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SHORT COMMUNICATION

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Molecular characterization of canine parvovirus type 2 (CPV2) reveals a high prevalence of the CPV2c genotype among dogs suffering from diarrhea

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Abstract

Canine parvovirus 2 (CPV-2) is a highly contagious virus in dogs that typically causes hemorrhagic enteritis and a high mortality rate in unvaccinated puppies. The genetic variability and antigenic diversity of CPV-2 hinder its effective prevention of infection by vaccination. To investigate the epidemiology and genetic characteristics of CPV-2 in China, rectal swabs from affected dogs were collected from different animal clinics in Kunshan from 2022 to 2023. Preliminary detection and capsid gene sequencing of CPV-2 were performed using previously described primers and protocols. The overall detection rate for CPV-2 was 16.5% (33/200). A significant association was found between the CPV-2-positivity and clinical signs, age, breed and vaccination status. Sequence analysis revealed the presence of CPV-2c genotypes in all positive samples, which were genetically similar to other Asian CPV-2c strains. Notably, four key mutations (A5G, F267Y, Y324I and Q370R) were detected in all isolates, and one novel mutation (I447M) was detected in three CPV-2 isolates. These mutations in the CPV-2 strains could impact vaccine efficacy and the effectiveness of the virus immune evasion. Surprisingly, no recombination events were observed between the identified CPV-2c strains and reference strains from China. Our data revealed that amino acid residues 324, 426 and 440 of VP2 may under strong selection pressure. This pattern of genetic variation in the CPV-2 lineage warrants continuous laboratory-based surveillance programs in other parts of China to better understand the pattern of seasonal distribution and association between emerging genotypes and the intensity of disease severity.

Keywords Canine parvovirus, Dogs, Epidemiology, Genetic diversity, Phylogenetic analysis

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Main text

Viral diarrhea poses a serious health threat to dogs worldwide, including in China. The important viruses that cause viral diarrhea in dogs include canine distemper virus (CDV), canine coronavirus (CCoV), canine parvovirus-2 (CPV-2), canine kobuviruses (CKoV) and canine bocavirus (CBoV) (Qi et al. 2020). CPV-2 is a single-stranded DNA virus and belongs to the species *Protoparvovirus carnivoran 1* (family *Parvoviridae*, subfamily *Parvovirinae*), together with feline panleukopenia virus (FPV) (Cotmore et al. 2019). CPV-2 is believed to have evolved from the FPV-like virus of domestic cats during the 1970s after specific mutations were acquired that enhanced its ability to interact with canine cell receptors (Parrish et al. 1991). The viral genome of CPV-2 is approximately 5.2 kb in length and consists of two major open reading frames (ORF1 and ORF2). ORF1 (nonstructural ORF) encodes two nonstructural proteins (NS1 and NS2), and ORF2 (structural ORF) encodes three main capsid proteins (VP1, VP2 and VP3). VP2 is a key capsid protein that contains 584 amino acid residues. The VP2 protein defines the host range and plays a pivotal role in inducing neutralizing antibodies against CPV-2 (Cotmore et al. 2019). CPV-2 causes severe enteric disease in affected dogs, which can lead to bloody diarrhea, myocarditis and death. CPV-2 affects dogs of all ages, although CPV-2 infections are more severe in puppies (Hoang et al. 2019; Castillo et al. 2020; Decaro et al. 2020; Qi et al. 2020; Fu et al. 2022).

In 1979, a new antigenic variant of CPV-2 emerged in US dogs; this variant had five key mutations (M87L, I101T, A300G, D305Y and V555I) in the VP2 protein and was named CPV-2a (Hoelzer et al. 2008). After the emergence of CPV-2a, several new mutations were reported for this virus, leading to another two variants or genotypes (CPV-2b and CPV-2c). These antigenic variants were classified by changes in amino acid 426 of VP2 (N in CPV-2a, D in CPV-2b, and E in CPV-2c) and shown to infect felines (Franzo et al. 2023). The original CPV-2 strain no longer circulates among dogs and is quickly replaced by CPV-2 antigenic variants (Shao et al. 2021; Jing et al. 2022; Lina et al. 2022).

In 1982, the first confirmed case of CPV-2 was reported in China; since then, it has become endemic, and new variants of CPV-2 have been continuously reported among dogs in China (Zhao et al. 2013; Qi et al. 2020; Hao et al. 2022). The first case of CPV-2c was reported in Vietnam in 2004 (Hoang et al. 2019). During the last decade, the Asian lineage of CPV-2c has spread worldwide, including Africa, USA, South America, and European countries (France, Italy and Germany), and is no longer considered to be Asian (Franzo et al. 2023). Until 2015, the genotypes CPV-2a and CPV-2b remained predominant in China, although an increasing number of cases of CPV-2c were reported in Europe and the Americas (Zhao et al. 2013, 2017; Zhong et al. 2014; Zhou et al. 2016; Wu et al. 2018; Li et al. 2019; Qi et al. 2020; Chen et al. 2021; Jiang et al. 2021; Liu et al. 2021; Fu et al. 2022; Hao et al. 2022; Huang et al. 2022; Wang et al. 2022). Recently, the incidence of CPV-2 infections associated with CPV-2c genotypes has increased in several provinces of China; seemingly, this new CPV-2c variant is quickly replacing the previous CPV-2a and CPV-2b in China (Hao et al. 2020, 2022; Hu et al. 2020; Qi et al. 2020; Chen et al. 2021; Fu et al. 2022; Jing et al. 2022).

Although vaccination against CPV-2 is common in practice, clinical cases still occur worldwide. Understanding the distribution of CPV-2 genotypes and virus evolution is essential for better preventive and control measures. Therefore, surveillance of CPV-2 in different geographical locations is needed to prepare and design more effective vaccines and therapeutic measures. To date, there are no published data on the distribution and genetic characteristics of CPV-2 in Kunshan city, China. We performed this epidemiological study to investigate the prevalence and genetic diversity of CPV-2 in Kunshan city, China.

