Infection, Genetics and Evolution 73 (2019) 242–247

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

A divergent canine parvovirus type 2c (CPV-2c) isolate circulating in China

Chuanfeng Li[a,*,1], Jingyu Tang[a,1], Zongyan Chen[a], Guangbin Niu[b], Guangqing Liu[a,*]

[a] Innovation Team of Small Animal Infectious Disease, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences(CAAS), Shanghai 200241, China
[b] Shanghai Center for Animal Disease Control and Prevention, Shanghai 201103, China

ARTICLE INFO

Keywords:
Canine parvovirus
Genomic characterization
Phylogenetic analysis
Purifying selection

ABSTRACT

The variability and the intrinsically high mutation rate of canine parvovirus type 2(CPV-2) increased the diversity of CPV-2 in canine populations. Since the first occurrence of CPV-2, three antigenic variants (2a, 2b and 2c) were detected and distributed worldwide. CPV-2c infection has been detected and increasingly reported in China. Here, a CPV-2c strain CPV-SH1516 was isolated and its complete genome sequence was first characterized. Compared with other CPV-2c isolates, CPV-2c isolates from China continued to evolve into divergent CPV-2c variants with specific unique amino acid substitutions under purifying selection. Emergence of CPV-2c isolates from China was driven by the unique gradual point mutations in key sites of VP2 rather than introduction from outside China. Combining sequence comparison with phylogenetic analysis based on the amino acid sequences of VP2, the vast majority of CPV-2c isolates from China formed a monophyletic cluster and CPV-SH1516 was a representative isolate of CPV-2c circulating in China. Overall, our study provides valuable insight into the evolutionary mechanism of CPV-2c.

1. Introduction

Canine parvovirus type 2 (CPV-2), belonging to the genus *Protoparvovirus* in the *Parvoviridae* family (http://ictvonline.org/virusTaxonomy.asp), can cause severe gastroenteritis and myocarditis in dogs, cat and wild carnivores. It contains a linear, single-stranded, negative-sense DNA genome of~5200 nucleotides that encodes four proteins containing two structural proteins (VP1 and VP2) and non-structural proteins (NS1 and NS2). Although CPV-2 is a DNA virus, it shows a very high genomic substitution rate similar to those of RNA viruses, with values of about 10^{-4} substitutions per site per year, which is responsible for rapid and continual antigenic evolution and emergence of new antigenic variants (Shackelton et al., 2005). Since its first emergence in 1978, CPV-2 has evolved giving rise to three main antigenic variants termed CPV-2a, CPV-2b and CPV-2c.

Although CPV-2c was first detected in Italy in 2000, it has circulated in Germany in 1996 on the basis of a retrospective analysis (Decaro et al., 2007). CPV-2c has also been increasingly detected in China in recent years (Geng et al., 2015; Zhao et al., 2017). Nevertheless, until now, there is no systematic analysis of the evolutionary dynamics of CPV-2c in China. Moreover, no full-length genome of CPV-2c has been reported in China as yet. In this study, we isolated a CPV-2c strain and determined its complete genome sequence for the first time. Further, the evolutionary characterization of CPV-2c isolates from China was elucidated in detail by comparing with other CPV-2c isolates available in the GenBank database.

2. Materials and methods

2.1. Clinical case and virus isolation

In October 2015, a clinical case characterized by depression, anorexia, vomiting and diarrhea was observed in a diseased dog (aged two months and unvaccinated) from Shanghai of China suspected CPV-2 infection by using a canine parvovirus antigen rapid test kit (Quicking biotech, Shanghai, China). To isolate the pathogen, the clarified small intestinal tissue homogenate was filtered through a 0.22-μm Millipore filter and inoculated into the Crandell-Rees feline kidney (CRFK) cells by using the synchronous inoculation method. The inoculated cells were maintained at 37 °C in a humidified 5% CO_2 incubator and monitored daily for cytopathic effect (CPE). The culture supernatants were harvested when CPE appeared in 70% of the cells.

2.2. Antigenic typing of CPV by PCR

To determine the antigenic subtype of the isolate, Total viral DNA

* Corresponding authors at: Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, No. 518 Ziyue Rd, Shanghai 200241, China.
E-mail addresses: lichuanfeng@shvri.ac.cn (C. Li), liugq@shvri.ac.cn (G. Liu).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.meegid.2019.05.004
Received 2 February 2019; Received in revised form 29 April 2019; Accepted 6 May 2019
Available online 08 May 2019

was extracted from the harvested cell cultures using an E.Z.N.A. ® Tissue DNA Kit (OMEGA, USA) according to the manufacturer's instructions. PCR reaction was performed as per the thermocycling conditions previously described by Buonavoglia et al. (2001).

