (Wang et al. 2013). In addition, the CPV suspensions were titrated using a 50% tissue culture infective dose (TCID₅₀) assay.

Animal infection experiment and histopathology

To determine the pathogenicity of the isolates, animal experiments were performed with 20 beagles, 9-10 weeks old, unvaccinated, healthy, and that did not have antibodies against CPV, obtained from Nanjing University Experimental Animal Center. The beagles were randomly divided into four groups of 5 dogs each. Each beagle in Group 1 was orally inoculated with 2 ml of the 20160810-BJ-81 stock, whereas Group 2 was inoculated with 20170322-BJ-26 and Group 3 was inoculated with 20170320-BJ-11 using a virus stock of 10^{5.0} TCID₅₀. The remaining five beagles in the control group were orally inoculated with 2 ml of sterile PBS. To avoid contact transmission, each dog was maintained alone in its cage. After infection, each dog was clinically observed for three weeks. After infection, clinical indices, including body weight, body temperature, the presence of vomiting and diarrhea, and mental status, were monitored and recorded for 15 days or until the inoculated dogs died from virus infection. Rectal swabs were collected from dogs every day and tested by PCR as described above. Tissue samples, including livers, spleens, lungs, kidneys, small intestines, and lymph nodes, were collected from the challenged beagles, within 12 h of death, fixed by submersion in Bouin's solution, and then embedded in paraffin wax. Serial 4-µm sections were prepared for hematoxylin and eosin staining.

Results

Detection of the VP2 gene

Of the 89 samples, 77 (86.5%) tested positive by PCR. A 1775-bp fragment covering the full-length sequence of the VP2 gene was amplified from 36 (46.7%) samples. Detailed information for the 36 samples is shown in Table 2.

Table 2. Genotypes and sources of 36 clinical samples collected from dogs and cats.

No	Strain	Date of collection	Place of collection	Source	GenBank no.	Genetic type
1	20161107-BJ-2	Nov-2016	Beijing, China	Feline	MF347716	FPV
2	20161107-BJ-3	Nov-2016	Beijing, China	Feline	MF347717	FPV
3	20161107-BJ-5	Nov-2016	Beijing, China	Feline	MF347718	FPV
4	20170320-BJ-3	Mar-2017	Beijing, China	Feline	MF347719	FPV
5	20170320-BJ-5	Mar-2017	Beijing, China	Feline	MF347720	FPV
6	20170322-BJ-19	Mar-2017	Beijing, China	Feline	MF347721	FPV
7	20170322-BJ-33	Mar-2017	Beijing, China	Feline	MF347722	FPV
8	20160810-BJ-36	Aug-2016	Beijing, China	Feline	MF347726	FPV
9	20170405-BJ-B	Apr-2017	Beijing, China	Feline	MF347727	FPV
10	20160810-BJ-81	Aug-2016	Beijing, China	Canine	MF347723	CPV-2c
11	20170322-BJ-26	Mar-2017	Beijing, China	Canine	MF347724	CPV-2c
12	20170405-BJ-K	Apr-2017	Beijing, China	Canine	MF347725	CPV-2c
13	20160810-BJ-19	Aug-2016	Beijing, China	Canine	MF347728	new CPV-2a
14	20160810-BJ-59	Aug-2016	Beijing, China	Canine	MF347729	new CPV-2a
15	20161020-BJ-11	Oct-2016	Beijing, China	Canine	MF347730	new CPV-2a
16	20161107-BJ-4	Nov-2016	Beijing, China	Canine	MF347731	new CPV-2a
17	20161107-BJ-10	Nov-2016	Beijing, China	Canine	MF347732	new CPV-2a
18	20170320-BJ-4	Mar-2017	Beijing, China	Canine	MF347733	new CPV-2a
19	20170320-BJ-11	Mar-2017	Beijing, China	Canine	MF347734	new CPV-2a
20	20170322-BJ-9	Mar-2017	Beijing, China	Canine	MF347735	new CPV-2a
21	20170322-BJ-13	Mar-2017	Beijing, China	Canine	MF347736	new CPV-2a
22	20170322-BJ-16	Mar-2017	Beijing, China	Canine	MF347737	new CPV-2a
23	20170322-BJ-25	Mar-2017	Beijing, China	Canine	MF347738	new CPV-2a
24	20170322-BJ-31	Mar-2017	Beijing, China	Canine	MF347739	new CPV-2a
25	20170322-BJ-32	Mar-2017	Beijing, China	Canine	MF347740	new CPV-2a
26	20170405-BJ-A	Apr-2017	Beijing, China	Canine	MF347741	new CPV-2a
27	20170405-BJ-D	Apr-2017	Beijing, China	Canine	MF347742	new CPV-2a
28	20170405-BJ-E	Apr-2017	Beijing, China	Canine	MF347743	new CPV-2a
29	20170405-BJ-F	Apr-2017	Beijing, China	Canine	MF347744	new CPV-2a
30	20170405-BJ-I	Apr-2017	Beijing, China	Canine	MF347745	new CPV-2a
31	20160810-BJ-10	Aug-2016	Beijing, China	Canine	MF347746	new CPV-2b
32	20160810-BJ-18	Aug-2016	Beijing, China	Canine	MF347747	new CPV-2b
33	20160810-BJ-42	Aug-2016	Beijing, China	Canine	MF347748	new CPV-2b
34	20160810-BJ-77	Aug-2016	Beijing, China	Canine	MF347749	new CPV-2b
35	20161109-BJ-12	Nov-2016	Beijing, China	Canine	MF347750	new CPV-2b
36	20170320-BJ-12	Mar-2017	Beijing, China	Canine	MF347751	new CPV-2b

