VP2 Gene of a Canine Parvovirus Isolate from Stool of a Puppy

Kentarou HIRAYAMA1), Rui KANO1), Tomoko HOSOKAWA-KANA13), Koutarou TUCHIYA3), Shingo TSUYAMA4), Yuka NAKAMURA2), Yoshihide SASAKI2) and Atsuhiko HASEGAWA3)

1)Departments of Pathobiology, 2)Internal Medicine, Nihon University School of Veterinary Medicine, 1866, Kameino, Fujisawa, Kanagawa, 252–8510, 3)Nippon Institute for Biological Science, 9–2221–1, Shin-machi, Ome, Tokyo, 198–0024 and 4)Tsuyama Animal Hospital, 1–39–1 Yahata, Chigasaki, Kanagawa, 253–0085, Japan

*CORRESPONDENCE TO: Dr. KANO, R., Department of Pathobiology, Nihon University School of Veterinary Medicine, 1866, Kameino, Fujisawa Kanagawa, 252–8510, Japan.

NOTE Internal Medicine

Abstract. VP2 gene of a canine parvovirus (CPV) isolate from the feces of a puppy which was diagnosed to be CPV infection was analyzed. The result indicated that this clinical isolate was phylogenetically close to the isolate of wild-type CPV (strain CPV-T37) prevailing in Taiwan rather than isolates from Japan.

Key words: canine parvovirus, molecular analysis, VP2.

Canine parvovirus type2 (CPV-2) emerged as a new pathogen of dogs in 1978, almost simultaneously in Europe and North America, and was responsible for hemorrhagic gastroenteritis and myocarditis in puppies [14, 15]. The close antigenic and genomic relationships among CPV-2, feline panleukopenia virus and mink enteritis virus suggested that CPV-2 might have appeared by genetic mutation in a wild carnivore host receptive to one of the feline panleukopenia virus-like paroviruses [7, 18].

From 1979 to 1981, monoclonal antibodies, restriction enzyme analysis, and DNA sequencing demonstrated the antigenic variant or biotype (CPV-2a) that differs from the original strain CPV-2 in three coding regions of the gene for the VP2 capsid protein. A second biotype (CPV-2b) was detected around 1984, and the significant differences from CPV-2 were the substitution of two amino acid (Asn→Asp, Ile→Val) in the VP2 protein. Both of these biotypes are now prevailing throughout the canine population worldwide, replacing the original strain, CPV-2 [7, 13, 18]. Nevertheless, epidemiological study of CPV-2a and CPV-2b in Japan has not been well investigated.

In this study, we isolated CPV-2b from a diarrheic fecal sample from a puppy and investigated the phylogenetic relation between the clinical isolate and the other CPVs by molecular analysis of the VP2 capsid gene.

A 5-month-old male Miniature Dachshund weighing 3.5 kg was referred to Nihon University Animal Medical Center with complaints of depression, fever, anorexia, diarrhea and vomiting. Complete blood cell count revealed leukopenia (4700 cells/µl) and the results of a serum biochemical analysis were within the normal range (Table 1). A paroviral infection was confirmed by a fecal examination with a paroviral antigen detection kit (Sinovus Biotech Inc., N.Y., U.S.A.).

The patient dog was treated with enrofloxacin (5 mg/kg) for 3 days. After a 7-day treatment, the dog recovered from vomiting and had a good appetite.

The fecal sample was collected from the patient dog and suspended in phosphate-buffered saline (PBS). After centrifugation at 900G for 20 min, the supernatant fluid was collected. This supernatant was filtered with a 0.45-µm filter (Millipore Co., Mass, U.S.A.). The filtered sample was overlaid on a Crandell feline kidney (CRFK) cell line grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (at final concentrations of 100 units/ml of penicillin G potassium and 0.5 mg/ml of streptomycin sulfate). This CRFK had been seeded about 24 hr earlier at a density of 4 × 104 cells/ml before overlaying. After several days, a cytopathic effect (CPE) in the cells was observed. The cells were frozen and thawed three times. The cell debris was removed by centrifugation at 900G for 20 min. The supernatant fluid was filtered with a 0.45-µm filter and the filtered sample was collected. The virus in the collected sample was passaged several times in the CRFK cells before examination.