2.3. Transmission electron microscopy

To confirm the morphology and size of the isolate, the harvested cell cultures were partially purified by ultracentrifugation through a 20% (wt/vol) sucrose cushion at 35000 rpm for 3 h at 4 °C. The purified sample was stained with 3% phosphotungstic acid (PTA) and observed by transmission electron microscope (H-7500, Hitachi, Japan).

2.4. Analysis of western blotting and indirect immunofluorescence assay

To confirm viral proliferation in CRFK cells, western blotting (WB) and Indirect immunofluorescence assay (IFA) were conducted with rabbit anti-VP2 polyclonal antibodies (diluted 1:200, prepared in our laboratory) and/or with anti β-Actin mouse monoclonal antibody in this study. The bar graph for WB was also drawn according to the relative gray values of VP2 /β-actin by using the softwares Image J and GraphPad Prism 6.

2.5. Complete genome sequencing and analysis

The complete genome sequence of the isolate was determined using five pairs of specific primers, described in our previous study (Li et al., 2018) and submitted to GenBank with accession number MG013488. The secondary structures at the both ends of CPV-SH1516 were predicted using the mfold Web Server (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form) and modified using RnaViz 2.0 software. The number of the reiteration of DNA sequences in the right end UTR of CPV-SH1516 was analyzed using the online program Tandem Repeats Finder (http://tandem.bu. edu/trf/ trf.basic. submit. html). Multiple Sequence alignments were performed using DNAStar software (DNASTAR Inc., Madison, WI, USA) by the Clustal W method. The analysis of Entropy values for NS1 and VP2 proteins was performed by the Entropy plot method using the BioEdit software. To examine the selection pressures during the CPV-2c genome evolution, the non-synonymous (dN) and synonymous (dS) ratio (dN/dS) was assessed using the Datamonkey web interface (http://classic.datamonkey.org/dataupload. php), a maximum-likelihood-based tool for the identification of sites selection pressure, with HKY85 as nucleotide substitution bias model. The single likelihood ancestor counting (SLAC) method was selected for analysis of sites subject to positive or negative selection.

2.6. Phylogenetic analysis of VP2 protein sequences

The phylogenetic relationship between CPV-2c isolates from China and other prototype CPV-2c isolates from outside China was evaluated by using the Molecular Evolutionary Genetics Analysis (MEGA) software (version 6). A phylogenetic tree based on CPV-2c VP2 protein sequences was constructed by using the neighbor-joining method with a Poisson model, based on 1000 bootstrap duplicates. Bootstrap values > 60% were considered to be significant (Fig. 3).

3. Results and discussion

3.1. Identification of CPV-SH1516

After two passages, CPE characterized by cell rounding, floating, increased granularity, and disintegration (Fig. 1A and B), was observed in infected CRFK cells at 72 hpi. Herein, this isolate was designated as CPV-SH1516. Based on the key aa mutation at residue 426 (Asp426Glu) in the VP2 protein of CPV-SH1516 (Buonavoglia et al., 2001), CPV-SH1516 was considered as the antigenic subtype CPV-2c. The isolated virus is small icosahedral, non-enveloped viral particles of~25 nm in diameter, typical of parvovirus under transmission electron microscope (Fig. 1C). The WB and IFA results all showed that the expression of capsid protein VP2 can be detected in virus infected cell culture specifically (Fig. 1D, E and F). These results indicated that a Chinese CPV-2c strain CPV-SH1516 was isolated and identified in CRFK cells successfully.

