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Recombination Between Vaccine and Field Strains of Canine Parvovirus is Revealed by Isolation of Virus in Canine and Feline Cell Cultures

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ABSTRACT. Canine parvovirus type 2 (CPV) is a pathogen that causes severe hemorrhagic gastroenteritis with a high fatality rate in pups worldwide. Since CPV emerged in the late 1970s, its origin has been explored with the conclusion that CPV originated from feline panleukopenia virus or a closely related virus. Both high mutation rate and recombination are assumed to be key factors in the evolution of parvoviruses. Here we provide evidence for natural recombination in CPV isolated from dogs in cell culture. Antigenic and genetic properties of isolates from 10 diseased pups were elucidated. Six pups had been vaccinated beforehand with live combined vaccine containing original antigenic type CPV (CPV-2). Six isolates recovered from 4 vaccinated pups in cell cultures were found to contain either CPV-2 or CPV-2-like viruses. The other isolates, including all those from non-vaccinated pups, were CPV-2b viruses. Antigenic typing of two CPV-2-like isolates, 03–029/M and 1887/f, with a monoclonal antibody panel suggested they were a mixture of CPV-2 and CPV-2a (03–029/M) and a recombinant of CPV-2 and CPV-2b (1887/f). Genetic analysis of the VP1 gene indicated that isolate 03–029/M was a mixture of CPV-2, CPV-2a and a recombinant of CPV-2 and CPV-2a viruses, while isolate 1887/f was composed of a recombinant of CPV-2 and CPV-2b viruses. This is the first demonstration of natural CPV recombination in the field and suggests that recombination in the evolution of CPV is a more frequent and important process than previously believed.
KEY WORDS: canine parvovirus, cell culture, dog, parvovirus, recombination.

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Canine parvovirus type 2 (CPV), one of the feline parvovirus (FPV) subspecies, emerged suddenly throughout the world in the late 1970s as a new pathogen that caused severe hemorrhagic gastroenteritis and myocarditis in domestic dogs [1, 2, 7, 20, 23, 40]. Mortality rate is usually high, particularly in non-immune pups. The origin of the virus is still not clear but the most likely hypothesis from phylogenetic analysis is that CPV originated from feline panleukopenia virus (FPLV) or a very closely related carnivore parvovirus of feral canids, such as foxes and mink [52, 55]. There has been speculation that, during circulation for some time in an European local dog population, this ancestral CPV gradually adapted for domestic dogs and the emergent original CPV (antigenic type 2: CPV-2) rapidly spread globally as a new pathogen of domestic dogs.

In the early 1980s, this first CPV-2 disappeared from the field having been replaced by a new antigenic variant designated CPV-2a, and another antigenic variant CPV-2b appeared soon afterwards. These variants can be distinguished by monoclonal antibodies (MAbs) [35, 43, 45–47]. CPV-2a and 2b use both canine and feline transferrin receptors for binding to cells both *in vitro* and *in vivo* [17, 42] and consequently can infect dogs as well as cats [34, 56]. In contrast, CPV-2 can infect both feline and canine cells *in vitro* but infects only dogs *in vivo* [57].

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During the period between the late 1980s and the early 1990s these prototype CPV-2a and 2b viruses were further replaced by "new CPV-2a and 2b" variants in which the amino acid (aa) residue at site 297 of the VP2 protein was changed from Ser to Ala [4, 28, 58]. This is a non-synonymous substitution that does not result in an antigenic change and thus new CPV-2a and 2b are genetic but not antigenic variants of the prototype CPV-2a and 2b. Today, the new CPV-2a and 2b have become the predominant CPV throughout the world, although the relative proportion of antigenic types varies from country to country [8, 9, 13, 15, 21, 28, 33, 38, 48, 56, 59]. Our recent study indicates that the new CPV-2b has been predominant since 1997 over the field of Japan [39]. In addition to the current major antigenic types CPV-2a and 2b, some novel antigenic variants, CPV-2c (a) and CPV-2c (b) [18, 19], and "Glu-426" [6, 38] also called CPV-2c [10], have been found in field samples from cats and dogs [9, 15, 21, 22, 48], suggesting that CPV is still evolving in some environments. (Hereafter, "new CPV-2a and 2b" is designated "CPV-2a and 2b", unless it is necessary to distinguish them genetically from the prototype CPV-2a and 2b).

Parvoviruses are fastidious viruses which are difficult to isolate in cell culture. Parvovirus replication is basically host cell dependent and takes place only in actively dividing S-phase cells where cellular DNA polymerase is synthesized abundantly [5, 53]. In addition, CPV shows minimum cytopathic effect (CPE) during a productive infection *in vitro*. Therefore, virus isolation (VI) for the detection of

CPV from dogs in diagnostic laboratories has gradually become uncommon and has been replaced by simpler methods, particularly PCR, which has become the principal diagnostic tool for CPV infection [12]. On the other hand, for discovering new biological changes of CPV in the field antigenic typing by hemagglutination-inhibition (HI) and neutralization tests with MAbs is necessary [35, 37, 38, 43, 45–47], which requires that virus be grown in cell culture. Thus, VI is indispensable for a complete analysis of CPV properties. Recent genetic methods designed for "CPV typing" [10, 11] make presumptions of known antigenic types; that is, they detect only mutation sites in the capsid gene sequence previously recognized as resulting in antigenic change. Although such genetic methods are suitable for epidemiological studies that target known antigenic types of CPV, only a small nucleotide change in primers may result in mismatches that give false negative results [15, 22].

