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New genotype classification and molecular characterization of canine and feline parvoviruses

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ABSTRACT

Background: Canine parvovirus (CPV) and feline panleukopenia (FPV) cause severe intestinal disease and leukopenia.
Objectives: In Korea, there have been a few studies on Korean FPV and CPV-2 strains. We attempted to investigate several genetic properties of FPV and CPV-2.
Methods: Several FPV and CPV sequences from around world were analyzed by Bayesian phylo-geographical analysis.
Results: The parvoviruses strains were newly classified into FPV, CPV 2-I, CPV 2-II, and CPV 2-III genotypes. In the strains isolated in this study, Gigucheon, Rara and Jun belong to the FPV, while Rachi strain belong to CPV 2-III. With respect to CPV type 2, the new genotypes are inconsistent with the previous genotype classifications (CPV-2a, -2b, and -2c). The root of CPV-I strains were inferred to be originated from a USA strain, while the CPV-II and III were derived from Italy strains that originated in the USA. Based on VP2 protein analysis, CPV 2-I included CPV-2a-like isolates only, as differentiated by the change in residue S297A/N. Almost CPV-2a isolates were classified into CPV 2-III, and a large portion of CPV-2c isolates was classified into CPV 2-II. Two residue substitutions F267Y and Y324I of the VP2 protein were characterized in the isolates of CPV 2-III only.
Conclusions: We provided an updated insight on FPV and CPV-2 genotypes by molecular-based and our findings demonstrate the genetic characterization according to the new genotypes.

Keywords: Canine parvovirus; feline parvoviruses; classification; genotype

INTRODUCTION

Canine parvovirus (CPV) and feline panleukopenia virus (FPV) are members of the genus *Protoparvovirus* [1], belonging to the family *Parvoviridae*, and are small, non-enveloped DNA viruses with a single-stranded linear genome of approximately 5.2 kb [1,2]. The CPV and FPV cause severe intestinal disease and leukopenia in canine and feline [3,4].

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: Chung HC, Kim SJ, Lim SK, Park YH, Park B; Formal analysis: Chung HC, Kim SJ, Nguyen VG, Shin S, Kim JY, Park YH; Investigation: Chung HC, Nguyen VG; Writing - original draft: Chung HC, Kim SJ; Writing - review & editing: Chung HC, Kim SJ, Nguyen VG.

Canine parvovirus represents the cross-species viral transmission between felines and canines. Parvovirus CPV type 2 (CPV-2) emerged during the 1970s and rapidly spread worldwide [5,6]. Although there is still some controversy surrounding the origin of CPV-2, most researchers and clinicians accept that CPV-2 is derived from FPV of domestic cats [3]. Subsequently, two new antigenic types of CPV-2, type 2a (CPV-2a) and type 2b (CPV-2b), emerged and have virtually replaced the original CPV-2 strain worldwide [7]. Another antigenic type, CPV type 2c (CPV-2c) emerged in the 2000s and spread globally [4]. The novel antigenic categories appeared consecutively after the initial discovery of CPV-2 as a variant of FPV, and the genomes of the carnivore parvoviruses are 98% homologous to each other [6] with few nonsynonymous and synonymous changes of nucleotides [8]. Extensive phylogenetic studies on CPV-2 and FPV isolates reveal that there are 2 distinct clusters represented by FPV type from cats (FPV), raccoons, and mink, and by CPV type from dogs and raccoon dogs [3,7-9].

Two open reading frames exist in the parvovirus genome. One codes for non-structural proteins (NS1 and NS2) and has low nucleotide substitution, and the other codes for structural viral proteins (VP1 and VP2) [9]. The 2 structural viral proteins are splice variants and are identical in sequence except for a 143 amino acid (aa) long N-terminal stretch that is unique to VP1. The VP2 is the major capsid protein that comprises about 90% of the entire viral capsid [10]. This capsid is a determinant of host range as a main target for neutralizing antibodies against parvovirus [9]. So far, most studies have investigated on the evolution of the VP2 gene instead of studies that are focused on VP1.

Although DNA viruses have a lower mutation rate as compared to RNA viruses, the genomic substitution rate for both CPV and FPV is comparable to that observed for RNA viruses rather than other types of DNA viruses [11]. Although it can be postulated that consecutive genetic evolution has occurred, there are scarce data on genetic analysis and the topology of FPV and CPV worldwide. Moreover, no comprehensive genetic analysis on Korean CPV and FPV isolates has been carried out, although there are a few studies that attempted to do so [12-16]. We performed an extensive genetic analysis using the Bayesian method on over 200 previously published CPV and FPV sequences including recent Korean CPV and FPV isolates. From our novel study perspective, we suggest a new genetic classification for CPV (CPV 2-I, -II and -III) as an alternate to the conventional genotype classification (CPV-2a, -2b, and -2c) as proposed by other genetic analyses.

MATERIALS AND METHODS

Sample collection and polymerase chain reaction (PCR)-based detection of FPV and CPV-2

For detection of FPV and CPV-2, rectal swab samples were collected from cats and dogs showing viral clinical symptoms (less than 6 months) that were hospitalized at three different animal clinics (Rara, Gigucheon, and Rachi) in Seoul and one animal clinic (Jun) in Suwon from June 2017 to October 2017 (**Table 1**). The collected four samples were suspended in 1 mL of 0.1M phosphate-buffered saline (PBS) at pH 7.4 and centrifuged at 2,500 g for 15 min at 4°C. The supernatant from each sample was collected and used for PCR amplification.

DNA extraction was performed using an RNA/DNA Extraction kit (Invitrogen, USA) according to the manufacturer's instructions and the extracted samples were stored at −20°C.