3.2. Genomic characterization of CPV-SH1516

The full-length genome of CPV-SH1516 was 5059 nucleotides (nts) long with G + C content of 37.05%. The left and right untranslated region (UTR) is 269nts and 521nts in length, respectively. The left ORFs encoded NS1 (668 aa) and NS2 (165 aa) proteins, and the right ORFs encoded VP1 (727 aa) and VP2 (584 aa) proteins (Fig. 2 A). The left-end inverted terminal repeat (ITR) (120 nt) of CPV-SH1516, similar with those of UFMT, CPV-b, Y1, CPV-SH14, LZ1 and LZ2 but different from CPV-N, CPV2a, and Laika-1993, can form a Y-shaped secondary structure with two small internal palindromes (“ears”) and a mismatched “bubble” region in the duplex stem region (Fig. 2 B). The left-end hairpin contains a mutation at nt 49(C49G) and a GC insertion at nt 53–54 compared with CPV-N, Laika-1993 and CPV2a. The left-end hairpin ears are essential for establishing a functional intranuclear transcription template and for progeny genome encapsidation (Cotmore and Tattersall, 2013). Additionally, the presence of this mismatched “bubble” has been proved to be necessary for efficient parvoviral DNA replication (Costello et al., 1995). Remarkably, the right-end ITR (197 nt) of CPV-SH1516 can form a U-Shaped structure with a small asymmetric “bubble” region of three unpaired nts, AGA, in the duplex stem region(Fig. 2 B), and has no any tandem repeat DNA sequences, while some CPV isolates, such as UFMT, CPV-N, Y1 and CPV-b, have three, two, one and one repeat sequence, respectively.

3.3. Evolutionary analysis of CPV-2c isolates from China

Currently, the NS1 genes of CPV-2c strains CPV-SH1516 and WANGQING-1 from China are available in the GenBank database. Compared with NS1 genes of other known CPV-2c isolates, the four aa substitutions (I60V, YE 544–545 FV, and L630P) were observed in CPV-SH1516 and WANGQING-1. These aa mutations have been reported in Chinese new CPV-2a isolates (Wang et al., 2016). The analysis of entropy of NS1 protein also showed that these aa substitutions have higher entropy values (data not shown), indicating their high variability and residue 351 has the highest values (0.55615), suggesting the potential mutation site in Chinese isolates. Based on a previous study about the organization of functional domains in CPV NS1(Niskanen et al., 2010), the aa residues 60 and 630 were located at the N-terminal origin of replication (aa residues from 16 to 275) and the C-terminal transactivation domain (aa residues from 600 to 667), respectively, while the aa residues 544 and 545 were positioned at the predicted helicase domain of the Parvovirus NS1 protein (aa residues from 279 to 546) using the protein sequence analysis & classification program InterPro (http://www.ebi.ac.uk/interpro/scan.html, data not shown). Although limited CPV-2c isolates from China available for analysis, it can be inferred that the mutations mentioned may affect the viral replication and transcription efficiency.

To trace the evolutionary process of VP2 genes of CPV-2c isolates from China, the discrepancy of aa mutations at sites 5, 267, 324 and 370 of VP2 protein was analyzed in this study (Table 1). Compared with the original CPV-2c isolates including strains G7/97, G172/97, 56/00, 136/00, 03B10 and 67/06 from European countries, the earliest Chinese CPV-2c isolate 08/09 in 2009 had no any aa mutation, whereas the Chinese isolates G1 and 06/09 had aa substitutions F267Y and Y324I in 2009 respectively, which were also appeared in subsequent CPV-2c isolates from China. From the year 2014, the CPV-2c isolates from China WANGQING-1, YANJI-1 and HRB-A6 possessed additional aa