Detection of CPV-2

Of the 200 specimens collected from dogs in Kunshan city, 33 (16.5%) were found to be positive for CPV-2. A total of six vaccinated dogs (6/131, 4.6%) and 27 (27/69, 39.13%) nonvaccinated dogs were positive for CPV-2 in this study (Table 1). The severity of clinical signs was variable among vaccinated and nonvaccinated dogs; however, clinical signs were generally more severe in unvaccinated dogs (Table 2). Notable clinical signs were lethargy, anorexia, depression, fever, vomiting and hemorrhagic diarrhea among the affected dogs. Among the dogs (n = 42) that exhibited diarrhea in this study (11 with watery diarrhea, 31 with bloody diarrhea), 29 were positive for CPV-2 (6 with watery diarrhea, 23 with bloody diarrhea). Chi-square test analysis revealed significant associations between age and breed and between clinical signs ($p < 0.05$) and CPV-2-positive dogs, while a nonsignificant association ($p > 0.05$) was observed for sex (Table 1). CPV-2-positive specimens were negative for coinfections with CCoV and CDV.

Nucleotide identity and phylogenetic analysis

Of the 33 positive specimens, 12 could not yield good quality sequences and were therefore not selected for nucleotide analysis. The 21 sequences that yielded good quality reads were selected for analysis and deposited in GenBank (accession numbers OR399576-OR399596). A total of 21 nucleotide sequences of the VP2 gene

Table 1 Statistical analysis of the percentage of patients with a positive CPV-2 test

Parameter	Total number of samples	CPV-2 positive number	CPV-2 positive rate (%)	CPV-2 negative number	CPV-2 negative rate (%)	X^2	*p*	OR	95% CI
Clinical sign	*n* = 200	33	16.5	167	83.5			-	-
Cases with diarrhea	42	29	69.04	13	30.9	106.551	< 0.0001	11.289	(5.32–23.98)
Cases without diarrhea	158	4	2.5	154	97.4			0.1314	(0.045–0.3497)
Gender	*n* = 200							-	-
Male	122	19	15.6	103	84.4	0.1948	0. 659	0.934	(0.5044- 1.7276)
Female	78	14	17.9	64	82.05			1.107	(0.5562- 2.2034)
Breed	*n* = 200							-	-
Pure	88	21	23.9	67	76.1	6.1846	0 .0129	1.586	(0.8565–2.9373)
Mixed	112	12	10.7	100	89.3			0.6073	(0.2999–1.2298)
Age	*n* = 200							-	-
≤ 6 M	117	26	22.2	91	77.8	6.7003	0.0096	1.4459	(0.8145- 2.5668)
> 6 M	83	7	8.4	76	91.6			0.4661	(0.1973–1.101)
Vaccination status	*n* = 200							-	-
Vaccinated	131	6	4.6	125	95.4	39.1582	< 0.00001	0.2429	(0.0987- 0.5976)
Non vaccinated	69	27	39.1	42	60.9			3.2532	(1.7661- 5.9926)

were compared for nucleotide homology with reference strains retrieved from the NCBI GenBank. Comparison analysis revealed 99–100% identity at the nucleotide level between the CPV-2 strains in this study. OR399582 had the least nucleotide identity (98.5%) with OR399591. Notably, all the CPV-2 strains from Kunshan shared the highest nucleotide identity with domestic CPV-2 strains from Shanghai, Nanjing, Henan and Beijing, and the nucleotide identities were all greater than 99%. Notably, OR399579, OR399590 and OR399594 exhibited the highest nucleotide identity with CPV-2 strains from Thailand. The CPV-2 strains detected in the present study exhibited a nucleotide sequence identity of 97–98% compared to that of foreign vaccine strains (Vanguard-Pfizer, Parvodog-Merial, Nobivac-Intervet, Duramune-Zoetis, Quantum-Shering).

Genetic and phylogenetic analysis of the *VP2* gene

Genetic and phylogenetic analysis of the VP2 gene revealed that all 21 CPV-2 strains included in the present study formed a new clade within the reference CPV-2c sequences. All the CPV-2c genotypes showed the highest identity with domestic CPV-2c genotypes reported from Nanjing, Shanghai, Henan and Beijing, suggesting a close phylogenetic relationship. In addition, these strains were closely related to the CPV-2c genotypes reported from Vietnam and Thailand. However, the CPV-2c genotypes detected in this study were genetically distinct from those of vaccine strains and CPV-2c strains reported from Europe and North America (Fig. 1).

Deduced amino acid analysis of the *VP2* gene

Amino acid sequence analysis inferred and classified all the CPV-2 strains detected in the present study into CPV-2c. We identified substitutions in the VP2 protein when compared with those in the reference and vaccine strains. Deduced amino acid sequence analysis revealed several substitutions, including four key mutations (A5G, F267Y, Y324I, and Q370R) and one novel mutation (I447M), in three CPV-2 isolates at nucleotide positions 1340–1342 of the *VP2* gene (OR399582, OR399583 and OR399588). All the sequences contained isoleucine (I) at residue 447, except for OR399582, OR399583 and OR399588, which contained methionine (M) instead of isoleucine. In addition, amino acid substitutions at residues 5 (A → G), 87 (M → L), 101 (I → T), 267 (F → Y), 297 (S → A), 300 (A → G), 305 (D → Y), 324 (Y → I), 370 (Q → R), 375 (N → D), and 426 (N → E) were observed in all 21 sequences when compared to the reference genes (accession number: M38245) (Table 3). The presence of asparagine (Vanguard-Pfizer, Parvodog-Merial and Nobivac-Intervet), aspartic acid (Duramune-Zoetis and Quantum-Shering) and glutamic acid (all 21 CPV-2c genotypes of the present study) was observed at residue 426 in the VP2 gene for the vaccine and Kunshan CPV-2c genotypes, respectively.