Both PCR and VI have been used routinely for detection of CPV from clinical specimens in our laboratory [36]. Recently, we experienced a novel case that led us to reassess VI as the method of choice for detection of CPV. In that case each virus isolated in either canine or feline cell cultures from the same material was finally determined to be of the same CPV-2b antigenic type, but the virus grew very poorly in the feline cell culture until it adapted after more than 10 times of sequential subculturing. Neither genetic analysis of the VP1 gene nor antigenic typing of the isolates clarified differences between the viruses that replicated either in MDCK or fcwf-4 cells. Therefore, it was considered that recent field CPV-2b viruses might have become more difficult to replicate in feline cells than before. They may be new 'host range' variants, which can only be demonstrated in cell culture and that this property does not seem to have its origin in the VP1 gene. To explore this possibility, 10 additional previous clinical samples were re-examined by VI, and the isolates were investigated in detail by antigenic typing with MAbs and molecular methods, including nucleotide (nt) sequencing. This study produced the unexpected results, presented here, which provide the first evidence for genetic recombination between different antigenic types of CPV in dogs. VI was essential for this discovery as recombinants could be selectively isolated in cell culture from clinical specimens that contained antigenically and genetically heterogeneous CPVs.

MATERIALS AND METHODS

Viruses and cell cultures: The following FPVs were used as references: strain TU 1 of FPLV [25]; strains CPV-b [46], CPV-31 [47], FPV-314 [34]; CPV-39 [47], MD97–008 [18]; and HNI-4–1 [38] of CPV. The antigenic types of strains CPV-b, CPV-31 and FPV-314, CPV-39 and MD97–008, and HNI-4–1 are CPV-2, CPV-2a, CPV-2b, and CPV-2b variant "Glu-426", respectively.

Madin-Darby canine kidney (MDCK), *Felis catus* whole fetus-4 (fcwf-4) (ATCC CRL2787), and Crandell feline kidney (CRFK) cells were used for cultivation of the reference viruses and for VI from the clinical specimens as described previously [25, 35, 36].

Clinical specimens: The 10 samples listed in Table 1 were examined. They were chosen randomly from clinical specimens that had been stored at –80°C during the period 2003 to 2006. All samples had been taken in animal hospitals from pups clinically suspected of having CPV infection and were found to contain either new CPV-2a or new CPV-2b virus by PCR. Six of the pups had been vaccinated 4 to 15 days before the incident with live vaccines containing a CPV-2 fraction. The remaining 4 pups had no history of vaccination.

CPV isolation: VI from the clinical specimens was performed by the method described previously [36]. Briefly, the supernatant obtained from either a rectal swab extract or 10% homogenized tissue suspension was filtered through a 220 nm pore size filter and 0.2 m*l* of the filtrate was inoculated into a 60 mm plastic plate containing 4×10^5 of either MDCK or fcwf-4 cells. The plate was incubated at 37°C for 4 to 6 days and the presence of CPV was determined by both PCR and cell staining. The cells were examined with Giemsa staining and by immunofluorescence (IF) with the FPV-specific MAb 2C7 [35]. When no positive result was obtained, the cells were trypsinized and subcultured at a ratio of 1:4.

Serology: Antigenic typing was performed by a HI test [35] with a panel of MAbs. The specificity of MAbs

Table 1. Clinical specimens examined

Sample no.	Specimen	Sampling year	Age of dog (mo.)	Vaccination record[a)]	Detection of CPV-2a or 2b[b)]
03–021	Rectal swab	2003	3	none	+
03–029	Spleen	2003	2	15 days prior to death	+
04–013	Rectal swab	2004	2	6 days prior to death	+
04–030	Rectal swab	2004	2	none	+
04–032	Rectal swab	2004	2	none	+
1862	Rectal swab	2005	7	4 days before sampling	+
1887	Rectal swab	2005	2	7 days prior to death	+
1921	Rectal swab	2006	4	11 days prior to death	+
1936	Rectal swab	2006	2	none	+
1953	Rectal swab	2006	2	4 days prior to death	+

a) Canine live combined vaccines containing an antigenic type CPV-2 fraction.
b) Either new CPV-2a or new CPV-2b genetic type by the PCR with primers CPV-05 and CPV-02 (Table 2).

Table 2. CPV specific primers used in PCR and sequencing

Primer	Sequence	Binding site[a)]
1	5'-GTACATTTAAATATGCCAGA-3'	nt 2,991–3,010
52	5'-ATTAATGTTCTATCCCATTG-3'	nt 3,423–3,442
CPV-05	5'-GAAGAGTGGTTGTAAATAATT-3'	nt 3,025–3,045
CPV-02	5'-CCTATATAACCAAAGTTAGTAC-3'	nt 3,685–3,706
F4	5'-CATACATGGCAAACAAATAGAGCATTGGGC-3'	nt 3,615–3,644
R4	5'-ATTAGTATAGTTAATTCCTGTTTTACCTCC-3'	nt 4,096–4,124
CPV-10	5'-GGAATTCCGGCAAAGAGAGCCAGGA-3'	nt 2,289–2,313
2R2	5'-CTAATATAATTTTCTAGGTGCTAG-3'	nt 4,518–4,541
2F2	5'-GCCACCATGAGTGATGGAGC-3'	nt 2,781–2,800
2F3	5'-AATACTATGCCATTTACTCCAGC-3'	nt 3,327–3,349

a) The nt numbering followed the sequence of CPV-b strain of CPV-2 (accession no. M38245).

obtained from Cornell University are as follows: A3B10 reacts approximately equally with all FPV subspecies including CPV; A2F8 reacts with CPV; B4E1 reacts with CPV-2 but much less well with CPV-2a and 2b; B4A2 reacts with FPLV, CPV-2, and CPV-2a but at a much lower level with CPV-2b; C1D1 and C7D6 react with CPV-2a and 2b. MAbs A3B10, A2F8, B4E1, B4A2, C1D1, and C7D6 have been described as MAbs 8, 7, D, I, 1D1, and 7D6 in previous papers [43, 47]. MAb P2–215, obtained from Hokkaido University, recognizes FPLV and mink enteritis virus [16]. MAb 21C3 was obtained from Tokyo University and reacts only with CPV-2b but at a much lower level with CPV-2b variant "Glu-426" [38].

