Infection, Genetics and Evolution 73 (2019) 269–275

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

Research paper

The first isolation and identification of canine parvovirus (CPV) type 2c variants during 2016–2018 genetic surveillance of dogs in Mongolia

Uyangaa Temuujin[a,1], Ariunaa Tserendorj[a,1], Jumpei Fujiki[b], Yoshihiro Sakoda[c], Erdene-Ochir Tseren-Ochir[a], Masatoshi Okamatsu[c], Keita Matsuno[c], Tumenjargal Sharav[a], Motohiro Horiuchi[d], Takashi Umemura[e], Tungalag Chultemdorj[a,*]

[a] Department of Infectious Disease and Microbiology, School of Veterinary Medicine, Mongolian University of Life Sciences, Ulaanbaatar, Zaisan 17024, Mongolia
[b] Laboratory of Veterinary Biochemistry, Department of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
[c] Laboratory of Microbiology, Faculty of Veterinary Medicine, Hokkaido University, Hokkaido 060-0818, Japan
[d] Laboratory of Veterinary Hygiene, Faculty of Veterinary Medicine, Hokkaido University, Hokkaido 060-0818, Japan
[e] JICA Expert in School of Veterinary Medicine, Mongolian University of Life Sciences, Mongolia

ARTICLE INFO

Keywords:
Canine parvovirus
Sequencing
Phylogenetic analysis
Molecular characterization

ABSTRACT

Canine parvovirus type 2 (CPV-2) causes a highly contagious and fatal disease, developing into acute hemorrhagic enteritis and myocarditis, in dogs. CPV-2 has evolved, generating antigenic variants CPV-2a/2b/2c that are globally distributed. However, investigating molecular characterization of CPV-2 among dog populations in Mongolia has been limited. Herein, 42 stool samples were collected from dogs with clinical signs of infection, and conventional PCR assays were employed to detect CPV-2 in 23. Our results indicated that during 2016–2018, the new CPV-2a and 2c subtypes were detected in 34.7% of the samples, and the new CPV-2b subtype was detected in 30.4% of samples. VP2 protein sequence analysis and next-generation sequencing of the complete viral genome confirmed these antigenic types. However, sequence analysis indicated new and unreported mutations, Pro580Thr, and Tyr584His in the CPV-2c subtype. From a PCR-positive sample, CPV-2c was successfully isolated, and we performed an immunofluorescence assay for antigen detection. Additionally, we performed genetic characterization and phylogenetic analysis to investigate genetic diversity among isolates from the region, resulting in high CPV-2 genetic diversity in the Mongolian dog population. Striking similarities were also observed between sequences of the strains isolated from Mongolia and China over a similar time span.

1. Introduction

Canine parvovirus type 2 (CPV-2) is a significant virus of canines worldwide and was first described in 1978 (Carmichael, 2005). This non-enveloped single-stranded DNA virus belongs to the *Parvoviridae* family and has an approximately 5.2 kb long genome, which encodes two capsid proteins (VP1 and VP2) and two non-structural proteins (NS1 and NS2). There is also a third protein, VP3, which is produced by proteolytic processing of VP2 (Mohan Raj et al., 2010). CPV-2 is closely related to feline panleukopenia virus (FPV) and to parvoviruses isolated from raccoons, minks, and arctic foxes (Parrish, 1999; Truyen and Parrish, 1995), with nucleotide variation from FPV being lower than 0.5%.

Since its global emergence, highly contagious CPV-2 causes acute gastroenteritis and lymphopenia in young dogs and is endemic in the most wild and domestic dog populations. Now, new antigenic types, CPV-2a, 2b, and 2c, are circulating worldwide (Decaro et al., 2006; Mohan Raj et al., 2010; Decaro and Buonavoglia, 2012; Sharma et al., 2016; Zhao et al., 2017; Mira et al., 2018; Clark et al., 2018).

Genetic variation among CPV-2 isolates has been used to further classify the virus among these three antigenic variants that differ in their amino acid sequences and VP2 gene structure (Amrani et al., 2016; Buonavoglia et al., 2001; Mira et al., 2018; Mohan Raj et al., 2010). All the three antigenic variants differ from wild-type CPV-2, except for a few amino acids in the VP2 protein, whereas genetic differences among the variants are located at amino residue 426, with types 2a, 2b, and 2c displaying Asn, Asp, and Glu residues, respectively (Martella et al., 2006). Additional amino acid changes at position 297

* Corresponding author at: Department of Infectious Disease and Microbiology, School of Veterinary Medicine, Mongolian University of Life Sciences, 17024 Khan-Uul district, Zaisan, Ulaanbaatar, Mongolia.
E-mail addresses: uyangaa_vet@muls.edu.mn (U. Temuujin), tungalag_vet@muls.edu.mn (T. Chultemdorj).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.meegid.2019.05.006
Received 7 January 2019; Received in revised form 5 April 2019; Accepted 7 May 2019
Available online 09 May 2019
1567-1348/

(Ser → Ala) were observed in both CPV-2a and CPV-2b. Residue 297 is located in a minor antigenic site close to epitope B, and substitutions at this position may be responsible for changes in antigenicity of CPV variants (Truyen, 2006). Canine parvovirus type 2a/2b with a mutation at 297 (Ser → Ala) is designated as New CPV-2a/2b (Decaro et al., 2006; Martella et al., 2005; Mohan Raj et al., 2010; Ohshima et al., 2008). These CPV variations are present worldwide (Zhang et al., 2010; Decaro and Buonavoglia, 2012; Wang et al., 2015; Parker et al., 2017).