Recombination analysis and estimation of selection pressure on *VP2* gene

Based on the RDP4, no potential recombination events were found within the 21 selected strains in this study.

Table 2 Information on the clinical signs, sex, breed, age and vaccination status of 33 CPV-2-positive specimens in Kunshan

CPV-2 strains	Time	Age (months)	Sex	Breed	Clinical signs	Vaccination status	Genetic type	GenBank No.
KS-1	2022	2	M	Hybrid	Anorexia, depression, fever	No	CPV-2c	OR399576
KS-2[a]	2022	3	F	Purebred	Anorexia, Fever, vomiting, watery diarrhea	No	CPV-2c	Not available
KS-3	2022	3	M	Purebred	Anorexia, fever, Bloody diarrhea	No	CPV-2c	OR399577
KS-4[a]	2022	5	M	Purebred	Anorexia, fever, Bloody diarrhea	No	CPV-2c	Not available
KS-5	2022	7	F	Purebred	Anorexia, fever, Bloody diarrhea	No	CPV-2c	OR399578
KS-6	2022	4	M	Purebred	Bloody diarrhea, vomiting	No	CPV-2c	OR399579
KS-7	2022	3.5	F	Purebred	Fever, watery diarrhea	No	CPV-2c	OR399580
KS-8	2022	8	F	Purebred	Anorexia, depression, fever	Yes	CPV-2c	OR399581
KS-9[a]	2022	5	M	Purebred	Anorexia, watery diarrhea	No	CPV-2c	Not available
KS-10[a]	2022	4	M	Purebred	Anorexia, depression, fever, Bloody diarrhea	No	CPV-2c	Not available
KS-11	2022	8	M	Hybrid	Lethargy, anorexia	Yes	CPV-2c	OR399582
KS-12	2022	3	F	Purebred	Anorexia, fever, Bloody diarrhea	No	CPV-2c	OR399583
KS-13	2022	2.5	M	Hybrid	Bloody diarrhea, vomiting, Bloody diarrhea	No	CPV-2c	OR399584
KS-14	2022	3	F	Hybrid	Bloody diarrhea, vomiting, Bloody diarrhea	No	CPV-2c	OR399585
KS-15	2022	9	F	Purebred	Depression, anorexia, watery diarrhea, vomiting	Yes	CPV-2c	OR399586
KS-16	2022	6	M	Hybrid	Bloody diarrhea, vomiting	No	CPV-2c	OR399587
KS-17	2022	6	F	Hybrid	Bloody diarrhea, vomiting	No	CPV-2c	OR399588
KS-18	2022	2.3	M	Purebred	Anorexia, depression, fever, Bloody diarrhea	No	CPV-2c	OR399589
KS-19[a]	2022	4	M	Purebred	Bloody diarrhea, vomiting	No	CPV-2c	Not available
KS-20[a]	2022	5	M	Purebred	Bloody diarrhea, vomiting	No	CPV-2c	Not available
KS-21	2022	3	F	Purebred	Bloody diarrhea, vomiting	No	CPV-2c	OR399590
KS-22[a]	2022	9	M	Hybrid	Lethargy, anorexia, vomiting, Bloody diarrhea	No	CPV-2c	Not available
KS-23[a]	2022	10	M	Hybrid	Bloody diarrhea, vomiting	No	CPV-2c	Not available
KS-24[a]	2022	3	F	Purebred	Bloody diarrhea, vomiting	No	CPV-2c	Not available
KS-25[a]	2022	4	F	Purebred	Bloody diarrhea, lethargy	No	CPV-2c	Not available
KS-26	2023	6	M	Hybrid	Lethargy, anorexia, vomiting	Yes	CPV-2c	OR399591
KS-27	2023	5	F	Hybrid	Anorexia, fever, Bloody diarrhea	No	CPV-2c	OR399592
KS-28	2023	3	M	Purebred	Anorexia, fever, Bloody diarrhea	No	CPV-2c	OR399593
KS-29	2023	3.5	F	Hybrid	Anorexia, fever, Bloody diarrhea	No	CPV-2c	OR399594
KS-30[a]	2023	7	M	Purebred	Bloody diarrhea, lethargy	No	CPV-2c	Not available
KS-31	2023	6	F	Hybrid	Lethargy, anorexia, vomiting, watery diarrhea	Yes	CPV-2c	OR399595
KS-32[a]	2023	6	M	Purebred	Lethargy, anorexia, watery diarrhea	Yes	CPV-2c	Not available
KS-33	2023	4	M	Purebred	Bloody diarrhea, lethargy	No	CPV-2c	OR399596

Note: [a]12 sequences did not yield good quality sequences, hence not submitted to NCBI GenBank platform

FUBAR analysis revealed positive selection at two sites (426 and 440) and negative selection at 41 sites, while MEME analysis revealed positive selection at site 324 with a posterior probability of 0.0987. The FEL method detected positive selection at site 0 and negative selection at site 33.

In the present study, the overall detection rate of CPV-2 was 16.5% (33/200) (Table 1). Since its first confirmation in 1983 in China, CPV-2 incidence in clinical animal hospitals has varied between 3.90% and 95.8%, and the mortality rate has varied between 20.17% and 73.47% (Qi et al. 2020). As expected, most dogs suffering from diarrhea were positive for CPV-2. Diarrhea, especially in the blood, is a main clinical finding in CPV-2 infection and is caused by acute gastric hemorrhagic enteritis and bleeding in the gastrointestinal tract (Decaro and Buonavoglia 2012; Qi et al. 2020). CPV-2 detection rates were significantly greater ($p < 0.05$) in dogs that were purebred and aged less than 6 weeks. It has been reported that CPV-2 infections are more prevalent in young puppies and purebred dogs than in older and native or hybrid breeds in China. Higher percentages of CPV-2-positive animals were found in animals ranging from two to four months old in previous studies (Zhao et al. 2016; Qi et al. 2020). The low morbidity in dogs aged < 1 month and more than 4 months is most likely due to acquired