PCR: The primers used are listed in Table 2. Throughout the study nt numbering followed the sequence of the strain CPV-b of CPV-2 [46]. Primers 1 and 52 [34] were used for detection of CPV and primers CPV-05 and CPV-02 for CPV-2a and 2b. Primers F4 and R4 were used for a PCR based restriction fragment length polymorphism (PCR-RFLP) analysis of the VP2 gene.

PCR conditions were the same as those described previously [36]. Briefly, the sample was diluted 1:10 with sterile distilled water and applied to the PCR. Amplification was performed with 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 55°C for 2 min, and extension at 72°C for 2 min with a recombinant *Taq* DNA polymerase (Takara Biotechnology Co., Ltd.). To obtain a PCR product for sequencing, *Taq* DNA polymerase "Expand Long Range" (Roche Diagnostics K.K.) was used.

PCR-RFLP analysis of the VP2 gene: A PCR product of 510 bp obtained by PCR from the clinical specimens was digested with restriction endonuclease *Alu* I as described previously [39]. This PCR-RFLP can detect nt substitutions in the VP2 gene at positions 3,675 (from T to G) and 3,684 (from C to T) which cause an aa change at sites 297 and 300, respectively. Thus, the PCR-RFLP differentiates genetic types of CPV. The PCR product of CPV-2 was divided into 4 fragments of 388, 84, 70 and 18 bp; the product of prototype CPV-2a and 2b was divided into 3 fragments of 388, 154 and 18 bp; and the product of new CPV-2a and 2b was divided into 4 fragments of 388, 93, 61 and 18 bp.

DNA sequencing: The nucleotide sequence of the VP1 gene of CPV isolates from samples 03–029 and 1887 (Table 4) was determined. The region from nt 2,289 to 4,541 was amplified by a PCR with primers CPV-10 and 2R2 and the product size was 2,253 bp. The PCR product was cloned into pCR4-TOPO plasmid vectors (Invitrogen) and was sequenced by using an ABI PRISM Big Dye Terminator version 3.0 cycle sequencing kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Primers 2F2, 2F3, T7 promoter and T3 were used for sequencing. Sequence analysis was performed by GENETYX-Win version 4.0 (Software Development Co.).

Recombination and phylogenetic analyses: Seven different detection programs were used for analysis of recombination. These were RDP3 [31] which contains 6 separate programs: Bootscan [30], Chimaera [49], GENECONV [41], MaxChi [32], RDP [29], and SiScan [14]; and Genetic Algorithms for Recombination Detection (GARD)[26]. To exclude the possibility of detecting false-positive recombination, putative recombinant regions were considered only if 3 or more different programs detected recombination within the same general region of the alignment, as adopted previously by Shackelton *et al.* [51]. For recombination analysis, the 2,253 bp alignment corresponding to nt positions 2,289 to 4,541 was used, which includes the almost entire VP1 gene.

Phylogenetic and molecular evolutionary analyses were performed using MEGA version 3.1 [27]. Multiple alignments were carried out using CLUSTAL X version 1.83 [54]. A phylogenic tree was constructed using the neighbor-joining method [50] and distances were determined using the Kimura two-parameter method [24].

GenBank accession numbers: The nucleotide sequence accession numbers in the database of the indicated virus used in this study are as follows: CPV-b (M38245), CPV-d (M23255), CPV-15 (M24003), CPV-31 (M24000), CPV-39 (M74849), CPV-133 (M74852), CPV-435 (AY742953), FPV-314 (D78585), CPV-436 (AY742955), MD97–008 (AB115504), 56/00 (AY380577), and HNI-4–1 (AB120727). The nucleotide sequence data originally reported in this paper will appear in the GenBank/EMB/DDBJ nucleotide sequence databases under accession numbers AB437433 and AB437434 for the VP1 gene of CPV strains 1887/M/2 and 1887/f/3. (Hereafter, the isolate in the present study was represented as follows: sample number /

either MDCK (M) or fcwf-4 (f) cells used for VI / passage level / clone number)

RESULTS

Antigenic and genetic mixtures were recognized in CPV isolated in cell culture from clinical specimens: The RFLP analysis of the VP2 gene amplified from the clinical specimens is presented in Fig. 1. Apart from sample 1921, which showed a CPV-2 characteristic pattern, all were new CPV-2a and 2b type.

As a preliminary to the antigenic analysis of the clinical CPV isolates, the reaction profile of MAbs against known mixtures of different antigenic types of CPV was examined (Table 3). The panel of MAbs specifically typed only the sample consisting of a single antigen. Only MAb A3B10 which recognizes an epitope commonly existing in FPV subspecies reacted in all cases. The remaining MAbs apart from C7D6 were not useful for the typing of an antigenic mixture. MAb C7D6 reacted with CPV-2a and 2b in proportion to their respective proportions of antigen.

The results of the VI and antigenic typing of the isolates are presented in Table 4. CPV was recovered from 9 of the samples in both MDCK and fcwf-4 cell cultures. The MDCK cells were generally more efficient than fcwf-4 cells for isolation of field CPV, and the antigenic type of which was exclusively CPV-2b, as for samples 04–030, 04–032, 1862, and 1953. From sample 1936, parvovirus was not isolated in fcwf-4 cell culture, but a CPV-2b virus was finally recovered in MDCK cells after 3 blind passages. It is presumed that the virus content in the original sample was very low.