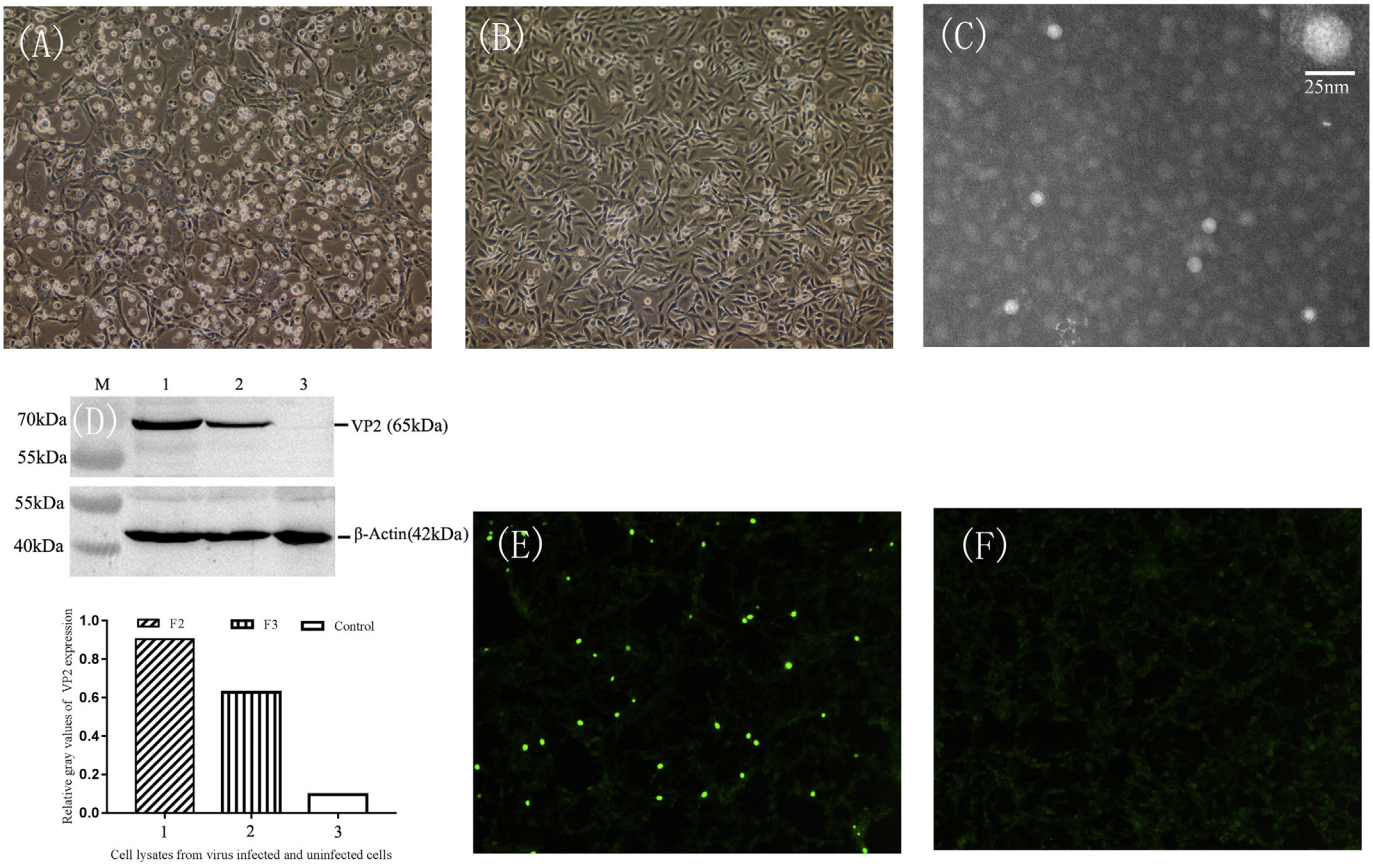

Fig. 1. Isolation and identification of CPV strain CPV-SH1516. (A) Cytopathic effect (CPE) of CRFK cells infected with the isolated virus at 72 hpi (100×). (B) Morphology of the control CRFK cells mock-infected with the isolated virus (100×). (C) Transmission electron microscopic image of the purified isolate negatively stained with 3% phosphotungstic acid (PTA). The scale bar represents 100 nm. (D) Western blot analysis of the total cell lysates from virus infected cells (Lane 1 and 2 for the second and third passages, respectively) and uninfected cells (Lane 3) using the rabbit anti-VP2 polyclonal antibody (the above panel) and anti-β-Actin mouse monoclonal antibody (the below panel, a protein- loading control), respectively. Lane M, prestained protein molecular weight marker. The corresponding bar graph for Western blotting was drawn according to the relative gray values of VP2 /β-actin by using the softwares Image J and GraphPad Prism 6. Its marks were the same as that of Western blotting. (E) Immunofluorescence staining of CRFK cells infected with the isolated virus using the rabbit anti-VP2 polyclonal antibody. CPV-specific green fluorescence was observed in the infected cells (100×). (F) Uninfected CRFK cells conserved as a negative control. No any fluorescence was observed in the uninfected cells (100×).

substitution Q370R. In the year 2014 and later, all known CPV-2c isolates from China, such as BJ14–8 and CPV-SH1516, had an additional aa mutation A5G, which was not included in the mutations of CPV-2c strains by Zhao et al. (2017). Until now, the four aa mutations together appeared in CPV-SH1516 and other recent CPV-2c isolates from China (Zhuang et al., 2019). Previous study has shown that the aa residue 5 was one of the surface and core residues in the antigenic sites (Langeveld et al., 1993). Therefore, this mutation of A5G may have changed the antigenicity and immunogenicity. The other aa residues, F267Y, Y324I and Q370R, were located in the greatest variable GH loop (loops 3 and 4) comprising aa 267–498 of VP2 protein (Agbandje et al., 1995). The last two aa residues were considered to be associated with hemagglutinating activity or canine TfR binding ability. In addition, the analysis of entropy of VP2 protein also showed that the entropy values of the above four aa sites were top four with higher values (data not shown). These results indicated that CPV-2c isolates from China including CPV-SH1516 continued to evolve by the unique gradual point mutations in key sites of VP2 during the progress of local adaption.

