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J Vet Res 64, 477-486, 2020
DOI:10.2478/jvetres-2020-0076

Genetic characterisation and local genotypes of canine parvovirus strains collected from pet dogs in central and eastern China during 2018–2019

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Received: June 11, 2020 *Accepted: October 26, 2020*

Abstract

Introduction: Canine parvovirus type-2 (CPV-2) causes acute infectious diseases in puppies, which show high morbidity and mortality. Better effect of vaccination against these diseases could be achieved with deeper knowledge of CPV-2 genotype dissemination and mutation history. This study investigated CPV-2–positive samples collected recently over a wide region of China. **Material and Methods:** A total of 118 faecal samples from dogs identified as CPV-positive were collected from veterinary clinics in central and eastern China. Overall, 16 strains collected from Anhui, 29 from Henan, and 16 from Zhejiang Province were sequenced to determine the genotypic composition of CPV-2 and mutational complexity of CPV-VP2. **Results:** The CPV-2a, CPV-2b, and CPV-2c genotypes were detected in Anhui and Henan Provinces, while CPV-2c alone was detected in Zhejiang Province. Sequence analysis of all strains showed 98.5%–99.8%, 98.3%–99.9%, and 98.7%–99.8% identity among the 16 Anhui, 29 Henan, and 16 Zhejiang strains, respectively. Strains collected from Anhui and Henan Provinces showed lower identity (97.0%), suggesting greater genetic divergence in central China. The mutation rates of Henan and Anhui strains were lower than that of Zhejiang strains. Major amino acid mutations occurred at sites 5, 370, 426, and 440. Epitope and entropy analyses implied these sites' likely conformance to the principles of mutation tendency, complexity, and diversity. **Conclusion:** The findings for the evolutionary structure of CPV-2 strains collected from three provinces in central and eastern China advance trend monitoring of the genetic variation in canine parvovirus and point to its implications in the development of novel vaccines.

Keywords: amino acid mutation site, canine parvovirus, phylogenetic tree, VP2 identity.

Introduction

Canine parvovirus (CPV) emerged in the late 1970s as a host-range variant of feline panleukopenia virus (FPV) (21). CPV belongs to the *Parvoviridae* family and is a nonenveloped virus with a single-stranded DNA genome (5,000 nt) comprising two open reading frames (ORFs). The first ORF encodes two nonstructural proteins, NS1 and NS2; the second ORF encodes two structural proteins, VP1 and VP2 (31). Since its discovery, this virus has been detected worldwide, primarily as CPV type-2 (CPV-2), and has become one of the most serious infectious pathogens among dogs. CPV-2 is among the most common aetiological agents of severe gastroenteritis, particularly in 6–20-week-old puppies, unvaccinated puppies, or puppies with poor maternal protection through passive immunity, and replicates mainly in intestinal crypts and the lymphoid organs but may reach any organ in susceptible animals. The most characteristic signs of this illness are diarrhoea, emesis, anorexia, depression, pyrexia, or hypothermia (5, 7, 32).

Currently, only CPV-2 has been thoroughly investigated. The VP2 capsid protein, accounting for 90% of the CPV nucleocapsid and 426 amino acids in size, plays a crucial role in the structure of this virus and serves as the main protective antigen - two characteristics which are the classification criteria for

CPV viral typing. Owing to variations in the antigenicity of VP2 across strains, multiple genotypes have emerged, including CPV-2a, CPV-2b, and CPV-2c. CPV-2a, CPV-2b, and CPV-2c differ at the 426th amino acid (Asn in 2a, Asp in 2b, and Glu in 2c) of the parvovirus VP2 protein (11, 17, 18). In general, a novel CPV-2 variant replaces the old variants rapidly (11, 15). In recent decades, CPV-2c has become widespread in European countries (6, 11), the United States (11, 12), South America (2, 11, 19, 20), and Africa (1, 11, 23). In Asia, CPV-2c was first reported from Vietnam in 2004 (11, 16); however, since then, this strain has not been prevalent in Asia (4, 11). Surprisingly, in the past few years, novel CPV-2c isolates from Asia have been identified in mainland China (8, 11, 25, 28), Taiwan (4, 11, 14), Laos (11, 24), and Thailand (3, 11). Therefore, in the present study, VP2 sequence analysis of strains collected from central and eastern China was performed to determine the mutation tendency of CPV-2 in infected dogs sampled from October 2018 to April 2019.

Material and Methods

Sample collection and DNA extraction. During the major CPV-2 infection seasons lasting from October 2018 to April 2019, 118 samples of canine faeces from pets confirmed to be CPV-positive using a colloidal gold strip test (Immunochromatographic Rapid Test Kit, BioNote, Hwaseong-si, Korea), were collected from pet hospitals in Henan, Anhui, and Zhejiang Provinces. Detailed clinical information was recorded for each sampled dog. Each faecal sample was suspended in phosphate-buffered saline, and virus isolation from feline kidney (F81) cells was performed as described previously (27). The virus and F81 cells were incubated until cytopathic effects were apparent. The buffer solution containing the virus was centrifuged, and then 500 μL of the supernatant was inoculated in a cell culture flask of F81 and cultured in cell culture tank at 37°C for 2 h. After that, all the liquid in the culture flask was poured out and replaced by 5mL cell culture medium, and the culture was continued for 3 days to collect the virus until the 5th passage, and the virus infection of F81 cells was detected. In the whole process of cell inoculation, on average 3 passages were completed. DNA was extracted from the infected faeces or F81 cells using the EasyPure Viral DNA/RNA Kit (TransGen Biotechnology, Inc., Beijing, China) according to the manufacturer's instructions. The extracted DNA samples were stored at −80°C.