Unexpectedly, CPV-2 or CPV-2-like viruses were recovered in either MDCK or fcwf-4 cells, or in both from 4 of the 6 vaccinated dogs (03–029, 04–013, 1887, and 1921). In the case of sample 04–013, typical CPV-2b virus was isolated in MDCK cells, but the virus isolated in fcwf-4 cells was a typical CPV-2. In the case of sample 1921, only CPV-2 virus was isolated in both MDCK and fcwf-4 cell cultures, in accordance with the result of the PCR-RFLP analysis (Fig. 1). Since the viruses isolated from samples 04–013 and 1921 showed typical HI reaction profiles against the MAb panel, they were not genetically analyzed further.

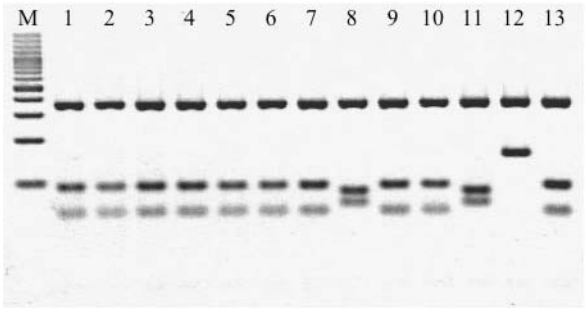

Fig. 1. RFLP analysis of the PCR products obtained from the clinical specimens. A 510 bp of the PCR product amplified with primers F4 and R4 was digested with restriction endonuclease *Alu* I. Lanes: M, 100-bp ladder size markers; 1 to 10, sample no. 03–021, 03–029, 04–013, 04–030, 04–032, 1862, 1887, 1921, 1936, and 1953; 11 to 13, RFLP type references CPV-2 type (strain CPV-b), prototype CPV-2a and 2b type (strain CPV-31), and new CPV-2a and 2b type (strain MD97–008).

Detection of recombinant VP1 sequence between CPV-2 and CPV-2a: The sample studied was 03–029 which was from a pup vaccinated 6 days prior to death. Either new CPV-2a or 2b virus was involved in this case (Fig. 1). Typical CPV-2 virus was isolated in fcwf-4 cells, but the virus isolated in MDCK cells (03–029/M/2) did not show a typical CPV-2 reaction to MAb B4E1 (Table 4). However, it was suspected that both CPV-2 and CPV-2a viruses were present in isolate 03–029/M/2 from the HI titers against

Table 3. Antigenic typing of the experimental mixtures with different antigenic CPVs, and novel isolates 03–029/M/2 and 1887/f/3

Mixed ratio (%)			HI titers against the MAb						
CPV-2[a)]	CPV-2a	CPV-2b	A3B10	B4E1	B4A2	C1D1	C7D6	21C3	Antigenic typing
100	0	0	25,600	10,240	32	< 10	< 2	< 100	CPV-2
75	25	0	12,800	40	16	< 10	< 2	< 100	CPV-2 ?
50	50	0	12,800	40	16	< 10	128	< 100	CPV-2a?
25	75	0	12,800	20	16	< 10	256	< 100	CPV-2a?
0	100	0	12,800	20	16	1,280	256	< 100	CPV-2a
75	0	25	25,600	80	< 4	< 10	< 2	< 100	CPV-2 ?
50	0	50	12,800	40	< 4	< 10	32	< 100	CPV-2b?
25	0	75	12,800	40	< 4	< 10	256	< 100	CPV-2b?
0	0	100	12,800	40	< 4	1,280	256	12,800	CPV-2b
0	75	25	12,800	40	4	1,280	256	< 100	CPV-2a ?
0	50	50	12,800	40	< 4	1,280	256	< 100	CPV-2a or 2b ?
0	25	75	12,800	40	< 4	1,280	256	< 100	CPV-2a or 2b ?
		03–029/M/2	12,800	10	16	< 10	4	< 100	CPV-2 + CPV-2a ?
		1887/f/3	12,800	5,120	< 4	< 10	2	12,800	CPV-2 + CPV-2b ?
		MAb specificity	Parvovirus	FPLV CPV-2	FPLV, CPV-2 CPV-2a	CPV-2a CPV-2b	CPV-2a CPV-2b	CPV-2b	

a) Strains CPV-b, CPV-31, and CPV-39 were used for the antigenic types CPV-2, CPV-2a, and CPV-2b, respectively.

Table 4. Antigenic typing of CPV isolates obtained from clinical specimens in cell culture