The selective pressures on the NS1 and VP2 genes of CPV-2c were assessed by calculating the ratio of dN/dS. The mean dN/dS values for NS1 and VP2 were 0.145911 and 0.08 (dN/dS $<$ 1), respectively. This result indicates that NS1 and VP2 were under purifying selection. For aa sites pressure analysis of NS1 and VP2, no positively selected site was identified in all CPV-2c isolates by the SLAC method with the default significance level of 0.1. And three (residues 137, 242 and 528) and twelve (residues 12, 45, 69, 82,101,197,260,273,321,366,503 and 553) negatively selected sites were respectively predicted when a significance level of 0.1. These results suggested that those specific aa mutations involved in NS1 and VP2 were not induced by host immune pressure and have been fixed to make them to be CPV-2c variants with higher fitness. In previous studies, A few positively selected sites of VP2 were found when selection pressure was analyzed between feline panleukopenia virus (FPLV) and CPV or among distinct antigenic subtypes of CPV (Hoelzer et al., 2008). This indicated that these Chinese isolates were only divergent CPV-2c variants.

3.4. Phylogenetic analysis of CPV-2c isolates

Although it had been reported that CPV-2c isolates can form a monophyletic group in the VP2 phylogenetic tree (Zhao et al., 2017; Zhuang et al., 2019), this result was based on a limited number of CPV-2c isolates from China or CPV-2c isolates from Asia. Therefore, it can't reflect the phylogenetic relations between CPV-2c isolates from China and other countries. In this study, phylogenetic analysis of the VP2 amino acid sequence showed that all CPV-2c isolates from China except for the earliest Chinese CPV-2c strain 08/09 formed a monophyletic cluster distant from the prototypical CPV-2c strains from European countries. Interestingly, CPV-2c strain 08/09 was clustered with