Table 1. Detailed information of the parvovirus-isolated samples in this study

GenBank	Strain	Sampling date	Site	Host	Old	Vaccine	Sample type	Clinical presentation
MN400981	Rachi	06-June-2017	Seoul	Canine	1 year	Yes (DHPP)	Rectal swab	Hemorrhagic diarrhea
MN400978	Gigucheon	16-July-2017	Seoul	Feline	1 year	Yes (RCP)	Rectal swab	Panluekopneia (depression, loss of appetite, high fever, vomiting, severe diarrhea)
MN400980	Rara	18-August-2017	Seoul	Feline	1 year	Yes (RCP)	Rectal swab	Panluekopneia (depression, loss of appetite, high fever, vomiting, severe diarrhea)
MN400979	Jun	10-Octber-2017	Gyeonggi; Suwon	Feline	1 year	Yes (RCP)	Rectal swab	Panluekopneia (depression, loss of appetite, high fever, vomiting, severe diarrhea)

DHPP, dog annual core vaccine of Distemper, Hepatitis, Parainfluenza, and Parvovirus; RCP, cat annual core vaccine of Rhinotracheitis, Calicivirus, and Panleukopenia.

Table 2. List of primers used in this study

Primer names	Detection method	Sequence (5′-3′)	Location (nt)	Purpose	Size (bp)	References
CPV-F1	PCR	AGATAGTAATAATACTATGCCATTT	531-1250	Detection	719	[28]
CPV-R2		TTTTGAATCCAATCTCCTTCTGGAT				
CaKV-F	RT-PCR	CCCTGGAACACCCAAGGCCGCT	-	Detection	504	[29]
CaKV-R		TCTGGTTGCCATAGATGTGGTG				
CDV-F	RT-PCR	ACTTTCTGGCACTGTGATT	-	Detection	669	[29]
CDV-R		ACACCTTCAATNGCATGNAT				
Herpesvirus type 1F	PCR	CTATGTTTCTTATGGATATGAGACTTTGTGAT	359-665	Detection	307	[30]
Herpesvirus type 1R		ATAGTTTTAACATTTCGACACCATTCATGTAG				
CPV-1F	PCR	CAACGTAATGAGAACTAT	1-1253[a]	Genome sequencing	1,253	In this study
CPV-1R		CCCACAGCTTGTGCTATGGC				
CPV-2F	PCR	GACAAGGTGGTAAAAGAAATACAG	1163-2445	Genome sequencing	1283	In this study
CPV-2R		ATGAGGTGGTGGTCTACTTC				
CPV-3F	PCR	GAGCTAAAAAGGCAATTGCTCC	2345-3644	Genome sequencing	1,300	In this study
CPV-3R		CTTGGATCACCATCTGCTGC				
CPV-4F	PCR	TGAGGCGTCTACACAAGGGC	3549-4269	Genome sequencing	721	In this study
CPV-4F		TTAGTATAATTTTCTAGGTGCTAGTT				
FPV-1F	PCR	CTTTAGAACCAACTGACCAA	1-1419[b]	Genome sequencing	1,419	In this study
FPV-1R		CACATGCTATAGCGTGACAAAC				
FPV-2F	PCR	GAATGCACGGATGGAATTGG	1372-2827	Genome sequencing	1456	In this study
FPV-2R		CTGACAGCAGGTTGACCACC				
FPV-3F	PCR	AAAAGCCGGTGCAGGACAAG	2738-4240	Genome sequencing	1,503	In this study
FPV-3R		GGTGCATTTACATGAAGTCTTGG				
FPV-4F	PCR	CCACCAGTTTATCCAAATGGTC	4161-4837	Genome sequencing	677	In this study
FPV-4R		CATATTCTAAGGGCAAACCAAC				

CPV, canine parvovirus, CDV, canine distemper virus, CaKoV, canine kobuvirus; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.
[a]Site location, reference, GenBank accession number KR002799; [b]Site location, reference, GenBank accession number MH559110.

All rectal swab samples were tested using the primer sets for FPV, CPV-2, and other viral pathogens listed in **Table 2**. The PCR thermal profile was as follows: initial denaturation at 94°C for 5 min, then 35 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 45 sec, and a final extension phase at 72°C for 7 min.

Isolation of FPV and CPV-2

A monolayer of CRFK cells (ATCC® CCL-94™) was grown overnight to approximately 80–100% confluence in a 25-cm^2 cell culture flask. The cells were washed twice with 1× PBS prior to inoculation with a 10% suspension of the samples (Rara, Gigucheon, Rachi, and Jun), which were previously diluted in 500 mL PBS and filtered through a 0.02 μm filter. After two hours incubation at 37°C and 5% CO_2, 4.5 mL of cell growth media (Dulbecco's minimum essential medium) supplemented with trypsin (10 μg/mL) and antibiotic-antimycotic solution (5 μL/mL) were added at a ratio of 1:10. After inoculation, the cells were monitored daily for

cytopathic effects (CPEs). After 4–5 days observation for CPE, cells were harvested to confirm the presence of each virus type by reverse transcription (RT)-PCR. Serial passages of the Rara, Gigucheon, Rachi, and Jun isolates of FPV and CPV-2, were continued at 37°C in 5% CO_2 atmosphere to passage number 13.

Immunofluorescence of assay of CRFK cells infected with FPV and CPV-2

An immunofluorescence assay was performed for the 4 isolated strains (Rachi, Gigucheon, Rara, and Jun) with a negative control and positive control against canine parvovirus live vaccine (NeoPar, NeoTech) to confirm FPV or CPV-2 infection from passage level 13 in CRFK cells. After 1 day of fixation based on alcohols 100% with incubation −20°C. Approximately 300 µL of monoclonal primary antibody specific for parvovirus (CPV1-2A1) from Abcam Ltd. (cat No. ab140431), diluted 1:100, was applied to cells, and cells were then transferred to a 37°C incubator for 1 h. Afterwards, the primary antibody was rinsed and cells were incubated with the secondary antibody goat anti-mouse IgG H&L Alexa Fluor® 488 (cat No. ab150113) for 1 h.

Complete genome sequencing of FPV and CPV-2

For genetic characterization, the four isolates recovered in this study (Rachi, Rara, Gigucheon, and Jun) were completely sequenced by the primer walking method. Four pairs of overlapping primers were utilized for sequencing the Rara, Gigucheon, and Jun strains (FPV 1F to 4R), and for the Rachi strain (CPV 1F to 4R), as shown in **Table 2**. Specific PCR products were purified by the gel extraction method and further processed for TA cloning and transformation [17]. The full-length genomes of Gigucheon, Jun, Rara, and Rachi strains were registered in GenBank (accession numbers MN400978 to MN400981).