Cloning of the VP2-coding sequence of CPV. The complete VP2 sequences of CPV-positive samples were amplified from the extracted viral DNA via PCR using a high-fidelity enzyme (TransGen Biotechnology, Inc.) and the primer pair of CPV-F (5′-AGAGACAAT CTTGCACCAAT-3′) and CPV-R (5′-ATGTTAATA TAATTTTCTAGGTGCT-3′) (from nucleotides 2761–4536 of the sequence deposited at GenBank under accession number MF805797) was designed using Primer Premier 5.0 software (Premier Biosoft, San Francisco, CA, USA). Each amplicon was then analysed by electrophoresis, and the amplified VP2 sequences were cloned and sequenced. The vaccination status of each dog from which the samples was noted from records, as was the specific vaccine strain administered. That was the Vanguard Plus 5 classical CPV-2 commercial vaccine strain (Pfizer Inc., Lincoln, NE, USA).

Characterisation and genotyping of CPV-2. Sequences were assembled by overlapping and analysed using the SeqMan module of Lasergene 7 (DNASTAR, Inc., Madison, WI, USA). The NCBI nucleotide database was searched for typical VP2 gene sequences to identify mutations in CPV-VP2 sequences in various regions of China in recent years. The sequences obtained were compared using the NCBI nucleotide database to analyse the genotypes.

Mutation and phylogenetic analyses of CPV-2. The VP2 sequences of the investigated strains were compared with those of reference strains using Clustal X (22) to analyse identity and amino acid sequence variation. The CPV-VP2 sequences tested in this study and reference sequences in GenBank were aligned using MEGA 7.0 (13), and phylogenetic trees were constructed to analyse relationships between the strains tested in this study and reference strains. The details of the reference sequences incorporated in the phylogenetic tree are shown in Table 1. Differences in nucleotide and amino acid sequences between the collected and reference strains from China and overseas were analysed using MegAlign (DNASTAR, Inc.). EvolView 2 (10) was adopted for visualising and annotating phylogenetic trees with geographic location and phylogenetic clustering. Sequence identity between the collected and reference strains was determined using Lasergene 7 to examine genotypic variations across different regions of the study area.

Epitope and selection pressure analyses of CPV-2. Antigenic epitopes of the CPV-2c VP2 protein were predicted using Lasergene 7. Selection pressure on CPV based on entropy was analysed using VP2 sequences of the 61 collected strains using BioEdit (9).

Results

Virus identification and genotypes. All collected samples tested positive for CPV on the basis of virus isolation and PCR assays. The complete sequence (1755 nt) of the VP2 structural protein of 61 strains was obtained from 118 strains tested and deposited in GenBank (where strains had 100% sequence identity and were from the same site, only one was retained for analysis). Accession numbers and clinical information for each strain are summarised in Table 2. Sequence analysis of strains collected from the three provinces showed that the infection rates of CPV-2a, CPV-2b, and CPV-2c were 3/29, 4/29 and 22/29, respectively, in Henan Province and 2/16, 3/16, and 11/16, respectively, in Anhui Province; all CPV genotypes collected from

Zhejiang Province were CPV-2c. The vaccination rate was 50% in Anhui Province, 58.62% in Henan Province, and 87.5% in Zhejiang Province. From the above distribution data, CPV-2c is evidently the predominant CPV genotype in central and eastern China. Genotypes and geographical distribution of the 61 strains are shown in Fig. 1.

Nucleotide identity of the VP2 gene. Comparison of the nucleotide acid sequences of the VP2 gene between the 61 strains obtained in this study and reference strains in GenBank revealed 98.3%–99.99% identity. The nucleotide identity was 98.5%–99.8% for the 16 strains collected from Anhui Province, 98.3%–99.8% for the 29 strains from Henan Province, and 98.7%–99.8% for the 16 Zhejiang Province strains. Between provinces, the identity was 97.0%–99.9% comparing strains collected in Anhui and Henan Provinces, 97.3%–99.8% for strains collected in Henan and Zhejiang Provinces, and 97.6%–99.8% when Anhui and Zhejiang Province strains were examined.