Most of the commercial vaccines currently available are based on CPV-2 or CPV-2b, and several studies have demonstrated that they are able to cross-protect against all antigenically distinct types (Spibey et al., 2008; Siedek et al., 2011; Reitzenstein et al., 2012; Hernandez-Blanco and Catala-Lopez, 2015). However, CPV-2 infections are still ubiquitous in dogs, which could potentially indicate failure of vaccination (Mittal et al., 2014). Therefore, the identification and monitoring of CPV-2 types circulating in the canine population are important in understanding its evolution and the development of control measures, including vaccination enhancements (Pinto et al., 2012). However, molecular characterization of CPV-2 viruses among dog populations in Mongolia is limited. To date, studies on CPV-2 in Mongolia include high infection rates reported via rapid diagnostic kits (Tserendorj, A., unpublished data) and CPV-2a and 2b types first detected by conventional PCR from stool samples collected in 2013 (Sharav et al., 2015).

Here, we analyzed molecular characteristics and genetic diversity of CPV-2 isolates from stool samples collected between 2016 and 2018 in Ulaanbaatar, Mongolia, to better understand CPV-2 epidemiology and shed light on better control measures. In addition, this is the first report of the isolation and full genome characterization of CPV-2c variant CPV/MGL/A16–5/2016 in Mongolia.

2. Material and methods

2.1. Sample collection and viral DNA detection

Stool samples ($n = 42$) were collected from dogs showing symptoms of fever, diarrhea, or vomiting, or hemorrhagic symptoms, from various animal hospitals in Ulaanbaatar, Mongolia, between September 2016 and January 2018. The collected stool samples were kept in coolers during transport to the laboratory and immediately stored at −20 °C until analysis. Viral DNA was extracted from dog stool samples using Wizard® genomic DNA purification kit (Promega, USA) according to the manufacturer's protocol.

Conventional PCR using primer sets (Table 1) amplifying a 452 bp fragment of the CVP-2 VP2 gene and GoTaq® Green Master Mix (Promega, USA) was performed to detect presence of viral DNA (Kariatsumari et al., 1991). Reactions, each 25 μL, were performed using a SimpliAmp™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA) and the following program: 5 min at 98 °C and 40 cycles of 10 s at 98 °C, 15 s at 55 °C, 30 s at 68 °C, and 3 min at 68 °C. Negative control (DNA from a stool sample of an apparently healthy dog) and positive control (DNA from pMEV; Horiuchi et al., 1994) were used.

PCR was employed to perform CPV-2 subtyping of positive samples. Briefly, GoTaq® DNA Polymerase (Promega, USA) was used along with CPV-2 primers designed to amplify specific regions of the VP2 gene (Table 1), which were previously described in (Pereira and Durigon, 2000; Sharma et al., 2016).

2.2. VP2 gene sequence and phylogenetic analyses

DNA from positive samples was amplified via PCR using TaKaRa Ex Taq TM DNA Polymerase (Takara Bio Inc.) and Hfor/Hrev and 555for/555rev primers designed to amplify a specific region of the capsid gene that is critical to biological properties of the virus (Parker and Parrish, 1997; Parrish et al., 1991) (Table 1). These primers were previously described (Buonavoglia et al., 2001). Nucleotide sequencing was performed at Macrogen Inc. (Seoul, Rep. of Korea), and DNA sequence raw data were aligned and edited with Geneious 8.1.9 (Biomatters Limited, Auckland, New Zealand). Nucleotide sequences of the VP2 gene and deduced protein sequences were compared with other parvovirus isolates retrieved from the GenBank database. They were aligned and analyzed using the Clustal Omega multiple sequence alignment in EMBL-EBI.

A preliminary phylogenetic tree was constructed with 98 sequences (> 900 nucleotides) that corresponded to the partial coding sequence of VP2 gene (available on GenBank as of 10/09/2018) and 11 isolates from this study. The sequences were aligned using BioEdit 7.2.5, and manual editing and tree reconstruction were performed with Molecular Evolutionary Genetics Analysis (MEGA) 7.0 software (Kumar et al., 2016) using a neighbor-joining (NJ) method with the Kimura 2-parameter model. A total of 1000 replicates were used to generate bootstrap values.

We selected VP2 gene sequences (> 900 nucleotides) from 11 CPV-2 isolates based on our preliminary phylogenetic analysis, antigenic diversity, and genetic relations. These were analyzed together with the sequences of the 11 CPV-2 viruses collected from 2016 to 2018 to construct NJ tree with MEGA 7.0 software using the Maximum Composite Likelihood method. Bootstrap analyses were performed with 1000 replicates (Kumar et al., 2016).

2.3. Complete genome sequencing

On the basis of VP2 gene sequence analysis, DNA from a selected CPV-2c strain was used to prepare sequencing libraries with NEB Next Ultra DNA Library Prep Kit and Multiplex Oligos for Illumina (New England Biolabs, Ipswich, MA, USA), and the complete genomic sequences of selected viruses were determined by next-generation sequencing (NGS) using the MiSeq (Illumina)™ sequencer platform. The annotation was performed by CLC Genomics Workbench 11.0.1 (CLC bio, Aarhus, Denmark) using a de novo assembly method.

Table 1
Primers used in this study.

Primer	Detection of genotype	Primer sequence (5′-3′)	Position (nt)	Genome location	Amplicon size (bp)	Reference
V1	VP2	GTACATTTAAATATGCCAGA	3029–3048	VP2	452	Kariatsumariet al. (1991)
V52		ATTAATGTTCTATCCCATTG	3459–3479			
CPV-2abs	CPV-2a	GAAGAGTGGTTGTAAATAATT	3025–3045		681	Pereira et al. (2000)
CPV-2abas		CCTATATAACCAAAGTTAGTAC	3685–3706			
CPV-2bs	CPV-2b	CTTTAACCTTCCTGTAACAG	4043–4062		427	Pereira et al. (2000)
CPV-2bas		CATAGTTAAATTGGTTATCTAC	4449–4470			
Hfor	Only used for sequencing	CAGGTGATGAATTTGCTACA	3556–3575		629	Buonavoglia et al. (2001)
Hrev		CATTTGGATAAACTGGTGGT	4166–4185			
555for		CAGGAAGATATCCAGAAGGA	4003–4022		583	Buonavoglia et al. (2001)
555rev		GGTGCTAGTTGATATGTAATAAACA	4561–4585			

Table 2
Age, sex, breed, vaccination status, and genotypes of Mongolian dogs positive for canine parvovirus.

