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Genetic analysis of canine parvovirus vaccine strains in Korea

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Abstract : After the original identification of canine parvovirus (CPV) type 2 (CPV-2) in 1978, new antigenic variants such as CPV-2a, CPV-2b and CPV-2c have become widespread in the most countries. In this study, the genetic analysis of canine parvovirus was investigated in a total of 13 CPV vaccines, which have been licensed in Korea since late 1980s, and a field isolate of CPV from a dog with CPV infection clinical symptom. The partial VP2 gene of CPV was amplified and sequenced from 13 vaccine strains and one field isolate. The results showed that of the 13 vaccine strains, 10 strains belong to the CPV-2, 2 strains to CPV-2b, the remaining and one isolate to CPV-2a type, respectively. Several mutations of amino acids were detected at residues of the critical region of the commercial vaccine strains. These data suggest that new type of vaccines containing CPV-2a or CPV-2b/2c type may be required for the better prevention of new CPV infection in dog population in Korea, because CPV-2 contained in most licensed vaccines has been replaced by antigenic variants designated CPV-2a or CPV-2b/c in the worldwide dog population.

Keywords : canine parvovirus, genetics, Korea, phylogeny

Introduction

Infection of canine parvovirus (CPV) is one of the most fatal viral diseases, showing hemorrhagic enteritis, leukopenia, nausea and myocarditis in puppies over the age of 2 months [1]. CPV belongs to a member of the genus *Parvovirus* of the family *Parvoviridae* along with the feline panleukopenia virus (FPV) and mink enteritis virus (MEV) and contains negative single strand DNA about 5.2 kb in length. Since CPV was identified in late 1970s in dogs, new CPV has been spread out worldwide and named as CPV-2 type to distinguish it from CPV prototype (CPV-1). An antigenic variant of CPV-2 type was identified by using specific monoclonal antibodies [18]. In the early 1980s, two CPV-2 variants characterizing that amino acid residue at positions 297 and 426 of VP2 gene changed from serine to alanine and from asparagine to aspartic acid respectively emerged and were named as CPV-2a and CPV-2b [2, 9, 14, 17]. Currently, CPV-2a is the main genotype circulating in the dog population in India, Germany and Korea, while CPV-2b is detected commonly in USA, Taiwan and Japan [6, 10, 14, 16]. Recently, a new antigenic type (CPV-2c) was reported in several countries including Italy, Spain, Germany, Vietnam, Japan and USA [6, 8, 11]. The CPV-2c was first detected as a genetic variant of CPV-2b and of which amino acid residue at position 426 of VP2 gene changed from aspartic acid to glutamic acid in Italian dogs [3]. Many dogs infected with CPV-2c had mucoid yellow diarrhea, hemorrhagic diarrhea, leucopenia, and lymphopenia [5, 11].

The non-enveloped capsid of CPV is composed of VP1, VP2 and VP3, respectively. Among these proteins, VP2 is the main capsid protein and induces protective antibody. Since substitutions of a few amino acids in VP2 are liable for the antigenic feature and the control of host range, the deduced amino acid sequence of VP2 may make us to differentiate between variants such as CPV-2 and CPV-2a/b/c. At this stage, CPV subtyping in commercial CPV vaccine strains has not been identified. Thus, we investigated nucleotide sequence analysis of important partial VP2 gene of CPVs in thirteen commercial vaccines available in

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Korea as well as in a field isolate.

Materials and Methods

Virus isolation

Fecal samples were collected from puppies (N = 7) aged 70 day in Chungju province of Korea in 2009. The dogs showed the typical symptoms of CPV infection with nausea and hemorrhagic diarrhea and four of them died. For the virus isolation, the fecal samples were checked with one step CPV antigen test kit (Anigen, Korea) for CPV. Two samples showing strong positive reaction were filtered using 0.45 μm filter and inoculated into A72 cells (A72, derived from canine fibroblast cell) grown in alpha minimum essential medium with 10% fetal bovine serum. The cells were incubated at CO_2 incubator for 7 days and cytopathic effects (CPE) were observed in the cells. The supernatant of the cells was screened by hemagglutination test and the cells fixed with cold aceton were tested by indirect fluorescent assay (IFA) using CPV specific monoclonal antibody (Jeno Biotech, Korea). The isolate was propagated on A72 cells and the viral titer was measured by IFA.

