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<Original Article>

Isolation and identification of canine parvovirus type 2b in Korean dogs

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Abstract : Canine parvovirus (CPV) is a major diarrhea-causing agent in puppies. Since CPV type 2 (CPV-2) emerged in 1978, new antigenic variants including CPV-2a, CPV-2b, and CPV-2c have been identified in many countries. Two puppies died suddenly at a veterinary clinic in Gyeonggi province, South Korea. Two viruses were isolated in A72 cells, confirmed as CPV strains based on a CPV rapid kit and an indirect fluorescence test and designated QIACP1403 and QIACP1404. The nucleotide sequences of complete *VP2* genes of QIACP1403 and QIACP1404 were determined, and the corresponding amino acid sequences were deduced. Molecular analyses revealed that the QIACP1403 and QIACP1404 isolates were type CPV-2b. Several mutated amino acids were detected on *VP2* gene residues of the two isolates. Phylogenetic analyses showed that the two isolates were most closely related to strain CPV-BM11, which was isolated from Chinese dogs in 2011. Our results suggest that these isolates may be a candidate for a vaccine to prevent CPV infection in dogs after conducting passages of the isolates in an *in vitro* culture system.

Keywords : CPV-2b, canine parvoviral enteritis, virus isolation

Introduction

Canine parvovirus (CPV) is a diarrhea-causing pathogen that affects puppies and kittens older than 2 months with symptoms of hemorrhagic gastroenteritis, lethal myocarditis, leucopenic, nausea, vomiting, and diarrhea [1, 12, 13]. CPV is in the family *Parvoviridae* (parvovirus), and is closely related to the feline panleukopenia virus and parvovirus that originated in raccoons, minks, and foxes [9]. CPV contains negative-sense single-stranded DNA with a genome of approximately 5.2 kb in length and consists of nonstructural proteins NS1 and NS2 and structural proteins VP1 and VP2. The VP2 protein (585 residues) is the main capsid protein and induced neutralizing antibody [8].

A new type of CPV was identified in the late 1970s and spread to Europe, Asia, and North America. This type has been referred to as CPV-2 to distinguish it from canine parvovirus virus minute variant (CPV-1) [4]. In the early 1980s, CPV-2 variants were identified using specific monoclonal antibodies, restriction enzyme analysis, and DNA sequencing [20]. Studies of two CPV-2 variants called CPV-2a and CPV-2b revealed that amino acid residues at positions 297 and 426 of the *VP2* gene changed from serine to alanine and from asparagine to aspartic acid, respectively [2]. Since a new antigenic variant designated CPV-2c with glutamic acid at position 426 was first identified in Italy in 2000, many researchers in Italy, Spain, Germany, Vietnam, Japan, Mexico, and the USA have reported CPV-2c in naturally infected animals [3, 7, 11, 17].

Although the original CPV is no longer present in the canine population based on surveillance studies, CPV-2a has been circulating mainly in India, Germany, and Korea. CPV-2b has also been detected in the USA, Taiwan, and Japan [10, 14, 17, 22]. However, a CPV-2c infection with symptoms of mucoid yellow or hemorrhagic diarrhea, leucopenia, and lymphopenia has been reported worldwide in adult dogs and in dogs inoculated with a commercial CPV vaccine [8]. Currently, CPV2a/b/c shows antigenic variance in five or six amino acid residues of the VP2 protein. Thus, CPV sub-typing of wild CPV infections is necessary to understand the evolution of the virus and provide information about field status. It is also important to develop a new CPV vaccine. In this study, we isolated two wild CPV-2b strains circulating in a Korean dog population using a cell culture system and investigated their genetic relationship with other CPV strains.