Virus strains	Cell type	When CPE positive; Passage level	When CPE positive; HA titer	HI titers against the MAb[a)] A3B10	A2F8	P2/215	B4E1	C1D1	21C3	Antigenic typing
TU 1	CRFK		32	3,200	<10	16,000	640	<10	<100	FPLV
CPV-b	CRFK		512	12,800	320	<1,000	5,120	<10	<100	CPV-2
CPV-31	CRFK		256	6,400	160	<1,000	10	1,280	<100	CPV-2a
CPV-39	CRFK		512	12,800	160	<1,000	10	1,280	12,800	CPV-2b
HNI-4–1	CRFK		1024	25,600	160	<1,000	10	1,280	400	"Glu-426"
03–021	MDCK	P[b)]	1,024	12,800	160	<1,000	<10	1,280	6,400	CPV-2b
	fcwf-4	P	4,096	12,800	160	<1,000	<10	1,280	12,800	CPV-2b
03–029	MDCK	2	32	12,800	160	<1,000	10	<10	<100	CPV-2 + 2a ?
	fcwf-4	2	1,024	25,600	320	<1,000	5,120	<10	<100	CPV-2
04–013	MDCK	P	1,024	25,600	160	<1,000	10	2,560	12,800	CPV-2b
	fcwf-4	3	64	6,400	160	<1,000	2,560	<10	<100	CPV-2
04–030	MDCK	P	2,048	6,400	160	<1,000	<10	640	6,400	CPV-2b
	fcwf-4	P	64	6,400	160	<1,000	<10	640	12,800	CPV-2b
04–032	MDCK	P	8,192	12,800	160	<1,000	10	640	12,800	CPV-2b
	fcwf-4	P	1,024	12,800	160	<1,000	10	1,280	12,800	CPV-2b
1862	MDCK	P	4,096	12,800	160	<1,000	<10	1,280	6,400	CPV-2b
	fcwf-4	P	1,024	12,800	160	<1,000	10	1,280	12,800	CPV-2b
1887	MDCK	2	4,096	12,800	160	<1,000	10	1,280	12,800	CPV-2b
	fcwf-4	3	8	12,800	640	<1,000	5,120	<10	12,800	CPV-2 + 2b ?
1921	MDCK	2	4,096	12,800	320	<1,000	5,120	<10	<100	CPV-2
	fcwf-4	2	2,048	12,800	320	<1,000	5,120	<10	<100	CPV-2
1936	MDCK	4	2	12,800	160	<1,000	10	1,280	6,400	CPV-2b
	fcwf-4	No virus isolation								
1953	MDCK	2	64	25,600	160	<1,000	10	1,280	6,400	CPV-2b
	fcwf-4	6	8	12,800	160	<1,000	10	1,280	12,800	CPV-2b
		MAb specificity		Parvovirus	CPV	FPLV	FPLV/CPV-2	CPV-2a/2b	CPV-2b	

a) A square outlined shows a novel HI titer.
b) Primary culture.

MAbs B4A2 and C7D6 (Table 3).

Six clones of the VP1 gene from isolate 03–029/M/3 were sequenced and the predicted aa variations of the VP2 protein are shown in Table 5. As expected from the antigenic typing data (Tables 3 & 4), the isolate was a mixture of CPV-2 and new CPV-2a viruses. However, the Asn (N) residue at site 560 of the clones 03–029/M/3/1and 03–029/M/3/3 was substituted for Asp (D), and thus they were tentatively designated as "new CPV-2a variant" clones. Clone 6 (03–029/M/3/6) was a recombinant sequence between the VP1 genes of CPV-2 and new CPV-2a origin. Predicted recombination regions were located around sites 3,651 and 3,995 of the alignment (Fig. 2A). As shown in Table 5, the aa residues between 297 and 426 were those of CPV-2a while the adjacent sequences at both ends were those of CPV-2. Phylogenetic analysis of clone 03–029/M/3/6 was presented in Fig. 3. Two fragments of both ends belonged to the clade with the same nt regions of CPV-2 viruses (Fig. 3A & C), and the middle fragment belonged into the clade of new CPV-2a viruses (Fig. 3B).

Clone 03–029/M/3/5 was not predicted to be a putative recombinant sequence by the programs. However, aa characteristics (Table 5) and phylogenetic analysis of this clone suggested that this was also a recombinant sequence between CPV-2 and new CPV-2a variant. The first two-thirds of the alignment belonged to the clade of CPV-2 viruses (Fig. 3A & B) and the remaining portion belonged to the clade of new CPV-2a viruses (Fig. 3C). It was concluded that the major part of the virus from sample 03–029 was CPV-2 virus, and additionally contained new CPV-2a virus, new CPV-2a variant and a few different recombinants of them.

Isolation of a recombinant between CPV-2 and CPV-2b: The sample analyzed was 1887 which was from a pup vaccinated 7 days prior to death. Either new CPV-2a or 2b virus was involved in the case (Fig. 1). Typical CPV-2b virus was isolated in MDCK cells, but the virus isolated in fcwf-4 cells (1887/f/3) showed not only a typical CPV-2b reaction to MAb 21C3 but also a typical CPV-2 reaction to B4E1 (Tables 3 & 4). In addition, the reactions were not those of the antigenic mixtures experimentally determined in Table 3, which suggested that the isolate 1887/f/3 might be a chimeric virus between CPV-2 and CPV-2b that preserves each specific antigenic epitope.

Three clones of the VP1 genes from each isolate 1887/f/3 and 1887/M/2 were sequenced. Since all 3 clones from each isolate showed almost identical sequences, the consensus sequence was obtained for each isolate. Isolate 1887/M/2 had a typical new CPV-2b sequence, while the isolate 1887/f/3 had a recombinant sequence between those of CPV-2 and new CPV-2b viruses. Predicted recombination regions were located around sites 3,755 and 4,435 of the alignment

Table 5. Amino acid sequence variation in the VP2 protein in each clone obtained from isolate 03–029/M/3 and 1887/f/3