№	Sample ID	Date	Age (months)	Sex	Breed	Vaccination status	Genotype	Animal location/animal hospital	Accession number
1	A16–1	5-Sep-16	3	M	Unknown	−	2b	Khan-uul/ Amar	
2	A16–4	8-Sep-16	2	M	Unknown	−	2b	Bayanzurkh/Amar	
3	A16–5	15-Sep-16	3	F	Unknown	−	2c	Sukhbaatar/Amar	MH660909 /NGS/ MK241946 /Partial/
4	A16–6	3-Oct-16	4	M	Caucasian shepherd	−	2a	Khan-uul/Amar	MK241940
5	A16–8	8-Oct-16	3	M	German shepherd	+	2b	Bayangol/Amar	
6	A16–9	11-Oct-16	4	M	Unknown	N/A	2c	Chingeltei /Amar	MK241944
7	A16–10	14-Oct-16	4	F	Beagle	−	2b	Chingeltei /Amar	
8	A16–11	11-Nov-16	4	F	Unknown	±	2b	Khan-uul/Amar	
9	S17–2	3-Jun-17	4	F	Caucasian shepherd	−	2c	Bayangol/SOS	MK241945
10	S17–3	3-Jun-17	4	F	Rottweiler	−	2a	Bayangol/SOS	
11	S17–5	9-Jun-17	3	M	German shepherd	−	2b	Bayanzurhk/SOS	MK241943
12	S17–7	15-Jun-17	6	M	Caucasian shepherd	+	2b	Bayangol/SOS	MK241942
13	S17–10	16-Jun-17	3	M	German shepherd	−	2a	Bayangol/SOS	
14	S17–12	19-Jun-17	4	F	Tibetan mastiff	±	2a	Suhkbaatar/SOS	
15	S18–1	4-Jan-18	2	F	Siberian husky	N/A	2c	Chingeltei /SOS	MK241947
16	S18–2	5-Jan-18	2	F	Central Asian Ovcharka	+	2a	Bayangol/SOS	
17	S18–3	8-Jan-18	3	M	Rottweiler	+	2a	Songinokhairkhan/SOS	MK241941
18	S18–4	9-Jan-18	4	F	Mongolian Bankhar	+	2c	Sukhbaatar/SOS	MK241948
19	S18–5	14-Jan-18	4	F	Central Asian Ovcharka	N/A	2c	Bayanzurkh/SOS	
20	S18–6	16-Jan-18	4	M	Central Asian Ovcharka	+	2a	Chingeltei /SOS	
21	S18–7	22-Jan-18	3	M	Siberian husky	+	2a	Bayanzurkh/SOS	
22	S18–8	26-Jan-18	3	M	Mongolian	+	2c	Bayanzurkh/SOS	MK241949
23	S18–9	27-Jan-18	3	M	Golden retrievers	−	2c	Bayanzurkh/SOS	MK241950

N/A = not available; ‘+’ = complete vaccination; ‘ ± ’ = incomplete vaccination; ‘-‘= unvaccinated; M = male; F = female.

Table 3
Amino acid residues in the VP2 protein.

Country, virus	Year	Amino acid at residue							Accession number
		274	297	300	426	555	580	584	
USA, FPV	1967	Arg	Ser	Ala	Asn	Val	Pro	Tyr	EU659111
USA, CPV-2	1979	Arg	Ser	Ala	Asn	Val	Pro	Tyr	EU659116
USA, CPV-2a	1984	Arg	Ser	Gly	Asn	Ile	Pro	Tyr	M24003
USA, CPV-2b	2000	Arg	Ala	Gly	Asp	Val	Pro	Tyr	EU659119
CPV-2a subtype									
Italy	2014	Arg	Ala	Gly	Asn	Val	Pro	Tyr	MH491880
China	2011	Arg	Ala	Gly	Asn	Val	Pro	Tyr	JX660690
	2010	Arg	Ala	Gly	Asn	Val	Pro	Tyr	MH685920
	2013	Arg	Ala	Gly	Asn	Val	Pro	Tyr	KF676668
	2015	Arg	Ala	Gly	Asn	Val	Pro	Tyr	MG583676
India	2012	Arg	Ala	Gly	Asn	Val	Pro	Tyr	KF772940
	2018	Arg	Ala	Gly	Asn	Val	Pro	Tyr	MH545963
Thailand	2015	Arg	Ala	Gly	Asn	Val	Pro	Tyr	KP715681
Singapore	2016	Arg	Ala	Asp	Asn	Val	Pro	Tyr	KX618915
Mongolia	2016	Arg	Ala	Gly	Asn	Val	Pro	Tyr	MK241940
	2018	Arg	Ala	Gly	Asn	Val	Pro	Tyr	MK241941
CPV-2b subtype									
China	2011	Arg	Ala	Gly	Asp	Val	Pro	Tyr	JQ268284
	2016	Arg	Ala	Gly	Asp	Val	Pro	Tyr	KY937663
Mongolia	2017	Arg	Ala	Gly	Asp	Val	Pro	Tyr	MK241942, MK241943
CPV-2c subtype									
Vietnam	2013	Arg	Ala	Gly	Glu	Val	Pro	Tyr	LC214969
Indonesia	2013	Arg	Ala	Gly	Glu	Val	Pro	Tyr	LC216910
China									
	2016	Arg	Ala	Gly	Glu	Val	Pro	Tyr	MF001435, MF001437
	2017	Arg	Ala	Gly	Glu	Val	Pro	Tyr	MF347724, MH155193
Tailand	2016	Arg	Ala	Gly	Glu	Val	Pro	Tyr	MH711902
	2016	Arg	Ala	Gly	Glu	–	–	–	MH711893
Italy	2008	Arg	Ala	Gly	Glu	Val	Pro	Tyr	FJ005245
Greese	2009	Arg	Ala	Gly	Glu	Val	Pro	Tyr	GQ865519
Uruguay	2011	Arg	Ala	Gly	Glu	Val	Pro	Tyr	KM457142
Croatia	2014	Arg	Ala	Gly	Glu	Val	Pro	Tyr	KP859576
Brazil	2015	Arg	Ala	Gly	Glu	Val	Pro	Tyr	KY073269
USA	2015	Arg	Ala	Gly	Glu	Val	Pro	Tyr	MF457594
Mongolia	2016	Arg	Ala	Gly	Glu	Val	Pro	Tyr	MK241946
	2017	Lys	Ala	Gly	Glu	Val	Pro	Tyr	MK241945
	2016	Arg	Ala	Gly	Glu	Val	Thr	Tyr	MK241944
	2018	Arg	Ala	Gly	Glu	Val	Pro	Tyr	MK241947, MK241949, MK241950
	2018	Arg	Ala	Gly	Glu	Val	Pro	His	MK241948