Materials and Methods

Sample preparation and virus isolation

Two 3-month-old puppies were received from a veterinary clinic in Gyeonggi province, Korea, in 2014. The dogs showed typical symptoms of a CPV infection, including nausea and hemorrhagic diarrhea and eventually died. Fecal samples were obtained from the intestines of each of puppy and subjected

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to a CPV rapid diagnostic kit (BioNote, Korea). For virus isolation, the two samples were filtered through a 0.45 μm syringe filter and inoculated into A72 cells, which were derived from canine fibroblasts, and grown in alpha minimum essential medium with 10% fetal bovine serum. The cells were incubated in a CO_2 incubator and their morphology was observed for 7 days. The cell supernatant was screened using the hemagglutination assay (HA), and then the cells were fixed in cold 80% acetone, subjected to an indirect fluorescent assay (IFA) using a specific CPV monoclonal antibody (MEDIAN Diagnostics, Korea), and stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG + IgM. After a wash in phosphate-buffered saline (pH 7.2), specific fluorescence was visualized in the infected cells using a fluorescence microscope (Nikon, Japan). The isolates were propagated in A72 cells and titrated as 10-fold serial dilutions in 96-well plates. The IFA viral titers were calculated according to the Reed and Muench method and expressed as the 50% fluorescent assay infectious dose per mL ($FAID_{50}$/mL).

Hemagglutination assay

The HA test was carried out by preparing serial two-fold dilutions of the isolates in 50 μL Sorensen buffer (pH 6.8) with 50 μL 0.6% pig erythrocytes. The HA titer was expressed as the reciprocal of the highest dilution of the isolate that hemagglutinated.

DNA extraction and polymerase chain reaction (PCR) analysis

Virus DNA was extracted from the two CPV strains using a DNA extraction kit (iNtRON, Korea) according to the manufacturer's instructions. PCR was conducted for gene amplification using specific primers (Table 1) for the CPV *VP2* gene. The PCR was carried out in PCR premix (Bioneer, Korea) containing 10 μL denatured DNA, 1 μL each primer (50 pmol), and 38 μL distilled water in a 50 μL final volume. The cycling profile was denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were visualized after 1.8% agarose gel electrophoresis containing the Redsafe Nucleic acid staining solution (iNtRON).

Cloning and DNA sequencing

All PCR products purified with the gel extraction kit were ligated with the pGEM-T easy vector (Promega, USA) according to the manufacturer's protocol. Plasmid DNA was isolated from amplified *Escherichia coli* (DH5α), and recombinant plasmids were identified using *EcoR*1 enzyme digestion (Bioneer). The sequences of the purified plasmids were determined using an MJ Research PTC-225 Peltier Thermal Cycler and ABI PRISM BigDye Terminator Cycle Sequencing kits with AmpliTaq DNA polymerase (FS enzyme; Applied Biosystems, USA) according to the manufacturers' protocols. Single-pass sequencing was performed with each template using universal primers (*e.g.*, SP6 and T7). The fluorescent-labeled fragments were purified from the unincorporated terminators using an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis on an ABI 3730xl sequencer (Applied Biosystems). Both DNA strands were sequenced for verification.

Phylogenetic analysis

The nucleotide sequences, accession numbers, and names of the strains used for the phylogenetic analysis were obtained from the GenBank database (National Center for Biotechnology Information, USA). The full nucleotide sequences of the *VP2* genes from the isolates were compared to those of other known CPV strains using Clustal W2 [14]. Genetic distances were calculated using the Kimura-2 correction parameter, and a phylogenetic tree was constructed using the neighbor-joining method with 1,000 bootstrap replicates in MEGA4 [21].

