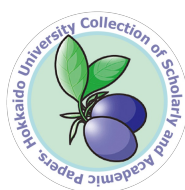




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Prevalence and molecular characterization of parvovirus in domestic kittens from Northeast China during 2016–2017

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Abstract

Feline panleukopenia virus (FPV) and Canine parvovirus (CPV) are significant enteric viruses in cats, causing acute diarrhoea, hemorrhagic enteritis and leukopenia. To better understand the prevalence and molecular epidemiology of parvovirus in domestic kittens in China, we tested for these viruses in 143 faecal samples collected from diarrhoea-affected/healthy domestic kittens during 2016–2017 and analysed the sequences of parvovirus-positive samples. According to the results, 55 samples (38.46%) were positive for parvovirus; among these, 1 (named as CC-04/16-05) was identified as a new CPV-2a strain, 1 (LY-02/16) as a new CPV-2b strain and 53 as FPV. These findings show CPV-2a/2b and FPV to be circulating in domestic kittens in Northeast China. Genetic analysis shown that all of the FPV strains isolated in China can be grouped into the FPV G1 cluster, whereas two FPV vaccine strains are classified into the G2 cluster, revealing that this may be an important reason for the limited effectiveness of vaccines against FPV. Furthermore, a common mutation (Tyr324Ile) was present in strains CC-04/16-05 and LY-02/16, and Phe267Tyr and Thr440Ala mutations were present only in strain LY-02/16. The emergence of novel CPV-2a/2b strains with critical mutations, which related to the host-ranges of CPV, suggesting that there are a potential risk of the outbreak of CPV in *felid*.

Key Words: Canine parvovirus, Feline panleukopenia virus, Genetic analysis, Kitten, Northeast China

Introduction

Feline panleukopenia virus (FPV) and *Canine Parvovirus* (CPV), which belong to the genus *parvovirus*, subfamily *Parvovirinae* in the family *Parvoviridae*, are small, non-enveloped viruses

with approximately 5.2-kbp linear single-stranded DNA genome²⁷⁾. FPV and CPV are the most significant viral pathogens of severe enteric disease in cats and dogs, respectively, characterized by acute diarrhoea, haemorrhagic enteritis and leukopenia. FPV-induced disease in cats was

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recognized in 1920s; in the late 1970s, a host-range variant of FPV, CPV-2, emerged as a new virus in dogs and then circulated rapidly worldwide²⁶. Between 1979 and 1985, two novel genetic and antigenic variants of CPV, CPV-2a (426Asn) and CPV-2a (426Asp), were found and identified in dogs²⁹. Soon after they emerged, a new mutation was observed in CPV-2a/2b at amino acid 297 (Ser to Ala) of the VP2 protein, and these variants were designated as new strains of CPV-2a/2b^{8,26}. Another antigenic variant, CPV-2c (426Glu), was first reported in Italy in 2000⁴, and it has since become the predominant type in several regions, including Italy, Argentina⁵, Portugal¹⁵, India²⁵, China and North America^{30,31}. At present, the antigenic variants CPV-2a/2b/2c have completely replaced original CPV-2, and appear to be co-circulating in many countries.

Identity and similarity between FPV and CPV-2 are more than 98% at the nucleotide and amino acid sequence levels. CPV-2 is distinguished from FPV based on changes in six VP2 protein residues, namely, 80, 93, 103, 323, 564 and 568¹³. A capsid protein, VP2 is an important ligand that binds to the host cell transferrin receptor (TfR)¹⁶, and the host ranges of the above two parvoviruses are determined by residues 93, 103 and 323¹³. Previous research demonstrates that FPV cannot replicate in the gut of dogs; CPV-2 is able to replicate in feline cell cultures but is incapable of replicating in feline tissues⁶. After the mid-1980s, three antigenic variants, CPV-2a/2b/2c, containing five amino acid changes (residues 87, 300, 305, 426, and 555) compared with the original CPV-2 emerged successively in dogs and rapidly spread worldwide. With the emergence of subtypes, the host range of CPV had also changed. Unlike the original CPV-2, antigenic variants can bind to feline TfR and infect cats, causing the same clinical signs as feline panleukopenia (FPL)^{9,22}. Since the first report of cats infected with CPV-2a in the late 1980s, CPV-2a/2b has been reported in domestic cats in Japan²², Turkey²⁴,

Germany³³, India²³ and other countries. Recent reports have shown that CPV-2c can also infect cats and cause more serious symptoms²⁰.