Bayesian phylogenetic analyses

For the genetic analyses, we downloaded different datasets from GenBank of 200 complete genome sequences, and 200 NS1, 200 VP1, and 275 VP2 protein-coding genes. These sequences originated from Asia (China, Thailand, Mongolia, India, Korea, and Japan), America (Uruguay, Argentina, USA, and Brazil), and Europe (Italy, Albania, New Zealand, United Kingdom, Germany, France, and Canada), that were sampled from 1964–2018.

This dataset was chosen for the Bayesian coalescent-based Markov chain Monte Carlo (MCMC) analysis. All analyses were performed using BEAST package v1.8.4 under the following assumptions: (i) HKY+G4 nucleotide substitution model in codon-based SRD06 [18], (ii) a constant population size for the coalescent prior, and (iii) the three molecular clock models of uncorrelated relaxed clock model (UCLD). The phylogenic trees were summarized with TreeAnnotator v1.7.2 to determine the maximum clade credibility (MCC) tree, which was depicted using FigTree v1.4.3.

Antigenic analysis on a B cell epitope of Korean FPV and CPV-2 strains

B cell epitopes on the VP2 protein of FPV and CPV-2 strains epitope were already determined site of the 1 to 23 aa [19]. Also, the same position was predicted as the B cell epitope by BepiPred-2.0 version in this study [20]. The Jameson–Wolf antigenic index implemented using Lasergene Protean software (DNASTAR, Inc., USA) was used to predict whether aa substitutions would affect the antigenic properties of neutralizing epitopes. The antigenic index was calculated for each aa site and plotted using Microsoft Excel 2017 (Microsoft, USA).

RESULTS

Feline and Canine parvovirus detection and isolation

In the samples collected from the four clinics (Rachi, Rara, Jun, and Gigucheon), FPV or CPV-2 parvovirus was detected by CPV-specific PCR target band size of 719 bp. Other pathogens (canine kobuvirus, canine herpesvirus, and canine distemper virus) were not detected.

As shown in **Table 1**, the four strains (Rachi, Rara, Jun, and Gigucheon) were isolated successfully from the CRFK cell line. For the Rachi strain, cytopathic effects on the cells were observed earlier (passage 1, 48 h after inoculation) than for the other three strains (passage 2, 72 h after inoculation). Among the isolated strains, the highest infective titers were for the Rachi isolate, increasing the median tissue culture infectious dose (TCID50) from $10^{4.5}$ at passage 3 to a TCID50 of $10^{7.5}$ at passage 13 (**Table 3**). Beginning at passage level 10, the four strains showed the strongest CPE with cell rounding and detachment (not shown). To confirm the viral isolation, an indirect immunofluorescence assay was performed to identify CRFK cells (from passage 10) infected by FPV and CPV-2. All four strains in CRFK cells showed positive nuclear green fluorescence at 24 h post-inoculation (**Fig. 1**).

Table 3. Titration of parvoviruses isolates from passage 1 to passage 13 in CRFK cells

Strains	Passages																							
	1		2		3		4		5		6		7		8		9		10		11		12		13	
	CPE	Titer[b]	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer
Rachi	+[a]	N	+	N	++	4.5	+++	N	+++	5.5	+++	N	+++	N	+++	6.0	+++	N	+++	6.5	+++	N	+++	7.0	+++	7.5
Gigucheon	-	N	+	N	+	2.0	++	N	++	2.5	++	N	++	N	++	4.0	++	N	+++	4.5	+++	N	+++	5.0	+++	6.0
Rara	-	N	+	N	+	3.5	++	N	+++	4.0	+++	N	+++	N	+++	4.5	+++	N	+++	5.0	+++	N	+++	6.0	+++	6.5
Jun	-	N	+	N	++	3.0	++	N	++	4.5	+++	N	+++	N	+++	6.0	+++	N	+++	6.5	+++	N	+++	7.0	+++	7.5

CPE, cytopathic effect; N, not determined

[a]+ small, ++ medium, +++ strong; [b]Infection titer: $\log_{10}$ $TCID_{50}$/mL.

