Virology 278, 13–19 (2000)
doi:10.1006/viro.2000.0653, available online at http://www.idealibrary.com on IDEAL®

RAPID COMMUNICATION

Predominance of Canine Parvovirus (CPV) in Unvaccinated Cat Populations and Emergence of New Antigenic Types of CPVs in Cats

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Received July 17, 2000; returned to author for revision August 9, 2000; accepted September 19, 2000

Serological, sequence, and *in vitro* host range analyses of feline parvovirus (FPV) isolates in Vietnam and Taiwan revealed that more than 80% of the isolates were of the canine parvovirus (CPV) type, rather than feline panleukopenia virus (FPLV). Although parvovirus isolates from three Vietnamese leopard cats were genetically related to CPV type 2a or 2b, they had a natural mutation of VP2 residue 300 Gly to an Asp, resulting in remarkable changes in their antigenic properties. These results indicated the possibility that CPV-2a/2b-type viruses can spread in cats more efficiently than conventional FPLV under natural conditions and that CPV-2a/2b viruses are further evolving in cats.

Introduction. The appearance of new viruses with pathogenic potential is a constant threat to humans and animals. The diseases caused by feline parvovirus (FPV) were known to occur only in cats (feline panleukopenia virus, FPLV) or raccoons until the mid-1940s, when a similar disease with a high mortality was observed in mink (*1*). Although the mink agent, named mink enteritis virus (MEV), was indistinguishable from FPLV by conventional methods, a panel of monoclonal antibodies (MAbs) classified FPLV and MEV isolates into three antigenic types, FPLV and MEV type 1 (MEV-1), MEV type 2 (MEV-2) and MEV type 3 (*1*). In the late 1970s, another parvovirus emerged in dogs. The new virus, canine parvovirus (CPV) [designated CPV type 2 (CPV-2) to distinguish it from an unrelated parvovirus, minute virus of canines], rapidly spread worldwide and initially killed thousands of unprotected dogs (*2*). Since CPV-2 was shown to be antigenically and genetically very close to FPLV (*3*), FPLV and CPV are classified as host-range variants of FPV.

At present, the most widely accepted hypothesis on the ancestor of CPV-2 is the emergence from a variant of FPLV or of a closely related virus infecting another carnivore, such as mink or fox (*4*). Since the emergence of CPV-2, two new antigenic types of CPV, designated CPV types 2a and 2b (CPV-2a and CPV-2b), arose consecutively and the new virus types almost completely replaced CPV-2-type viruses worldwide (*5*). These observations set FPVs as a significant model for natural viral evolution. CPV strains replicate in both canine and feline cells *in vitro,* whereas FPLV strains efficiently replicate only in feline cells (*6–8*). In contrast to the *in vitro* characteristics, CPV-2 viruses do not have the *in vivo* feline host range (*8, 9*). Recently, we and others reported the isolation of CPV-2a- or CPV-2b-like viruses from domestic cats in Japan and the United States (*9–11*), suggesting that CPV-2a and CPV-2b gained a feline host range, while the proportion of CPV-like viruses from domestic cats was at most 10% in these countries.

In the present study, we examined 18 recent parvovirus isolates from domestic and leopard cats in Vietnam and Taiwan. Since few animals are vaccinated against FPV infection, especially in Vietnam, the isolates might show the natural evolutionary patterns of FPV subfamilies. Elucidation of how FPVs evolve may help to prevent a possible outbreak of a new type of FPV.

Results. Growth properties of the isolates in feline FL74 and canine CL-1 cells. Although the virus stocks of all the isolates were shown to have more than 10^5 $TCID_{50}$ (50% tissue culture infective doses)/ml in FL74 cells,

