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Prevalence and genetic characterization of canine parvoviruses in Korea

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Abstract The prevalence of canine parvovirus (CPV) variants in dog was investigated in a total of 51 fecal samples submitted over a 2-year period (2005–2007) in Korea. The CPV VP2 gene was amplified and sequenced from the fecal samples, and the results indicated that of the 51 samples, 49 samples belong to the CPV-2a family, 1 to CPV-2b, and the remaining 1 to CPV-2a variant. The VP2 gene of 20 isolates was sequenced and phylogenetic analysis was conducted. With one exception, all of the isolates were closely related to a Taiwanese isolate (CPV T37) and they formed geographical patterns of VP2 gene nucleotide sequences. Our finding showed that CPV-2a was the predominant type and CPV-2b and CPV-2a variant also existed in Korea. Using the hemagglutination inhibition (HI) and the neutralization (Nt) test, the animals inoculated with CPV-2 developed low antibody titers against the CPV-2 variants in laboratory animal was also identified.

Nucleotide sequence data reported is available in the GenBank database under the accession No. EF599096, EF599097, EF599098.

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Canine parvovirus type 2 (CPV-2) was identified as a new pathogen of dogs in 1978 and is the major cause of hemorrhagic gastroenteritis and myocarditis in puppies [1]. CPV-2 is antigenically and genetically related to feline panleukopenia virus (FPLV). However, CPV-2 infects dogs and other *Canidae*, but not cats, whereas FPV infects cats, mink, and raccoons, but not dogs. A few amino acid differences between CPV and FPV determine the species specificity of these viruses [2, 3]. In the period from 1979 to 1981, CPV-2a was demonstrated to be an antigenic variant that differs from CPV-2, and subsequently, around 1984, a second variant, CPV-2b, was described [4]. Both of these variants can infect or replicate and be transmitted between dogs and cats, and they now prevail throughout the population worldwide [5]. Recently, CPV-2a- and CPV-2b-related viral strains were isolated from leopard cats in Vietnam and Taiwan [6]. A novel CPV mutant (Glu-426) as a new type called CPV-2c was detected and is progressively replacing other CPV types in Italy [7, 8]. CPV-2c has also been found in Vietnam [9], Spain [10], Germany [11], and United Kingdom [12]. However, the prevalence of CPV variants has not been well investigated in Korea.

CPV-2 is a non-enveloped and single strand DNA virus, with two nonstructural proteins (NS1 and NS2) and two structural proteins (VP1 and VP2). VP2 is a major determinant of host immune response and host range [13, 14]. There were five amino acid differences at residues 87, 101, 300, 305, and 555 of VP2 between CPV-2 (Met-87, Ile-101, Ala-300, Asp-305, and Val-555) and CPV-2a (Leu-87,

Thr-101, Gly-300, Tyr-305, and Ile-555), and also five amino acid differences at residues 87, 101, 300, 305, and 426 of VP2 between CPV-2 (Met-87, Ile-101, Ala-300, Asp-305, and Asn-426) and CPV-2b (Leu-87, Thr-101, Gly-300, Tyr-305, and Asp-426) [9, 14]. The significant difference between CPV-2a (Asn-426 and Ile-555) and CPV-2b (Asp-426 and Val-555) was the substitution of two amino acids in the major antigenic VP2 capsid protein [14]. A new CPV mutant called CPV-2c had a Glutamate substitution in the 426th residue of VP2 protein [7, 10].

In this study, we isolated three types of CPV—CPV-2a, CPV-2b, and CPV-2a variant—from 51 dog diarrheic fecal samples. On the basis of their VP2 gene sequences, we investigated the types of CPV of the fecal samples and phylogenetic relationship between virus isolates from these fecal samples and other CPVs including two vaccine strains commercialized in Korea. We also compared the serological cross-reactivity among CPV-2 variants including CPV-2 using the isolates.

A total of 57 fecal samples were submitted to the Green Cross Veterinary Institute in Yong-in, Korea, from 2005 to 2007. The fecal samples were first tested using a commercially available, rapid CPV antigen detection kit (Animal Genetics, Inc., Suwon, Korea) that can detect 1HA unit of CPV and has 100% specificity (data not shown). Using this approach, 51 of the 57 samples were identified as being CPV-positive. Vaccine history of the animals for each isolate was not available.