Table 1. Reference strains used in the study

Strain	Accession number	Genotype	Place of isolation	Year of isolation
nn171025	MK332005	CPV-2a	Guangxi	2017
nn17101	MK332003	CPV-2a	Guangxi	2017
nn1693	MK332002	CPV-2b	Guangxi	2016
nn1681	MK331996	CPV-2b	Guangxi	2016
nn171105	MK332007	CPV-2c	Guangxi	2017
nn171024	MK332004	CPV-2c	Guangxi	2017
CPV-411b.us.9	EU659121	CPV-2b	The USA	1998
CPV-13.us.81	EU659118	CPV-2a	The USA	1981
CPV-6.us.80	EU659117	CPV-2	The USA	1980
Raccoon/ WI/ 37/ 10	JN867618	CPV-2a	The USA	2010
110/ 07-27	FJ005236	CPV-2c	The USA	2007
08-B	GU362934	CPV-2a	Italy	2008
260-00	MF177231	CPV-2a	Italy	2000
140/ 05	FJ005265	CPV-2b	Italy	2005
CPV /IZSSI /25835/ 09	KU508407	CPV-2c	Italy	2009
56/00	FJ222821	CPV-2c	Italy	2000
CPV/dog/HCM/20/2013	LC216910	CPV-2c	Indonesia	2013
Pome	EF599098	CPV-2c(a)	South Korea	2007
DH326	EF599097	CPV-2b	South Korea	2007
DH426	EF599096	CPV-2a	South Korea	2007
16M130	MH643886	CPV-2	South Korea	2016
2670/CPV-2c/2010/Ind	KX425920	CPV-2c	India	2010
CU267	MH711901	CPV-2c	Thailand	2017
TH011401	KT364589	CPV-2c	Thailand	2014
T37	CPU72698	CPV-2a	Taiwan	1996
T10	CPU72696	CPV-2b	Taiwan	1996
2017090801	MH127909	CPV-2c	Taiwan	2017
Protein (VP2)	KU244254	CPV-2c	Taiwan	2015
PV/PL/HeN02/08	EU441280	CPV-2a	Henan	2008
Henan42	KJ438805	CPV-2a	Henan	2013
CPV-HN1617	MF467229	CPV-2c	Henan	2016
CPV-zj18	KM386948	CPV-2b	Zhejiang	2014
CPV-zj7	KM386937	CPV-2a	Zhejiang	2014
Beijing	HQ883267	CPV-2a	Beijing	2010
BJ-1	MN101726	CPV-2a	Beijing	2018
2011-BJ-B43	KF803527	CPV-2b	Beijing	2011
2011-BJ-B6	KF803606	CPV-2b	Beijing	2011
CPV-SH1516	MG013488	CPV-2c	Shanghai	2017
Shanghai/04g/2016	KY937646	CPV-2a	Shanghai	2016
ShangHai/3-2/2016	KY937640	CPV-2a	Shanghai	2016
Shanghai/03g/2016	KY937637	CPV-2c	Shanghai	2016
CPVpf/2007(vaccine)	FJ197847	CPV-2	South Korea	2007
29/97(vaccine)	FJ222823	CPV-2b	N.I.[a]	2008
CPV-GX1581	MF467242	CPV-2c	Guangxi	2015

[a] no information

Table 2. Clinical information on sources of Chinese canine parvovirus type 2 (CPV-2) obtained in this study