Results

Detection and isolation of CPV

Filtered fecal samples were applied to a rapid diagnostic kit, and the DNA extracted from the fecal samples was amplified with a CPV diagnostic primer set. Both the rapid kit and PCR results showed strong positive reactions against CPV titers in the two samples (data not shown). The A72 cells inoculated with the fecal samples were examined under a microscope for 7 days and were passaged blindly. No cytopathic effects (CPEs) were observed in the cells. The HA test was performed on infected A72 cells at 5 days post-infection

Table 1. List of the oligonucleotide primers used for polymerase chain reaction against for canine parvovirus (CPV)

Primer designated	Oligonucleotide sequence (5'-3')	Target gene	Size of amplicon
CVPDF	GAG CAT TGG GCT TAC CAC CA	*VP2*	793 bp
CPVDR	GGA TTC CAA GTA TGA GAG GC		
CPVSF1	CCA ACT AAA AGA AGT AAA CC		708 bp
CPVSR1	TGG TTG GTT TCC ATG GAT AAA AAC C		
CPVSF2	AGA TAG TAA TAA TAC GCC ATT T		719 bp
CPVSR2	TTT TGA ATC CAA TCT CCT TCT GGA T		
CPVSF3	ACA GGA GAA ACA CCT GAG AGA TTT A		736 bp
CPVSR3	CCT ATA TCA AAT ACA AGT ACA ATA		

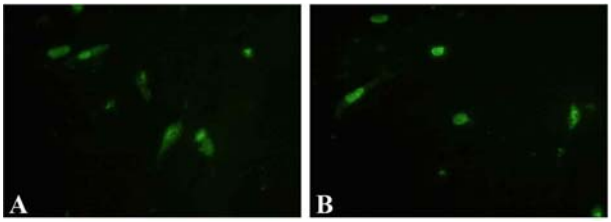

Fig 1. Immunofluorescence of QIACP1403 (A) and QIACP1404 isolates (B) by indirect fluorescent assay test using monoclonal antibody against CPV in A72 cells.

to determine whether the isolates could hemagglutinate. The two isolates showed HA titers of 32. The A72 cells infected with the isolates were stained with a CPV specific antibody. As shown in Figure 1, fluorescence was detected in A72 cell nuclei. The viral titers of the isolates were $10^{3.5}$ $FAID_{50}$/mL and $10^{4.5}$ $FAID_{50}$/mL at the third passage. The two isolates were designated QIACP1403 and QIACP1404 and deposited in the Korea Veterinary Culture Collection (accession no. KVCC-VR1500038 and KVCC-VR1500039).

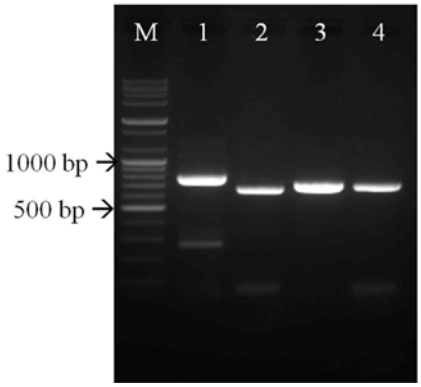

Fig 2. Three specific primer sets targeted *VP2* gene of the QIACP1403 strain were used for reverse transcription polymerase chain reaction. M, 1 kb DNA ladder; Lane 1, QIACP1403 strain with CPVDFR primer sets; Lane 2, QIACP1403 strain with CPVSFR1 primer sets; Lane 3, QIACP1403 strain with CPVSFR2 primer sets; Lane 4, QIACP1403 strain with CPVSFR3 primer sets.

Nucleotide sequence and amino acid analyses of the CPV *VP2* gene

PCR using specific CPV primers amplified three partial *VP2* genes from QIACP1403 and QIACP1404 (Table 1). As shown in Figure 2, three *VP2* gene PCR products were detected in QIACP1403 with sizes of 708, 719, and 736 bp on 1.8% agarose gel electrophoresis. A total length of 1756 bp nucleotides was determined for the complete *VP2* gene of both strains, and their amino acid sequences were deduced. The nucleotide sequence data of the two strains were deposited in GenBank with accession no. KP893077 and KP893078, respectively. The *VP2* gene sequences of 35 other CPVs obtained from GenBank were compared to those of the two strains to identify molecular epidemiological relationships. The nucleotide sequence analysis showed that the two strains had identical homology, and the highest sequence similarity of the two strains corresponded to that of the CPV-BM11 strain (99.5% at the nucleotide level and 99.3% at the amino acid level) isolated from Chinese dogs in 2011 (Fig. 3). As shown in Table 2, the QIACP1403 and QIACP1404 strains had glycine, asparagine, and valine at positions 300, 426, and 555 of the *VP2* gene, respectively. The amino acid sequence analysis revealed that the newly isolated viruses were type CPV-2b.