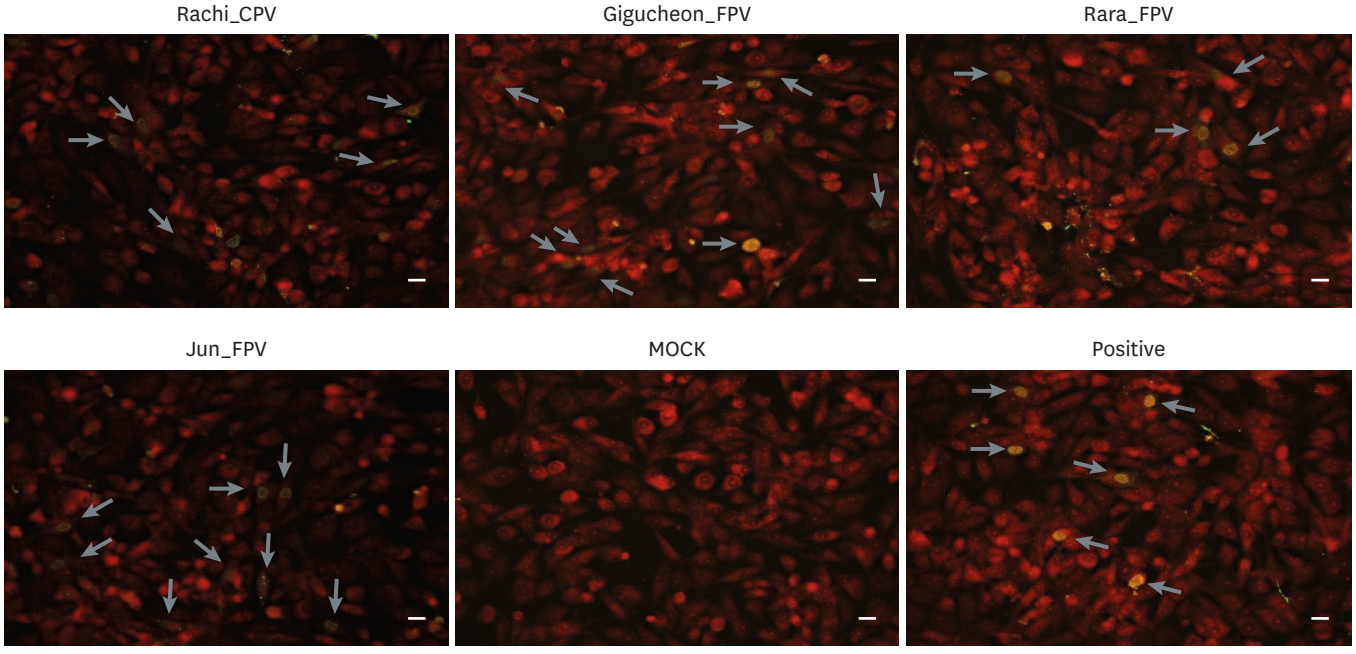

Fig. 1. Results of IFA based on fluorescence microscopy. Specific IFA staining of CRFK cells infected by CPV (Rachi) and FPV (Gigucheon, Rara, and Jun) isolates at passage level 10. The specific Alexa Fluor® 488 (Green) images (arrow marked; gray color) were taken 24 h post-inoculation. The white scale bar in the micrographs = 100 μm.

IFA, immunofluorescence assay; CPV, canine parvovirus.

Table 4. Estimated nucleotide substitution rates of complete genomes for each gene

Gene	No. of sequences used	The clock model[a]	Mean rate[b]	95% HPD interval
Complete	200	UCLD	2.85×10^{-4}	2.23×10^{-4} to 3.64×10^{-4}
NS1	200	UCLD	1.96×10^{-4}	1.46×10^{-4} to 2.56×10^{-4}
NS2	200	UCLD	3.92×10^{-4}	2.50×10^{-4} to 5.44×10^{-4}
VP1	200	UCLD	2.47×10^{-4}	1.77×10^{-4} to 3.33×10^{-4}
VP2	277	UCLD	2.95×10^{-4}	2.32×10^{-4} to 3.69×10^{-4}

UCLD, uncorrelated relaxed clock model; HPD, highest posterior density.
[a]UCLD with an underlying lognormal distribution. [b]The geometric mean nucleotide substitution rate (substitutions/site/year).

Evolutionary rates of CPV-2 and FPV

The evolutionary rates of the genes CPV-2 and FPV were inferred from the UCLD molecular clocks. As shown in **Table 4**, the investigated complete genomes, NS1, VP1, and VP2 genes were estimated to be rapidly evolving on the order of 10^{-4} nucleotide substitutions/site/year. The magnitude of the differences between the highest and lowest geometric mean rates were approximately 2-fold, within the range of 3.92×10^{-4} (NS2 gene) and 1.96×10^{-4} (NS1 gene). Notably, the 95% highest posterior density evolutionary rates observed among the genes revealed that the NS2 gene was the most varied ($2.50 \times 10^{-4} - 5.44 \times 10^{-4}$).

Bayesian geographical phylogenetic analyses of FPV and CPV-2

Of the phylogenetic tree topology (**Fig. 2A-D**), among the four datasets the FPV and CPV-2 strains were consistently divided into three genotypes as CPV 2-I, 2-II and 2-III with a posterior probability of 85%–99%. Of the viruses circulating in South Korea in 2007 and in 2014–2018, the phylogenetic trees reconstructed from three datasets indicated that they were grouped within the FPV, CPV 2-I, and CPV 2-III genotypes (**Fig. 2D** in VP2 protein, highlighted in gray color). Of these, the Rara, Gigucheon, and Jun isolates were included in FPV clade and the isolate Rachi was included in the CPV 2-III clade.

Focusing on the VP2 protein (**Fig. 2D**) of branching pattern of CPV-2, the CPV 2-I genotype included only the isolates from the USA, China and Korea. The CPV 2-II genotype did not included the strains from Asian countries including China, Thailand and Korea. The CPV 2-III genotype included the isolates from most countries located in Asia, Europe, North and South America. Interestingly, USA strains were not included in the CPV-2 III genotype. According to branch cascade, it is inferred that CPV 2-I isolates diverged from an ancestral USA strain. The CPV 2-II and -III isolates were regarded as being more recently diverged from Italian isolates originated from an ancestral USA strain (**Fig. 2A-D**).

Variations at the neutralizing epitope in the VP2 of CPV and FPV Korean strains

Using the B cell epitope prediction program, this study predicted potential linear epitopes on the VP2 protein site of 1 to 23 aa. The aa variations in the B cell epitopes (**Fig. 3**) were observed between three Korean FPV field strains (Jun, Rara, and Gigucheon) and reference FPV and CPV-2 strains. To access the influence of aa substitutions, the Jameson–Wolf antigenic index of VP2 were analyzed. As shown in **Fig. 3A** (VP2 gene) with gray color highlights, the predicted epitope (1 to 23 aa) of the three Korean CPV and FPV strains (Jun, Rara, and Gigucheon) showed significant differences in antigenic index with the other Korean strains. For example, epitope regions in 3, 4, 11, 12, and 13 aa had a lower antigenic index than the other Korean FPV and CPV-2 strains in the comparison. The aa substitution of Rara (3Asp→3Glu, 6Val→6Phe, 9Asp→9Asn, 16Arg→16Lys, 19Arg→19Lys) and Gigucheon (6Val→6Phe, 9Asp→9Asn, 16Arg→16Lys, 19Arg→19Lys, 20Ala→20Pro) resulted in the alteration of the antigenic index due to aa substitutions.