Strain	Genotype	Site	Date of sampling	Age	Breed	Sex	Vaccinated status	Accession number
CH-AH-D1	CPV-2b	Hefei	Oct. 7, 2018	5 months	Poodle	Female	Unvaccinated	MN119560
CH-AH-D2	CPV-2c	Anqing	Oct. 7, 2018	2 years	Poodle	Male	1 dose	MN119561
CH-AH-D3	CPV-2c	Suzhou	Oct. 8, 2018	1 year	Mixed	Female	2 doses	MN119562
CH-AH-D4	CPV-2c	Suzhou	Oct. 11, 2018	3 months	Poodle	Male	Unvaccinated	MN119563
CH-AH-D5	CPV-2b	Wuhu	Nov. 9, 2018	1 year	Mixed	Female	2 doses	MN119564
CH-AH-D6	CPV-2c	Anqing	Nov. 11, 2018	4 months	Poodle	Female	1 dose	MN119565
CH-AH-D7	CPV-2c	Suzhou	Nov. 13, 2018	45 days	Schnauzer	Male	Unvaccinated	MN119566
CH-AH-D8	CPV-2c	Hefei	Nov. 30, 2018	7 months	Mixed	Male	1 dose	MN119567
CH-AH-D9	CPV-2c	Wuhu	Dec. 1, 2018	6 months	Retriever	Male	Unvaccinated	MN119568
CH-AH-D10	CPV-2c	Suzhou	Dec. 3, 2018	3 months	Schnauzer	Female	Unvaccinated	MN119569
CH-AH-D11	CPV-2c	Anqing	Jan. 7, 2019	8 months	Mixed	Male	2 doses	MN119570
CH-AH-D12	CPV-2c	Suzhou	Feb. 7, 2019	4 months	Mixed	Female	Unvaccinated	MN119571
CH-AH-D13	CPV-2c	Wuhu	Mar. 11, 2019	2 months	Poodle	Female	1 dose	MN119572
CH-AH-D14	CPV-2a	Hefei	Mar. 16, 2019	52 days	Mixed	Female	Unvaccinated	MN119573
CH-AH-D15	CPV-2a	Anqing	Apr. 2, 2019	4 months	Retriever	Male	1 dose	MN119574
CH-AH-D16	CPV-2b	Suzhou	Apr. 3, 2019	3 months	Poodle	Female	Unvaccinated	MN119575
CH-HN-D1	CPV-2c	Zhengzhou	Oct. 3, 2018	40 days	Mixed	Male	Unvaccinated	MN119576
CH-HN-D2	CPV-2c	Hebi	Oct. 4, 2018	4 months	Mixed	Female	1 dose	MN119577
CH-HN-D3	CPV-2c	Nanyang	Oct. 8, 2018	10 months	Mixed	Male	1 dose	MN119578
CH-HN-D4	CPV-2c	Nanyang	Nov. 6, 2018	5 months	Retriever	Female	1 dose	MN119579
CH-HN-D5	CPV-2b	Luoyang	Nov. 7, 2018	4 months	Mixed	Male	Unvaccinated	MN119580
CH-HN-D6	CPV-2c	Xinxiang	Nov. 12, 2018	5 months	Schnauzer	Male	Unvaccinated	MN119581
CH-HN-D7	CPV-2a	Xinyang	Nov. 14, 2018	4 months	Mixed	Female	1 dose	MN119582
CH-HN-D8	CPV-2c	Luoyang	Nov. 16, 2018	2 months	Mixed	Female	1 dose	MN119583
CH-HN-D9	CPV-2c	Zhengzhou	Nov. 28, 2018	2 months	Schnauzer	Male	Unvaccinated	MN119584
CH-HN-D10	CPV-2c	Anyang	Nov. 29, 2018	4 months	Mixed	Female	1 dose	MN119585
CH-HN-D11	CPV-2c	Xinyang	Dec. 3, 2018	50 days	Mixed	Female	1 dose	MN119586
CH-HN-D12	CPV-2c	Nanyang	Jan. 1, 2019	3 months	Retriever	Male	Unvaccinated	MN119587
CH-HN-D13	CPV-2c	Shangqiu	Jan. 3, 2019	3 months	Mixed	Female	1 dose	MN119588
CH-HN-D14	CPV-2a	Anyang	Jan. 4, 2019	4 months	Mixed	Female	1 dose	MN119589
CH-HN-D15	CPV-2a	Zhengzhou	Jan. 5, 2019	1 month	Mixed	Male	Unvaccinated	MN119590
CH-HN-D16	CPV-2b	Anyang	Jan. 5, 2019	2 months	Poodle	Male	Unvaccinated	MN119591
CH-HN-D17	CPV-2c	Zhengzhou	Feb. 3, 2019	1 year	Mixed	Female	2 doses	MN119592
CH-HN-D18	CPV-2b	Nanyang	Feb. 4, 2019	4 months	Poodle	Male	Unvaccinated	MN119593
CH-HN-D19	CPV-2c	Xinxiang	Feb. 5, 2019	5 months	Mixed	Female	1 dose	MN119594
CH-HN-D20	CPV-2b	Hebi	Mar. 8, 2019	2 months	Poodle	Female	Unvaccinated	MN119595
CH-HN-D21	CPV-2c	Xinxiang	Mar. 9, 2019	5 months	Mixed	Male	1 dose	MN119596
CH-HN-D22	CPV-2c	Nanyang	Mar. 10, 2019	8 months	Retriever	Male	2 doses	MN119597
CH-HN-D23	CPV-2c	Hebi	Mar. 12, 2019	3 months	Mixed	Male	1 dose	MN119598
CH-HN-D24	CPV-2c	Luoyang	Mar. 15, 2019	2 months	Mixed	Female	1 dose	MN119599
CH-HN-D25	CPV-2c	Anyang	Mar. 29, 2019	2 months	Schnauzer	Female	Unvaccinated	MN119600
CH-HN-D26	CPV-2c	Zhengzhou	Mar. 31, 2019	4 months	Mixed	Male	Unvaccinated	MN119601
CH-HN-D27	CPV-2c	Luoyang	Apr. 1, 2019	1 year	Mixed	Female	1 dose	MN119602
CH-HN-D28	CPV-2c	Nanyang	Apr. 4, 2019	9 months	Mixed	Female	1 dose	MN119603
CH-HN-D29	CPV-2c	Zhengzhou	Apr. 5, 2019	4 months	Poodle	Male	Unvaccinated	MN119604
CH-ZJ-D1	CPV-2c	Huzhou	Oct. 2, 2018	1 month	Mixed	Female	1 dose	MN119605
CH-ZJ-D2	CPV-2c	Hangzhou	Oct. 3, 2018	3 months	Mixed	Male	Unvaccinated	MN119606
CH-ZJ-D3	CPV-2c	Jinhua	Oct. 4, 2018	2 months	Mixed	Female	1 dose	MN119607
CH-ZJ-D4	CPV-2c	Ningbo	Oct. 8, 2018	4 months	Retriever	Male	1 dose	MN119608
CH-ZJ-D5	CPV-2c	Hangzhou	Nov. 10, 2018	3 months	Mixed	Male	1 dose	MN119609
CH-ZJ-D6	CPV-2c	Ningbo	Nov. 11, 2018	6 months	Mixed	Female	2 doses	MN119610
CH-ZJ-D7	CPV-2c	Hangzhou	Nov. 17, 2018	2 months	Mixed	Female	Unvaccinated	MN119611
CH-ZJ-D8	CPV-2c	Jinhua	Dec. 1, 2018	5 months	Mixed	Male	1 dose	MN119612
CH-ZJ-D9	CPV-2c	Huzhou	Dec. 1, 2018	3 months	Mixed	Male	1 dose	MN119613
CH-ZJ-D10	CPV-2c	Shaoxing	Dec. 3, 2018	4 months	Retriever	Female	1 dose	MN119614
CH-ZJ-D11	CPV-2c	Jinhua	Jan. 4, 2019	7 months	Mixed	Male	2 doses	MN119615
CH-ZJ-D12	CPV-2c	Hangzhou	Feb. 6, 2019	2 months	Mixed	Male	1 dose	MN119616
CH-ZJ-D13	CPV-2c	Jinhua	Mar. 14, 2019	3 months	Mixed	Male	1 dose	MN119617
CH-ZJ-D14	CPV-2c	Shaoxing	Mar. 14, 2019	1 year	Mixed	Female	1 dose	MN119618
CH-ZJ-D15	CPV-2c	Hangzhou	Apr. 4, 2019	8 months	Mixed	Male	2 doses	MN119619
CH-ZJ-D16	CPV-2c	Jinhua	Apr. 4, 2019	9 months	Retriever	Female	2 doses	MN119620

Table 3. Statistics of the main amino acid mutation sites in the VP2 capsid protein of canine parvovirus type 2 in Chinese and reference strains