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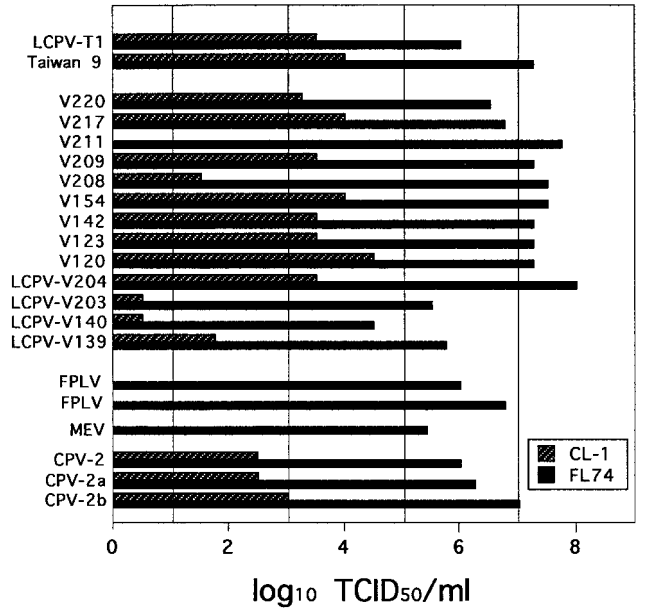

FIG. 1. Comparison of the titers of FPV isolates in feline FL74 and canine CL-1 cells. As reference FPLV isolates, Japanese TU-1 and FPLV No402 isolates were used. MEV M-1 isolate was used as an MEV reference strain. Cp49, CPV97-003, and CPV97-008 were used as CPV-2, CPV-2a, and CPV-2b, respectively. The titers are shown as $TCID_{50}$/ml.

FPLV and MEV reference strains as well as one domestic feline isolate V211 did not replicate in the CL-1 cells (Fig. 1). In contrast, like CPV reference strains, two Taiwanese and most of the Vietnamese isolates had more than 10^3 $TCID_{50}$/ml in the canine cells, while the titers of V208, leopard cat parvovirus (LCPV)-203, LCPV-139, and LCPV-140 in CL-1 cells were relatively low. These results suggest that most of the recent Asian feline isolates have the *in vitro* canine host range.

Reactivity of MAbs against FPVs by the Hemagglutination-Inhibition (HI) Assay. As shown in Table 1, the HI analyses with the panel of 13 MAbs revealed that most of the parvovirus isolates from domestic and leopard cats in Vietnam and Taiwan were of either the CPV-2a or the CPV-2b type, while only three isolates, V142, V208, and V211, were shown to be of the FPLV type. Interestingly, three Vietnamese wild feline isolates (LCPV-V139, LCPV-V140, and LCPV-V203) were demonstrated to be new antigenic types, while the reactivity against MAbs P2-215 and A4E3 indicated that the three viruses were of the CPV type. Thus, we designate the new antigenic types of CPV—which do not react with MAbs A3B10, B6D5, C1D1, 2D9, 3G5, and 4G1—as CPV-2c viruses. In addition, the