2.3.1. Database accession numbers

Eleven ($n = 11$) sequences for the VP2 gene were submitted to GenBank under accession numbers MK241940-MK241950, and complete genome sequences were submitted with accession number MH660909 (Table 2).

2.4. Virus isolation in cell culture and immunofluorescence assay (IFA)

Crandell feline kidney (CRFK) cells were grown in 60 mm Petri dishes containing Eagle's Minimal Essential Medium (*E*-MEM, Corning USA) with 10% fetal bovine serum (FBS, Invitrogen, USA) at 37 °C with 5% CO_2. For isolation of CPV-2 using cell culture, three PCR-positive samples were inoculated onto CRFK monolayers, 70–80% confluency. The infected monolayers were incubated for 48–72 h and harvested and screened by PCR assays and immunofluorescence to confirm the presence of infectious virus.

IFA was conducted to detect specific CPV-2 antigen in CRFK cells. Low-density CRFK cells were prepared 24 h after incubation. Then, the medium was removed from each dish, and 400 µL of isolated parvovirus was added. After incubation at 37 °C for 48 h, CRFK cells were harvested; each sample was suspended with 100 µL PBS. The negative controls of PBS and parvovirus samples were added onto slides. Acetone fixation was performed at −30 °C for 30 min. For the blocking process, 5% FBS was added, and slides were incubated at room temperature for 30 min. IFA was performed using the P2–284 (1 µg/mL) monoclonal antibody (Horiuchi et al., 1997) as the primary antibody and Alexa Fluor 488 goat anti-Mouse IgG (1 µg/mL) for the secondary antibody. Finally, coverslips were mounted with a drop of mounting medium. The image of infected cells were taken using a Nikon Eclipse E200 fluorescence microscope under a 100× objective lens.

3. Results

3.1. Detection and characterization of CPV-2

A total of 23 CPV-2 positive samples were detected from 42 stool samples based on PCR between 2016 and 2018 in the capital city of Mongolia (Table 2). Known breeds were the Caucasian shepherd, Rottweiler, Tibetan mastiff, Siberian husky, Central Asian shepherd, and Mongolian mastiff (Bankhar). According to data from pet owners, six (26.08%) of the positive dogs were living in the Bayangol district, four (17.39%) were living in the Chingeltei district, and three (13%) were living in the Sukhbaatar district of Ulaanbaatar, centrally located. The overall apparent genetic surveillance in dogs was 54.7% (95% CI 38.7–70.2) in stool samples as discerned by conventional PCR assay. The amplicons detected in 34.7% (8/23) of the samples were CPV-2a, and in 30.4% (7/23) and 34.7% (8/23) of the samples, CPV-2b and CPV-2c were detected, respectively. Among the positive samples, 39.13% (9/23) of the dogs were 3 months old and 43.47% (10/23) of the dogs were 4 months old. Ten (43.47%) of the positive dogs were female and 13 (56.52%) were male. All dogs were aged between 2 and 6 months, with or without a vaccination history, of different breeds, and of both genders. For more information about the dogs used in this study, see Table 2.

3.2. Amino acid sequence analysis and phylogeny

The VP2 proteins were aligned with sequences of related variants/strains obtained from GenBank. The asparagine or aspartic acid at VP2 residue 426 resulted in identification of CPV-2a or 2b, respectively. The genetic characterization of CPV-2c declare glutamic acid change at residue 426, was detected in all positive PCR samples. However, the amino acid residue at position 297 and 555 was alanine and valine, respectively, suggesting all samples were new strains of CPV-2a and 2b. In contrast, analysis of the VP2 protein has shown that three mutations in amino acids residues 274, 580, and 584 for only CPV-2c in Mongolian field isolate strains (Table 3). Our data indicated that the currently circulating CPV-2 in Ulaanbaatar were the 2c subtype with minor amino acid mutations.

We investigated a partial CPV VP2 gene sequence to determine genetic variability of field strains circulating in the capital city of Mongolia. The phylogenetic tree was constructed using partial VP2 gene nucleotide sequences of CPV strains obtained from the GenBank database and Mongolian isolated CPV strains (Fig. 1). The CPV-2a and 2b isolates from Mongolia sequenced as part of this study were clustered in the phylogeny with sequences for other virus sampled in various Eurasian countries such as Italy, China, India and Thailand. The Mongolian CPV-2c strains were more-closely related to CPV-2c strains isolated from Asian region than to earlier strains isolated in the 1970–2000s in the Europe and America.