Vaccines

The commercial CPV vaccines produced by Korean animal vaccine companies and used in this study were as follows: Greencross DHPPL (Greencross, Korea); Daesung DHPPL (Daesung, Korea); Canishot DHPPL (ChoongAng, Korea); Komipharm DHPPL (Komipharm, Korea); Himmvac DHPPL (KoreaBNP, Korea). The eight CPV vaccines imported from several countries such as USA, France, Czech and Japan were as follows: Bayovac DHPPL (Bioveta, Czech); Canine-9 DHPPL (Kyotobiken, Japan); Canvac DHPPiL (Dyntec, Czech); Duramune Max Pv (Fort Dodge, USA); Eurican DHPPI2 (Merial, France); Nobivac DHPPi (Intervet, Netherland); Quantum dog DA2PPv (Schering-Plough, USA); Vanguard plus (Pfizer, USA). Twelve commercial vaccine strains employed in this study contained modified live virus. The other Daesung DHPPL was an inactivated virus vaccine.

DNA extraction and PCR condition

Viral DNA was extracted from thirteen commercial CPV vaccines using a DNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions. A PCR using specific primers (Table 1), which amplify an important portion of VP2 region of CPV, was carried out for the gene amplification. The PCR was performed in PCR premix (Bioneer, Korea) containing 5 μL of denatured DNA, 1 μL of each primer (50 pmol) and 43 μL of distilled water, for a 50 μL final volume. The cycling profile was as follows: denaturation at 95°C for 5 min, followed by 35 cycles with denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were visualized using electrophoresis on 1.5% agarose gel containing ethidium bromide. Purified PCR products were ligated with the pGEM-T easy vector (Promega, USA).

Sequencing and phylogenetic analysis

After cloning the VP2 gene of CPV, sequencing reactions of the purified recombinant plasmid DNA were performed with ABI PRISM 7900/ Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, USA). The phylogenetic analysis was carried out on nucleotide sequence data of 793 base pair in VP2 gene from CPV vaccine and reference CPV strains. Phylogenetic tree and homology analysis were obtained using neighbor-joining methods of the DNAStar software program (DNAStar, USA) and DNASIS software program (Hitachi, Japan).

Results

Identification of isolate

Of the two fecal samples inoculated into A72 cells, one was selected. Following second blind passage of the isolate, the cells infected with the isolate were checked by IFA test. CPV-specific fluorescence appeared in the nucleus of the infected cells (data not

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

Primer designated	Oligonucleotide sequence (5'-3')	Target gene	Size of amplicon
CPVDF	GAG CAT TGG GCT TAC CAC CA	VP2	793 bp
CPVDR	GGA TTC CAA GTA TGA GAG GC		