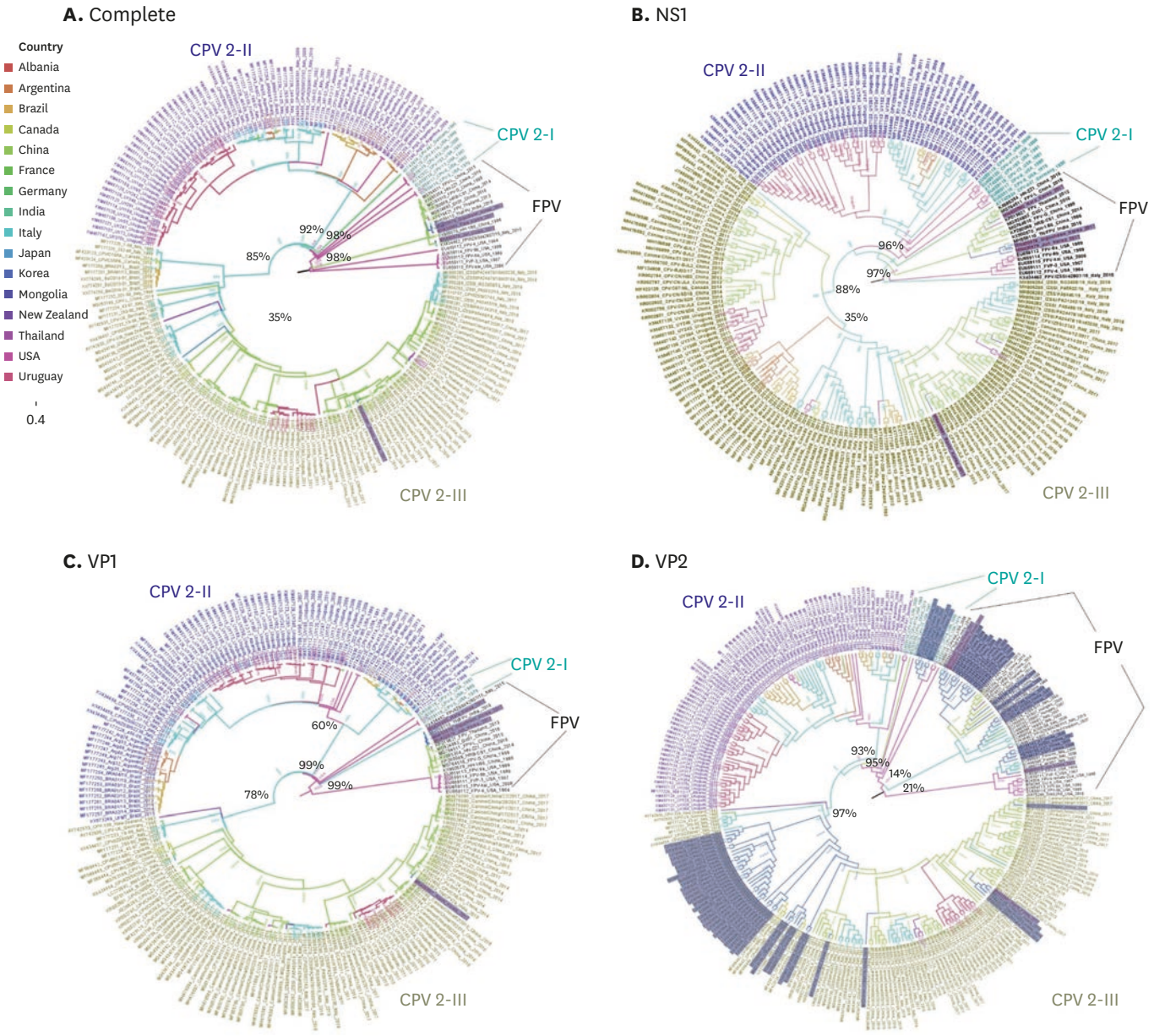

Fig. 2. Bayesian phylo-geographical analysis and classification between FPVs and CPVs. The phylogenetic trees were reconstructed from (A) complete genome, (B) NS1, (C) VP1, and (D) VP2 genes. Three genotypes of CPV-2 were indicated as CPV 2-I (green color), CPV 2-II (blue color), and CPV 2-III (yellow color) and 1 genotype FPV (black color). Number shown on the node of phylogenetic tree were posterior probability. In this study, the CPV and FPV strains were highlighted with red color. Moreover, Korea strains were presented with gray color.
CPV, canine parvovirus; FPV, feline panleukopenia virus.

Amino acid mutation analysis based on the VP2 protein

The results of aa analysis based on the VP2 protein are shown in **Table 5**. There were common residue substitutions between the FPV and CPV-2 isolates. The FPV isolates presented K-80, K-93, V-103, D-323, N-564, and A-568, but the CPV-2 isolates exhibited R-80, N-93, A-103, N-323, S-564, and G-568 at the same position except for only one isolate 9985-46 (Japan, 2017) depicted by K-93. Within the FPV isolates, three recent FPV isolates Gigucheon (Korea, 2017), Rara (Korea, 2017) and TN/FPV (India, 2018) had residue substitutions in the front region (residues 2 to 20) of the VP2 sequence as compared to other FPV isolates. The FPV