Strains/GenBank Accession number	Mutation sites: amino acid residue					
	5	30	130	370	426	440
EU659117-2/the USA/1980	A	G	V	Q	N	T
FJ197847-2/South Korea/2007/Vaccine	\	\	\	\	\	\
MH643886-2/South Korea/2016	\	\	\	\	\	\
EU659118-2a/the USA/1981	\	\	\	\	\	\
MF177231-2a/Italy/2000	\	\	\	\	\	\
GU362934-2a/Italy/2008	\	\	\	\	\	\
EU441280-2a/Henan/2008	\	\	\	\	\	\
HQ883267-2a/Beijing/2010	\	\	\	\	\	A
KJ438805-2a/Henan/2013	\	\	\	\	\	A
KY937646-2a/Shanghai/2016	\	\	\	\	\	A
MK332005-2a/Guangxi/2017	\	\	\	\	\	A
MN101726-2a/Beijing/2018	\	\	\	\	\	A
CPU72696-2b/Taiwan/1996	\	\	\	\	D	\
EU659121-2b/the USA/1998	\	\	\	\	D	\
FJ005265-2b/Italy/2005	\	\	\	\	D	\
KF803606-2b/Beijing/2011	\	\	\	\	D	\
MK332002-2b/Guangxi/2016	\	\	\	\	D	A
MK331996-2b/Guangxi/2016	\	\	\	\	D	A
KU508407-2c/Italy/2009	\	\	\	\	E	\
KX425920-2c/India/2010	\	\	\	\	E	\
KU244254-2c/Taiwan/2015	\	\	\	\	E	\
LC216910-2c/Indonesia/2013	G	\	\	R	E	\
MF467242-2c/Vaccine/2015	G	\	\	R	E	\
MF467229-2c/Henan/2016	G	\	\	R	E	\
MGo13488-2c/Shanghai/2017	G	\	\	R	E	\
CH-AH-D1	G	\	\	\	D	A
CH-AH-D2	\	\	\	R	E	\
CH-AH-D3	\	W	\	R	E	\
CH-AH-D4	G	\	\	R	E	\
CH-AH-D5	\	\	\	\	D	A
CH-AH-D6	G	\	\	R	E	\
CH-AH-D7	\	\	\	R	E	\
CH-AH-D8	\	\	\	R	E	\
CH-AH-D9	G	\	\	R	E	\
CH-AH-D10	G	\	\	R	E	\
CH-AH-D11	G	\	\	R	E	\
CH-AH-D12	G	\	\	R	E	\
CH-AH-D13	G	\	\	R	E	\
CH-AH-D14	\	\	\	\	\	A
CH-AH-D15	\	\	\	\	\	A
CH-AH-D16	\	\	\	\	D	A
CH-HN-D1	G	\	\	R	E	\
CH-HN-D2	G	\	\	R	E	\
CH-HN-D3	G	\	\	R	E	\
CH-HN-D4	G	\	\	R	E	\
CH-HN-D5	\	\	\	\	D	A
CH-HN-D6	G	\	\	R	E	\
CH-HN-D7	\	\	A	\	\	A
CH-HN-D8	\	\	\	R	E	\
CH-HN-D9	G	\	\	R	E	\
CH-HN-D10	G	\	\	R	E	\
CH-HN-D11	G	\	\	R	E	\
CH-HN-D12	G	\	\	R	E	\
CH-HN-D13	G	\	\	R	E	\
CH-HN-D14	\	\	\	\	\	A
CH-HN-D15	\	\	\	\	\	A

Continued on next page

Table 3 (continued)

CH-HN-D16	\	\	\	\	D	A
CH-HN-D17	G	\	\	R	E	\
CH-HN-D18	\	\	\	\	D	A
CH-HN-D19	G	\	\	R	E	\
CH-HN-D20	\	\	\	\	D	A
CH-HN-D21	G	\	\	R	E	\
CH-HN-D22	G	\	\	R	E	\
CH-HN-D23	G	\	\	R	E	\
CH-HN-D24	G	\	\	R	E	\
CH-HN-D25	G	\	\	R	E	\
CH-HN-D26	G	\	\	R	E	\
CH-HN-D27	G	\	\	R	E	\
CH-HN-D28	G	\	\	R	E	\
CH-HN-D29	G	\	\	R	E	\
CH-ZJ-D1	G	\	\	R	E	\
CH-ZJ-D2	G	\	\	R	E	\
CH-ZJ-D3	G	\	\	R	E	\
CH-ZJ-D4	G	\	\	R	E	\
CH-ZJ-D5	G	\	\	R	E	\
CH-ZJ-D6	G	\	\	R	E	\
CH-ZJ-D7	G	\	\	R	E	\
CH-ZJ-D8	G	\	\	R	E	\
CH-ZJ-D9	G	\	\	R	E	\
CH-ZJ-D10	G	\	\	R	E	\
CH-ZJ-D11	G	\	\	R	E	\
CH-ZJ-D12	G	\	\	R	E	\
CH-ZJ-D13	G	\	\	R	E	\
CH-ZJ-D14	G	\	\	R	E	\
CH-ZJ-D15	G	\	\	R	E	\
CH-ZJ-D16	G	\	\	R	E	\

Strains with designations starting with “CH” are Chinese strains obtained in this study. (A:Ala, D:Asp, E: Glu, G: Gly, N:Asn, Q:Gln, R:Arg, T:Thr, V: Val, W:Try)