In addition, co-infection of CPV subtypes and FPV has been detected in a variety of domestic cats as well as wild cats, including tigers, leopard, and lions, and also in pandas^{2,37}. This mixed infection increases the chance of genetic mutation and recombination of CPV and FPV and suggests that cats act as carriers of different parvoviruses and as a source of new variants of parvovirus that infect cats, dogs and other animals.

Therefore, risk assessment of new parvovirus variants will be helpful for carrying out molecular aetiological investigations in domestic and wild cats worldwide. In China, a number of CPV strains isolated from dogs have been identified, sequenced and analysed, though genetic analysis of CPV and FPV in domestic cats is lacking^{36,38}. In the present study, we investigated the molecular characterization of parvovirus in domestic kittens from northeast China between 2016 and 2017. The genetic analysis of epidemic isolates and comparison among isolates and foreign isolates are discussed to allow a better understanding of the prevalence status of parvovirus in domestic cats in China.

Materials and Methods

Sample collection: A total of 143 faecal samples/anal swabs were collected from 87 diarrhoea-affected and 56 apparently healthy domestic kittens, aged from one to six months, from animal shelters, private veterinary clinics and rural households in Northeast China between January 2016 and June 2017. The faecal samples/anal swabs were homogenized in 1 ml of phosphate-buffered saline (PBS, pH = 7.4) with ampicillin and streptomycin, centrifuged at 8000 g for 10 min at 4°C, and the supernatant was collected and stored at -70°C until use.

DNA extraction and preliminary screening by PCR and RFLP: Genomic DNA of samples was extracted using Viral DNA Extraction Kit I (OMEGA Bio-tek., China) according the manufacturer's instructions. Preliminary parvovirus screening was carried out using the parvovirus consensus primers (P1: 5'-GGATGGGTGGAAATCACAGC-3'; P2: 5'-ATAACCAACCTCAGCTGGTC-3')³⁹⁾, which amplify an 845-base-pair (bp) fragment of the VP2 gene and CPV and FPV were differentiated by PCR-RFLP, as described previously⁴⁰⁾. Preliminary screening were performed in triplicate to ensure screening accuracy.

Amplification, sequencing and phylogenetic analysis of the full VP2 gene: Genomic DNA of PCR-positive samples was used as the template for amplification of the full-length VP2 gene of parvovirus using primer pairs (P3: 5'-ATGAGT GATGGAGCAGTTCAACCAGAC-3'; P4: 5'-CTA GGTGCTAGTTGATATGTAATAAAC-3')³⁹⁾. The amplified products were separated by 1.0% agarose gel electrophoresis and visualized using gel documentation system (Wealtec, USA). The PCR products were purified using a DNA Gel Extraction Kit (OMEGA Bio-tek., China), cloned into the PMD-18T vector, and transformed into DH-5 α cells according the manufacturer's instructions. Positive clones were selected, and plasmids were purified using a plasmid mini kit (OMEGA Bio-tek., China) and identified using the universal primer pair M13/M17 for PMD-18T. Positive plasmids were sequenced by Sangon Biotech (Shanghai, China). To ensure sequence accuracy, each sample was sequenced using at least three positive clones and each specific base was sequenced at least four times.

The full-length nucleotide and amino acid sequences of the parvovirus VP2 gene obtained in this study were compared with other parvovirus reference sequences in GenBank. Sequence analysis was performed with Lasergene software using the MegAlign function (DNASTAR, Madison, WI, USA). Phylogenetic analysis of the full-length VP2 gene was inferred using the neighbour-

joining method in MEGA 7.0, and evolutionary distances were computed using the maximum composite likelihood method.