Prevalence of the CPV genotype

All 36 strains were typed according to key amino acid residues at positions 87, 101, 297, 300, 305, 426, and 555, and detailed information is provided in Table 2. CPV-2 (n = 9, 25%, all detected in felines), new CPV-2a (n = 18, 50%), new CPV-2b (n = 6, 16.7%), and 3 CPV-2c (n = 3, 8.3%) were detected; new CPV-2a was predominant in Beijing. In addition, amino acid substitutions were identified. Compared with reference

strains, 23 amino acid substitutions (Table 3) were observed. Of the 36 strains, 27 (75%) had a Phe267Tyr substitution, 24 (66.67%) had a Thr440Ala substitution, and 27 (75%) had a Tyr324Ile substitution. Comparing the three CPV-2c strains isolated with CPV-2c strains distributed across the world, substitutions Phe267Tyr, Tyr324Ile, and Gln370Arg seemed to be specific to Chinese 2c strains isolated in recent years.

To define the evolutionary relationships between viruses, a maximum likelihood phylogenetic tree of

Table 3. Amino acid substitution sites, types, and rates in canine parvovirus VP2 genes

No.	Strain	Genetic type	Amino acid sites in the VP2 gene																											
			29	30	80	87	93	103	232	251	262	267	297	300	305	322	323	324	345	370	426	440	555	564	568					
			Amino acid substitution																											
			G→D	G→V	R→K	L→M	N→K	A→V	L→V	R→S	A→V	F→Y	S→A	A→G	D→Y	T→A	D→N	F→S	Q→R	N→D/E	T→A	L→V	N→S	A→G						
1	CPV-2	/	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					
2	CPV-2a	/	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	-	I	-	-	-	A	-	-	-					
3	CPV-2b	/	-	-	-	-	-	-	-	-	-	-	A	G	Y	-	-	-	-	-	D	A	-	-	-					
4	new CPV-2a	/	-	-	I	-	-	-	-	-	-	-	A	G	Y	-	-	I	-	-	-	-	-	-	-					
5	new CPV-2b	/	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	-	-	-	-	D	-	-	-	-					
6	CPV-2c	/	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	-	I	-	R	E	-	-	-	-					
7	20161107-BJ-2	CPV-2	-	V	K	M	K	V	V	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-					
8	20161107-BJ-3	CPV-2	-	-	K	M	K	V	V	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-					
9	20161107-BJ-5	CPV-2	D	-	K	M	K	V	V	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-					
10	20170320-BJ-3	CPV-2	-	-	K	M	K	V	V	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-					
11	20170320-BJ-5	CPV-2	-	-	K	M	K	V	V	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-					
12	20170322-BJ-19	CPV-2	-	-	K	M	K	V	V	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-					
13	20170322-BJ-33	CPV-2	-	-	K	M	K	V	V	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-					
17	20160810-BJ-36	CPV-2	-	-	K	M	K	V	V	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-					
18	20170405-BJ-B	CPV-2	-	-	K	M	K	V	V	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-					
14	20160810-BJ-81	CPV-2c	-	-	-	-	-	-	-	-	-	Y	A	G	Y	A	N	I	-	R	E	-	V	S	G					
15	20170322-BJ-26	CPV-2c	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	R	E	-	V	S	G					
16	20170405-BJ-K	CPV-2c	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	R	E	-	V	S	G					
19	20160810-BJ-19	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
20	20160810-BJ-59	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
21	20161020-BJ-11	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
22	20161107-BJ-4	new CPV-2a	-	-	-	-	-	-	S	V	Y	A	G	Y	-	N	I	S	-	-	-	A	V	S	G					
23	20161107-BJ-10	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
24	20170320-BJ-4	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
25	20170320-BJ-11	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
26	20170322-BJ-9	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
27	20170322-BJ-13	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
28	20170322-BJ-16	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
29	20170322-BJ-25	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
30	20170322-BJ-31	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
31	20170322-BJ-32	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
32	20170405-BJ-A	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
33	20170405-BJ-D	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
34	20170405-BJ-E	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
35	20170405-BJ-F	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
36	20170405-BJ-I	new CPV-2a	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	-	A	V	S	G					
37	20160810-BJ-10	new CPV-2b	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	D	A	V	S	G					
38	20160810-BJ-18	new CPV-2b	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	D	A	V	S	G					
39	20160810-BJ-42	new CPV-2b	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	D	A	V	S	G					
40	20160810-BJ-77	new CPV-2b	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	D	A	V	S	G					
41	20161109-BJ-12	new CPV-2b	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	D	A	V	S	G					
42	20170320-BJ-12	new CPV-2b	-	-	-	-	-	-	-	-	-	Y	A	G	Y	-	N	I	-	-	D	A	V	S	G					
	Sample numbers		1	1	9	9	9	9	9	9	1	1	27	27	27	27	1	27	27	1	3	9	24	36	27	27				
	Mutation rates (%)		2.78	2.78	25	25	25	25	25	25	2.78	2.78	75	75	75	75	2.78	75	75	2.78	8.33	25	66.7	100	75	75				

CPV-2 (M38246); CPV-2a (M24003); CPV-2b (M748749); new CPV-2a (JX048605); new CPV-2b (JX048607); CPV-2c (KT156832).