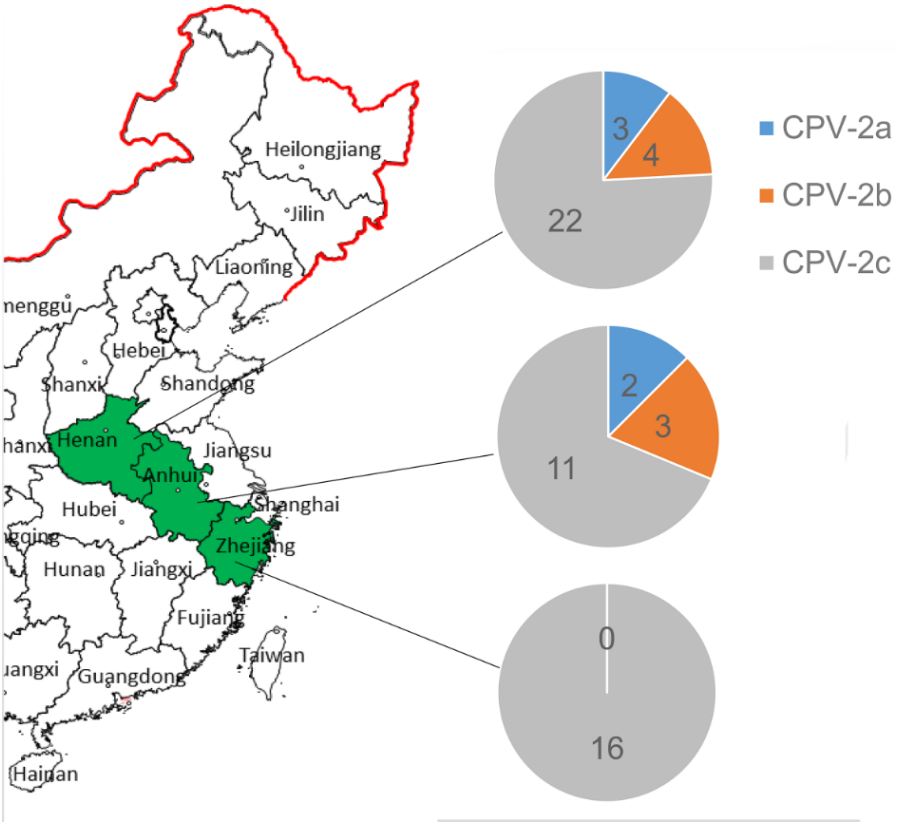

Fig. 1. Genotypes and geographical distribution of the canine parvovirus (CPV) strains tested in this study

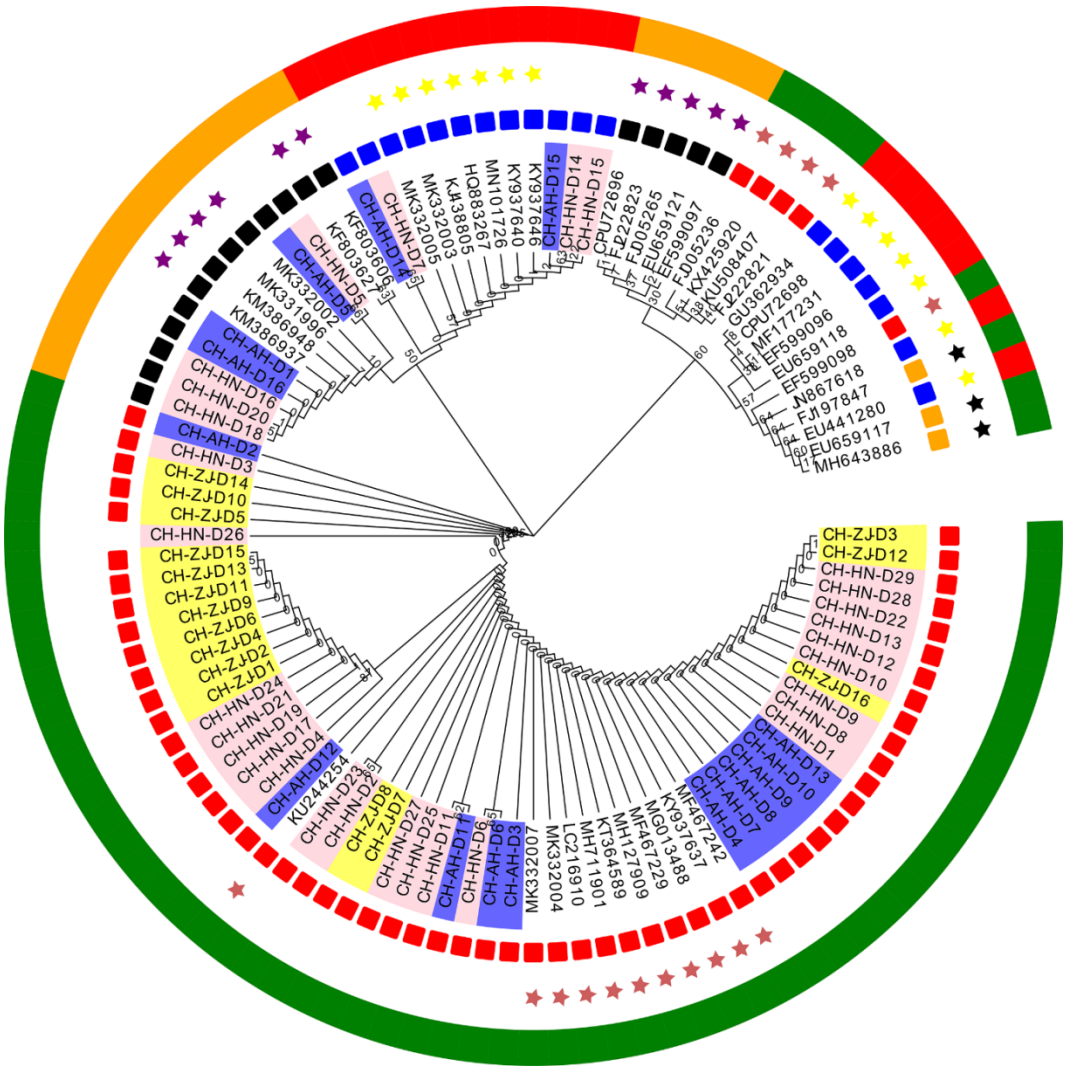

Fig. 2. Evolutionary tree of canine parvovirus type-2 (CPV-2). Blue areas represent the 16 strains collected from Anhui Province, pink areas represent the 29 strains collected from Henan Province, and yellow areas represent the 16 strains collected from Zhejiang Province. Yellow stars denote the CPV-2a reference strain; purple stars denote the CPV-2b reference strain; and red stars denote the CPV-2c reference strain. Red strips indicate the CPV-2a strain; orange strips indicate the CPV-2b strain; and green strips indicate the CPV-2c strain