Discussion

We have described the isolation of CPV-2b from mongrel puppies with hemorrhagic diarrhea. The two samples were positive for CPV on a rapid kit and by PCR. CPV has been isolated from several carnivores, such as dogs, raccoon dogs, masked civets, foxes, and minks [5, 16]. Previous studies have demonstrated that CPV originating from several species will replicate efficiently in CRFK, primary dog kidney, and the A72 cell lines, but not in bovine or human cell lines [15, 18, 23]. The CPV strains in this study did not induce

Table 2. Amino acid sequence differences of *VP2* gene in parvovirus

Genotype isoates	Codon and amino acid positions													Remark variants
	80	87	93	103	232	297	300	305	323	426	555	564	568	
FPLV	Lys	Met	Lys	Val	Val	Ser	Ala	Asp	Asp	Asn	Val	Asn	Ala	
CPV-2	Arg	Met	Asn	Ala	Ile	Ser	Ala	Asp	Asn	Asn	Val	Ser	Gly	
CPV-2a	Arg	Leu	Asn	Ala	Ile	Ser/Ala	Gly	Tyr	Asn	Asn	Ile	Ser	Gly	
CPV-2b	Arg	Leu	Asn	Ala	Ile	Ser/Ala	Gly	Tyr	Asn	Asp	Val	Ser	Gly	
CPV-2c	Arg	Leu	Asn	Ala	Ile	Ala	Asp	Tyr	Asn	Asn/Asp	Val	Ser	Gly	
KV0901*	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asn	Val	Ser	Gly	CPV-2a
QIACP1403*	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asp	Val	Ser	Gly	CPV-2b
QIACP1404*	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asp	Val	Ser	Gly	CPV-2b

*Korean CPV isolate.