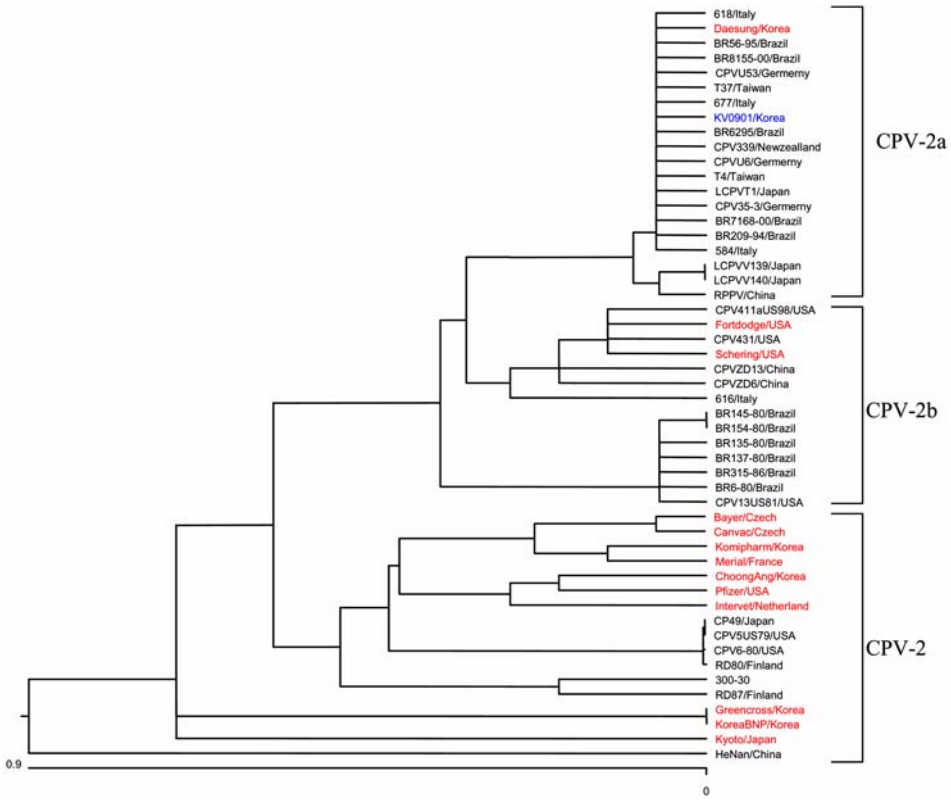

Fig. 1. Phylogenetic tree constructed from the partial VP2 gene nucleotide sequences of the vaccine strains and other sequences obtained from the GenBank database showing its genetic relationship among canine parvovirus (CPV) strains.

shown). Viral titer of $10^{5.5}$ $FAID_{50}$/mL in the second passage of the isolate was obtained. The isolate did not show CPE in A72 cells and was designated as KV0901.

Sequence analysis of VP2 gene of CPV

A total of 793 nucleotide sequences for the partial VP2 genes encoding capsid protein from 13 CPV vaccine strains and one isolate were identified and their amino acids were deduced. The VP2 gene sequences of 37 CPVs obtained from GenBank (NCBI) were compared with those of the thirteen vaccine strains licensed in Korea and one isolate to analyze the antigenic and evolutionary relationship of CPV. Based on the nucleotide sequence analysis of VP2 gene, one vaccine strain was classified into CPV-2a type, another two vaccine strains were divided into CPV-2b type, and the remaining ten vaccine strains were identified as CPV-2 type (Fig. 1). In addition, one isolate, KV0901, was classified into CPV-2a type. Compared with those of reference strains of CPV, homology of the VP2 gene nucleotide sequence of vaccine strains was ranged from 97 to 100%.

In order to find out any similarities among 13 vaccine strains, multiple sequence alignment of 263 amino acid residues of the critical region was carried out (Fig. 2). Alignment with deduced amino acid sequences showed 97.3 to 100% similarity among 13 vaccine strains. Ten vaccine strains identified as CPV-2 had unique amino acid sequence at positions 297, 300 and 305 of VP2 gene. Two strains, Fortdodge and Schering-plough strains, classified as CPV-2b had the same amino acid sequence at residues 323 and 426. Amino acid sequence of Daesung strain showed the complete identity with that of KV0901 isolate designated as consensus. In addition, several mutations of amino acid were identified at residues 301, 316, 323, 344, 349, 375, 380, 387, and 391 in the critical VP2 region of the commercial CPV vaccine strains.