Isolates	Amino acid residue at[a]												Typing of sequence[c]
	87	101	219	297	300	305	321	347	375	386	426	560	
Reference viruses													
CPV-2[b]	M	I	V	S	A	D	N	T	N	K	N	N	
new CPV-2a	L	T	I	A	G	Y	K	A	D	Q	N	N	
new CPV-2b	L	T	I	A	G	Y	K	A	D	Q	D	N	
Clones from the isolate 03–029/M/3													
03–029/M/3/1	L	T	I	A	G	Y	K	A	D	Q	N	D	new CPV-2a variant
03–029/M/3/2	M	I	V	S	A	D	N	T	N	K	N	N	CPV-2
03–029/M/3/3	L	T	I	A	G	Y	K	A	D	Q	N	D	new CPV-2a variant
03–029/M/3/4	M	I	V	S	A	D	N	T	N	K	N	N	CPV-2
03–029/M/3/5	M	I	V	S	A	D	N	T	N	K	N	D	CPV-2 + newCPV-2a variant?
03–029/M/3/6	M	I	V	A	G	Y	K	A	D	Q	N	N	CPV-2 + new CPV-2a
Clones from the isolate 1887/f/3													
1887/f/3/1	M	I	V	S	A	D	N	A	D	Q	D	N	CPV-2 + new CPV-2b
1887/f/3/2	M	I	V	S	A	D	N	A	D	Q	D	N	CPV-2 + new CPV-2b
1887/f/3/3	M	I	V	S	A	D	N	A	D	Q	D	N	CPV-2 + new CPV-2b

a) A square outlined shows a putative recombinant position.
b) Typical amino acid residues observed for the reference CPV-2, new CPV-2a and new CPV-2b strains.
c) Typing based on nucleotide and amino acid sequence data. The clones 03–029/M/3/6 and 1887/f/3/1~/3 were considered to be a recombinant between CPV-2 and CPV-2a, and CPV-2 and CPV-2b, respectively. The clones 03–029/M/3/1 and /3 were tentatively named as "new CPV-2a variant" clone since aa residue at position 560 was changed from N to D. The clone 03–029/M/3/5 may be a recombinant between CPV-2 and the new CPV-2a variant.

Table 6. Archives of CPV detection from 203 clinical specimens during the last 8 years from 2000 to 2007 in the author's laboratory

PCR[a]	Virus isolation by cell culturing with		Number of samples
	MDCK	CRFK/fcwf-4[b]	
+	+	+	27 (62.8%)[c]
+	–	–	10 (23.3%)
+	+	–	2 (4.7%)
+	–	+	2 (4.7%)
–	+	+	1 (2.3%)
–	–	+	1 (2.3%)
–	–	–	160

a) Routine PCR with primers 1 and 52 (Table 2).
b) Either CRFK or fcwf-4 cells were used.
c) Percentage of positive samples.

(Fig. 2B). The region between the sites 3,755 and 4,435 was of CPV-2b origin and the rest was of CPV-2 origin. Predicted aa variations of the VP2 protein are shown in Table 5. All clones showed the same aa variations. The first half (~at site 321) had aa characteristics of CPV-2 and the latter half had those of new CPV-2b. Phylogenetic analysis of the isolate 1887/f/3 is presented in Fig. 3. The first half of the alignment (2,289–3,755) belonged to the clade with the same nt regions of CPV-2 viruses (Fig. 3D), and the latter fragment (3,756–4,541) belonged to the clade of new CPV-2b viruses (Fig. 3E). Thus, together with the MAb typing results, isolate 1887/f/3 was considered to be a recombinant between CPV-2 and CPV-2b viruses. In conclusion, the major composition of sample 1887 was CPV-2b virus, but additionally contained a recombinant of CPV-2 and CPV-2b viruses.

DISCUSSION

Our principal finding was that recombination events occur among CPVs in the field. Previously, considering the rapid rates of nucleotide substitution (about 1×10^{-4} substitutions per site per year) which is closer to those of RNA viruses than to that of other double-stranded DNA viruses, Shackelton *et al.* [52] concluded that the high mutation rate of DNA genome and the positive selection of mutations in the major capsid gene, but not recombination, were the major mechanism for the emergence and evolution of CPV. And recently, using the genetic data deposited in databases, natural recombination among porcine, Aleutian mink disease, and several rodent parvoviruses was described and it was suggested that recombination is an important mechanism also for the natural evolution of parvoviruses infecting human, wild or agricultural animals [51]. Actually, until this study, any type of recombination has not been documented for any FPV subspecies, including CPV, in nature and this is the first demonstration of clear evidence of recombination events between CPV-2 and CPV-2a, and between CPV-2 and CPV-2b viruses in dogs. The CPV-2 strain detected simultaneously in the samples is considered to be a parental strain and most probably of vaccine origin since all the canine vaccine products in Japan contain a CPV-2 that became extinct in the field worldwide more than 20 years ago.

In this study, the use of VI for CPV detection contributed greatly to the successful demonstration of these natural CPV recombinants. The isolation of CPV from clinical specimens has become less popular with the advent of alternative diagnostic methods. This is owing to the relatively low sen-