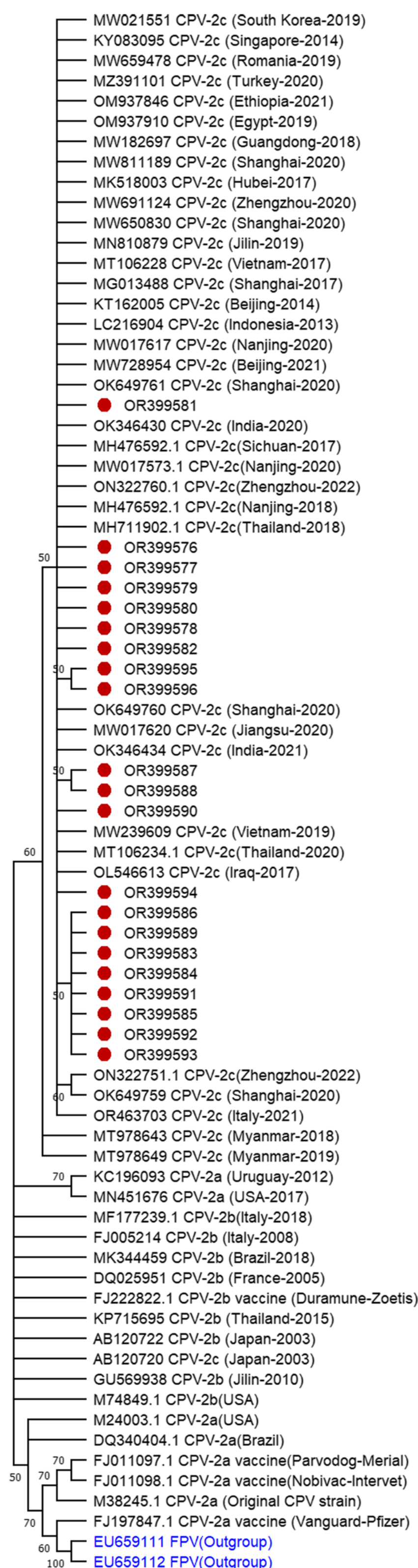

Fig. 1 Phylogenetic analysis of complete *VP2* gene sequences of CPV-2 strains circulating in Kunshan. The evolutionary history was inferred by using the maximum likelihood method and the Tamura–Nei model with 1,000 bootstrap replicates via MEGA 11 software. The present study CPV-2c genotypes are indicated by "red circles" followed by their accession number, genotype, location, and detection year. CPV-2 reference strains representing known genotypes (CPV-2a, CPV-2b and CPV-2c) were retrieved from GenBank and included in the tree

maternal antibodies and the development of adaptive immune responses later in life, respectively. There were more male dogs with CPV-2 infections than female dogs in this study, although these associations were not statistically significant ($p > 0.05$). Several researchers have reported a greater incidence of CPV-2 in male dogs than in female dogs (Zhao et al. 2017; Qi et al. 2020). This difference appears to be related to the dog market in China, as people prefer male dogs over female dogs in China (Zhao et al. 2016; Qi et al. 2020). The second possible reason might be the highly aggressive nature and stress levels of male dogs compared to those of female dogs. Native or mixed breeds have greater resistance to CPV and can better adapt to local climates and environmental changes than purebred or nonnative breeds (Huang et al. 2018; Qi et al. 2020). Interestingly, positive cases were also observed among some vaccinated dogs, which is disturbing and could be linked to vaccine failure.

In this research, all CPV-2c genotypes showed the highest nucleotide identity with domestic CPV-2c genotypes from Shanghai, Nanjing, Henan and Beijing and were clustered into the Asian CPV-2c clade (Fig. 1), suggesting a close phylogenetic relationship. Several reports agree with our findings, which suggest an increasing number of cases of CPV-2c in China in recent years (Wang et al. 2016, 2022; Wu et al. 2018; Hao et al. 2020, 2022; Hu et al. 2020; Qi et al. 2020; Chen et al. 2021; Jiang et al. 2021; Liu et al. 2021; Fu et al. 2022; Jing et al. 2022). Recently, Liu reported a positive detection rate of 40.78% (84/206) and a predominance of CPV-2c in Shanghai in 2020 (Liu et al. 2021). This also indicates that the currently circulating CPV-2c genotype in Kunshan might have been introduced from these regions of China. Second, according to the information of local veterinarians in Kunshan, the number of pet dogs in Kunshan has increased rapidly over the last few years, and these dogs were brought to Kunshan from different regions of China. Based on the latest reports, it can be inferred that CPV-2c has gradually become the predominant genotype in China in recent years.

Genetic analysis inferred that all 21 Kunshan CPV-2 strains belong to the Asian lineage of CPV-2c strains, which contains glutamic acid at site 426 of the VP2

Table 3 Summary table of amino acid substitutions in the VP2 region of CPV-2 detected in this study (2022–2023) compared with reference CPV-2 strains. The gray shaded area shows the locations of the amino acid substitutions