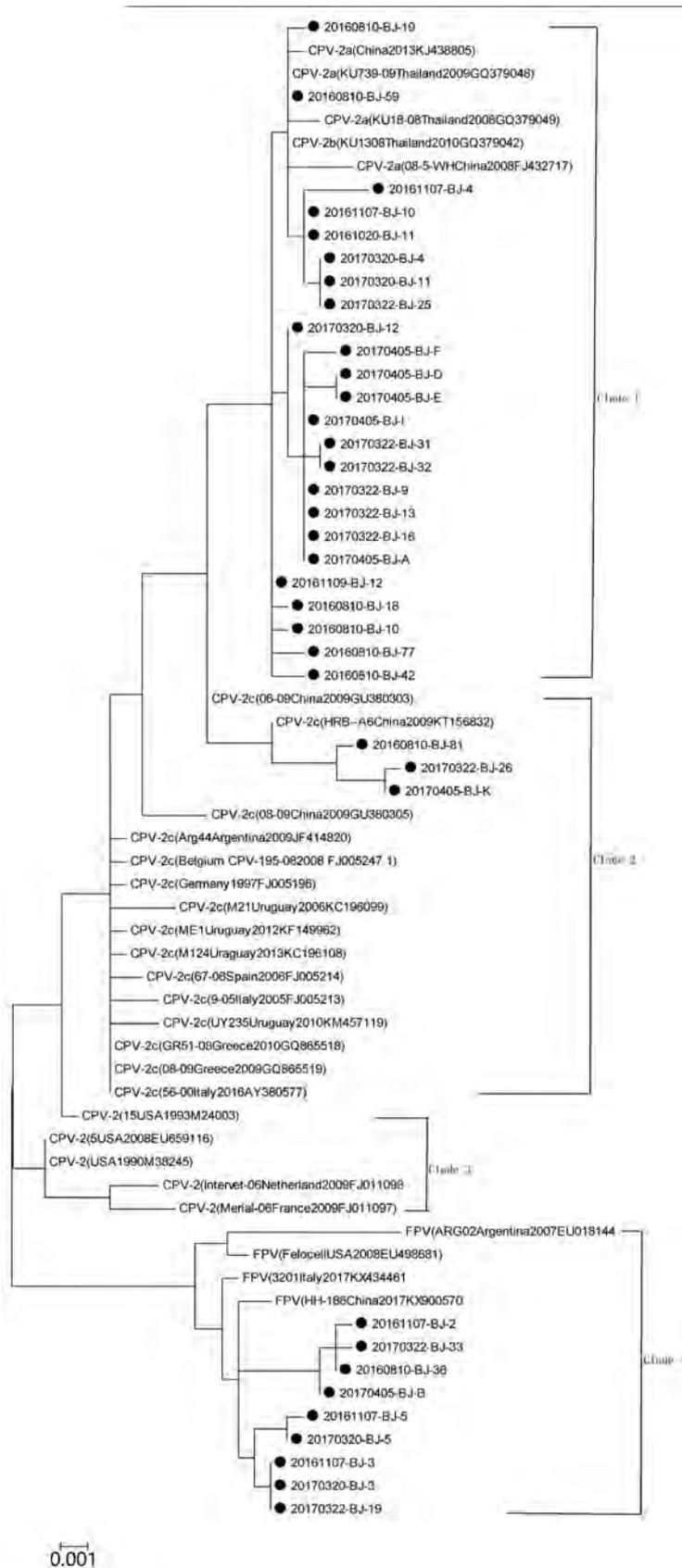


Fig. 1. Maximum-likelihood tree constructed from complete VP2 nucleotide sequences (1755 bp). Note that the CPV strains under study are marked with solid circles (●). Nucleotide sequences were analyzed in MEGA5.0, and bootstrap values were calculated from 1000 replicates.

