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

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ABSTRACT. VP2 gene of a canine parvovirus (CPV) isolate from the feces of a puppy which was diagnosed to be CPV infection was analysed. 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.

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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 parvoviruses [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-2a 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/ μ l) and the results of a serum biochemical analysis were within the normal range (Table 1). A parvoviral infection was confirmed by a fecal examination with a parvoviral 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- μ m 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 4×10^4 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- μ m 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'-CCAACTAAAAGAAGTAAACC-3')
F1 (5'-AGATAGTAATAATACTATGCCATTT-3')
F2 (5'-ACAGGAGAAACACCTGAGAGATTTA-3')
R1 (5'-TGGTTGGTTTCCATGGATAAAAACC-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

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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 μ l) 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 μ g 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].

Table 1. Results of complete blood cell count and serum biochemical analysis

ALB	g/dl	2.3	WBC	/ μ l	4700
ALKP	U/L	191	RBC	$\times 10^4/\mu$ l	621
ALT	U/L	55	HGB	g/dl	14.2
AMYL	U/L	11010	HCT	%	39.7
BUN	mg/dl	11.3	MCV	fl	63.9
Ca	mg/dl	9.4	MCH	pg	22.9
CHOL	mg/dl	186.9	MCHC	g/dl	35.8
CREA	mg/dl	0.6	PLT	$\times 10^4/\mu$ l	38
GLU	mg/dl	121.7	CRP	mg/dl	0.25
LIPA	U/L	2910			
NH3	umol/L	25	Na	mmol/L	139
TP	g/dl	5.1	K	mmol/L	4.9
GLOB	g/dl	2.7	Cl	meq/L	101

Table 2. Comparison of CPV hemagglutination (HA) and hemagglutination-Inhibition (HI) titers

Test virus	at first admission		after 1 week
	HA	HI	HI
CP49	1280	512	256
TDK	1280	512	256
Y-1	640	512	256
Clinical Isolate	160	1024	256

CP49 (strain of CPV-2), TDK (strain of CPV-2a), Y-1 (strain of CPV-2b), Clinical Isolate (strain of CPV-2b).

Table 3. Phylogenetically informative nucleotide sequence of VP2 gene. The coding sequence differences in the VP2 protein gene which were found in the various isolates

nucleotide Biotype	239	246	259	279	308	699	871	889	899	913	967	1038	1167	1276	1623	1691	1703
FPLV	A	A	A	A	T	T	T	C	C	G	G	A	T	A	A	A	C
CPV-2	G	G	A	C	C	C	C	C	C	G	A	G	C	A	A	G	G
CPV-2a	G	G	T	C	C	C	C	G	G	T	A	G	C	A	C	G	G
CPV-2b	G	G	T	C	C	C	C	G	G	T	A	G	C	G	C	G	G
CPV-2c	G	G	T	C	C	C	C	G	A	T	A	G	C	A/G	C	G	G
Clinical Isolate	G	G	T	C	C	C	C	G	G	T	A	G	C	G	C	G	G

Table 4. Phylogenetically informative amino acid sequence of VP2 gene

Amino acid Biotype	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
Clinical Isolate	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asp	Val	Ser	Gly

FPLV=Feline panleukopenia virus; CPV=Canine parvovirus.