ID number	strains	genotypes	Year	Origin	Amino acid position of VP2																
					4	5	13	87	101	267	297	300	305	324	370	375	426	440	447	555	557
Reference																					
M38245	CPV-b	Original CPV2	1978	USA	**G**	**A**	**P**	**M**	**I**	**F**	**S**	**A**	**D**	**Y**	**Q**	**N**	**N**	**T**	**I**	**V**	**K**
M24003	CPV-15	CPV2a	1984	USA	G	A	P	L	T	F	S	G	Y	Y	Q	D	N	T	I	I	K
DQ340404	BR6-80	CPV2a	1980	Brazil	G	A	P	L	T	F	S	G	Y	Y	Q	D	N	T	I	V	K
M74849	CPV-39	CPV2b	1984	USA	G	A	P	L	T	F	S	G	Y	Y	Q	D	D	T	I	V	K
DQ340411	BR8-90	New CPV2a	1990	Brazil	G	A	P	L	T	F	A	G	Y	Y	Q	D	N	T	I	V	K
MF134808	BJ03/17	New CPV2a	2017	China	G	A	P	L	T	Y	A	G	Y	I	Q	D	N	A	I	V	K
JQ268284	LZ2	New CPV2b	2011	China	G	A	P	L	T	Y	A	G	Y	I	Q	D	D	A	I	V	K
MF177239	288-01	CPV2c	2001	Italy	G	A	P	L	T	F	A	G	Y	Y	Q	D	E	T	I	V	K
MH476592	Canine/China/23	CPV2c	2011	China	G	A	P	L	T	Y	A		Y	I	R	D	E	T	I	V	K
FJ197847.1	Vaccine strain (Vanguard-Pfizer)	CPV2a	2009	Korea	G	A	P	M	I	F	S	A	D	Y	R	E	N	T	I	V	K
FJ011097.1	Vaccine strain (Parvodog-Merial)	CPV2a	2009	China	G	A	P	M	I	F	S	A	D	Y	R	N	N	T	I	V	T
FJ011098.1	Vaccine strain (Nobivac-Intervet)	CPV2a	2009	China	G	A	P	M	I	F	S	A	D	Y	R	N	N	T	I	V	T
FJ222822.1	Vaccine strain (Duramune-Zoetis)	CPV2b	2009	Italy	G	A	P	L	T	F	A	G	Y	Y	R	N	D	T	I	V	E
GU212792.1	Vaccine strain (Quantum-Shering)	CPV2b	2010	Thailand	G	A	P	M	I	F	S	A	D	Y	R	E	D	T	I	V	K
This study																					
OR399576	KS-1	CPV2c	2022	China	G	G	P	L	T	Y	A (GCT)	G	Y	I (ATT)	R	D	E	T	I	V	K
OR399577	KS-3	CPV2c	2022	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399578	KS-5	CPV2c	2022	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399579	KS-6	CPV2c	2022	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399580	KS-7	CPV2c	2022	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399581	KS-8	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399582	KS-11	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	M	V	K
OR399583	KS-12	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	M	V	K
OR399584	KS-13	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399585	KS-14	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399586	KS-15	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399587	KS-16	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399588	KS-17	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	M	V	K
OR399589	KS-18	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399590	KS-21	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399591	KS-26	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399592	KS-27	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399593	KS-28	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399594	KS-29	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399595	KS-31	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K
OR399596	KS-33	CPV2c	2023	China	G	G	P	L	T	Y	A	G	Y	I	R	D	E	T	I	V	K

protein (Table 3). Deduced amino acid sequence analysis revealed several substitutions, including four key mutations (A5G, F267Y, Y324I and Q370R) and one novel mutation (I447M), in some CPV-2 isolates. Amino acid residue 5 is one of the fundamental residues of the neutralizing antigenic site, and any substitution at this site might change the antigenic and immunological behaviors of the virus (Li et al. 2019). The amino acid site 267 has minimal or no effect on the antigenicity of the virus because it is not exposed on the capsid surface (Shackelton et al. 2005). Alexis reported that amino acid positions 324, 426 and 440 appear to be associated with immune evasion via antigenic drift and could impact protective immunity induced by traditional vaccines (Alexis et al. 2021). Csagola reported early cytopathic effects in MDCK cells for CPV-2c genotypes carrying isoleucine at residue 324 compared with CPV-2c carrying tyrosine at residue 324 (Cságola et al. 2014). In 2014, the amino acid substitution Q370R appeared in Chinese isolates (KP749851) and is thought to play an active role in mediating receptor binding together with other neighboring residues. Notably, we observed one novel mutation (I447M) in three CPV-2 isolates (OR399582, OR399583 and OR399588), which was first reported in China and Vietnam. However, the impact of this mutation on the behavior of CPV-2c is still unknown and has yet to be elucidated. Since residue 447 is not exposed on the capsid surface, mutations at this site may not affect the antigenicity of the virus. However, confirmational changes in the virus protein due to this mutation cannot be ignored (Hoang et al. 2019; Fu et al. 2022). Surprisingly, we did not observe evidence of recombination events in CPV-2 lineages in this study, which is in line with previous findings from Brazil (Silva et al. 2017). However, genetic recombination plays a key role in the evolution of parvoviruses and has been reported previously (Pérez et al. 2014; Tang et al. 2022). Moreover, our data revealed that amino acid residues 324, 426 and 440 of VP2 are under strong selection pressure. It has been reported that residue 324 is adjacent to site 323 and could impact virus receptor binding and host range (Alexis et al. 2021; Liu et al. 2021).

Our study has several limitations. First, we could not collect data from other cities in Jiangsu Province; therefore, a clear prevalence and comparison of CPV-2 genotypes in Jiangsu Province cannot be drawn. Second, our findings may not be conclusive for the overall burden of CVP-2 in Kunshan populations and may not represent the overall burden of CPV-2 in this region because we only collected samples from dogs that visited clinics for treatment or vaccination purposes. Finally, due to limited resources, we could not perform an analysis of other pathogens commonly associated with diarrhea and coinfections.

Conclusions

This is the first molecular epidemiology study to provide useful information about the epidemiology and antigenic diversity of CPV-2c lineages in Kunshan. Our findings revealed a predominance of CPV-2c genotypes in Kunshan. The presence of some unique mutations and strong selection pressure on some amino acid residues in the VP2 protein could enhance immune evasion and pathogenesis in these new CPV-2c lineages. This pattern of genetic variation warrants further systematic epidemiological studies in China to monitor and understand the antigenic diversity and behavior of new CPV-2 variants and lineages.

Methods

Specimen collection

During the canine disease surveillance program, rectal swabs ($n = 200$) were collected from dogs suffering from clinical diseases at different veterinary clinics in Kunshan city from April 2022 to April 2023. In addition, we collected fecal samples from dogs with watery or bloody diarrhea. The samples were suspended in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, San Jose, CA, USA). Clinical staff recorded data about breed, age, sex, clinical symptoms, residence and vaccination status of all the sampled dogs. Ethical approval from the Institutional Animal Care and Use Committee (IACUC) was not needed for this study as long as the animals investigated in this study were not identifiable in the retrospective records.