Results

Screening results

Of the total samples, 55 (38.46%) tested positive for parvovirus. Parvovirus-positive kittens were found across different age groups in all populations, with high positive rate ranging from 28.00% to 46.38%. Faecal samples/anal swabs collected from different regions showed different positive rate ranging from 22.22% to 45.57%. Female and male kittens had a similar positive rate (37.97% and 39.06%), and those with diarrhoea had a higher positive rate (55.17%) than healthy kittens (12.5%), and unvaccinated kittens also have a higher positive rate (48.21%) than vaccinated kittens (28.21%) (Table 1). In addition, two parvovirus-positive samples tested positive for CPV in a PCR-RFLP assay, yielding 602-bp and 414-bp specific products. Other samples were positive for FPV, generating 602-bp, 302-bp and 102-bp specific products in PCR-RFLP reactions (data not shown). Detailed information on the parvovirus-positive samples, including the names, sources and viral types of samples and the ages, sexes, vaccination status and clinical signs of kittens, are shown in supplementary table 1.

Sequence analysis

The full-length VP2 gene was successfully amplified from the parvovirus-positive samples, yielding a 1755-bp PCR fragment, and 24 different sequences (GenBank accession no. MF541119-MF541142) were obtained from 55 positive faecal samples/anal swabs. Sequence analysis showed these sequences to be highly related to each other, with nucleotide sequence identity ranging from 97.9% to 99.9%; moreover, had high identity of 97.7%–99.9% with the VP2 gene of reference FPV and CPV-2a/2b/2c strains in GenBank were

observed. Among these positive samples, 53 were identified as FPV, one as CPV-2a (CC-04/16-05), and one as CPV-2b (LY-02/16), considering changes in residues 87, 297, 300, 305, 426, and 555 of the VP2 protein. These results were in accord with the results of PCR-RFLP screening.

To further analyse mutations in these positive samples, we determined amino acid changes in the VP2 protein of parvovirus-positive samples (Table 2). All FPV-positive samples presented G171A, T302C and G1521A mutations, with corresponding amino acid changes of Gly57Gly, Ile101Thr and Thr507Thr. Four non-synonymous mutations were present in partial FPV sequences obtained in this study, but these mutations were invalid for the classification of these FPVs. Samples, such as JL-04/16, with the only mutation of Ile101Thr in VP2 protein compared with reference strain (M38246) were predominant (49/53) in all FPV-positive samples, suggesting that this FPV strain was predominant strain in Northeast China. For CPV-positive samples, CC-04/16-05 presented Leu87Met, Ala297Ser and Gly300Ala substitutions and was characterized as a new CPV-2a strain; LY-02/16, with its Ala297Ser and Asp426Asn mutations, was identified as a new CPV-2b strain. Furthermore, residue 267 (Phe→Tyr), 324 (Tyr→Ile) and 440 (Thr→Ala) changes, which had been described in CPV-2a and CPV-2b isolated from China, South Korea and Japan, were also found in CC-04/16-05 and LY-02/16^{7,25,32,40}.

Phylogenetic analysis

A phylogenetic tree based on the full-length VP2 nucleotide sequence was constructed using 24 parvovirus-positive samples with different sequences in this study and 64 reference FPV and CPV strains from Europe, America and Asia (Fig. 1). The phylogenetic analysis indicated that all of the parvoviruses can be grouped into two large branches: the CPV branch and the FPV branch. All of the FPV strains were classified into three clusters, G1 (1521A), G2 (1521G) and G3 (246G, 699C and 1602G), and all CPV strains