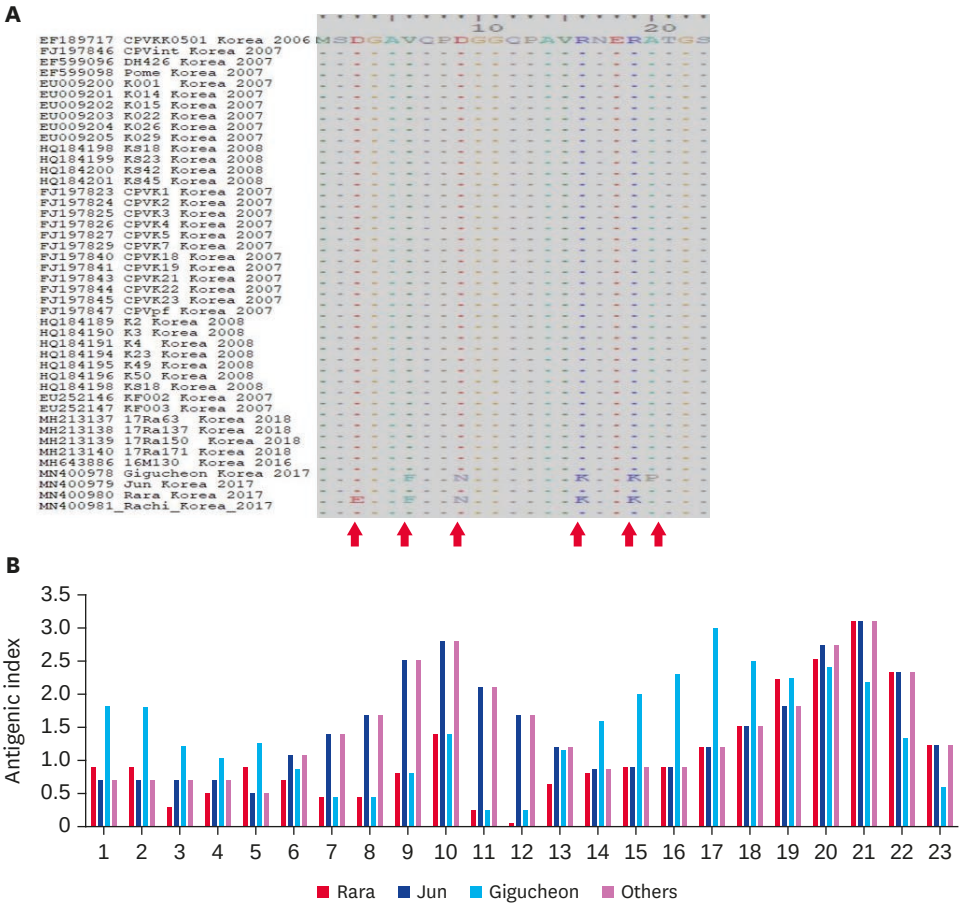

Fig. 3. Amino acid substitutions in epitope in this strains (Rachi, Rara, Jun, and Gigucheon) 40 field Korean strains. (A) Amino acid alignment of the VP2 protein between CPVs and FPVs. The 23 aa already known B cell linear epitope [19] on the VP2 protein are shaded in gray. The aa substitutions attenuation are indicated by solid arrows. (B) Shaded areas in aa (1 to 23 aa) are regions where antigenic indexes were different due to aa variations between in this study strains (Rara, Jun, Gigucheon) and the other Korean FPV and CPV strains.
aa, amino acid; CPV, canine parvovirus; FPV, feline panleukopenia virus.

isolate Gigucheon (Korea, 2017), Rara (Korea, 2017) and TN/FPV (India, 2018) were noted as V6F/D9N/R16K/R19K/A20P, D3E/V6F/D9N/R16K/R19K, and A5P/V6F/Q7H/D9N/R16K/R19K in this region, respectively.

The CPV 2-I isolates were completely differentiated from each other by the change at residue 297 on the VP2 protein. The CPV 2-I isolates exhibited S-297 only, but the CPV 2-II and -III isolates presented A- or N-297 and A-297, respectively. Residue 297-309 (SEGATNFDGVE) forms the exposed surface of the capsid protein that neutralizing antibody binds to [21]. In this antigenic region, the CPV 2-I isolate CPV-13 (USA, 1981) and CPV-L (China, 2014) presented residue changes of A300G, D305Y and A300S, and D305Y respectively, as compared to the other CPV 2-I isolates. The CPV 2-II isolate Arg9 (Argentina, 2008), Arg50 (Argentina, 2008), Arg68 (Argentina 2010), Bra03/13 (Brazil, 2013), and Bra05/13 (Brazil, 2013) displayed a residue change of A300N as compared to other CPV 2-II isolates. In an intra-group comparison of CPV 2-III, the isolate Pome (Korea, 2007) and 9985-46 (Japan, 2017) had residue changes of G300D and G300V, respectively. The isolate RC14/BC (Canada, 2010), RC19/BC (Canada, 2016), and RC20/BC (Canada, 2016) presented respective residue changes of G300D, T301A, and Y305H. Two characterized residue substitutions appeared

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Table 5. Amino acid mutations in VP2 protein of FPV and CPV isolates