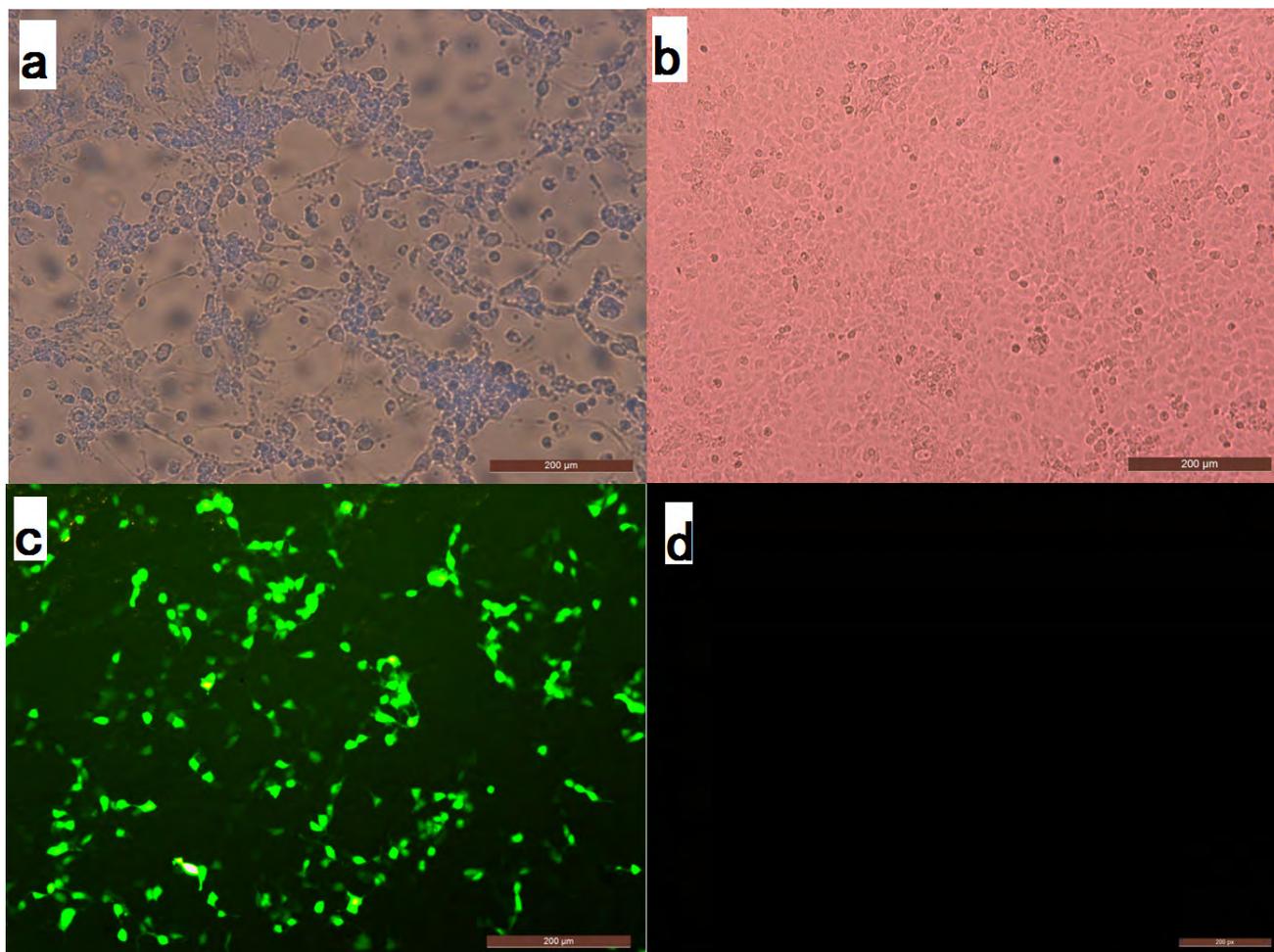


Fig. 2. Isolation and identification of CPV in F81 cells. a: F81 cells infected with the isolated virus. b: Uninfected F81 cells. c: Indirect immunofluorescence assay of infected F81 cells. d: Indirect immunofluorescence assay of uninfected F81 cells.

CPV strains based on complete VP2 nucleotide sequences was constructed (Fig. 1). The strains were divided into four clades, with all of the new CPV-2a and new CPV-2b strains clustered in clade 1, the three CPV-2c strains and reference CPV-2c strains forming cluster 2, a monophyletic group of the CPV-2 genotype forming clade 3, and the classical feline parvovirus strains and nine epidemic strains in this study forming clade 4. In the CPV-2c cluster, the three CPV-2c strains were closely related to the HRB-A6 strain, sharing 99.5–99.9% identity at the nucleotide level and 99.8–100% identity at the amino acid level and revealing 10 non-synonymous codons changes; however, Thr322Ala (nt: A→G) was first detected in 20160810-BJ-81.