DNA extraction and CPV-2 screening

We performed total DNA extraction from the specimens using commercially available kits for viral DNA extraction (TaKaRa minibest, Cat#9766, Takara Biotechnology, Dalian, China). Preliminary screening of CPV-2 in the samples was performed using CPV-F (CAGGTGATG AATTTGCTACA) and CPV-R (CATTTGGATA AAC TGGTGGT) as described previously (Guo et al. 2013). PCR amplification was performed by using a LifeEco Bioer Thermal Cycler (LifeEco TC 96, Bioer, Hangzhou, China) and 2×rapid Taq master mix (Vazyme, Nanjing, China). PCR amplicons (611 bp) were analyzed using a GenoSens 1880 gel imaging analysis system (GenoSens 1880, Clinx, Shanghai, China). CPV-2-positive specimens were also tested for CCoV and CDV coinfections by using protocols described previously (Frisk et al. 1999; Xiu et al. 2020).

VP2 Gene amplification and Sequencing

A pair of primers was designed to amplify the nearly full length of *VP2* gene (1755 bp) as described previously (Hoang et al. 2019). All PCR runs included a negative template control (nuclease-free water) and a corresponding vaccine positive control sample. PCR was performed with 2×Rapid Taq Master Mix (Vazyme, Nanjing, China). The positive PCR products were sequenced by the commercial sequencing company GeneWiz (GeneWiz, Suzhou, China) by using the BigDye Terminator 3.1 Kit and an ABI-PRISM 3730XL DNA sequencer (Applied Biosystems).

Sequence analysis

For phylogenetic analysis, we edited and aligned the nucleotide sequences using the ClustalW alignment tool in BioEdit Software (Ibis Biosciences, Carlsbad, CA, USA). To understand the genetic diversity of the 21 CPV-2 isolates in this study, 56 reference sequences of the VP2 gene were retrieved from the National Center for Biotechnology Information (NCBI) nucleotide database (http://www.ncbi.nlm.nih.gov). In addition, we compared CPV-2 strains from Kunshan with those of five of the most commonly used vaccines, Vanguard-Pfizer (FJ197847.1), Parvodog-Merial (FJ011097.1), Nobivac-Intervet (FJ011098.1), Duramune-Zoetis (FJ222822.1), and Quantum-Shering (GU212792.1). Deduced amino acid analysis was performed to identify the amino acid change at residue 426 in the CPV-2 variants.

Recombination analysis and estimation of selection pressure on the VP2 gene

The VP2 gene sequences were aligned and subsequently screened for potential recombination events by using the recombination detection program 4 (RDP4) package v. 4.101 software, which contains various recombination detection methods (RDP, GENECONV, Bootscan, MaxChi, Chimera, SiScan and 3Seq). The highest acceptable P value was set at 0.05 (Tang et al. 2022). Selection pressure on the VP2 protein in CPV-2 was estimated by using different methods, such as single-likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), fast unconstrained Bayesian approximation (FUBAR) and mixed effects model of evolution (MEME), which are available on the Datamonkey web server (http://www.datamonkey.org). For the evaluation of selective pressures at individual sites of codon alignment, the synonymous (dS) and nonsynonymous (dN) substitution rates were estimated, where a dN/dS ratio < 1 indicates negative selection and a dN/dS ratio > 1 represents positive selection (Liu et al. 2021).

The abbreviations used are canine parvovirus (CPV), canine distemper virus (CDV), canine coronavirus (CCoV), parvoviruses, canine kobuviruses (CKoV), canine bocavirus (CBoV), feline panleukopenia virus

(FPV), open reading frame (ORF), and polymerase chain reaction (PCR).

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Authors' contributions
S.U., D.G., S.K., Y.C., and Z.F. collected the samples and conducted the laboratory and statistical analysis. S.U. designed the study, guided the sampling plan, and supported manuscript development. QZ and WY collected specimens and clinical data from dogs at veterinary clinics. B. A guided this study and supported manuscript development and editing.

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Availability of data and materials
The data used to support the findings of this study are included within the article.

Declarations

Ethics approval and consent to participate
Ethical approval was not needed for this study.

Consent for publication
Not applicable.

Competing interests
The authors declare that there are no conflicts of interest.

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References

Alexis, V.A., V. Sonia, Daniela, S., Miguel, G., Timothy, H., Valentina, F., Lisette, L., Leonardo, S. 2021. Molecular analysis of full-lengt h VP2 of canine parvovirus reveals antigenic drift in CPV-2b and CPV-2c variants in Central Chile. *Animals* 11(8): 2387. https://doi.org/10.3390/ani11082387.

Castillo, C., V. Neira, P. Aniñir, S. Grecco, R. Pérez, Y. Panzera, N.A. Zegpi, A. Sandoval, D. Sandoval, S. Cofre, and R. Ortega. 2020. First molecular identification of canine parvovirus type 2 (CPV2) in Chile reveals high occurrence of CPV2c antigenic variant. *Frontiers in Veterinary Science* 7: 194. https://doi.org/10.3389/fvets.2020.00194.

Chen, B., X. Zhang, Zhu, J., Liao, L., Bao, E. 2021. Molecular epidemiological survey of canine parvovirus circulating in China from 20 14 to 2019. *Pathogens (Basel, Switzerland)* 10(5): 588. https://doi.org/10.3390/pathogens10050588.

Cotmore, S.F., M. Agbandje-McKenna, M. Canuti, J.A. Chiorini, A.M. Eis-Hubinger, J. Hughes, M. Mietzsch, S. Modha, M. Ogliastro, J.J. Pénzes, et al. 2019. ICTV virus taxonomy profile: parvoviridae. *The Journal of General Virology* 100: 367–368. https://doi.org/10.1099/jgv.0.001212.