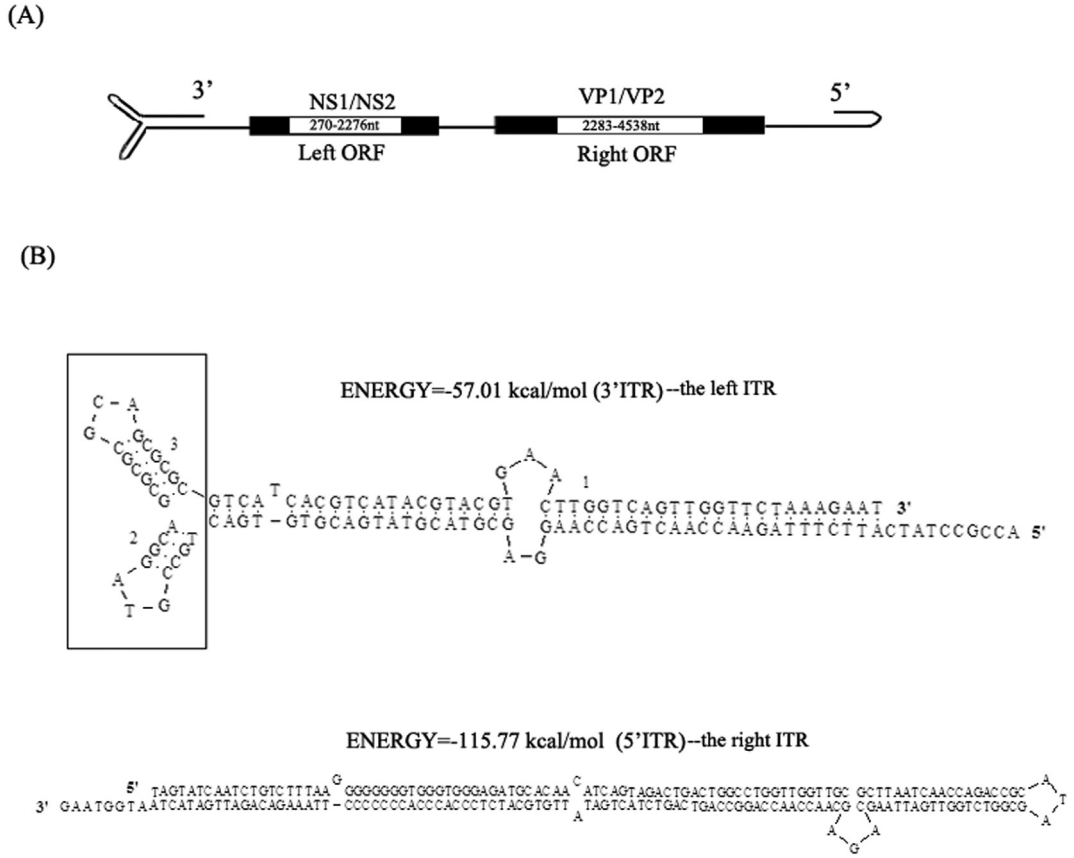

Fig. 2. Genome organization schematic diagram and the predicted secondary structures at the both ends of CPV-SH1516. (A) The CPV-SH1516 genome is represented in the 3′ → 5′ orientation and similar to the classic CPV genome organization. The 3′(left-end) and 5′(right-end) inverted terminal repeats (ITRs) of CPV-SH1516 form two hairpin structures, respectively. The left ORFs located between nt 270 and 2276 encoded NS1 (668 aa) and NS2 (165 aa) proteins, and the right ORFs ranged from nt 2283 to nt 2276 encoded VP1 (727 aa) and VP2 (584 aa) proteins. (B) The secondary structures of the ITRs located at each end of the CPV-SH1516 were predicted based on the minimal free energy (△G). The left-end ITR (120 nt) of CPV-SH1516 can fold into a Y-shaped hairpin while the right-end one (197 nt) can fold into a U-Shaped structure.

Table 1
Amino acid mutations in the VP2 genes of representative CPV-2c isolates analyzed in this study.

Strains	Origin	Year	Position of amino acid substitution				Accession number
			5	267	324	370	
G7/97	Germany	1997	Ala (A)	Phe (F)	Tyr (Y)	Gln (Q)	FJ005196
G172/97	Germany	1997	A	F	Y	Q	FJ005199
56/00	Italy	2000	A	F	Y	Q	FJ222821
136/00	Italy	2000	A	F	Y	Q	FJ005195
03B10	France	2005	A	F	Y	Q	DQ025951
67/06	Spain	2006	A	F	Y	Q	FJ005214
08/09	China	2009	A	F	Y	Q	GU380305
G1	China	2009	A	Tyr (Y)	Y	Q	KF482468
06/09	China	2009	A	Y	Ile (I)	Q	GU380303
WANGQING-1	China	2014	A	Y	I	Arg (R)	KP749851
YANJI-1	China	2014	A	Y	I	R	KP749854
HRB-A6	China	2014	A	Y	I	R	KT156832
BJ14-8	China	2014	Gly (G)	Y	I	R	KT162005
GY-4	China	2015	G	Y	I	R	KY386853
CPV-GX1581	China	2015	G	Y	I	R	MF467242
CPV-JS1592	China	2015	G	Y	I	R	MF467225
CPV-SH1516	China	2015	G	Y	I	R	MG013488

European original CPV-2c isolates (Fig. 3). These results indicated that CPV-2c isolates from China were likely to derive from local adaption of original CPV-2c isolates rather than introduction from other countries and CPV-SH1516 isolated in this study was a representative isolate of CPV-2c circulating in China.

4. Conclusions

In conclusion, the first complete genome sequence of a CPV-2c isolate from China was determined and characterized in this study. Genetic and selective pressure analysis based on the NS1 and VP2 showed that CPV-2c isolates from China continued to evolve into divergent CPV-2c variants with specific unique aa substitutions under purifying selection, which might provide a selective advantage during viral local adaptation. Sequence comparison showed that the local adaption of CPV-2c isolates from China was driven by the unique gradual point mutations in key sites of VP2. Combining the phylogenetic analysis based on the VP2 aa sequences, the vast majority of CPV-2c isolates from China formed a monophyletic cluster and CPV-SH1516 was a representative isolate of CPV-2c circulating in China.

Conflicts of interest

The authors declare that they have no competing interests.