grouped into four clusters, CPV-2, CPV-2a (426Asp), CPV-2b (426Asn) and CPV-2c (426Glu). All the FPV sequences in this study were placed in the G1 cluster, which consisted of three strains from Japan, three strains from South Korea, four strains from Italy, one strain from Hungary and nine strains from China, while two FPV vaccine strains (Pfizer and Merial vaccines) were classified in the G2 cluster. Four FPV sequences (BJ-03/16, CC-02/16, JL-04/16 and JL-01/17-03) formed a subcluster with the common synonymous mutation A1110G; two sequences (SP-01/16 and JL-10/17-06) and some reference strains from South Korea and Italy were placed in different clades, and the other 16 FPV sequences were grouped into a subcluster with V211, V142, HRB-CS1, Tiger/H7-163 and Changc2007, as well as other strains isolated from China and Japan. CC-04/16-05 was placed into a new CPV-2a cluster with JL4/2013 and HB3/2013 isolated from China and LY-02/16 into a new CPV-2b cluster with JL6/2013 and HB1/2013 (strains from China).

Discussion

Haemorrhagic enteritis and leukopenia caused by parvovirus is a highly contagious viral enteric disease in cats, with very high morbidity ranging from 20% to 100% and mortality of 25%–90%. Many risk factors, including age, breed, vaccination status and housing conditions, affect the prevalence of parvovirus in cats^{32,34}. It is widely believed that cats up to one year old have higher mortality than younger kittens, and considering the preservation of maternally derived antibodies (MADs), the vast majority of parvovirus infections in younger kittens are subclinical infections. Recent research has indicated that kittens aged 3–6 months are more vulnerable than younger kittens or older cats to parvovirus infection¹⁹. Moreover, the prevalence is closely related to the clinical signs. For example, the cat population with vomiting and

Table 1. The preliminary screening results of parvovirus infection in domestic kittens in Northeast China

Factor	Source		Age				Gender		Clinical symptom			Vaccination status		
	RH	PVC	AS	2 < months ≤ 6	1 < months ≤ 2	0 < months ≤ 1	Female	Male	Health	Diarrhea	Unvaccinated	Vaccinated	Unknown	
No. tested	27	37	79	25	69	49	79	64	56	87	56	39	48	
No. positive	6	13	36	7	32	16	30	25	7	48	27	11	17	
Positive rate (100%)	22.22%	35.14%	45.57%	28.00%	46.38%	32.65%	37.97%	39.06%	12.5%	55.17%	48.21%	28.21%	35.42%	

RH, Rural households; PVC, Private veterinary clinics; AS, Animal shelters.

Table 2. Amino acid mutations in the VP2 protein of parvovirus obtained from domestic kittens in this study

Strain	accession no.	Sample no. ^a	80 (K)	87 (M)	93 (K)	101 (I)	103 (V)	164 (V)	232 (V)	267 (F)	297 (S)	300 (A)	305 (D)	323 (D)	324 (Y)	334 (A)	426 (N)	440 (T)	564 (N)	Type
Reference strain																				
CU-4	M38246		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	FPV
CPV-b	M38245		R	-	N	-	A	-	I	-	-	-	-	N	-	-	-	-	S	CPV-2
CPV-15	M24003		R	L	N	T	A	-	I	-	-	G	Y	N	-	-	-	-	S	CPV-2a
CPV-39	M74849		R	L	N	T	A	-	I	-	-	G	Y	N	-	-	D	-	S	CPV-2b
JL4/2013	KR002797		R	L	N	T	A	-	I	-	A	G	Y	N	I	-	-	-	S	new CPV-2a
JL6/2013	KR002799		R	L	N	T	A	-	I	Y	A	G	Y	N	I	-	D	A	S	new CPV-2b
This study																				
JL-04/16 ^b	MF541126	49	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	FPV
BC-02/16	MF541119	1	-	-	-	T	-	I	-	-	-	-	-	-	-	-	-	-	-	FPV
JL-24/17-05	MF541133	1	-	-	-	T	-	I	-	-	-	-	-	-	-	T	-	-	-	FPV
SP-01/16	MF541142	1	-	-	-	T	-	-	-	-	-	-	-	-	-	T	-	-	-	FPV
JL-04/17-03	MF541127	1	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	S	FPV
CC-04/16-05	MF541122	1	R	L	N	T	A	-	I	-	A	G	Y	N	I	-	-	-	S	new CPV-2a
LY-02/16	MF541141	1	R	L	N	T	A	-	I	Y	A	G	Y	N	I	-	D	A	S	new CPV-2b

^aIndicates the number of samples with the same amino acid changes in the VP2 protein in this study.