Virus isolation

Three parvoviruses were isolated, 20170320-BJ-11 (new CPV-2a) and 20160810-BJ-81 and 20170322-BJ-26 (both CPV-2c). Typical CPE and IFA-positive results in F81 cells infected by 2017320-BJ-11 were obtained (Fig. 2a and 2c). Electron microscopy showed a large number of 20–25-nm virus particles with cubic symme-

try that is typical of this parvovirus (data not shown). As determined by the method of Reed and Munch, the titers of the three isolated viruses were $10^{6.03}$ TCID₅₀/ml, $10^{6.24}$ TCID₅₀/ml, and $10^{6.43}$ TCID₅₀/ml, respectively.

Animal infection experiment and histopathology

On days 5–14 p.i., the challenged beagles presented with typical clinical signs: mental depression, loss of appetite, fever, vomiting, and diarrhea (from mucoid to hemorrhagic) with the appearance of diarrhea, large quantities of mucus, intestinal casts, and bloody fluid were seen in fecal droppings. The morbidity rate of dogs in Group 1, inoculated with 20160810-BJ-81, was 100% (5/5), whereas that in Group 2 (inoculated with 20170322-BJ-26) and Group 3 (inoculated with 20170320-BJ-11) was 80% (4/5). Virus shedding from the inoculated animals was confirmed by PCR from days 1 to 14 p.i. The peak titers in fecal swab elutes from the challenged animals were $10^{3.8}$ – $10^{4.7}$ TCID₅₀/ml on days 7–10 p.i. Histopathologic lesions were observed in tested tissues of the beagles that were infected