The CPV-positive fecal samples were suspended in phosphate buffered saline. After centrifugation at 3,000*g* for 20 min, the supernatant fluids were collected and filtered with a 0.20-μm syringe filter (Sartorius, Hannover, Germany). Both filtered fecal samples and the KCPV strain were propagated in a Crandell feline kidney (CRFK) cell line grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and antibiotic solution (at final concentrations of 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B). Conditioned medium from these cultured cells was screened for CPV antigen detection.

PCR was performed using VP2 gene-specific primer pairs, as described previously [6]. Fecal samples were suspended in PBS and centrifuged at 6,000*g* for 5 min. For DNA extraction, cell lysis buffer (500 μl) and 200 μl/ml proteinase K was mixed with 200 μl of the sample solution and the mixture was thoroughly vortexed and incubated for 1 h at 65°C. The extraction of DNA was performed as previously described [15]. The reaction mixture contained DNA (2 μl), 10X Taq DNA polymerase buffer (2.5 μl), 1.5 mM $MgCl_2$, 2.0 μl dNTPs (2.5 mM/μl), 1 μl of each specific primer (10 pmol each), and 1 μl Taq DNA polymerase (Promega, USA). Distilled water was added to a total volume of 25 μl. The 1.75-kb sized PCR product, covering most of the VP2 coding sequence, was amplified in three parts with a 79-bp overlap, with fragment sizes being 708, 719, and 736 bp, respectively.

The PCR products were gel purified and sequenced on an ABI373 automated sequencer using a commercial service (Genotech, Co. Ltd., Daejeon, Korea), with the sequence of each product being determined twice. The type of the fecal samples and field isolates was determined on the basis of conserved nucleotide and amino acid differences in the VP2 gene, as reported previously [7, 16, 17].

The nucleotide and deduced amino acid sequences of the 20 isolate VP2 genes, of which dogs had been identified to have clinical symptoms associated with severe hemorrhagic enteritis and finally leading to death, were compared with each other, as well as with other CPV VP2 gene and amino acid sequences obtained from GenBank (Table 1). Alignments of individual CPV sequences were created

Table 1 CPV isolates from various area of Korea and GenBank accession numbers of other CPVs obtained from the GenBank

Serial no.	Isolate	Year	Origin	GenBank accession no.
1	KCPV	1986	Vaccine strain	
2	Quantum	?	Vaccine strain	
3	DH326	2005	Yong-in, Kyunggi	EF599097
4	DH426	2005	Yong-in, Kyunggi	EF599096
5	SNUB2	2005	Seoul	
6	Ani1	2005	Seong-nam, Kyunggi	
7	Ani2	2005	Seong-nam, Kyunggi	
8	Pome	2005	Suwon, Kyunggi	EF599098
9	Pome1	2005	Seong-nam, Kyunggi	
10	Mu3	2005	Seong-nam, Kyunggi	
11	JSK	2006	Chung-ju, Chungbuk	
12	SKW	2006	Kim-po, Kyunggi	
13	MR0603	2006	Seong-nam, Kyunggi	
14	MR0604	2006	Seong-nam, Kyunggi	
15	MR0605	2006	Seong-nam, Kyunggi	
16	MR0606	2006	Seong-nam, Kyunggi	
17	JS	2006	Chung-ju, Chungbuk	
18	GJ	2006	Seong-nam, Kyunggi	
19	JE	2006	Jeong-eup, Jeonnam	