3.3. Virus isolation and full-length viral genome sequence with NGS

CPV-2c was successfully isolated from one of the three positive samples that was inoculated in CRFK cells and sequenced. No

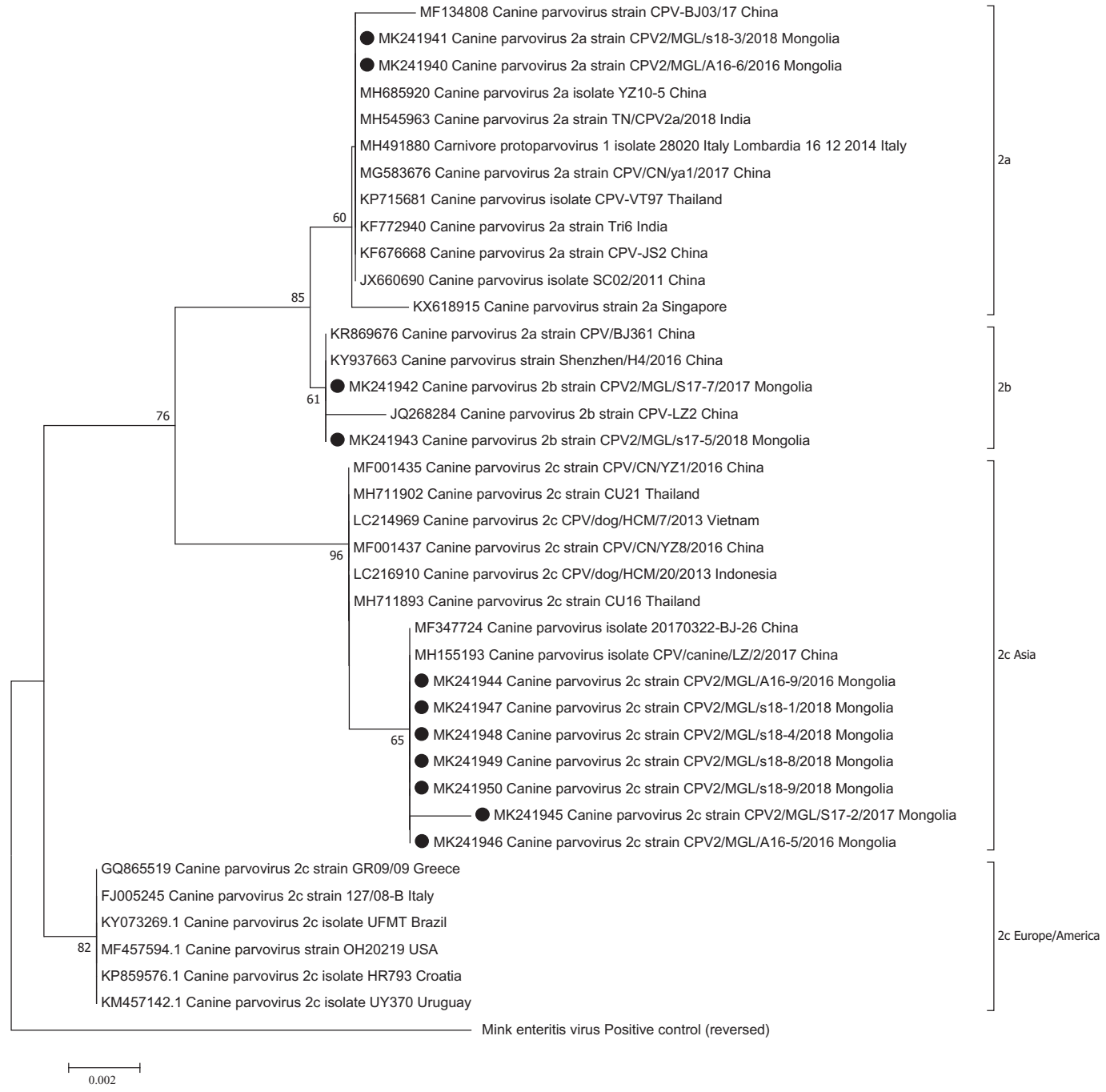

Fig. 1. CPV-2 phylogenetic tree analysis.
The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length, =0.037, is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood model and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 595 nucleotides in the final dataset. Evolutionary analyses were conducted in MEGA7.

cytopathic effect was observed in CRFK cells, at least up to the seventh passage. The presence of virus in harvested cell culture supernatant was detected by PCR and confirmed by IFA. Via IFA, the isolated virus, CPV2/MGL/A16–5/2016, in CRFK cells was confirmed using mAb P2–284 as the primary antibody and Alexa Fluor 488 goat anti-Mouse IgG for the secondary antibody at 48 h post-inoculation (100× magnification). At 48 h post-inoculation in the presence of CPV (Fig. 2), some fluorescence was observed in the nuclei of cells.

The isolated virus (CPV2/MGL/A16–5/2016) was sequenced in its entirety by NGS. The genomic sequence consisted of 5075 nucleotides (nt), containing two open reading frames (ORFs). ORF1 was located at nt positions 270–2276, and it encodes NS1 (668 aa) and NS2 (165 aa). ORF2 encoded two structural proteins: VP1 (727 aa) and capsid protein VP2 (584 aa). Genome sequencing revealed the key glutamic acid located at position 426. Other amino acids of importance detected were Leu, Thr, Ala, Gly, and Tyr located at positions 87, 101, 297, 300, and 305 of the full-length sequence of this virus.