GenBank accession no_Strain name_Year	Mutations sites: amino acid residue																					Genotype	
	3	5	6	7	9	16	19	20	80	93	103	267	297	300	301	305	323	324	426	564	567	Previous	This study
EU498680_Purevax_Italy_2008	D	A	V	Q	D	R	R	A	K	K	V	F	S	A	T	D	D	Y	N	N	A	FPV	FPV
EU498681_Felocell_Italy_2008	D	A	V	Q	D	R	R	A	K	K	V	F	S	A	T	D	D	Y	N	N	A		
MN400979_Jun_Korea_2017	D	A	V	Q	D	R	R	A	K	K	V	F	S	A	T	D	D	Y	N	N	A		
MN400978_Gigucheon_Korea_2017	D	A	F	Q	N	K	K	P	K	K	V	F	S	A	T	D	D	Y	N	N	A		
MN400980_Rara_Korea_2017	E	A	F	Q	N	K	K	A	K	K	V	F	S	A	T	D	D	Y	N	N	A		
MH559110_TN/FPV_India_2018	D	P	F	H	N	K	K	A	K	K	V	F	S	A	T	D	N	Y	N	N	A		
EU659116_CPV-5_USA_1979	D	A	V	Q	D	R	R	A	R	N	A	F	S	A	T	Y	N	Y	N	S	G	Old CPV-2a strain-like	CPV 2-I
EU659118_CPV-13_USA_1981	D	A	V	Q	D	R	R	A	R	N	A	F	S	G	T	D	N	Y	N	S	G		
FJ197846_CPVint_Korea_2007	D	A	V	Q	D	R	R	A	R	N	A	F	S	A	T	D	N	Y	N	S	G		
FJ197847_CPVpf_Korea_2007	D	A	V	Q	D	R	R	A	R	N	A	F	S	A	T	Y	N	Y	N	S	G		
MG763189_CPV-L_China_2014	D	A	V	Q	D	R	R	A	R	N	A	F	S	S	T	D	N	Y	N	S	G		
MH213140_17Ra171_Korea_2018	D	A	V	Q	D	R	R	A	R	N	A	F	S	A	T	Y	N	Y	N	S	G		
AY742932_CPV-193_USA_1991	D	A	V	Q	D	R	R	A	R	N	A	F	A	G	T	Y	N	Y	D	S	G	CPV-2b	CPV 2-II
AY742934_CPV-447_Germany_1995	D	A	V	Q	D	R	R	A	R	N	A	F	A	G	T	Y	N	Y	D	S	G	CPV-2b	
MF177241_Arg9_Argentina_2008	D	A	V	Q	D	R	R	A	R	N	A	F	N	G	T	Y	N	Y	N	S	G	CPV-2a	
MF177228_485-09_Italy_2009	D	A	V	Q	D	R	R	A	R	N	A	F	A	G	T	Y	N	Y	E	S	G	CPV-2c	
KM457118_UY190_Uruguay_2009	D	A	V	Q	D	R	R	A	R	N	A	F	A	G	T	Y	N	Y	E	S	G	CPV-2c	
MF177229_368-12-17_Albania_2012	D	A	V	Q	D	R	R	A	R	N	A	F	A	G	T	Y	N	Y	E	S	G	CPV-2c	
MF177258_BRA03/13_Brazil_2013	D	A	V	Q	D	R	R	A	R	N	A	F	N	G	T	Y	N	L	D	S	G	CPV-2b	
MK806279_IZSSI/PA24478/18/id3184_Italy_2018	D	A	V	Q	D	R	R	A	R	N	A	Y	A	G	T	Y	N	I	E	S	G	CPV-2c	CPV 2-III
MH545963_TN/CPV2a_India_2018	D	A	V	Q	D	R	R	A	R	N	A	Y	A	G	T	Y	N	I	N	S	G	CPV-2a	
MN400981_Rachi_Korea_2017	D	A	V	Q	D	R	R	A	R	N	A	Y	A	G	T	Y	N	I	D	S	G	CPV-2b	
MH660909_5 MGL_Mongolia_2017	D	G	V	Q	D	R	R	A	R	N	A	Y	A	G	T	Y	N	I	E	S	G	CPV-2c	
MH711894_CU24_Thailand_2016	D	G	V	Q	D	R	R	A	R	N	A	Y	A	G	T	Y	N	I	E	S	G	CPV-2c	
MF805789_Canine/China/01/2016_China_2016	D	G	V	Q	D	R	R	A	R	N	A	Y	A	G	T	Y	N	I	E	S	G	CPV-2c	
JQ268284_CPV-LZ2_China_2011	D	A	V	Q	D	R	R	A	R	N	A	Y	A	G	T	Y	N	I	D	S	G	CPV-2b	
KM457102_UY243_Uruguay_2010	D	A	V	Q	D	R	R	A	R	N	A	Y	A	G	T	Y	N	I	N	S	G	CPV-2a	
KY403998_CPV-YH_China_2008	D	A	V	Q	D	R	R	A	R	N	A	Y	A	G	T	Y	N	I	N	S	G	CPV-2a	
EU009204_K026_Korea_2007	D	A	V	Q	D	R	R	A	R	N	A	F	A	G	T	Y	N	I	N	S	G	CPV-2a	
MH213139_17Ra150 _Korea_2018	D	A	V	Q	D	R	R	A	R	N	A	Y	A	G	T	Y	N	Y	D	S	G	CPV-2b	
MG434740_IZSSI/PA3213_Italy_2017	D	A	V	Q	D	R	R	A	R	N	A	F	A	G	T	Y	N	L	N	S	G	CPV-2a	
LC270892_9985-46_Japan_2017	D	A	V	Q	D	R	R	A	R	K	A	F	A	V	T	Y	N	Y	D	S	G	CPV-2b	
MF069442_CPV/RC14/BC_Canada_2010	D	A	V	Q	D	R	R	A	R	N	A	F	A	D	A	H	N	Y	N	S	G	CPV-2a	
EF599098_Pome_Korea_2007	D	A	V	Q	D	R	R	A	R	N	A	F	A	D	T	Y	N	Y	N	S	G	CPV-2a	
EU659119_CPV-410_USA_2000	D	A	V	Q	D	R	R	A	R	N	A	F	A	G	T	Y	N	Y	D	S	G	CPV-2b	
AY742935_CPV-U6_Germany_1995	D	A	V	Q	D	R	R	A	R	N	A	F	A	G	T	Y	N	Y	N	S	G	CPV-2a	
AY742933_CPV-339_New Zealand_1993	D	A	V	Q	D	R	R	A	R	N	A	F	A	G	T	Y	N	Y	N	S	G	CPV-2a	

FPV and CPV isolates in this study were indicated by italics (bold indicates this study).
FPV, feline panleukopenia virus; CPV, canine parvovirus.

only in the CPV 2-III isolates. Eighty-four CPV 2-III isolates from several countries (China, Korea, India, Mongolia, Thailand, Uruguay, and Italy) presented Y-267 and I-324, whereas other all FPV and CPV-2 isolates had F-267 and Y-324 at the same position.

DISCUSSION

The emergence of consecutive FPV and CPV variants are being reported worldwide [19,22,23]. The FPV and CPV which represent carnivore parvovirus have high nucleotide substitution rates as approximately 10^{-4} per site per year as compared to those observed in other DNA viruses [11]. Our results indicate similar rates of 1.96 to 3.92×10^{-4} according to complete genomes or to each non-structural or structural gene (NS1, VP1, and VP2; **Table 4**). Thus, it seems that this relatively high mutation rate allows for the continuous appearance of variants.

Until now, CPV-2 has been categorized as either type 2a, 2b, or 2c according to aa changes on the VP2 protein, since the virus was regarded as a variant of FPV first discovered in the 1970s, and this genetic classification method has been widely accepted [8,19,23,24]. However, previous phylogenetic analyses of the VP2 gene did not highly support the genetic classification, as low bootstrap values determined for each node diverged to other genotypes due to low genetic variability [4,13,14,16,22]. In contrast, the high informative results elicited from the Bayesian phylogenetic analysis in this study, showed clear classified into three different genotypes (designated as CPV 2-I, -II, and -III).