Author contributions

CF Li and GQ Liu conceived and designed the details of the

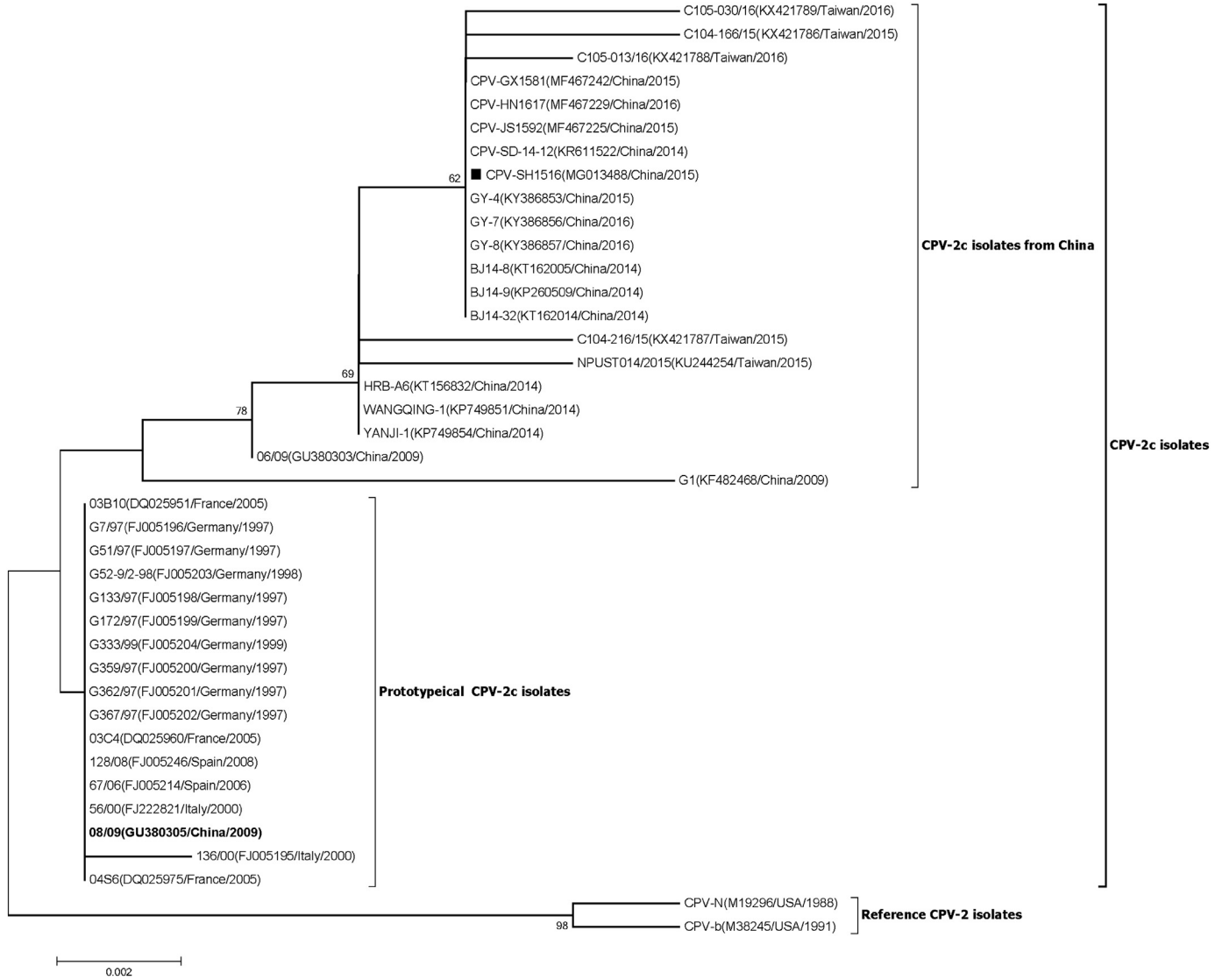

Fig. 3. Phylogenetic analysis based on the VP2 protein sequences of CPV-2c isolates from China and other prototype CPV-2c isolates. A phylogenetic tree was constructed based on the complete VP2 protein sequences using the neighbor-joining method with 1000 bootstrap replicates and Poisson model in the MEGA 6.0 software package. Scale bar indicates amino acid substitutions per site. CPV-SH1516 isolated in this study is labeled with a black solid square (■). The reference CPV-2 isolates, CPV-N and CPV-b, were used to root the evolutionary tree. GenBank accession numbers, locations and collecting time of CPV-SH1516 and the reference strains are indicated following by virus strains in the branches.

experiments. JY Tang performed the data collection and experiment. CF Li analyzed the data and wrote the manuscript; ZY Chen and GB Niu assisted in the experimental operations and revision of the manuscript.