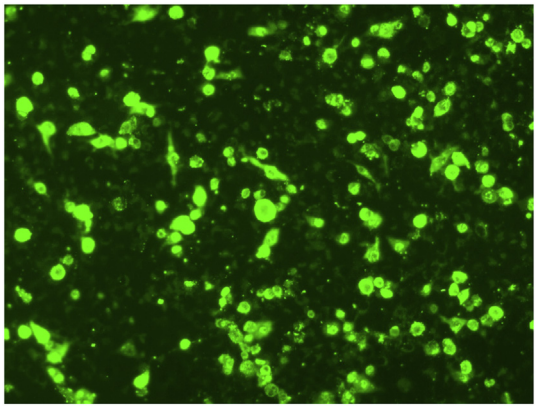

Fig. 2. Immunofluorescence assay of CRFK cells infected with CPV-2c. Samples were taken at 48 h post-infection. Monoclonal antibody (P2–284 IgG_1) efficiently detected virus-infected CRFK cells under fluorescence microscopy at 100× objectives. The bound antibody was visualized by a further reaction with a green fluorescent secondary antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

CPV-2 virus in dogs spread worldwide since 1970s, but antigenic variants CPV-2a emerged in1979; the other, CPV-2b appeared in 1984 had several amino acid substitution in VP2. Subsequently, CPV-2a/2b having a amino acid mutation at 297 (Ser to Ala) designated as New CPV-2a and -2b (Martella et al., 2005; Decaro et al., 2006; Ohshima et al., 2008).

During 2016–2018, we isolated and characterized CPV in vaccinated and unvaccinated dogs in Mongolia. By type-specific PCR of stool samples from dogs, we revealed the presence of CPV-2a and CPV-2c in equal proportions; however, the new CPV-2b subtype was predominant from 2016 to 2017. CPV-2c was not detected in Asia until 2012 (Decaro and Buonavoglia, 2012), and since then, CPV-2c has been detected in South Asia (Lin et al., 2017; Nandi et al., 2010; Vannamahaxay et al., 2017). Since 2018, the CPV-2b subtype was not detected in Ulaanbaatar, with CPV-2c becoming predominant. Our results evidenced prevalence of CPV-2c in the Mongolian dog population via our molecular epidemiology survey.

The sequence analysis of VP2 demonstrated that “new CPV-2a” and “new CPV-2b” subtypes were circulating in Mongolia during 2016–2018. These subtypes replaced the prototype CPV-2a/2b strains and became predominant types in many countries (Decaro et al., 2006; Martella et al., 2005; Mohan Raj et al., 2010; Ohshima et al., 2008). The VP2 amino acid mutation at position 297 from serine to alanine has reportedly been shown to not affect antigenicity (Zhang et al., 2010), whereas the CPV-2c subtype has been predominant in the dog population since 2018 instead of 2b. In addition, this study detected three different site mutations among CPV-2c-positive samples in Mongolia. One isolate possessed an amino acid mutation of arginine to lysine at position 274 of the VP2 protein. The GH loop comprised between the βG and βH strands of VP2 region, which is formed by residue 267 to 498. This region contains sites with the greatest variability, influenced by its presentation on the capsid surface (Decaro et al., 2009). The change was at R274K, which was encountered in one South Korean isolates (Kang et al., 2008). Other mutations were threonine and histidine at amino acid residues 580 and 584 in the capsid protein, respectively. These mutation never been detected before in CPV-2c. However, CPV-2b from Italian strain have mutation at 580 residue (Pro to Ser), it was conserved at the C-terminus of VP2 protein (Decaro et al., 2009). This was the first report that the CPV-2c variant have mutation in the residue 584 of VP2 protein. The result of our NJ tree demonstrated that CPV-2 ($n = 7$) isolates from Mongolia sequenced in this study clustered with isolates from the various Asian countries such as China, Thailand and Vietnam all of which belong to the 2c genotype. Striking similarities were also observed between sequences of strains isolated from Mongolia and China over a similar time span. NGS analysis showed the same aforementioned key residues of the CPV variants (Cavalli et al., 2008) and was characterized as CPV-2c on the basis of the presence of codon GAA at positions 4062–4064 of the viral genome, accounting for a glutamic acid residue at position 426 of the encoded protein (Amrani et al., 2016).

The commercial vaccines currently available worldwide, including Mongolia, are based on CPV and CPV-2b, and several studies have demonstrated that they are able to cross-protect against all antigenic types (Spibey et al., 2008; Siedek et al., 2011; Reitzenstein et al., 2012). However, in our work, 10 out of 23 CPV-2-positive dogs were vaccinated against CPV and yet demonstrated clinical symptoms. The vaccine against 5 viral antigen injected to the healthy dogs 6 weeks of age or older. Puppies should receive 3 doses, each administered 3 weeks apart for complete vaccination. The 8 dogs has been completed vaccination according to vaccine guidelines and before presentation of clinical symptoms and remained 2 dogs has been incomplete vaccination (Mouzin et al., 2004). Therefore, additional study regarding CPV vaccination methods, including strain selection, delivery time, and cold chain, is necessary to fully understand the dynamics of cross-protection efficacy and to enhance current vaccination strategies, being mindful of virus evolution.

5. Conclusions

In summary, the current findings present entirely new information. In 2016–2018, subtype CPV-2c was predominant in Ulaanbaatar, Mongolia. CPV-2c, which has displayed unreported mutation of amino acid residues at 580, and 584 position in VP2. Our results indicate that the recent CPV-2c isolate from Mongolian field strain shares a common evolutionary origin with Chinese strains.

Acknowledgments

We would like to thank the “Strengthening the Capacity for Human Resource Development in the field of Veterinary Medicine and Animal Husbandry” team, which was supported by JICA, for providing facilities and technical/methodological support. We would like to thank Baljidmaa Batmunkh and Nyamsuren Otgontogtokh for excellent technical assistance and collecting samples.