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ATGAGTGATGGAGCAGTTCAACCAGACGGTGGTCACTGCTGTCAGAAATGAAAGAGCT    60
M S D G A V Q P D G G Q P A V R N E R A
ACAGGATCTGGGAACGGGTCTGGAGCGGGGGTGGTGGTCTCTGGGGGTGTGGGGATT    120
T G S G N G S G G G G G G S G G V G I
TCTACGGGTACTTTCAATAATCAGACCTAAATTTAAATTTTGGAAAACGGATGGGTGGAA    180
S T G T F N N Q T E F K F L E N G W V E
ATCACAGCAAACTCAAGCAGACTTGTACATTAAATATGCCAGAAAGTAAAATTATAGA    240
I T A N S S R L V H L N M P E S E N Y R
AGAGTGGTTGTAATAATTTGGATAAACTGCAGTTAACGGAAACATGGCTTTAGATGAT    300
R V V V N N L D K T A V N G N M A L D D
ACCCATGCACAAATTGTAACACCTTGGTCATTGGTTGATGCAATGCTTGGGGAGTTGG    360
T H A Q I V T P W S L V D A N A W G V W
TTTAAATCCAGGAGATTGGCACTAATTGTTAATACTATGAGTGAGTGCATTAGTAGT    420
F N P G D W Q L I V N T M S E L H L V S
TTTGAACAAGAAATTTTAAATGTTGTTTAAAGACTGTTTCAGAACTCTGCTACTCAGCCA    480
F E Q E I F N V V L K T V S E S A T Q P
CCAACATAAGTTTATAATAATGATTAACTGCATCATTGATGGTTGCATTAGATAGTAAT    540
P T K V Y N N D L T A S L M V A L D S N
AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTATCCATGG    600
N T M P F T P A A M R S E T L G F Y P W
AAACCAACCATACCAACTCCATGGAGATATTATTTCAATGGGATAGAACATTAATACCA    660
K P T I P T P W R Y Y F Q W D R T L I P
TCTCATACTGGAAGTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT    720
S H T G T S G T P T N I Y H G T D P D D
GTTCAATTTTATACTATTGAAAATCTGTGCCAGTACACTTACTAAGAAGAGTGATGAA    780
V Q F Y T I E N S V P V H L L R T G D E
TTTGCTACAGGAACATTTTGTGTTGTAACCATGTAGACTAACACATACATGGCAA    840
F A T G T F F F D C K P C R L T H T W Q
ACAAATAGAGCATTGGGCTTACCACCATTTCTAAATCTTTGCCCAAGCTGAAGGAGGT    900
T N R A L G L P P F L N S L P Q A E G G
ACTAATTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGAATCAATCGGA    960
T N F G Y I G V Q Q D K R R G V T Q M G
AATACAAATATATATTACTGAAGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCA    1020
N T N Y I T E A T I M R P A E V G Y S A
CCATATTATTCTTTGAGGCGTCTACACAAGGCCATTTAAACACCTATTGCAGCAGGA    1080
P Y Y S F E A S T Q G P F K T P I A A G
CGGGGGGGAGCGCAACAGATGAAATCAAGCAGCAGATGGTGATCCAAAGATATGCATTT    1140
R G G A Q T D E N Q A A D G D P R Y A F
GGTAGACAACATGGTCAAAAACTACCACAACAGGAGAAACACCTGAGAGATTACATAT    1200
G R Q H G Q K T T T T G E T P E R F T Y
ATAGCACATCAAGATACAGGAAGATATCCAGAAGGAGATTGGATTCAAATATTAACTTT    1260
I A H Q D T G R Y P E G D W I Q N I N F
AACCTTCCTGTACAGATGATAATGTATTGTACCAACAGATCCAATTGGAGGTAAAACA    1320
N L P V T D D N V L L P T D P I G G K T
GGAATTAACATACTAATATATTTAATACTTATGGTCCTTTAACTGCATTAAATAATGTA    1380
G I N Y T N I F N T Y G P L T A L N N V
CCACCAGTTTATCCAAATGGTCAAATTTGGGATAAAGAAATTTGATACTGACTTAAACCA    1440
P P V Y P N G Q I W D K E F D T D L K P
AGACTTCATGTAAATGCACCATTTGTTTGTCAAAATAATTGTCCTGGTCAATTATTGTGA    1500
R L H V N A P F V C Q N N C P G Q L F V
AAAGTTGCACTAATTTAACAAATGAATATGATCCTGATGCATCTGCTAATATGTCAAGA    1560
K V A P N L T N E Y D P D A S A N M S R
ATTGTAACCTTACTCAGATTTTGGTGGAAAGGTAAATTAGTATTTAAAGCTAAACTAAGA    1620
I V T Y S D F W W K G K L V F K A K L R
GCCTCTCATACTTGGAAATCCAATTCAACAATGAGTATTAAATGTAGATAACCAATTTAAC    1680
A S H T W N P I Q Q M S I N V D N Q F N
TATGTACCAAGTAATTTGGAGGTATGAAAATGTATATGAAAATCTCAACTAGCACCT    1740
Y V P S N I G G M K I V Y E K S Q L A P
AGAAAAATATATTAA
R K L Y *