Antigenic analysis was performed by hemagglutinating (HA) test and hemagglutination-inhibition (HI) test according to Carmichael et al. [4].

The CPV VP2 gene specific primer pairs were used in this study as reported by Ikeda et al. [9]. The sequences of the primers were as follows:

51 (5′-CCAACTAAAAAGAAGTAAACC-3′)
F1 (5′-AGATAGTAAATATACATATCGCATT-3′)
F2 (5′-ACAGGAGAAAACCTGTGAGATAC-3′)
R1 (5′-TGGTGTGTTTCATGGATAAAACC-3′)
R2 (5′-TTTTGAATCCAATCTCCTTCTGGAT-3′)
R3 (5′-CCTATATCAAATACAAGTACAATA-3′)

To amplify the VP2 gene of CPV by PCR, the following primer pairs were used: primer pair A [primers 51 and R3] for amplifying the regions between nt 2687 and 4692, primer pair B [primers 51 and R1] for amplifying the regions between nt 2687 and 3395, primer pair C [primers F1 and R2] for amplifying the regions between nt 3316 and 4035, and primer pair D [primers F2 and R3] for amplifying...
the regions between nt 3956 and 4692.

Samples which contained the virus as a template were directly added to the PCR mixture without the extraction of the virus DNA. The PCR reaction mixture (30 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin and 2.5 mM each of deoxynucleoside triphosphate, 1.0 unit of Taq polymerase and 0.5 µg of a pair of primers. The PCR amplification was carried out for 30 cycles and consisted of template denaturation (0.5 min, at 94°C), primer annealing (2 min, at 55°C) and polymerization (2 min, at 74°C). The PCR products were electrophoresed through 2% agarose gel and then stained with ethidium bromide. The PCR products from the samples were sequenced by the dideoxy chain termination method with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA, U.S.A.).

Homology relationships among the VP2 gene of reference strains of CPV were examined by FASTA database analysis in the DNA data bank of Japan (DDBJ).

To examine the VP2 gene among clinical isolates and the other CPVs, we performed the neighbor-joining (NJ) method in the Clustal W multiple sequence alignment programs [17]. The phylogenetic tree was constructed by means of the TREEVIEW program [12]. Bootstrap analysis was performed on 1000 random samples taken from multiple alignment as described by Felsenstein [6], and analyzed by the Clustal W programs.

The results of serological analyses (HA test and HI test) of the clinical isolate are shown in Table 2. Amplification of isolate of CPV VP2 gene with 6 primers yielded 1755-bp fragments (Fig. 1), consistent with the expected sizes of the CPV VP2 gene reported previously [9].

| nucleotide | 239 | 246 | 259 | 279 | 308 | 699 | 871 | 889 | 899 | 913 | 967 | 1038 | 1167 | 1276 | 1362 | 1691 | 1703 |
| Biotype    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| FPLV       | A    | A    | A    | A    | T    | T    | T    | C    | G    | A    | T    | A    | A    | A    | A    | C    |
| CPV-2      | G    | G    | A    | C    | C    | C    | C    | C    | G    | A    | G    | C    | A    | A    | G    | G    |
| CPV-2a     | G    | G    | T    | C    | C    | C    | C    | G    | T    | A    | G    | C    | A    | C    | G    |
| CPV-2b     | G    | G    | T    | C    | C    | C    | C    | G    | T    | A    | G    | C    | G    | G    | G    |
| CPV-2c     | G    | G    | T    | C    | C    | C    | C    | A    | T    | A    | G    | C    | A/G | C    | G    |
| Clinical Isolate | G | G | T | C | C | C | C | G | T | A | G | C | G | G | G |