Funding

This work was supported by the Mongolian University of Life Sciences [grant number 7/2017].

References

Amrani, N., Desario, C., Kadiri, A., Cavalli, A., Berrada, J., Zro, K., Sebbar, G., Colaianni, M.L., Parisi, A., Elia, G., Buonavoglia, C., Malik, J., Decaro, N., 2016. Molecular epidemiology of canine parvovirus in Morocco. Infect. Genet. Evol. 41, 201–206. https://doi.org/10.1016/j.meegid.2016.04.005.

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/10.1099/0022-1317-82-12-3021.

Carmichael, L.E., 2005. An annotated historical account of canine parvovirus, in: journal of veterinary medicine series B: infectious diseases and veterinary. Public Health 52 (7–8), 303–311. https://doi.org/10.1111/j.1439-0450.2005.00868.x.

Cavalli, A., Martella, V., Desario, C., Camero, M., Bellacicco, A.L., De Palo, P., Decaro, N., Elia, G., Buonavoglia, C., 2008. Evaluation of the antigenic relationships among canine parvovirus type 2 variants. Clin. Vaccine Immunol. 15, 534–539. https://doi.org/10.1128/CVI.00444-07.

Clark, N.J., Seddon, J.M., Kyaw-Tanner, M., Al-Alawneh, J., Harper, G., McDonagh, P., Meers, J., 2018. Emergence of canine parvovirus subtype 2b (CPV-2b) infections in Australian dogs. Infect. Genet. Evol. 58, 50–55. https://doi.org/10.1016/j.meegid.2017.12.013.

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

Decaro, N., Martella, V., Desario, C., Bellacicco, A.L., Camero, M., Manna, L., D'Aloja, D., Buonavoglia, C., 2006. First detection of canine parvovirus type 2c in pups with haemorrhagic enteritis in Spain. J. Vet. Med. Ser. B Infect. Dis. Vet. Public Heal. 53, 468–472. https://doi.org/10.1111/j.1439-0450.2006.00974.x.

Decaro, N., Desario, C., Parisi, A., Martella, V., Lorusso, A., Miccolupo, A., Mari, V., Colaianni, M.L., Cavalli, A., Trani, L.D., Buonavoglia, C., 2009. Genetic analysis of canine parvovirus type 2c. Virol. J. 385, 5–10. https://doi.org/10.1016/j.virol.2008.12.016.

Horiuchi, M., Goto, H., Ishiguro, N., Shinagawa, M., 1994. Mapping of determinants of the host range for canine cells in the genome of canine parvovirus using canine parvovirus/mink enteritis virus chimeric viruses. J. Gen. Virol. 75, 1319–1328. https://doi.org/10.1099/0022-1317-75-6-1319.

Horiuchi, M., Mochizuki, M., Ishiguro, N., Nagasawa, H., Shinagawa, M., 1997. Epitope mapping of a monoclonal antibody specific to feline panleukopenia virus and mink enteritis virus. J. Vet. Med. Sci. 59, 133–136. https://doi.org/10.1292/jvms.59.133 ER.

Kang, B.K., Song, D.S., Lee, C.S., Jung, K.I., Park, S.J., Kim, E.M., Park, B.K., 2008. Prevalence and genetic characterization of canine parvoviruses in Korea. Virus Genes 36, 127–133. https://doi.org/10.1007/s11262-007-0189-6.

Kariatsumari, T., Horiuchi, M., Hama, E., Yaguchi, K., Ishigurio, N., Goto, H., Shinagawa, M., 1991. Construction and nucleotide sequence analysis of an infectious DNA clone of the autonomous parvovirus, mink enteritis virus. J. Gen. 72 (4), 867–875. Virol.. https://doi.org/10.1099/0022-1317-72-4-867.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA 7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33 (7), 1870–1874.

Lin, Y.C., Chiang, S.Y., Wu, H.Y., Lin, J.H., Chiou, M.T., Liu, H.F., Lin, C.N., 2017. Phylodynamic and genetic diversity of canine parvovirus type 2c in Taiwan. Int. J. Mol. Sci. 18, 1–11. https://doi.org/10.3390/ijms18122703.

Martella, V., Cavalli, A., Decaro, N., Elia, G., Desario, C., Campolo, M., Bozzo, G., Tarsitano, E., Buonavoglia, C., 2005. Immunogenicity of an intranasally administered modified live canine parvovirus type 2b vaccine in pups with maternally derived antibodies. Clin. Diagn. Lab. Immunol. 12 (10), 1243–1245. https://doi.org/10.1128/CDLI.12.10.1243-1245.2005.

Martella, V., Decaro, N., Buonavoglia, C., 2006. Evolution of CPV-2 and implicance for antigenic/genetic characterization. Virus Genes 33, 11–13. https://doi.org/10.1007/s11262-005-0034-8.

Mira, F., Dowgier, G., Purpari, G., Vicari, D., Di Bella, S., Macaluso, G., Gucciardi, F., Randazzo, V., Decaro, N., Guercio, A., 2018. Molecular typing of a novel canine parvovirus type 2a mutant circulating in Italy. Infect. Genet. Evol. 61, 67–73. https://doi.org/10.1016/j.meegid.2018.03.010.

Mittal, M., Chakravarti, S., Mohapatra, J.K., Chug, P.K., Dubey, R., Upmanuyu, V., Narwal, P.S., Kumar, A., Churamani, C.P., Kanwar, N.S., 2014. Molecular typing of canine parvovirus strains circulating from 2008 to 2012 in an organized kennel in India reveals the possibility of vaccination failure. Infect. Genet. Evol. 23, 1–6. https://doi.org/10.1016/j.meegid.2014.01.015.