Table 1 continued

Serial no.	Isolate	Year	Origin	GenBank accession no.
20	JYS26-1	2006	Jeong-eup, Jeonnam	
21	JYS26-4	2006	Jeong-eup, Jeonnam	
22	JYS26-6	2006	Jeong-eup, Jeonnam	
23	CPV 1	2003	India	DQ182612
24	CPV 15	2004	India	DQ182620
25	CPV 24	2005	India	DQ182624
26	CPV b	1978	USA	M38245
27	CPV-193	1991	USA	AY742932
28	CPV-436	2003	USA	AY742955
29	CPV-U6	1995	Germany	AY742935
30	CPV-447	1995	Germany	AY742934
31	CPV-U51	1997	Germany	AY742942
32	FPV 314	1993	Japan	D78585
33	97-008	2003	Japan	AB115504
34	CPV-W42	1995	Italy	AF306444
35	CPV-56-00	2000	Italy	AY380577
36	CPV-695	2001	Italy	AF401519
37	(L) CPV-V203	1997	Vietnam	AB054224
38	V 120	2001	Vietnam	AB054215
39	LCPV-T1	1998	Taiwan	AB054214
40	Taichung	2004	Taiwan	AY869724
41	China HN-3-2005	2005	China	DQ177497
42	CPV T37	1996	Taiwan	U72698
43	CPV T10	1996	Taiwan	U72696
44	LCPV V140	2000	Japan	AB054223
45	RPPV	2004	China	DQ354068

using the ClustalX program [18]. Sequence comparisons to reference viruses were edited and analyzed with the Bioedit v7.0.5.2 program and MEGALIGN program (DNASTAR, Madison, WI, USA). The phylogenetic relationships between the CPV isolate and selected reference viruses were estimated from their nucleotide sequences by employing the neighbor-joining method in the MEGA 3.1 program.

Twelve guinea pigs, weighted 300–350 g, were randomly divided to four groups (II, A, B, and C group) and housed four separated and isolated cages. All the animals were serologically negative to CPV before vaccination as determined by HI and neutralization test (Nt). All groups were inoculated with each type of CPV vaccine twice at the interval of 2 weeks as following. Group II animals ($n = 3$) were inoculated intramuscularly with 2 ml of the CPV2 vaccine (2^9 HAU of KCPV strain) and group A animals ($n = 3$) received 2 ml of the CPV2a isolate (2^9 HAU of DH426 passage level 15). Group B animals ($n = 3$) were injected 2 ml of the CPV2b isolate (2^9 HAU of DH326 passage level 15) and group C animals ($n = 3$) received 2 ml of the CPV2a variant isolate (2^9 HAU of Pome passage level 15) intramuscularly. At 14 days after the second vaccination, the antibody titers of each group were determined by HI and Nt using four viruses as previously described [19].

In CRFK cells, CPE were observed from 3 of 20 isolates, at first passage of sample DH426 and Pome, and the second passage of sample DH326. The cultivated supernatants of the 20 isolates were tested to be CPV-positive using rapid CPV antigen detection kit and PCR in the first passage. All isolates had passaged five times; however, three isolates of them that showed CPE in CRFK have passaged more than 15 times in CRFK.

Amplification of the isolates with the three primer pairs yielded 1,755-bp fragments, and the type of the fecal samples was characterized as CPV-2a, CPV-2b, or CPV-2a variant. Out of 51 fecal samples analyzed, 49 were identified as CPV-2a, 1 was identified as CPV-2b (designated DH326), and the remaining 1 was characterized as CPV-2a variant (designated Pome). The domestic vaccine strain (KCPV) was identified as CPV-2 and the imported vaccine strain (Schering-Plough Animal Health, USA) was characterized as CPV-2b (data not shown).

Type 2a and type 2a variant isolates were clustered together and closely related to a Taiwanese isolate (CPV T37), a Japanese isolate (FPV 314), and a Chinese isolate (RPPV); however, the type 2b isolate was closely related to a Taiwanese isolate (Taichung) on the basis of nucleotide sequences (Fig. 1).

The nucleotide differences were detected in 3 bases (G→A at 48, T→A at 318, and G→A at 405) between the isolate DH426 (accession no. EF599096) and CPV T37, 8 bases (T→A at 18, G→A at 36, G→A at 429, G→A at 627, G→A at 1,041, C→T at 1,466, A→G at 1,509, and C→T at 1,569) between DH326 (accession no. EF599097) and Taichung, and 12 bases (A→G at 36, A→G at 147, T→C at 303, G→A at 405, T→C at 537, G→A at 552, C→T at 630, C→T at 1,026, G→A at 1,276, C→T at 1,303, T→C at 1,695, and A→G at 1,722) between Pome (accession no. EF599098) and LCPV-V203.