Acknowledgements

This study was funded by the National Key Research and Development Program of China (No. 2016YFD0501003), the Shanghai Science and Technology Promotion Agriculture Innovation Program (2019No.3-3) and the Fundamental Research Funds for the Central Institutes Program (No. 2016JB12).

References

Agbandje, M., Parrish, C.R., Rossmann, M.G., 1995. The structure of parvoviruses. Semin. Virol. 6, 299–309.

Buonavoglia, C., Martella, V., Pratelli, A., Tempesta, M., Cavalli, A., Buonavoglia, D., Bozzo, G., Elia, G., Decaro, N., Carmichael, L., 2001. Evidence for evolution of canine parvovirus type 2 in Italy. J. Gen. Virol. 82, 3021–3025. https://doi.org/10.1099/0022-1317-82-12-302.

Costello, E., Sahli, R., Hirt, B., Beard, P., 1995. The mismatched nucleotides in the 5′-terminal hairpin of minute virus of mice are required for efficient viral DNA replication. J. Virol. 69, 7489–7496.

Cotmore, S.F., Tattersall, P., 2013. Parvovirus diversity and DNA damage responses. Cold Spring Harb. Perspect. Biol. 5, a012989. https://doi.org/10.1101/cshperspect.a012989.

Decaro, N., Desario, C., Addie, D.D., Martella, V., Vieira, M.J., Elia, G., Zicola, A., Davis, C., Thompson, G., Thiry, E., Truyen, U., Buonavoglia, C., 2007. Molecular epidemiology of canine parvovirus, Europe. Emerg. Infect. Dis. 13, 1222–1224. https://doi.org/10.3201/eid1308.070505.

Geng, Y., Guo, D., Li, C., Wang, E., Wei, S., Wang, Z., Yao, S., Zhao, X., Su, M., Wang, X., Wang, J., Wu, R., Feng, L., Sun, D., 2015. Co-circulation of the rare CPV-2c with unique Gln370Arg substitution, new CPV-2b with unique Thr440Ala substitution, and new CPV-2a with high prevalence and variation in Heilongjiang Province, Northeast China. PLoS One 10, e0137288. https://doi.org/10.1371/journal.pone.0137288.

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

Langeveld, J.P., Casal, J.I., Vela, C., Dalsgaard, K., Smale, S.H., Puijk, W.C., Meloen, R.H., 1993. B-cell epitopes of canine parvovirus: distribution on the primary structure and exposure on the viral surface. J. Virol. 67, 765–772.

Li, C., Tang, J., Chen, Z., Li, Q., Huang, Z., Wang, Q., Meng, C., Wang, Y., Liu, G., 2018. Genetic characterization of the complete genome of a mutant canine parvovirus isolated in China. Arch. Virol. 163, 521–525. https://doi.org/10.1007/s00705-017-3586-8.

Niskanen, E.A., Ihalainen, T.O., Kalliolinna, O., Hakkinen, M.M., Vihinen-Ranta, M., 2010. Effect of ATP binding and hydrolysis on dynamics of canine parvovirus NS1. J. Virol. 84, 5391–5403. https://doi.org/10.1128/JVI.02221-09.

Shackelton, L.A., Parrish, C.R., Truyen, U., Holmes, E.C., 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.

Wang, H., Jin, H., Li, Q., Zhao, G., Cheng, N., Feng, N., Zheng, X., Wang, J., Zhao, Y., Li, L., Cao, Z., Yan, F., Wang, L., Wang, T., Gao, Y., Yang, S., Xia, X., 2016. Isolation and sequence analysis of the complete NS1 and VP2 genes of canine parvovirus from domestic dogs in 2013 and 2014 in China. Arch. Virol. 161, 385–393. https://doi.org/10.1007/s00705-015-2620-y.

Zhao, H., Wang, J., Jiang, Y., Cheng, Y., Lin, P., Zhu, H., Han, G., Yi, L., Zhang, S., Guo, L., Cheng, S., 2017. Typing of canine parvovirus strains circulating in north-East China. Transbound. Emerg. Dis. 64, 495–503. https://doi.org/10.1111/tbed.12390.

Zhuang, Q., Qiu, Y., Pan, Z., Wang, S., Wang, B., Wu, W., Yu, J., Yi, Y., Sun, F., Wang, K., 2019. Genome sequence characterization of canine parvoviruses prevalent in the Sichuan province of China. Transbound. Emerg. Dis. 66, 897–907. https://doi.org/10.1111/tbed.13100.