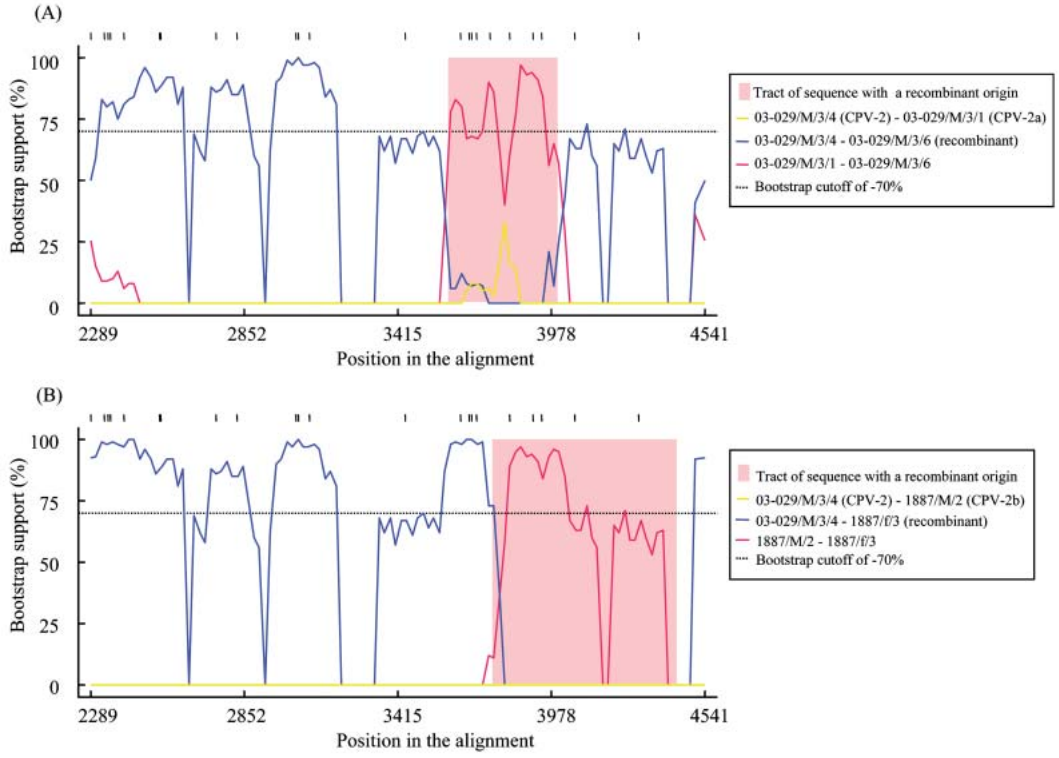

Fig. 2. Representative Bootscan analysis of the VP1 sequences of the recombinant clone 03–029/M/3/6 (A) and the CPV isolate 1887/f/3 (B). (A) The red line indicates the bootstrap support value (the y-axis) for nt sequence of the clone 03–029/M/3/6 which is on the same branch as the corresponding portion of clone 03–029/M/3/1 (CPV-2a characteristics). The blue line indicates the bootstrap support value for the nt sequences of clone 03–029/M/3/6 which is on the same branch as the corresponding portions of the clone 03–029/M/3/4 (CPV-2 characteristics). The nt sequence at sites 3,652–3,995 of the clone 03–029/M/3/6 shows similar characteristics to those of CPV-2a virus and the nt sequences at both sites 2,289–3,651 and 3,996–4,541 shows similar characteristics to those of CPV-2 virus. (B) The red line indicates the bootstrap support value for the nt sequence of the isolate 1887/f/3 which is on the same branch as the corresponding portion of isolate 1887/M/2 (CPV-2b characteristics). The blue line indicates the bootstrap support value for the nt sequences of isolate 1887/f/3 which is on the same branch as the corresponding portions of the clone 03–029/M/3. The nt sequence at sites 3,755–4,435 of the isolate 1887/f/3 shows similar characteristics to those of CPV-2b virus and the rest of nt sequences shows similar characteristics to those of CPV-2 virus.

sitivity of VI compared to DNA detection by PCR [12, 15]; because the nature of the clinical samples may result in contamination of cell cultures; and since specific confirmatory testing to demonstrate CPV in cell culture is required due to the minimal non-specific CPE produced by the virus. A summary of our own experience of CPV detection during the last 8 years is presented in Table 6, which confirms that the sensitivity of VI (75.6%) is indeed lower than that of PCR (93.9%) although specificity is higher with VI (98.8%) than with PCR (94.1%). Therefore, as already described by ourselves and others [12, 36], molecular methods such as PCR may be superior to others for routine detection of CPV and are very useful when known types of CPV are investigated in a large number of field samples [6, 8, 9, 11, 15, 21, 22, 28, 33, 48, 59].

In the present study, our routine PCR did reveal that the field CPV-2a or 2b viruses were decisively associated with all clinical cases (Table 1). In addition, PCR-RFLP also showed the same etiology in each sample, apart from in sample 1921 (Fig. 1). However, VI combined with antigenic and genetic analyses of the isolates from the vaccinated pups led to the recovery of unexpected viruses: a mixture of CPV-2 and CPV-2a viruses, and recombinants between CPV-2 and CPV-2a, and CPV-2 and CPV-2b viruses. These were not detected by our molecular methods of conventional PCR and PCR-RFLP.

It is almost certain that vaccine virus represented one of the parental viruses of the recombinants that were identified and it is likely that all the cases were due to a failure of vaccination timing. Since the pups had been vaccinated between 4 and 15 days before becoming ill, it may be assumed that the vaccine CPV-2 was replicating extensively in the dog's body and vaccine immunity was being generated. If the field CPV-2a or 2b viruses subsequently infected the dog, co-infection of different viruses in the same host cell occurred, which is the most fundamental