Mohan Raj, J., Mukhopadhyay, H.K., Thanislass, J., Antony, P.X., Pillai, R.M., 2010. Isolation, molecular characterization and phylogenetic analysis of canine parvovirus. Infect. Genet. Evol. 10, 1237–1241. https://doi.org/10.1016/j.meegid.2010.08.005.

Mouzin, D.E., Lorenzen, M.J., Haworth, J.D., King, V.L., 2004. Duration of serologic response to five viral antigens in dogs. J. Am. Vet. Med. Assoc. (1), 55–60. https://doi.org/10.2460/javma.2004.224.55.

Nandi, S., Chidri, S., Kumar, M., Chauhan, R.S., 2010. Occurrence of canine parvovirus type 2c in the dogs with haemorrhagic enteritis in India. Res. Vet. Sci. 88 (1), 169–171. https://doi.org/10.1016/j.rvsc.2009.05.018.

Ohshima, T., Hisaka, M., Kawakami, K., Kishi, M., Tohya, Y., Mochizuki, M., 2008. Chronological analysis of canine parvovirus type 2 isolates in Japan. J. Vet. Med. 70 (8), 769–775 (Sci. https://doi.org/JST.JSTAGE/jvms/70.769 [pii]).

Parker, J.S., Parrish, C.R., 1997. Canine parvovirus host range is determined by the specific conformation of an additional region of the capsid. J. Virol. 71 (12), 9214–9222.

Parker, J., Murphy, M., Hueffer, K., 2017. Investigation of a canine parvovirus outbreak using next generation sequencing. Sci. Rep. 7, 9633. https://doi.org/10.1038/s41598-017-10254-9.

Parrish, C.R., 1999. Host range relationships and the evolution of canine parvovirus. Vet. Microbiol. 69 (1–2), 29–40. https://doi.org/10.1016/S0378-1135(99)00084-X.

Parrish, C.R., Aquadro, C.F., Strassheim, M.L., Evermann, J.F., Sgro, J.Y., Mohammed, H.O., 1991. Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. J. Virol. 65, 6544–6552.

Pereira, C.A.D., Durigon, E.L., 2000. Genetic diversity of the VP1/VP2 gene of canine parvovirus type 2B amplified from clinical specimens in Brazil. Braz. J. Microbiol. 31, 312–314. https://doi.org/10.1590/S1517-83822000000400014.

Pinto, L.D., Streck, A.F., Gonçalves, K.R., Souza, C.K., Corbellini, Â.O., Corbellini, L.G., Canal, C.W., 2012. Typing of canine parvovirus strains circulating in Brazil between 2008 and 2010. Virus Res. 165, 29–33. https://doi.org/10.1016/j.virusres.2012.01.001.

Reitzenstein, M., Ludlow, D., Marcos, S., Kopta, L., Sandbulte, J., Mischnick, C., Slade, D., Hawkins, K.F., King, V., Inskeep, G., Sture, G., 2012. Cross protection of vanguard 5L4-CV vaccine against virulent canine parvovirus-2c circulating in the USA. Intern. J. Appl. Res. Vet. Med. 10 (3), 187–197. https://doi.org/10.1016/j.vaccine.2014.07.102.

Sharav, T., Tserendorj, A., Ganbayar, L., Ganbaatar, O., Bayasgalan, B., Chultemdorj, T., 2015. Predominance of canine parvovirus type 2b in dogs of Ulaanbaatar city. J. Agric. Sci. 15, 71–74.

Sharma, S., Dhar, P., Thakur, A., Sharma, V., Sharma, M., 2016. First detection of canine parvovirus type 2b from diarrheic dogs in Himachal Pradesh. Vet. World 9, 964–969 (10.14202/vetworld.2016.964-969).

Siedek, E.M., Schmidt, H., Sture, G.H., Raue, R., 2011. Vaccination with canine parvovirus type 2 (CPV-2) protects against challenge with virulent CPV-2b and CPV-2c. Berl. Munch. Tierarztl. Wochenschr. 124, 58–64. https://doi.org/10.2376/0005-9366-124-58.

Spibey, N., Greenwood, N.M., Sutton, D., Chalmers, W.S.K., Tarpey, I., 2008. Canine parvovirus type 2 vaccine protects against virulent challenge with type 2c virus. Vet. Microbiol. 128, 48–55. https://doi.org/10.1016/j.vetmic.2007.09.015.

Truyen, U., 2006. Evolution of canine parvovirus-a need for new vaccines? Vet. Microbiol. 117 (1), 9–13. https://doi.org/10.1016/j.vetmic.2006.04.003.

Truyen, U., Parrish, C.R., 1995. The evolution and control of parvovirus host ranges. Semin. Virol. 6 (5), 311–317. https://doi.org/10.1006/smvy.1995.0037.

Vannamahaxay, S., Vongkhamchanh, S., Intanon, M., Tangtrongsup, S., Tiwananthagorn, S., Pringproa, K., Chuammitri, P., 2017. Molecular characterization of canine parvovirus in Vientiane, Laos. Arch. Virol. 162, 1355–1361. https://doi.org/10.1007/s00705-016-3212-1.

Wang, J., Lin, P., Zhao, H., Cheng, Y., Jiang, Z., Zhu, H., Wu, H., Cheng, S., 2015. Continuing evolution of canine parvovirus in China: isolation of novel variants with an Ala5Gly mutation in the VP2 protein. Infect. Genet. Evol. 38, 73–78 (10.1016/j.meegid.2015.12.009).

Zhang, R., Yang, S., Zhang, W., Zhang, T., Xie, Z., Feng, H., Wang, S., Xia, X., 2010. Phylogenetic analysis of the VP2 gene of canine parvoviruses circulating in China. Virus Genes 40, 397–402. https://doi.org/10.1007/s11262-010-0466-7.

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.