Table 4. Phylogenetically informative amino acid sequence of VP2 gene

<table>
<thead>
<tr>
<th>Amino acid Biotype</th>
<th>80</th>
<th>87</th>
<th>93</th>
<th>103</th>
<th>232</th>
<th>297</th>
<th>300</th>
<th>305</th>
<th>323</th>
<th>426</th>
<th>555</th>
<th>564</th>
<th>568</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPLV</td>
<td>Lys</td>
<td>Met</td>
<td>Lys</td>
<td>Val</td>
<td>Val</td>
<td>Ser</td>
<td>Ala</td>
<td>Asp</td>
<td>Asp</td>
<td>Asn</td>
<td>Val</td>
<td>Asn</td>
<td>Ala</td>
</tr>
<tr>
<td>CPV-2</td>
<td>Arg</td>
<td>Met</td>
<td>Asn</td>
<td>Ala</td>
<td>Ile</td>
<td>Ser</td>
<td>Ala</td>
<td>Asp</td>
<td>Asp</td>
<td>Asn</td>
<td>Val</td>
<td>Ser</td>
<td>Gly</td>
</tr>
<tr>
<td>CPV-2a</td>
<td>Arg</td>
<td>Leu</td>
<td>Asn</td>
<td>Ala</td>
<td>Ile</td>
<td>Ser/Ala</td>
<td>Gly</td>
<td>Tyr</td>
<td>Asn</td>
<td>Asn</td>
<td>Ile</td>
<td>Ser</td>
<td>Gly</td>
</tr>
<tr>
<td>CPV-2b</td>
<td>Arg</td>
<td>Leu</td>
<td>Asn</td>
<td>Ala</td>
<td>Ile</td>
<td>Ser/Ala</td>
<td>Gly</td>
<td>Tyr</td>
<td>Asn</td>
<td>Asn</td>
<td>Ile</td>
<td>Ser</td>
<td>Gly</td>
</tr>
<tr>
<td>Clinical Isolate</td>
<td>Arg</td>
<td>Leu</td>
<td>Asn</td>
<td>Ala</td>
<td>Ile</td>
<td>Ala</td>
<td>Asp</td>
<td>Tyr</td>
<td>Asn</td>
<td>Asn/Asp</td>
<td>Val</td>
<td>Ser</td>
<td>Gly</td>
</tr>
</tbody>
</table>

FPLV=Feline panleukopenia virus; CPV=Canine parvovirus.
Fig. 1. Nucleotide sequence of the VP2 gene of the clinical isolate in this study. The boxed sequence indicates the specific differences between the nucleotide of a clinical isolate and another CPV-2b obtained from the GeneBank database.
The nucleotide differences in the VP2 gene between the clinical isolate in this study [DDBJ accession no. AB128923 (Fig. 1)] and common strains of CPV-2b were detected in 6 bases [nucleotide 36 (A→G), nucleotide 147 (A→G), nucleotide 969 (C→T), nucleotide 1291 (C→T), nucleotide 1509 (G→A) and nucleotide 1695 (T→C)]. The relation among antigenic typings of CPV and the specific nucleotide of VP2 protein is shown in Tables 3 and 4. These analyses confirmed that the isolated CPV was CPV-2b in the antigenic type.

The phylogenetic analysis of CPV for VP2 gene sequences of the clinical isolate and the others revealed that the clinical isolate is genetically close to the wild-type CPV-2a isolate from Taiwan (Fig. 2).

The puppy in this case had been vaccinated twice, but the patient was infected with CPV, showing the symptoms. The antigen type of the isolate was CPV-2b, which has become common throughout the world, including Japan, in recent years. The vaccination program has been designed to protect dogs from CPV-2b infection, but the commercial vaccines sometimes failed, as in this case. Therefore, it is speculated that there might be some differences in the antigen epitope among field isolates of CPV.

The homology of VP2 gene nucleotide sequences was 99.6% between the clinical isolate and 3 reference strains [CPV-T37 (dog, Taiwan), CPV-T10 (dog, Taiwan), V123 (dog, Taiwan)].
and was 99.5% between the clinical isolate and strain 97–008 (dog, Japan) isolated from Japanese domestic dogs in recent years. Moreover, the phylogenetic analysis of the VP2 gene indicated that this clinical isolate was close to the isolate of wild-type CPV from Taiwan (strain CPV T-37) rather than the isolates from Japan. This CPV-2b isolate might have been imported from Taiwan, since no isolates like the strains prevailing in Taiwan have been detected in Japan.

REFERENCES