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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.

Fig. 2. Phylogenetic tree constructed from the VP2 gene nucleotide sequences of a clinical isolate in this study and other sequences obtained from the GenBank 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 antigenic 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

(cat, Vietnam)], 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

1. Azetaka, M., Hirasawa, T., Konishi, S. and Ogata, M. 1981. *Jpn. J. Vet. Sci.* **43**: 243-255.
2. Battilani, M., Ciulli, S., Tisato, E. and Prosperi, S. 2002. *Virus Res.* **83**: 149-157.
3. Battilani, M., Scagliarini, A., Tisato, E., Turilli, C., Jacoboni, I., Casadio, R. and Prosperi, S. 2001. *J. Gen. Virol.* **82**: 1555-1560.
4. Carmichael, L. E., Joubert, J. C. and Pollock, R. V. H. 1980. *Am. J. Vet. Res.* **41**: 784-791.
5. Chalmers, W. S. K., Truyen, U., Greenwood, N. M. and Baxendale, W. 1999. *Vet. Microbiol.* **69**: 41-45.
6. Felsenstein, J. 1985. *Evolution* **39**: 783-791.
7. Ikeda, Y., Nakamura, K., Miyazawa, T., Tohya, Y., Takahashi, E. and Mochizuki, M. 2002. *Emerg. Infect. Dis.* **8**: 341-346.
8. Ikeda, Y., Miyazawa, T., Kurosawa, K., Naito, R., Hatama, S., Kai, C. and Mikami, T. 1998. *J. Vet. Med. Sci.* **60**: 973-974.
9. Ikeda, Y., Mochizuki, M., Naito, R., Nakamura, K., Miyazawa, T., Mikami, T. and Takahashi, E. 2000. *Virology* **278**: 13-19.
10. Mochizuki, M., Horiuchi, M., Hiragi, H., SanGabriel, MC., Yasuda, N. and Uno, T. 1996. *J. Clin. Microbiol.* **34**: 2101-2105.
11. Nakamura, K., Sakamoto, M., Ikeda, Y., Sato, E., Kawakami, K., Miyazawa, T., Tohya, Y., Takahashi, E., Mikami, T. and Mochizuki, M. 2001. *Clin. Diagn. Lab. Immunol.* **8**: 663-668.
12. Page, R. D. 1996. *Comput. Appl. Biosci.* **12**: 357-358.
13. Parker, J. S. L. and Parrish, C. R. 1997. *J. Virol.* **71**: 9214-9222.
14. Pratelli, A., Cavalli, A., Martella, V., Tempesta, M., Decaro, N., Carmichael, L. E. and Buonavoglia, C. 2001. *Clin. Diagn. Lab. Immunol.* **8**: 612-615.
15. Rypula, K., Chmielewski, R., Smielewska-Los, E. and Klimontowski, S. 2002. *J. Vet. Med. B. Infect. Dis. Vet. Public. Health.* **49**: 142-145.
16. Steinell, A., Munson, L., Van Vuuren, M. and Truyen, U. 2000. *J. Gen. Virol.* **81**: 345-350.
17. Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. *Nuc. Acid. Res.* **22**: 4673-4680.
18. Truyen, U. 1999. *Vet. Microbiol.* **69**: 47-50.