Based on the deduced amino acid sequences of VP2, 14 of the isolates characterized as CPV-2a were clustered close to known Indian isolates (CPV1, CPV24), while isolate DH426 was clustered with known German (CPV U6) isolate and isolate SKW was clustered with known Indian (CPV15) isolate and Taiwanese (CPV T37, LCPV T1) isolates.

Isolate DH326, identified as CPV-2b, was closely related to isolates from Taiwanese isolate (CPV T10). Isolate

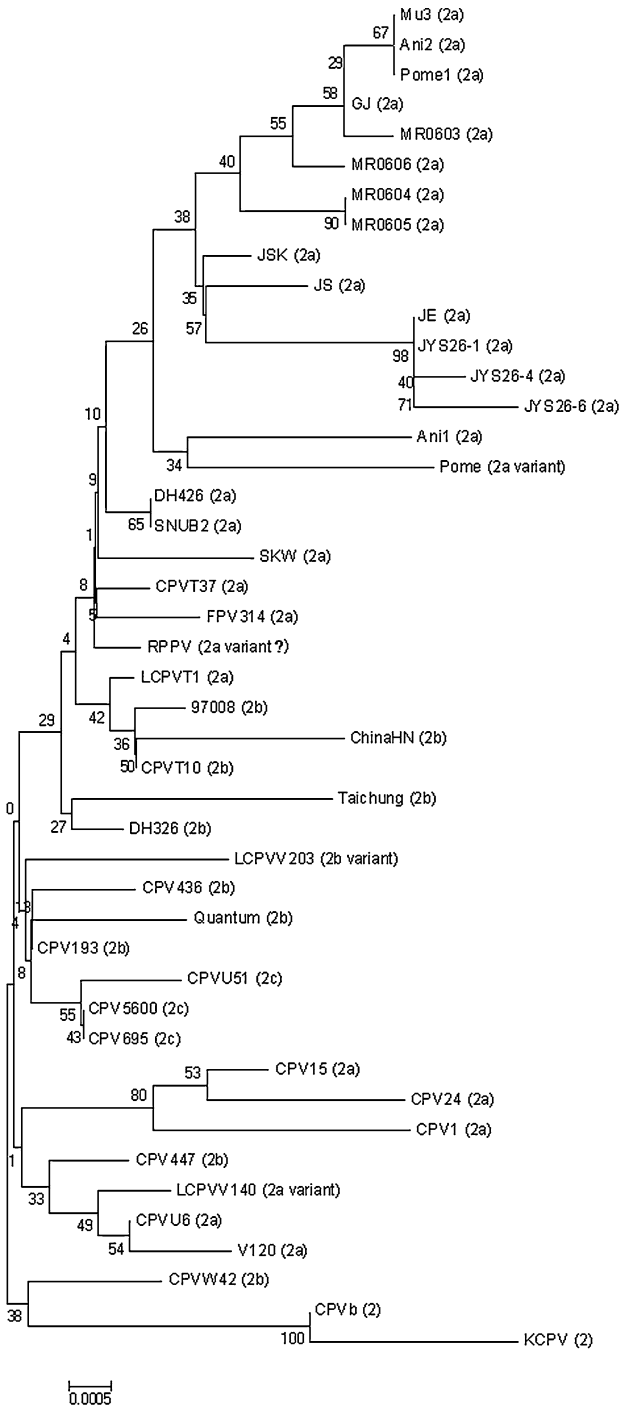

Fig. 1 Phylogenetic tree constructed from the VP2 gene nucleotide sequences of the field isolates in this study and other sequences obtained from the GenBank database