According to the conventional classification of CPV-2 divided into three variable types (CPV-2a, -2b, and -2c), the most relevant change was in residue 426 of the VP2 protein. Types CPV-2a, -2b, and -2c presented Asn (N), Asp (D) and Glu (E) at this position, respectively [19,23]. Our findings do not follow the previous genotyping pattern. The CPV 2-I clade included only CPV-2a isolates and CPV 2-II/III clades included each genetic isolate type (-2a, -2b and -2c; **Fig. 2A-D**).

The FPV isolates analyzed in this study formed one consolidated clade that were differentiated from the CPV-2 isolates. The residue changes in the VP protein differentiating FPV from CPV-2 were K- or Q80R, K90R, K93N, V103A, D323N, N564S, and A568G and these results were consistent with Ikeda et al. [25] except for residue 87, 232, and 297. The VP2 protein of FPV and CPV-2 encompasses major antigenic domains, and is regarded as a promising candidate immunogen with the capacity to induce neutralizing antibodies [19]. Residues A-91, V-92, N-93, and N-95 form the tip of a protruding area on the virus which also configures the antibody binding site in CPV-2 [21]. The change of K93N may contribute to an antigenic difference between FPV and CPV-2. There were no substitutions in this region throughout all CPV-2 isolates sequences analyzed. Interestingly, notable antigenic and genetic differences of VP2 protein were observed within residues 2 to 16 (SDGAVQPDGGQPAVR) which is one of four neutralizing antibody binding sites, although no other substitutions were observed in the other three binding sites, which are within residues 91-95 (AVNGN), 292-297 (NSLPQSS) and 297-309 (SEGATNFDGVE) [21]. Three recent FPV isolates derived from Korea (2017) and India (2018) showed the high variability in V6F/D9N/R16K (Gigucheon), D3E/V6F/D9N/R16K (Rara) and A5P/V6F/Q7H/ D9N/R16K (TN/FPV) in this region, respectively. Further serological investigation is needed on these strains, because these changes may affect the overall antigenicity of FPV.

In the classification of CPV-2 genotype, CPV 2-I isolates were completely differentiated from each other by the change in residue 297 on the VP2 protein. The CPV 2-I isolates

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presented only S-297, whereas the CPV 2-II and -III isolates presented A- or N-297 and A-297, respectively. Only residue 297 is under positive selection pressure when analyzing viruses identified after 1990, but there was no evidence of positive selection sites during the 1980s [26]. As a result, most recent CPV-2a/2b/2c isolates present Ala (A) instead of Ser (S) at residue 297 position [23,26,27]. Thus, CPV 2-I isolates were considered as "old" CPV-2a strain-like virus from an evolutionary view.

Residues 292-297 (NSLPQSS) and 297-309 (SEGATNFDGVE) of the VP2 protein are antigenic sites that are exposed to neutralizing antibody [21]. Additional residue substitutions were found around the residue 297 when comparing CPV 2-I isolates with CPV 2-II and -III isolates. The CPV 2-I isolates presented A-300 and D-305 except for two isolates (CPV-13 and CPV-L), but almost all CPV 2-II and -III isolates presented G-300 and Y-305. Within CPV 2-II or -III isolates, some strains showed residue substitutions in this same region. Five isolates (Arg9, Arg50, Arg68, Bra03/13, and Bra05/13) had the residue change of A300N within CPV 2-II isolates. One Korean isolate (Pome), one Japanese isolate (9985-46) and three Canadian isolates (RC14/BC, RC19/BC, and RC20/BC) had residue changes of G300D, G300V and G300D/T301A/Y305H, respectively within CPV 2-III isolates. These substitutions in this region may be responsible for antigenic changes of CPV variants [27].

The numbers of CPV-2a, -2b, and -2c isolates constituting the CPV 2-II clade were one, 13, and 64, respectively and the corresponding numbers for CPV-2a, -2b, and -2c isolates forming the CPV 2-III clade were 94, 20, and 27. It is noteworthy that almost all CPV-2a isolates analyzed were classified into the CPV 2-III clade and a large portion of CPV-2c isolates analyzed was classified into the CPV 2-II clade. Two residue substitutions of the VP2 protein were characterized only in the isolates of CPV 2-III, although these changes were not consistently presented across the isolates of CPV 2-III. The changes of F267Y and Y324I were observed in many CPV 2-III isolates (84 out of 141) and these changes appeared with either residue but were mostly observed together. The simultaneous change of F267Y and Y324I was first observed in the Chinese strain CPV-YH isolated in 2008, and afterwards appeared extensively throughout the isolates recovered from several countries including China, Korea, India, Mongolia, Thailand, Uruguay and Italy. It is presumed that these substitutions act as a positive selection through CPV evolution within CPV 2-III isolates. Therefore, extensive and long-term investigation is required to validate this assumption. Residues 267 to 498 in the VP2 protein form a GH loop located between the βG and βH strands and this region has greatest variability in the parvovirus genome due to its exposure on the capsid surface [4]. Antigenic variability of CPV may be induced by residue substitutions of VP2 protein such as F267Y and Y324I.

The present study performed extensive genetic and phylogenetic analysis on FPV and CPV-2 isolates from Korea and other countries by Bayesian method. In the genetic analysis of VP2 protein, these isolates were classified into FPV and three CPV genotypes (CPV 2-I, -II and -III). The recent FPV isolates Gigucheon (Korea, 2017), Rara (Korea, 2017) and TN/FPV (India, 2018) showed high variability of the antigenic site. The CPV 2-I included only CPV-2a-like isolates under the old classification and were differentiated by the change of residue S297A or N. Almost all CPV-2a isolates were classified into CPV 2-III and the large portion of CPV-2c was classified into CPV 2-II. The residue changes of F267Y and Y324I were further characterized in CPV 2-III isolates. Residue changes of F267Y and Y324I were observed in many CPV 2-III isolates recovered after 2008 from several countries (China, Korea, India, Mongolia, Thailand, Uruguay, and Italy). Such substitutions are presumed to act as a

positive selection in the evolution of CPV, so additional investigation is required to verify this assumption. Residue substitutions within the exposed surface of capsid protein may lead to antigenic changes in CPV. To counteract the emergence of new CPV variants, continued surveillance of viruses and possibly new vaccines may be required.

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