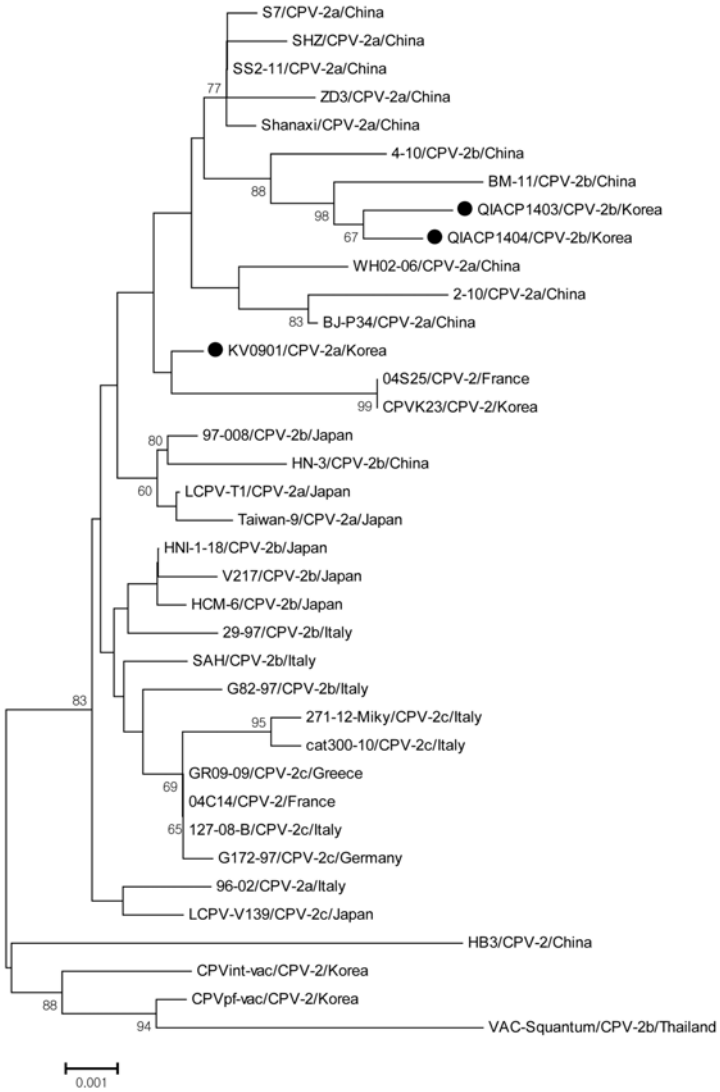

Fig. 3. Phylogenetic tree based on the complete *VP2* gene of the CPV isolates and other sequences of CPVs obtained from GenBank database.

distinct CPEs and grew moderately in A72 cells, with viral titers ranging from $10^{3.5}$ to $10^{4.5}$ $FAID_{50}$/mL. The IFA test enabled us to confirm the isolates as CPV. The HA activity of CPV has been tested in pig, green monkey, and dog red blood cells [1, 19]. The QIACP1403 and QIACP1404 strains had HA activity with 0.6% pig erythrocytes and showed a moderate HA titer, which may be responsible for the physical properties of low passage CPV.

A sequence comparison of several strains revealed surprising similarities at the nucleotide and amino acid levels (98.2–99.7%), although CPV strains have been isolated for > 50 years, and antigenic variants have been identified in many countries [16]. Many CPV strains have been isolated from naturally infected dogs, and their genetic data have accumulated. The QIACP1403 and QIACP1404 strains formed a close phylogenetic relationship with CPV-BM11, which was isolated from Chinese dogs in 2011, assuming that the recent CPV2b circulating in the Korean dog population originated in China [12]. Although many CPV strains isolated from different countries formed several clades in the phylogenetic tree, there seemed to be no distinguishable genotype within the nucleotide similarity among the CPV strains. Therefore, various methods, such as amino acid sequence analysis, single-nucleotide polymorphism, restriction fragment-length polymorphism, minor groove binder probe assay, real-time reverse transcription polymerase chain reaction (RT-PCR), and inhibition of hemagglutination with monoclonal antibodies have been developed to identify CPV variants and predict similarities in CPV antigens [7, 15]. Using the methods mentioned above, three antigenic variants, designated CPV2a/b/c, have been identified in dogs worldwide. In this study, the amino acid sequences of the complete *VP2* gene of two field isolates from puppies were compared to those of CPV strains reported previously. The two isolates had glycine, aspartic

acid, and valine at positions 300, 426, and 555, confirming the CPV variant as CPV2b. In addition, the KV0901 strain, which was isolated from a Korean puppy in 2009, had valine at position 555, inferring that CPV2a, like the KV0901 strain, continued to mutate at the same or other positions. In general, parvovirus mutations have low error rates, as parvovirus DNA is replicated by host cell DNA polymerase [6]. Amino acid substitutions can be found in residues of field CPVs, assuming it is a wild CPV avoiding acquired immunity by vaccination.

In conclusion, we isolated two CPVs designated QIACPV1403 and QIACPV1404 and confirmed that they were CPV2b. The amino acid residue analysis indicated that the two strains had identical homology and contained glycine, aspartic acid, and valine at positions 300, 426, and 555 of the *VP2* gene, respectively. However, we did not carry out pathogenicity studies of these isolates. Therefore, further studies are needed to determine if the CPV2b strains cause hemorrhagic diarrhea in puppies and whether it is a CPV2b vaccine candidate after passage *in vitro*.

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