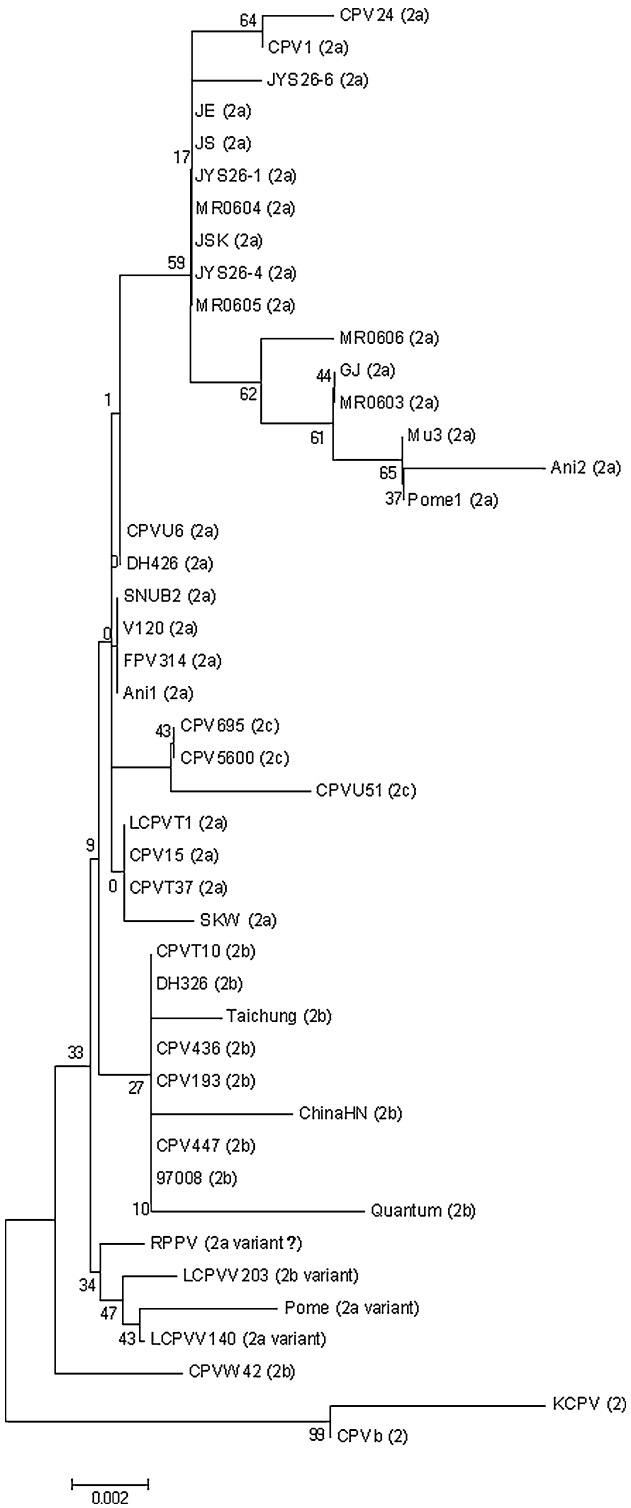

Fig. 2 Phylogenetic tree constructed from the VP2 amino acid sequences of the field isolates in this study and other sequences obtained from the GenBank database

Pome, identified as CPV-2a variant, was closely related to a Japanese isolate (LCPV V140) and a Vietnamese isolate (LCPV V203) (Fig. 2).

The HI and neutralization antibody titers of guinea pigs inoculated with CPV-2, CPV-2a, CPV-2b, and CPV-2a variant were displayed in Table 2. In case of group II, the homologous geometric mean antibody titer was 806 and the mean heterologous titers against the CPV-2a, CPV-2b, and CPV-2a variant were 320, 320, and 254, respectively, and the differences between CPV-2 and CPV-2b and

Table 2 Results of hemagglutination inhibition (HI) and neutralization test (Nt) on guinea pigs inoculated with CPV-2, CPV-2a, CPV-2b, and CPV-2a variant

Group	Test	Mean antibody titer			
		CPV-2	CPV-2a	CPV-2b	CPV-2a variant
Group II	HI	806.35	320.00	320.00	253.98
	Nt	507.97	126.99	100.79	80.00
Group A	HI	507.97	806.35	640.00	640.00
	Nt	640.00	806.35	640.00	806.35
Group B	HI	1,280.00	1,612.70	2,031.87	1,612.70
	Nt	806.35	640.00	1,015.94	806.35
Group C	HI	507.97	640.00	507.97	806.35
	Nt	403.17	403.17	403.17	507.97