^bIndicates other samples except BC-02/16, JL-24/17-05, SP-01/16, JL-04/17-03, CC-04/16-05, and LY-02/16 in this study.

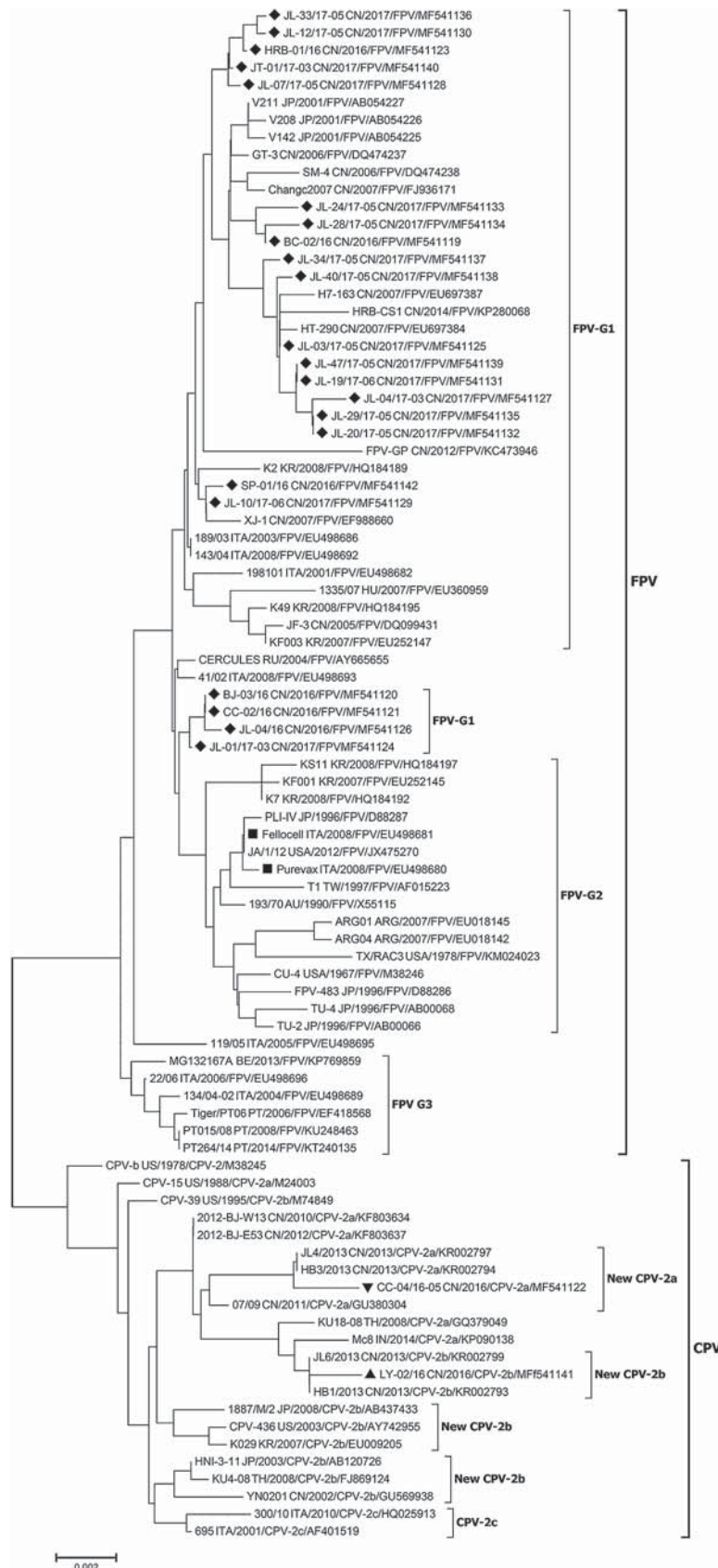


Fig. 1. A phylogenetic tree based on the full-length VP2 gene was constructed by the neighbour-joining method with 1000 bootstrap replicates using MEGA 7.0. Evolutionary relationships among 66 reference parvovirus sequences obtained from the GenBank database and 24 sequences isolated in this study and detailed information on each strain, including strain name, country and time of isolation, antigenic types, and GenBank accession number. Diamonds (◆), inverted triangles (▼) and triangles (▲) indicate FPV, CPV-2a and CPV-2b strains isolated from domestic kittens in this study, respectively. Squares (■) indicate FPV vaccine strains. Abbreviations: ARG, Argentina; AU, Australia; BE, Belgium; CN, China; HU, Hungary; ITA, Italy; IN, India; JP, Japan; KR, South Korea; PT, Portugal; RU, Russia; TH, Thailand; TW, Taiwan; USA, United States of America.

diarrhoea has a greater risk of parvovirus infection than other populations. In the current study, 38.46% (55/143) of cats were found to be positive for parvovirus, and kittens with diarrhoea had a higher prevalence (55.17%) than healthy kittens (12.5%). Furthermore, 44.83% of the kittens with diarrhoea had no parvovirus infection, suggesting that the diarrhoea in many of those cats may have been caused by other pathogens, such as bacteria, parasites, Feline Astrovirus, Feline Rotavirus and others, rather than by parvovirus. Although the difference in prevalence for kittens of different ages was not significant, those aged 0–1 and 1–2 months had higher mortality than those aged 2–6 months; this may be related to the sample source and health status. Indeed, the sample source reflects the housing conditions of the kittens, and the results suggested that kittens grouped together in crowded housing, such as animal shelters, were more susceptible to parvovirus. Vaccination against feline panleukopenia are considered the most effective measure providing protection from FPV infection, but many investigations discovered that the effectiveness of current vaccines against FPV is limited. For instance, one study investigated the prevalence of antibodies against FPV in client-owned cats in southern Germany and discovered that 27% of the vaccinated cats had no antibodies against FPV¹⁹. A recent study investigated the prevalence of FPV from blood samples collected from stray and housed cats in South Korea and discovered that the FPV-positive rate of vaccinated cats was 1.3%¹⁸. In the current study, FPV-positive rate of unvaccinated kittens is 48.21%, interestingly, vaccinated kittens also have a higher positive rate (28.21%), consistent with descriptions in previous investigations^{3,19}. These findings further reveal that the current vaccines can't provide complete protection from FPV infection.