Cságola, A., S. Varga, M. Lőrincz, and T. Tuboly. 2014. Analysis of the full-length VP2 protein of canine parvoviruses circulating in Hungary. *Archives of Virology* 159: 2441–2444. https://doi.org/10.1007/s00705-014-2068-5.

Decaro, N., and C. Buonavoglia. 2012. Canine parvovirus–a review of epidemiological and diagnostic aspects, with emphasis on type 2c. *Veterinary Microbiology* 155: 1–12. https://doi.org/10.1016/j.vetmic.2011.09.007.

Decaro, N., C. Buonavoglia, and V.R. Barrs. 2020. Canine parvovirus vaccination and immunization failures: are we far from disease eradication? *Veterinary Microbiology* 247: 108760. https://doi.org/10.1016/j.vetmic.2020.108760.

Franzo, G., F. Mira, Schirò, G., Canuti, M. 2023. Not asian anymore: reconstruction of the history, evolution, and dispersal of the "Asia n" lineage of CPV-2c. *Viruses* 15(9): 1962. https://doi.org/10.3390/v15091962.

Frisk, A.L., M. König, A. Moritz, and W. Baumgärtner. 1999. Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. *Journal of Clinical Microbiology* 37: 3634–3643. https://doi.org/10.1128/JCM.37.11.3634-3643.1999.

Fu, P., D. He, X. Cheng, X. Niu, C. Wang, Y. Fu, K. Li, H. Zhu, W. Lu, J. Wang, and B. Chu. 2022. Prevalence and characteristics of canine parvovirus type 2 in Henan Province China. *Microbiology Spectrum* 10: e0185622. https://doi.org/10.1128/spectrum.01856-22.

Guo, L., S.L. Yang, S.J. Chen, Z. Zhang, C. Wang, R. Hou, Y. Ren, X. Wen, S. Cao, W. Guo, Z. Hao, Z. Quan, M. Zhang, and Q.G. Yan. 2013. Identification of canine parvovirus with the Q370R point mutation in the VP2 gene from a giant panda (Ailuropoda melanoleuca). *Virol J* 10: 163. https://doi.org/10.1186/1743-422X-10-163.

Hao, X., Y. He, C. Wang, W. Xiao, R. Liu, X. Xiao, P. Zhou, and S. Li. 2020. The increasing prevalence of CPV-2c in domestic dogs in China. *PeerJ* 8: e9869. https://doi.org/10.7717/peerj.9869.

Hao, X., Y Li., Xiao, X., Chen, B., Zhou, P., Li, S. 2022. The changes in canine parvovirus variants over the years. *International Journal of Molecular Sciences* 23(19): 11540. https://doi.org/10.3390/ijms231911540.

Hoang, M., W.H. Lin, V.P. Le, B.T.T. Nga, M.T. Chiou, and C.N. Lin. 2019. Molecular epidemiology of canine parvovirus type 2 in Vietnam from November 2016 to February 2018. *Virol J* 16: 52. https://doi.org/10.1186/s12985-019-1159-z.

Hoelzer, K., L.A. Shackelton, C.R. Parrish, and E.C. Holmes. 2008. Phylogenetic analysis reveals the emergence, evolution and dispersal of carnivore parvoviruses. *The Journal of General Virology* 89: 2280–2289. https://doi.org/10.1099/vir.0.2008/002055-0.

Hu, W., X. Xu, Q. Liu, J. Ji, Y. Kan, L. Yao, Y. Bi, and Q. Xie. 2020. Molecular characterization and genetic diversity of canine parvovirus type 2 prevalent in Central China. *Journal of Veterinary Research* 64: 347–354. https://doi.org/10.2478/jvetres-2020-0056.

Huang, X.H., P. W, L.K. Liu, L. Ma, X.F. Fang, and B. Zhao. 2018. Epidemiological investigation of canine parvovirus disease in Nanning City. *Journal of Animal Husbandry and Veterinary Medicine* 34: 76–79.

Huang, S., X. Li, W. Xie, L. Guo, D. You, H. Xu, D. Liu, Y. Wang, Z. Hou, X. Zeng, S. Yang, H. Chai, and Y. Wang. 2022. Molecular detection of parvovirus in captive siberian tigers and lions in northeastern China from 2019 to 2021. *Frontiers in Microbiology* 13: 898184. https://doi.org/10.3389/fmicb.2022.898184.

Jiang, H., Y. Yu, R. Yang, S. Zhang, D. Wang, Y. Jiang, W. Yang, H. Huang, C. Shi, L. Ye, G. Yang, J. Wang, and C. Wang. 2021. Detection and molecular epidemiology of canine parvovirus type 2 (CPV-2) circulating in Jilin Province, Northeast China. *Comparative Immunology, Microbiology and Infectious Diseases* 74: 101602. https://doi.org/10.1016/j.cimid.2020.101602.

Jing, Z., P. Ji, Y. Wei, F. Hao, and Y. Wei. 2022. Isolation and identification of a novel canine parvovirus type 2c strain in domestic cats in Dalian China. *Frontiers in Veterinary Science* 9: 1001604. https://doi.org/10.3389/fvets.2022.1001604.

Li, C., J. Tang, Z. Chen, G. Niu, and G. Liu. 2019. A divergent canine parvovirus type 2c (CPV-2c) isolate circulating in China. *Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases* 73:242–247. https://doi.org/10.1016/j.meegid.2019.05.004.

Lina, Z., W. Kai, A. Fuyu, Z. Dongliang, Z. Hailing, X. Xuelin, G. Ce, Y. Hongmei, K. Yingjie, Z. Zhidong, L. Rongguang, and H. Yan. 2022. Fatal canine parvovirus type 2a and 2c infections in wild Chinese pangolins (Manis pentadactyla) in southern China. *Transboundary and Emerging Diseases* 69: 4002–4008. https://doi.org/10.1111/tbed.14703.