between CPV-2 and CPV-2a variant are statistically significant ($P = 0.033$ and 0.028). For animals inoculated with CPV-2a (group A), the homologous and heterologous geometric mean titers against CPV-2, CPV-2b, and CPV-2a variant were, respectively, 806, 508, 640, and 640; the differences are not statistically significant ($P = 0.225$, 0.308, and 0.239). For the animals inoculated with CPV-2b (group B), the homologous and heterologous geometric mean titers against CPV-2, CPV-2a, and CPV-2a variant were 2,032, 1,280, 1,613, and 1,613; the differences are not statistically significant ($P = 0.208$, 0.259, and 0.259). For guinea pigs given CPV-2a variant (group C), the homologous and heterologous geometric mean titers against CPV-2, CPV-2a, and CPV-2b were 806, 508, 640, and 508; the differences are not statistically significant ($P = 0.125$, 0.389, and 0.125).

The animals inoculated with CPV-2 (group II) had lower antibody titers than the guinea pigs inoculated with CPV variants (groups A, B, and C). The homologous geometric mean antibody titer in group A was 508, whereas the mean heterologous titers against CPV-2a, CPV-2b, and CPV-2a variant were 127, 101, and 80, respectively, which are statistically significant differences ($P = 0.011$, 0.009, 0.009). In other groups (A, B, and C), the differences between homologous antibody titer and heterologous antibody titers are not statistically significant ($P > 0.1$). These results indicated that the CPV-2 strain conferred a somewhat lower immunity against CPV-2 variants including CPV-2a, CPV-2b, and CPV-2a variant.

The two isolates identified as CPV-2b and CPV-2a variant had unique amino acid sequences at each key position of the VP2 region. Even though the isolate Pome has Asp at residue 300, the isolate Pome might be characterized as CPV-2a Asp-300 variant on the basis of the nucleotide and amino acid informative positions (Table 3). This is also evident from the phylogenetic trees constructed on nucleotide and amino acid sequences of VP2, where the

Table 3 Comparison of deduced amino acid sequences of the field isolates in this study

Isolate	87	101	297	300	413	418	426	435	440	555
MR0603	L	T	A	G	N	T	N	P	A	V
MR0604	L	T	A	G	D	I	N	P	A	V
MR0605	L	T	A	G	D	I	N	P	A	V
MR0606	L	T	A	G	D	T	N	P	A	V
Mu3	L	T	A	G	N	T	N	S	A	V
Pome	L	T	A	D	D	I	N	S	T	V
Pome1	L	T	A	G	N	T	N	S	A	V
SNUB2	L	T	A	G	D	I	N	P	T	V
Ani1	L	T	A	G	D	I	N	P	T	V
Ani2	L	T	A	G	N	T	N	S	A	V
DH326	L	T	A	G	D	I	D	P	T	V
DH426	L	T	A	G	D	I	N	P	T	V
JSK9	L	T	A	G	D	I	N	P	A	V
SKW	L	T	A	G	D	I	N	P	T	V
JS	L	T	A	G	D	I	N	P	A	V
GJ	L	T	A	G	N	T	N	P	A	V
JE	L	T	A	G	D	I	N	P	A	V
JYS26-1	L	T	A	G	D	I	N	P	A	V
JYS26-4	L	T	A	G	D	I	N	P	A	V
JYS26-6	L	T	A	G	D	I	N	P	A	V

isolate Pome clustered together with classical type 2a strains, including other CPV-2a isolates in Korea and FPV-314 that was isolated from a cat or type 2a variant (LCPV V140) (Fig. 1). The Asp-300 variants, detected in domestic and wild felids in southern Asia, displayed the substitution at residue 300 (Gly→Asp) irrespective of the antigenic type (2a or 2b) and were referred to as CPV-2a(c) or CPV-2b(c) on the basis of the amino acid encountered at residue 426 (Asn or Asp) of the VP2 protein [6]. There was a previous report of a mutant with the change at residue 426 (Asp→Glu) occurring in a strategic residue for the antigenicity of CPV has been detected in Italy [7]. The Glu-426 mutant was spreading among the canine population in Italy and has been reported in other countries [9, 10]. Moreover, while the mutation at residue 300 is located in a minor antigenic site, the mutation at residue 426 affects the major antigenic region that has been taken into account for classification of the variants CPV-2a and CPV-2b [14]. On the basis of the above considerations, Glu-426 mutant has been referred to as true antigenic variant CPV-2c, whereas the Asp-300 mutant should be regarded as mutants of CPV-2a and CPV-2b. This was the first report that the CPV-2a variant (Pome strain) with a mutation in the residue 300 of the VP2 gene was isolated in a dog; the same variant was found previously in leopard cats [6].