Since the first report of FPV infection in a cat with diarrhoea in 1920s, FPV and the host-range variants, CPV-2a/2b/2c have circulated rapidly in domestic and wild cats worldwide.

Although FPV and CPV antigenic variants can infect cats and cause the same clinical signs, the results of previous parvovirus investigations in cats showed that more than 95% of cases were caused by FPV. Regardless, the prevalence of CPV antigenic variants in cats has exhibited an increasing trend in many European and American countries in recent years, and the number of cases of FPV and CPV co-infection is also gradually rising^{2,10,21,24}. To date, only a few studies have reported CPV infection in cats in China, whereas reports of CPV in cats are common in Japan and South Korea. Wu *et al.* assessed CPV infection in domestic dogs and cats in Beijing during 2010–2013 and found two new CPV-2a strains in 16 samples collected from domestic cats; however, reports of CPV-2b/2c infection in cats in China are scarce³⁸. In recent research, two novel CPV-2a variants, which exhibit similar changes at residues 324 (Tyr→Ile) and 444 (Thr→Ala) to CPV strains isolated from dogs in many countries, including China, South Korea, Italy and Brazil, were detected in faeces collected from cats with diarrhoea in India²³. In our study, one novel CPV-2a variant, CC-04/16-05, and one CPV-2b variant, LY-02/16 (Tyr324Ile), were found in domestic kittens; this is the first report of the emergence of novel CPV variants in cats in China. It has been demonstrated that CPV-2 strains can replicate in canine and feline cells *in vitro* but cannot infect cats *in vivo*, whereas the CPV antigenic variants CPV-2a/2b/2c have an expanded host range and can infect domestic and wild cats *in vivo*. The host range of parvovirus is determined by the residues at positions 93, 103 and 323 of the VP2 protein^{13,26}. Muz *et al.* characterized the VP2 gene of CPV in domestic cats from Turkey and found Asn323Asp change in CPV-2a and CPV-2c strains, demonstrating a feline host²⁴. No changes at residues 93, 103 or 323 in the strains CC-04/16-05 and LY-02/16 strains were found, but a common mutation is present at residue 324, with a change from Tyr to Ile. Previous studies have noted that residue 324 in all carnivore parvoviruses is subject to