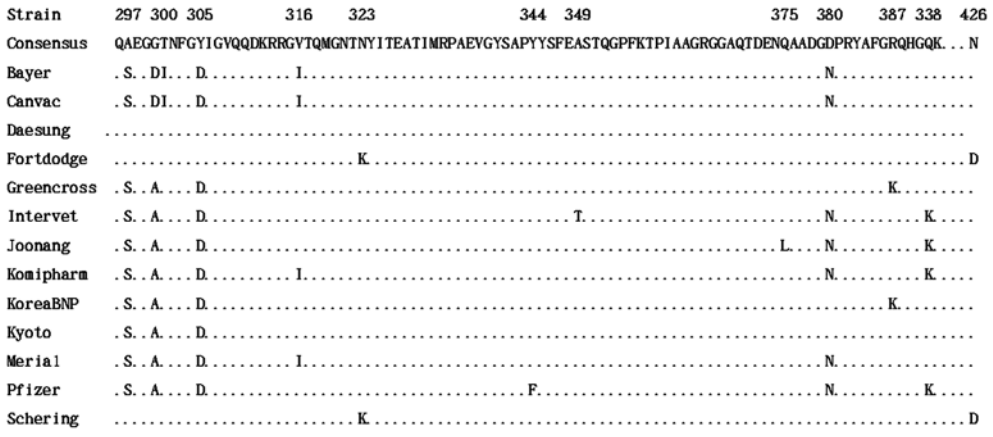

Fig. 2. Multiple sequence alignment of the deduced amino acid of VP2 protein among the CPV vaccine strains. The amino acids that are identical to consensus are indicated by dots, while different ones are indicated by abbreviated letters.

Discussion

The studies on CPV infection have been carried out intensively in a variety of canine and feline species since 1980. It has been known that the close genomic relationships exist among CPV-2, FPV and MEV [21]. In total, 42 vaccines associated with CPV have been licensed since the early 1980s. Of the 42 vaccines, 34 items are live attenuated vaccines with either single or combined form. Eight inactivated vaccines have been also used for the prevention of CPV infection [13].

Several methods such as hemagglutination inhibition with monoclonal antibodies, single-nucleotide polymorphisms, restriction fragment-length polymorphism, minor groove binder probe assay, and sequence analysis have been developed for the determination of CPV type and the prediction of CPV antigen similarity [5]. With the techniques mentioned above, two antigenic variants CPV-2a and CPV-2b have been identified as the prevalent virus in domestic dogs worldwide [11, 16, 19, 22]. It has been reported that CPV-2a is the predominant type and a small number of CPV-2b and CPV-2a variant are also detected in Korea [10, 14].

In this study, the nucleotide sequences of the partial VP2 gene of the thirteen vaccine strains and one field isolate from puppies were compared with those of other CPVs that had been previously reported. The results of sequence analysis showed that of the thirteen CPV vaccine strains, most CPV vaccine strains were identified as the CPV-2 type. In fact, there are reports that CPV type 2 vaccine protects dogs against virulent challenge with type 2a/2b/2c viruses [7, 20]. However, there are still severe clinical cases of parvovirus infection in Korean dog population. In addition to that, a previous study revealed that 99% (95/96) of Korean CPV isolates detected from fecal samples were classified into CPV-2a [12]. In a study comparing the pathogenicity of three different type of Korean CPV isolates (CPV-2a-I, CPV-2a-V, CPV-2b), the CPV-2a revealed stronger pathogenicity than CPV-2b [15]. Recently, Ohshima *et al.* [16] reported that the recent field CPV isolates reacted more efficiently to the antibodies produced in dogs vaccinated with the new CPV-2b vaccine strain than the conventional CPV-2 vaccine strain. In addition, it is considered that a CPV vaccine strain should be most closely related to the field antigenic type for the maximum potency of vaccine [21]. At this stage, no clear conclusion can be made why outbreaks of CPV in Korea are recurrent although dogs have been vaccinated. However, as implied in this study and elsewhere [16, 21], current vaccine strains used in Korea may not provide maximum protection to the prevalent CPV strains in dogs that need to be answered.

Mutation of the amino acid residues at positions 297, 300 or 305 distinguishes CPV-2 from CPV-2a/2b and the aspartic acid residue at position 426 also distinguishes CPV-2a from CPV-2b. It has been well known that mutational events of parvovirus have low error rates as parvovirus DNA is replicated by host cell DNA polymerase [4]. Amino acid changes in the

critical region of CPV vaccine strains were summarized in Fig. 2. Substitutions of amino acids are shown in the several residues of vaccine strains. It assumed that most of CPV vaccine strains have been modified after many passages using several cell lines such as A72, CRFK, and primary dog kidney cells. These amino acid changes could be served as "markers" of the each vaccine strain.

In conclusion, the genetic analysis of CPV commercial vaccine strains in Korea suggests that the efficacy of the conventional CPV-2 type vaccines against CPV-2a/2b/2c infection should be re-investigated in accordance with field circumstance. Furthermore, it is important to monitor continually emergence of new type of CPV in Korean dog population.

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