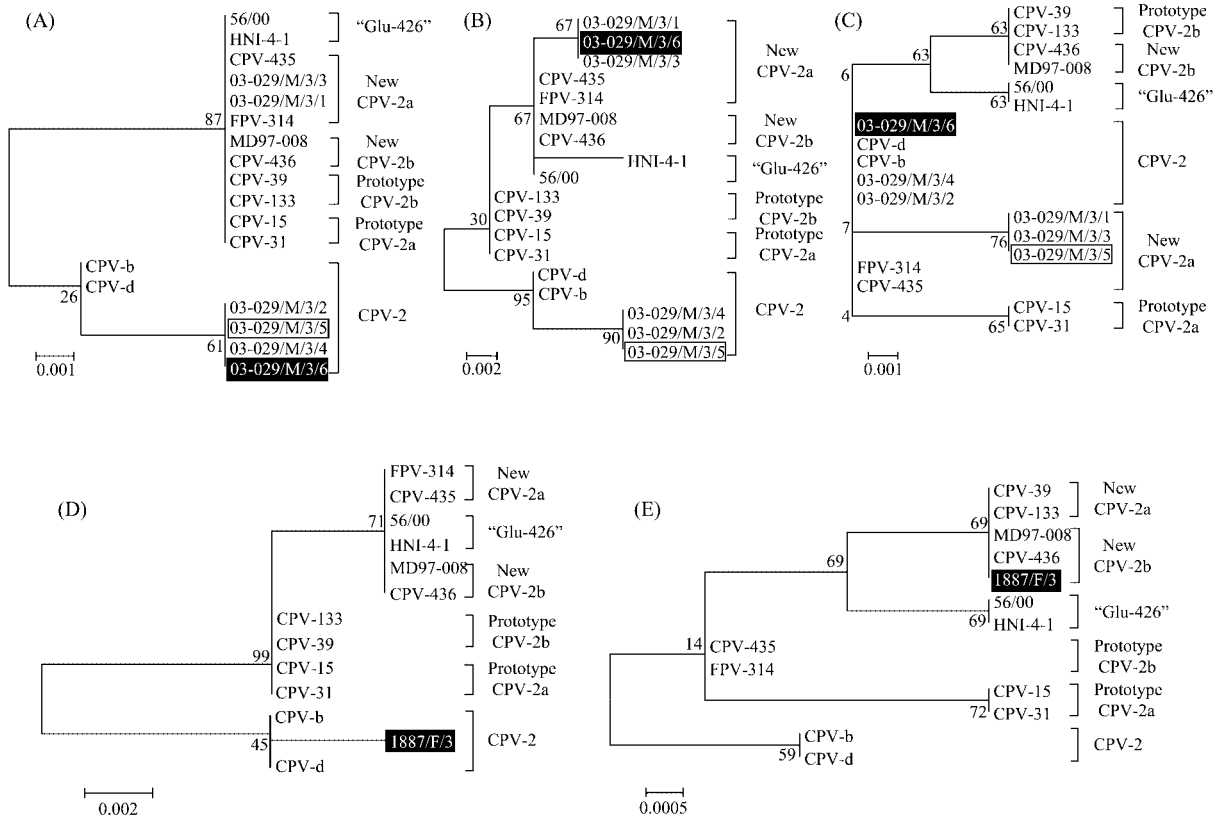

Fig. 3. Phylogeny for the recombinant clones CPV 03–029/M/3/5 and 03–029/M/3/6, and the CPV isolate 1887/f/3. Phylogenetic trees were inferred for nucleotides (A) 2,289–3,651, (B) 3,652–3,995 and (C) 3,996–4,541 of the clones 03–029/M/3/5 and 03–029/M/3/6, and for nucleotides (D) 2,289–3,755 and (E) 3,756– 4,541 of the isolate 1887/f/3. Each bootstrap value indicates the number of times that each branching was found in 100 bootstrap analyses.

requirement to promote virus recombination. Alternatively vaccine may have been administered during the incubation period of a field CPV infection. In either case, recombination between vaccine and field CPVs may occur more frequently than previously considered. This is a general potential hazard of the use of live vaccines, and is not limited to CPV.

Recombination was detected between CPV-2 and CPV-2a, and between CPV-2 and CPV-2b. However, each recombination site observed was not the same in isolates 03–029/M/2 and 1887/f/3, suggesting complexity of recombination in parvovirus species [51]. We assume that recombination occurred in the dogs and the pre-existing recombinants were recovered in the cell cultures. A specific PCR that could selectively detect the recombinant sequence from the original sample in which a large quantity of parental viruses were present was not available. Therefore, it cannot be completely ruled out that the recombination events occurred in the process of either VI or PCR *in vitro*. However, the likelihood of the generation of the recombinants in cell culture must be very low especially in the case of isolate 1887/f/3 because of its homogeneous antigenic and genetic nature after only 3 passages *in vitro* (Tables 3 & 5).

The biological significance of the recombination detected in the present study remains to be resolved. Two points should be considered further. The first is the pathogenic potential of the recombinants. The parental CPV-2 virus for both recombinants detected here is the attenuated vaccine strain. As neither specific genetic markers associated with attenuation of CPV by repeated passages in the cell culture [3, 44] nor specific break points of recombination have been revealed for parvoviruses [51], it is not possible to predict the virulence of the present recombinants compared to the other parental field CPV-2a or CPV-2b viruses. The second question is whether the current CPV vaccines could protect dogs from infection by such recombinants. An *in vitro* neutralization experiment by virus dilution method did show that both isolates 03–029/M/2 and 1887/f/3 were neutralized by the sera of dogs immunized with vaccines containing either CPV-2 or CPV-2b strains to the same extent as recent field CPV-2a and 2b viruses (data not shown). Although preliminary, this result suggests that the CPV vaccines containing either CPV-2 or CPV-2b may be effective against such recombinants since it is well known that the antibody-mediated immunity is effective against CPV infection. Further *in vivo* experiments are required to clarify these ques-

tions convincingly.

VI has been one of the indispensable technical skills for parvovirus studies. The art of isolation of FPV subspecies is to use both canine and feline cells at the same time regardless of the animal species of sample origin. Formerly in this way the CPV-2a etiology of a cat manifesting FPL-like disease was proven for the first time [34]. For sample1887 in the present study, both CPV-2b virus and the recombinant of CPV-2 and CPV-2b viruses were selectively isolated in canine and feline cell cultures, respectively. Although the utility of PCR is not at issue, based on the present results VI should be regarded as the gold-standard method for FPV detection from clinical specimens. Once isolated, the viruses are precious material for further analysis.

Although the recombination between CPV-2 and CPV-2a or 2b viruses demonstrated here is not likely to solve the origin of CPV, the proof that CPV causes recombination among mutually related viruses in nature, and also involves vaccine strains, is a stimulating observation for the study of the continuing evolution of FPV subspecies in the field.

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