strong positive selection. This residue is adjacent to residue 323, and together with residue 93, which affects Tfr binding, it plays an important role in the host range of parvovirus^{11,12,32}. This mutation is frequent in CPV strains isolated from dogs in many countries^{7,14,41}, though only one study reported the presence of a change at residue 324 (Tyr→Ile) in two new CPV-2a strains isolated from cats²³. Although this evidence was not sufficient to establish that residue 324 has impacted the parvovirus host range, the same mutation in CC-04/16-05 and LY-02/16 isolated from cats in China in this study provides support for the theory of position 324 being involved as a canine host-range mutation.

In addition, mutations Phe267Tyr and Thr440Ala, which have been described in many CPV-2a/2b/2c strains from dogs in various countries and in CPV strains isolated from cats, were present only in strain LY-02/16. Residue 440 is important because it is located at the main antigenic site of the virus, the top of the three-fold spike of the VP2 protein on the surface of the capsid^{17,28,35}. Mutation of this amino acid alters the antigenicity of CPV in its host and further explains the fact that it is not ideal for immunoprotection through CPV-2 vaccines.

Despite the many studies on FPV in China, analysis and molecular characterization of the VP2 gene and the complete genome are lacking, which may be related to the single genotype and serotype of FPV. In previous studies, some nucleotide mutations were used to denote phylogenetic classification for FPV strains globally¹. All FVPs grouped into three clusters, G1 (1521A), G2 (1521G) and G3 (246G, 699C and 1602G); the G3 cluster diverged first, followed by sequential divergence of the G2 and G1 clusters^{1,24}. In the current study, we first analysed mutations in the VP2 gene and the classification of FPV strains isolated in China. Phylogenetic analysis placed all FPV sequences in this study into the G1 cluster along with other FPV strains isolated in China, whereas two FPV vaccine strains were classified into the G2 cluster.

Although the antigenicity of FPV strains was not affected by these synonymous mutations, these mutations may increase the genetic complexity of the virus, and this might be an important reason for the limited effectiveness of vaccines against FPV^{3,18,19,32}. FPV strains isolated in China and FPV vaccine strains were placed into different branches, suggesting that the FPV strains in the G1 cluster are the dominant strains in China and that the dominant strains should be used as vaccine candidates to control the spread of FPV.

In conclusion, FPV, CPV-2a and CPV-2b were found in faecal samples and anal swabs collected from domestic kittens in China, and these strains show high homology with Asian epidemic parvovirus strains. Among them, FPV was the dominant strain causing severe enteric disease in kittens. Genetic analysis revealed that all of the FPV strains isolated in China can be grouped into the FPV G1 cluster and that novel CPV-2a/2b strains are present in cats in China. Therefore, genetic mutation and corresponding antigenic change, as a major mechanism of the emergence and evolution of newer parvovirus variants, should be monitored by large-scale molecular epidemiological investigation, which will be helpful for controlling the spread of FPV and CPV in cats worldwide.

Human and animal rights statement

This article does not contain any studies with animals performed by any of the authors.

Conflict of Interest

All authors declare that they have no competing interests.

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Supplemental data

Supplemental data associated with this article can be found, in the online version, at <http://dx.doi.org/10.14943/jjvr.66.3.145>

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