Liu, C., J. Gao, Li, H., Sun, F., Liang, H., Liu, H., Yi, J. 2021. Phylogenetic characteristics of canine parvovirus type 2c variant endemic in Shanghai, China. *Viruses* 13(11): 2257. https://doi.org/10.3390/v13112257.

Parrish, C.R., C.F. Aquadro, M.L. Strassheim, J.F. Evermann, J.Y. Sgro, and H.O. Mohammed. 1991. Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. *Journal of Virology* 65: 6544–6552. https://doi.org/10.1128/JVI.65.12.6544-6552.1991.

Pérez, R., L. Calleros, A. Marandino, N. Sarute, G. Iraola, S. Grecco, H. Blanc, M. Vignuzzi, O. Isakov, N. Shomron, et al. 2014. Phylogenetic and genome-wide deep-sequencing analyses of canine parvovirus reveal coinfection with field variants and emergence of a recent recombinant strain. *PLoS ONE* 9: e111779. https://doi.org/10.1371/journal.pone.0111779.

Qi, S., J. Zhao, D. Guo, and D. Sun. 2020. A mini-review on the epidemiology of canine parvovirus in China. *Frontiers in Veterinary Science* 7: 5. https://doi.org/10.3389/fvets.2020.00005.
Shackelton, L.A., C.R. Parrish, U. Truyen, and E.C. Holmes. 2005. High rate of viral evolution associated with the emergence of carnivore parvovirus. *Proc Natl Acad Sci U S A* 102: 379–384. https://doi.org/10.1073/pnas.0406765102.
Shao, R., C. Ye, Y. Zhang, X. Sun, J. Cheng, F. Zheng, S. Cai, J. Ji, Z. Ren, L. Zhong, Z. Lai, J. Ou, G. Lu, and S. Li. 2021. Novel parvovirus in cats China. *Virus Research* 304: 198529. https://doi.org/10.1016/j.virusres.2021.198529.
Silva, S.P., L. Silva, Rodrigues, E.D.L., Cardoso, J.F., Tavares, F.N., Souza, W.M., Santos, C.M.P., Martins, F.M.S., Jesus, I.S., Brito, T. C., Moura, T.P.C., Nunes, M.R.T., Casseb, L.M.N., Silva Filho, E., Casseb, A.R. 2017. Full-length genomic and molecular characterization of Canine parvovirus in dogs from North of Brazil. *Genetics and Molecular Research: GMR* 16(3): 1–8. https://doi.org/10.4238/gmr16039719.
Tang, Y., N. Tang, J. Zhu, M. Wang, Y. Liu, and Y. Lyu. 2022. Molecular characteristics and genetic evolutionary analyses of circulating parvoviruses derived from cats in Beijing. *BMC Veterinary Research* 18:195. https://doi.org/10.1186/s12917-022-03281-w.
Wang, J., P. Lin, H. Zhao, Y. Cheng, Z. Jiang, H. Zhu, H. Wu, and S. Cheng. 2016. Continuing evolution of canine parvovirus in China: isolation of novel variants with an Ala5Gly mutation in the VP2 protein. *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases* 38: 73–78. https://doi.org/10.1016/j.meegid.2015.12.009.
Wang, Z., Y. Jiang, T. Xin, W. Yuan, X. Guo, H. Zhou, H. Zhu, and H. Jia. 2022. Genetic characterization and evolutionary analysis of canine parvovirus in Tangshan, China. *Archives of Virology* 167: 2263–2269. https://doi.org/10.1007/s00705-022-05502-x.
Wu, H., X. Li, L. Wang, Y. Liu, and K. Tian. 2018. Molecular epidemiological survey of canine parvovirus in domestic dogs in four provinces, China. *Virusdisease* 29: 113–117. https://doi.org/10.1007/s13337-018-0427-7.
Xiu, L., R.A. Binder, N.A. Alarja, K. Kochek, K.K. Coleman, S.T. Than, E.S. Bailey, V.N. Bui, T.H. Toh, D.D. Erdman, and G.C. Gray. 2020. A RT-PCR assay for the detection of coronaviruses from four genera. *Journal of Clinical Virology* 128: 104391. https://doi.org/10.1016/j.jcv.2020.104391.
Zhao, Y., Y. Lin, X. Zeng, C. Lu, and J. Hou. 2013. Genotyping and pathobiologic characterization of canine parvovirus circulating in Nanjing China. *Virology Journal* 10: 272. https://doi.org/10.1186/1743-422X-10-272.
Zhao, Z., H. Liu, K. Ding, C. Peng, Q. Xue, Z. Yu, and Y. Xue. 2016. Occurrence of canine parvovirus in dogs from Henan province of China in 2009–2014. *BMC Veterinary Research* 12: 138. https://doi.org/10.1186/s12917-016-0753-1.
Zhao, H., J. Wang, Y. Jiang, Y. Cheng, P. Lin, H. Zhu, G. Han, L. Yi, S. Zhang, L. Guo, and S. Cheng. 2017. Typing of canine parvovirus strains circulating in north-East China. *Transboundary and Emerging Diseases* 64: 495–503. https://doi.org/10.1111/tbed.12390.
Zhong, Z., L. Liang, Zhao, J., Xu, X., Cao, X., Liu, X., Zhou, Z., Ren, Z., Shen, L., Geng, Y., Gu, X., Peng, G. 2014. First isolation of ne w canine parvovirus 2a from Tibetan mastiff and global analysis of the full-length VP2 gene of canine parvoviruses 2 in China. *International Journal of Molecular Sciences* 15(7): 12166–12187. https://doi.org/10.3390/ijms150712166.
Zhou, L., Q. Tang, L. Shi, M. Kong, L. Liang, Q. Mao, B. Bu, L. Yao, K. Zhao, S. Cui, and É. Leal. 2016. Full-length genomic characterization and molecular evolution of canine parvovirus in China. *Virus Genes* 52: 411–416. https://doi.org/10.1007/s11262-016-1309-y.

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