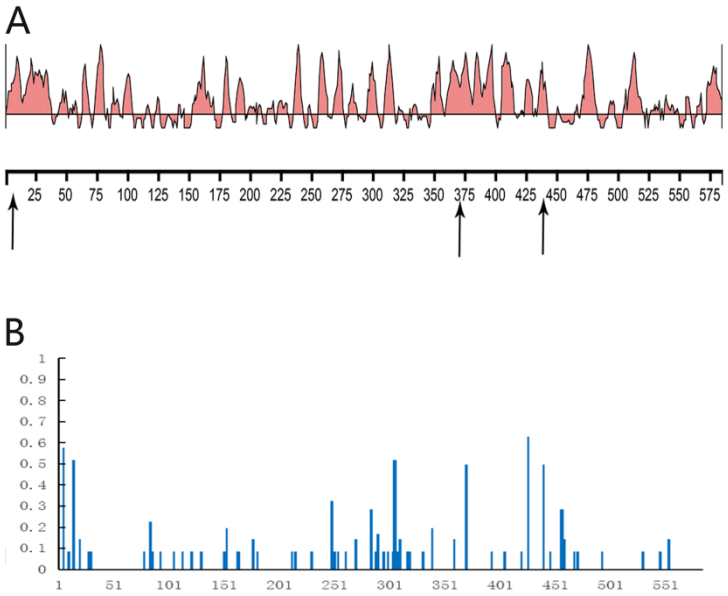

Fig. 3. Antigenic epitope prediction and amino acid entropy rates of the VP2 capsid protein of canine parvovirus type 2. A: Antigen epitope prediction for CPV-VP2; B: Amino acid entropy rates for CPV-VP2

Main amino acid mutation sites of the VP2 protein. The main amino acid mutation sites of the VP2 protein of the 61 strains were 5, 30, 130, 370, 426, and 440. In CPV-2, Ala5Gly mutation was evident at a rate of 73.77% (8 strains in Anhui Province, 21 in Henan Province, and 16 in Zhejiang Province). Gly30Trp mutation occurred only in one strain (CH-AH-D3), and Val130Al mutation likewise (CH-HN-D7). Gln370Arg mutation was noted in 80.33% of isolates (11 strains in Anhui Province, 22 in Henan Province, and 16 in Zhejiang Province). Two mutations occurred at site 426, with a rate of 11.48% for Asn426Asp (three strains in Anhui and four in Henan Province) and 80.33% for Asn426Glu (11 strains in Anhui Province, 22 in Henan Province, and 16 in Zhejiang Province). Thr440Ala had mutated 80.33% of strains (11 in Anhui Province, 22 in Henan Province, and 16 in Zhejiang Province). The specific mutations by strain are presented in Table 3.

Phylogenetic and evolutionary relationships. A phylogenetic tree was constructed based on VP2 sequences of the 61 strains tested in this study and 45 representative reference strains. Compared with the strains collected from countries such as the United States, Italy, Japan, South Korea, and India, the reference strains collected from China were distantly related. Overall, 49 CPV-2c strains (80.33%) in this study were closely related to CPV-2c reference strains collected from Thailand, Indonesia, Taiwan, Shanghai, Guangxi, and Henan Provinces. Among these 49 strains, 11 were from Anhui Province, 22 from Henan Province, and 16 from Zhejiang Province. Seven strains collected from Anhui (n = 3) and Henan (n = 4) Provinces were similar to CPV-2b. Five strains were closely related to CPV-2a, two of which were from Anhui Province and three from Henan Province. The specific interrelationships between the collected and reference strains are depicted in Fig. 2.

Epitope and selection pressure analyses of CPV-VP2. Antigenic epitope analysis of the VP2 protein of CPV-2c predicted several epitopes mainly located at amino acid sites 0–25 and 350–450 (Fig. 3A). In selection pressure analysis, the entropy was >0.5 mainly in two regions (0–51 and 251–451) (Fig. 3B). The results of epitope prediction and selection pressure analyses were consistent with one another.

Discussion

A CPV is widely distributed in various regions of China, and the virus undergoes rapid mutations. Since its identification, three important variations in the CPV genotype have occurred, resulting in the emergence of subtypes CPV-2a, CPV-2b, and CPV-2c. During 2009–2012, CPV-2a was the dominant genotype in the southern region of Nanjing (29). In this period and continuing until 2014, CPV-2c was not widespread in Henan Province (30). By 2015–2016, the novel CPV-2a strain had become the prevalent subtype in Henan, Guangxi, and Jiangsu Provinces and in these years Aln5Gly and Gln370Arg mutations in genotype 2c were reported for the first time (26). In this study, the epidemiological trend of CPV-2 was observed from 2018 to 2019 through sequencing the CPV-2 strains collected from three provinces (Henan, Anhui and Zhejiang). Variations in the CPV-2 genotype were strongly affected by vaccination and tended to be due to 2c mutations. In the present study, trends of CPV infections in Anhui, Henan, and Zhejiang Provinces revealed CPV-2c as the most widespread genotype. Three genotypes coexisted in Anhui and Henan Provinces, which are not perfectly covered by the vaccination program. In Zhejiang Province, in contrast, the vaccination rate was high and CPV-2c was the only strain detected. Based on genotypic and immunisation history, CPV-2c shows high transmissibility and strong adaptability. It is therefore the genotype most likely to cause vaccination failure when the program uses classical CPV-2, and this genotype's dominance may be explained by its having evolved under pressure from immunisation.