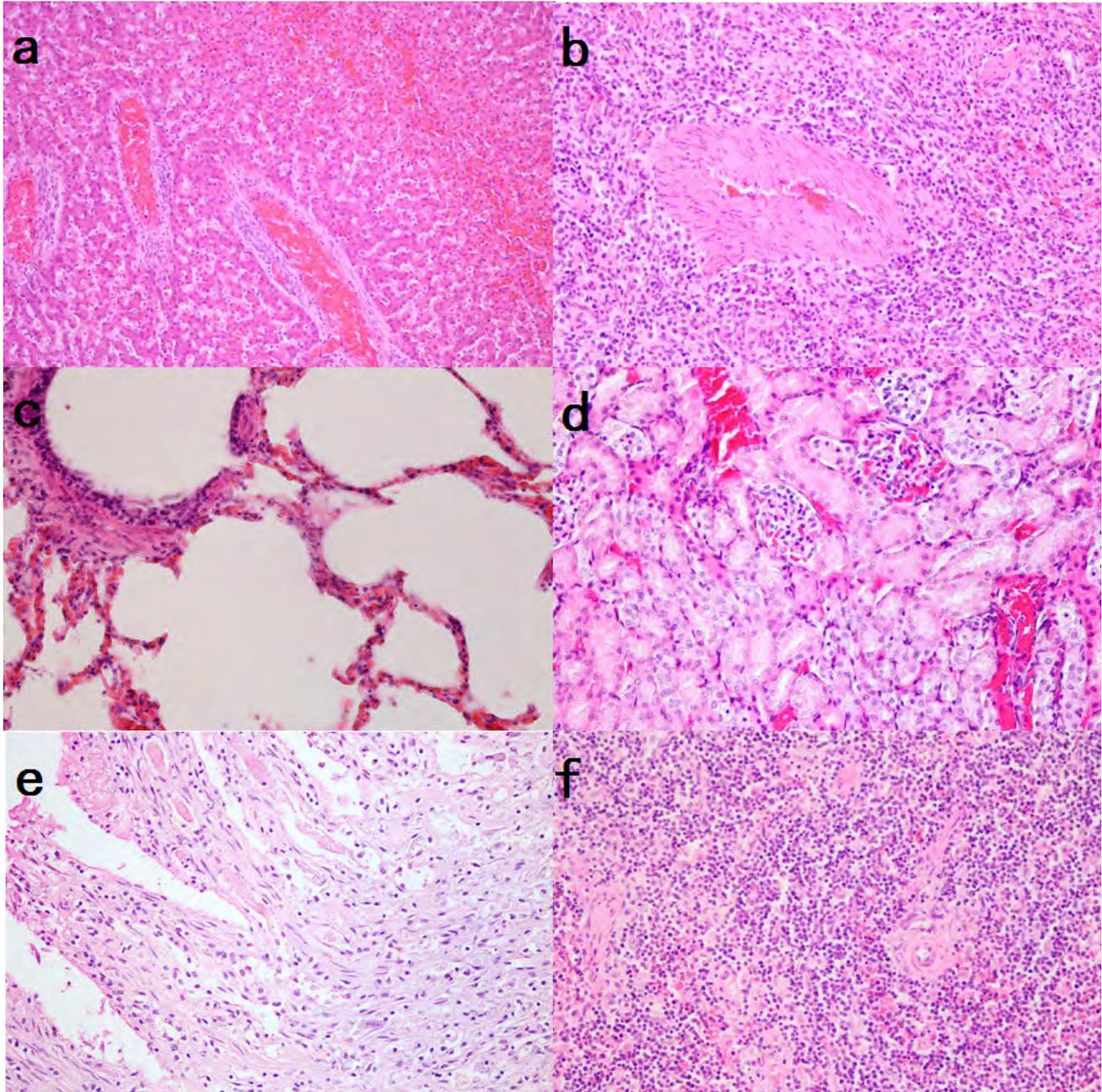


Fig. 3. Histopathologic appearance of hematoxylin and eosin-stained livers, spleens, lungs, kidneys, small intestines, and lymph nodes of beagle no. 2 experimentally infected with 20160810-BJ-81.

a: Liver, showing hyperemia of the hepatic sinusoid; 400×; b: Spleen, mild hemorrhage and congestion in the white pulp and medulla; 400×; c: Lung, a small number of detached epithelial cells and inflammatory cell infiltration in the alveoli; 400×; d: Kidney, renal tubules show epithelial degeneration and necrosis; 400×; e: Small intestine, epithelia of the mucosa display degeneration, necrosis, and desquamation; 400×; f: Lymph node, mild hemorrhage in the lymphoid cortex; 400×.

with 20160810-BJ-81 and died (shown in Fig. 3). In the livers of infected animals, the veins in the hepatic manifold were filled with blood, and hepatic sinusoids were hyperemic with erythrocytes (Fig. 3a). In spleens, mild hemorrhage and congestion were observed in the white pulp and medullary (Fig. 3b). In lungs, the alveoli contained a small number of detached epithelial cells and displayed inflammatory cell infiltration (Fig. 3c). In kidneys, we observed degeneration and necrosis

of the renal tubule epithelium, along with nuclear enrichment or nuclear disappearance and tubule interstitial congestion (Fig. 3d). In small intestines, disruption of the villous architecture was characterized by blunting and fusing of villi. The epithelium of the small intestinal villi was detached, the tissue underneath was exposed and loose, and the number of lymphocytes was increased (Fig. 3e). Mild hemorrhage was observed in the lymphoid node cortex (Fig. 3f).

The surviving animals were dehydrated and debilitated, but resumed eating and achieved complete clinical recovery. The hemagglutination inhibition titers of the serum samples collected from the animals on day 7 p.i. were 1:32-1:512, and, on day 15 p.i., were 1:256-1:1280. The control animals showed no clinical signs, virus shedding, or seroconversion. The control beagles did not show any symptoms during the experimental period, and all surviving beagles were friendly fed until now.