Since type 2b and type 2a variant were each identified in only one sample, it is not clear if CPV-2b and -2a variant are

true circulating CPV variants in Korea. All isolates identified as CPV-2a, however, had a substitution at residue 555 (Ile→Val) of VP2, which was different from the previously identified amino acid sequence of CPV-2a (Table 3). Nucleotide variation (A→G at 1,663) corresponded to this amino acid change at 555. The nucleotide variation at 1,663 (coding Val at residue 555) has occasionally caused the genotypic mischaracterization of these isolates as CPV-2b, using previously reported differential primer pairs, and as a consequence, Chinchkar et al. proposed that stringent PCR conditions should be used to discriminate variants [20–22]. The presence of Val at position 555 in type 2a was previously reported for Taiwanese and Indian isolates and it was proposed that these isolates were transition types between type 2a and type 2b or that they had evolved independently from CPV-2 [22, 23].

The deduced amino acid sequences from the amplified VP2 gene products revealed that among isolates in this study, variability existed at residues 413, 418, 435, and 440. Specifically, these isolates had Asp or Asn at position 413, Ile or Thr at position 418, Pro or Ser at position 435, and Thr or Ala at position 440 (Table 3). These residues are located in the GH loop; this large loop is composed of 267–498 residues and is located between the βG and βH strands. The GH loop intertwines with two other symmetry equivalents to form a protrusion around each threefold axis [24]. This region is exposed on the surface of the capsid and forms the 22 Å threefold spike; the greatest variability between parvovirus was observed in this antigenic region [25]. Mutations at 418 and 440 have also been reported previously for isolates from Italy [7, 26]. As reported previously in Italy, Taiwan, and India, we found that the greatest variability in the VP2 protein occurs (within the GH loop) at position 440. A high level of substitution in this region has been associated with the evolution of antigenic variants in circulating parvovirus types [6, 22, 26].

Phylogenetic analysis of VP2 revealed that the Korean isolates are genetically close to Asian isolates. Since type 2a isolates in this study were grouped according to geographical location, it appears that CPV-2a have evolved in a geographical manner in Korea. Interestingly, KCPV, which has been used as a vaccine strain in Korea, was clustered in a different group from the field isolates and was close to a USA isolate (CPVb) that had been characterized as CPV-2, and imported vaccine strain, even though identified as CPV-2b, was also clustered in a different group from the field isolates in this study. These findings and cross-reactivity between CPV-2 and CPV-2 variants suggest that the use of these vaccine strains in Korea might be reconsidered.

There is some discussion as to whether these new variants cause more pronounced hemorrhagic signs and lesions than the original CPV-2 in dogs [27]. Notably, in neutralization tests, antigenic differences have been demonstrated between the subtypes and it is feasible that these differences might have some clinical importance [19, 28]. Further investigation of CPV-2a, CPV-2b and -2a variant, with regard to their prevalence and pathogenesis in dogs, will be required to determine if these isolates are of clinical significance.

Previous studies reported that type 2b was the major CPV type in Brazil, Japan, and in the middle region of Taiwan [17, 23, 29], while type 2a was the most prevalent type in Italy and India [22, 30]. In this study, we found that in Korea, CPV-2a is the predominant type and that CPV-2b and -2a variant also exist.

In summary, three types of CPV were identified from the fecal samples examined in this study: CPV-2a, CPV-2b, and CPV-2a variant. CPV-2a was the predominant, circulating CPV in Korea and geographical differences were observed in the prevalence of circulating parvovirus variants. All type 2a isolates in Korea had substitution of Val at residue 555, as has been reported previously in Taiwan and India [22, 23]. Further studies will be required to determine the prevalence of CPV type 2b and type 2a variant and pathogenesis of CPV type 2a, type 2b, and type 2a variants in dog, and to establish the degree of cross-reactivity among these variants isolated in this study.

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