The main amino acid mutations in the VP2 protein of the 61 strains occurred at sites 5, 370, 426, and 440. In Anhui, Henan and Zhejiang Provinces, 45 (73.77%) strains harboured a similar Ala5Gly mutation to the reference strain CPV-2c. This finding indicates that the amino acid mutation at site 5 may determine the CPV subtype (CPV-2c), with such mutations gradually becoming increasingly common in China and overseas. The nearly three quarters proportion of strains collected from Anhui, Henan and Zhejiang Provinces with the Ala5Gly mutation in the CPV-VP2 protein suggests that it has become prevalent. A total of 49 (80.33%) strains in the three provinces displayed a similar Gln370Arg mutation to the reference strain CPV-2c. This site may alter the spatial structure of the VP2 protein to some extent, thereby affecting the pathogenicity of the virus (8). Mutations at the amino acid site 426 markedly affected the classification of CPV-2a, CPV-2b, and CPV-2c (30). The reference strain CPV-2a showed 426Asn, and mutation at this site occurred in 5 of the 61 strains (8.20%) tested in this study. Seven (11.48%) strains collected from Anhui and Henan Provinces were Asn426Asp-mutated in a similar way to the reference strain CPV-2b, although no mutation was detected in the strains collected from Zhejiang Province. A total of 49 (80.33%) strains revealed a similar Asn426Gln mutation to the reference strain CPV-2c. These findings indicate that the Asn426Gln mutation was highly prevalent in the three provinces studied. Furthermore, the strains collected from Henan and Anhui Provinces (19.67%) were like the reference strains CPV-2a and CPV-2b in having the Thr440Ala mutation. This result demonstrates that CPV strains carrying the Thr440Ala mutation are prevalent in Anhui and Henan Provinces. In terms of mutation rates, CPV-2c was the dominant mutant genotype in Anhui, Henan, and Zhejiang Provinces from October 2018 to April 2019.

Sequence identities and phylogenetic relationships of the 61 strains collected from the three provinces revealed in this study will contribute to a better understanding of the frequency and mutation tendencies of CPV strains in these provinces. The VP2 sequences of the CPV strains collected from Anhui, Henan, and Zhejiang Provinces shared high identity without any outstanding variations; however, these strains showed relatively distant relationships with the classical vaccine strains. Moreover, there were no obvious regional differences in CPV genotype distribution according to the evolutionary tree. This result indicates that CPV genotypes are characterised by mutations at individual amino acid sites which do not affect the overall evolutionary characteristics of CPV.

In this study, mutations at four major amino acid sites led to the emergence of different genotypes, which further evolved into the highly immune-resistant subtype CPV-2c under pressure from immunisation as previously described (4, 8). The epitope is the antigenic sequence that stimulates B cells to produce antibodies, leading to antigen–antibody binding. The VP2 protein of CPV-2c presents multiple epitopes; therefore, multiple amino acid mutations might be triggered via adaptive selection for survival under pressure from immunisation. The present study provides a theoretical and technical basis for evolutionary analysis and vaccine strain selection against CPV-2c. Selection pressure analysis revealed that the genomic regions carrying mutations were complex, diverse, and highly prone to mutations, which further explains the differentiation of CPV-2 into additional genotypes in recent years (4). Two regions (0–25 and 350–450) in epitope prediction (Fig. 3A) and two regions (0–51 and 251–451) in selection pressure analysis (Fig. 3B) showed a high degree of coincidence in their entropy values. Moreover, the mutations at the sites 5, 370, 426, and 440 in this study conform to the epidemic trends of the virus, further confirming the authenticity of the mutated sites in this study. Unfortunately, owing to the unavailability of specific sera for genotypic variants in our laboratory, we were unable to provide data for serological analysis in this study. Nonetheless, based on the results of epitope prediction and selection pressure analyses combined with vaccination history, the genotypic variations in strains collected from Anhui, Henan and Zhejiang Provinces in China may be attributed to pressure from immunisation. Animal inoculation experiments are needed to determine whether immunisation failure is a possible consequence of antibody pressure on evolution of CPV-2a, CPV-2b, and CPV-2c.

In conclusion, amino acid sequence analysis of the VP2 protein of 61 CPV strains collected from central and eastern China indicated that CPV-2c is the predominant genotype of CPV in the study regions, particularly in Zhejiang Province. In addition, strains with CPV-2a and CPV-2b genotypes harbouring novel mutations were also detected. These results highlight the need for further research targeting different CPV genotypes to develop vaccines and establish more effective vaccination programs that increase the scope of immunisation.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This study was supported by the National Natural Science Foundation of China (Grant nos. 31802185 and 31870917), the Scientific and Technological Project of Henan Province (Grant no. 182107000040), the program for Innovative Research Teams of Science and Technology in the University of Henan Province (Grant no. 20IRTSTHN024), the Key Scientific and Technological Project of the Education Department of Henan province (Grant no. 18A230012), and Technological Project of Nanyang Normal University (Grant nos. 18046 and 2019QN009).

Animal Rights Statement: None required.

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