Discussion

Since CPV emerged in 1978, it has spread in domestic and wild canine populations, where it is continuously evolving into new viral variants. The variability of the CPV genome and its high mutation rate have allowed CPV to diversify into these variants and to spread rapidly in the canine population (Shackelton et al. 2005). In China, CPV-2 was first reported in 1983, and several variants have appeared in recent years. A variety of isolates have since been characterized and their VP2 genes sequenced. In 2009, Yi et al. first detected CPV-2c (Yi et al. 2009), and J. Wang et al. first isolated CPV-2c in 2014 (Wang et al. 2016). In our study, we detected 18 new CPV-2a strains (accounting for 50% of our isolates), including six new CPV-2b, nine CPV-2, and three CPV-2c strains. This supports the idea that new CPV-2a is more prevalent than the new CPV-2b variant in Beijing. The prevalence of new CPV-2a and new CPV-2b determined in this study is in agreement with that reported by most studies in other provinces of China (Geng et al. 2015, Wu et al. 2015, Zhong et al. 2014) and other countries (Dogonyaro et al. 2013, Jeoung et al. 2008, Meers et al. 2007, Mukhopadhyay et al. 2014, Timurkan et al. 2015). Results of this study also showed that CPV-2 strains circulating in Beijing exhibit genetic variation.

VP2 encodes a viral capsid protein that is the major structural protein of CPV-2 and that is involved in the host immune response. Therefore, a small number of mutations in the VP2 gene may result in altered pathogenicity (Lin et al. 2014). Interestingly, in all CPV-2 samples, five substitutions (Arg80Lys, Leu87Met, Asn93Lys, Ala103Val, Ile232Val) co-occurred. Among them, Leu87Met was specific to CPV-2 strains. In contrast, the substitutions Arg80Lys, Asn93Lys, Ala103Val, and Ile232Val were found in our isolates but were not present in the reference CPV-2 strains. With the exception of the nine CPV-2 strains, all of the isolates showed eight distinctive amino acid substitutions: Phe267Tyr, Ser297Ala, Ala300Gly, Asp305Tyr, Asp323Asn, Tyr324Ile, Asn564Ser, and Ala568Gly. The substitution Thr440Ala appeared in all new CPV-2a and new

CPV-2b strains but not in CPV-2 and CPV-2c strains. All 36 sequences had the substitution Ile555Val.

Compared with reference strains from around the world, the three CPV-2c strains we isolated displayed four amino acid substitutions in VP2: Phe267Tyr, Thr322Ala, Ile324Tyr, and Gln370Arg. The Phe267Tyr substitution has been reported in China since 2013 (Wang et al. 2016), and the Tyr324Ile substitution has been observed in isolates from many Asia countries (Yi et al. 2016). The Gln370Arg substitution, which was previously found in some CPV-2a strains in Beijing and Harbin, China (Wang et al. 2016), was detected in CPV-2c strains for the first time in our study. Compared with CPV-2c reference strains, a Thr322Ala substitution in 20160810-BJ-81, caused by a A-G mutation at nt 964 in the VP2 gene, was found for the first time. It could be involved in a conformational change in VP2 that is required during replication. We observed some synonymous mutations in the VP2 gene, such as C-A (nt 408), T-C (nt 420), A-G (nt 780), G-A (nt 1290). Although these variations did not cause amino acid changes, the alterations likely affect the host range of the virus (Guo et al. 2013).

In an animal experiment, all infected beagles presented with typical clinical signs, whereas control beagles remained healthy. This indicates the pathogenicity of the isolated strains, 20170320-BJ-11, 20160810-BJ-81, and 20170322-BJ-26, and suggests they may pose a threat to domestic dogs. Parvovirus replication in the experimentally infected dogs was mainly seen in highly mitotically active tissues. We detected characteristic histopathological lesions, viral DNA, and antigen staining in multiple organs of the infected dogs, including livers, spleens, lungs, kidneys, small intestines, and lymph nodes. These results indicated that domestic pets are susceptible to these epidemic strains.

In summary, new CPV-2a was the most prominent type of CPV circulating in Beijing in 2016–2017, and its molecular characteristics were studied. Animal infection experiments with the isolated strains showed that they are pathogenic to domestic dogs.

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