TABLE 1

Reactivities of MAbs against FPVs by HI Assay

Isolates	(MAb8) A3B10	(MAbF) B6D5	(MAbG) B2F12	(MAbH) B2G11	P2-215	(MAbD) B4E1	(MAb14) A4E3	(1D1) C1D1	(7D6) C7D6	(MAbI) B4A2	2D9	3G5	4G1	Antigenic type
Taiwan 9	12800	NT	NT	NT	<1,000	8	128	1024	NT	16	NT	NT	NT	CPV-2a
LCPV-T1	12800	NT	NT	NT	<1,000	8	64	1024	NT	32	NT	NT	NT	CPV-2a
V142	12800	NT	NT	NT	128,000	1024	<4	<4	NT	16	NT	NT	NT	FPLV
V154	12800	NT	NT	NT	<1,000	8	128	1024	NT	16	NT	NT	NT	CPV-2a
V208	12800	NT	NT	NT	128,000	1024	<4	<4	NT	32	NT	NT	NT	FPLV
V211	12800	NT	NT	NT	128,000	1024	<4	<4	NT	32	NT	NT	NT	FPLV
V220	12800	NT	NT	NT	<1,000	8	128	1024	NT	16	NT	NT	NT	CPV-2a
LCPV-V139	<100	<4	<4	<4	<1,000	<4	128	<4	<4	32	<10	<100	<10	CPV-2c
LCPV-V140	<100	<4	<4	<4	<1,000	<4	128	<4	<4	32	<10	<100	<10	CPV-2c
LCPV-V203	<100	<4	NT	NT	<1,000	<4	64	<4	NT	<4	<10	<100	<10	CPV-2c
LCPV-V204	12800	NT	NT	NT	<1,000	4	64	1024	NT	<4	NT	NT	NT	CPV-2b
LCPV-V205	12800	256	NT	NT	<1,000	4	128	512	NT	<4	80	800	40	CPV-2b
FPLV	12800	NT	NT	NT	128,000	2048	<4	<4	NT	32	NT	NT	NT	
MEV-1	12800	2048	NT	64	256,000	2048	<4	<4	NT	32	NT	NT	NT	
MEV-2	200	512	NT	<4	256,000	4	<4	<4	NT	32	NT	NT	NT	Reference
CPV-2	12800	4096	<4	16	<1,000	2048	64	<4	<4	16	640	8000	2560	strains
CPV-2a	12800	512	<4	<4	<1,000	8	128	512	128	16	160	1000	320	
CPV-2b	12800	512	<4	<4	<1,000	8	64	512	128	<4	160	2000	320	
Specificity	FPL MEV-1 CPV-2 CPV-2a CPV-2b	FPL MEV-1 MEV-2 CPV-2 CPV-2a CPV-2b	FPL MEV-1	FPLV MEV-1	FPLV MEV-1 MEV-2	FPLV MEV-1 CPV-2	CPV-2 CPV-2a CPV-2b	CPV-2a&2b		FPLV MEV-1 MEV-2 CPV-2 CPV-2a	CPV-2 > CPV-2a, 2b			

Note. NT: not tested. TU-1, Abashiri, M-1, CPV-b, CPV-31, and CPV-39 were used as reference strains of FPLV, MEV-1, MEV-2, CPV-2, CPV-2a, and CPV-2b, respectively. No. 402 (FPLV), Cp49 (CPV-2), CPV 97-003 (CPV-2a), and CPV 97-008 (CPV-2b) were also used as reference isolates and similar reactivities were observed (data not shown).

TABLE 2

Phylogenetically Informative Amino Acid Sequences in the VP2 Gene

Isolates	80	87	93	103	232	297	300	305	323	426	555	564	568	FPV-type
Taiwan 9	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asn	Val	Ser	Gly	CPV-2a
LCPV-T1	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asn	Val	Ser	Gly	CPV-2a
V120	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asn	Val	Ser	Gly	CPV-2a
V123	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asp	Val	Ser	Gly	CPV-2b
V129	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asn	Val	Ser	Gly	CPV-2a
V142	Lys	Met	Lys	Val	Val	Ser	Ala	Asp	Asn	Asn	Val	Asn	Ala	FPLV
V154	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asn	Val	Ser	Gly	CPV-2a
V208	Lys	Met	Lys	Val	Val	Ser	Ala	Asp	Asp	Asn	Val	Asn	Ala	FPLV
V209	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asp	Val	Ser	Gly	CPV-2b
V211	Lys	Met	Lys	Val	Val	Ser	Ala	Asp	Asp	Asn	Val	Asn	Ala	FPLV
V217	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asp	Val	Ser	Gly	CPV-2b
V220	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asn	Val	Ser	Gly	CPV-2a
LCPV-V139	Arg	Leu	Asn	Ala	Ile	Ala	Asp	Tyr	Asn	Asn	Val	Ser	Gly	CPV-2c(a)
LCPV-V140	Arg	Leu	Asn	Ala	Ile	Ala	Asp	Tyr	Asn	Asn	Val	Ser	Gly	CPV-2c(a)
LCPV-V203	Arg	Leu	Asn	Ala	Ile	Ala	Asp	Tyr	Asn	Asp	Val	Ser	Gly	CPV-2c(b)
LCPV-V204	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asp	Val	Ser	Gly	CPV-2b
LCPV-V205	Arg	Leu	Asn	Ala	Ile	Ala	Gly	Tyr	Asn	Asp	Val	Ser	Gly	CPV-2b
FPLV/MEV-1	Lys	Met	Lys	Val	Val	Ser	Ala	Asp	Asp	Asn	Val	Asn	Ala	
MEV-2/BFPV	Lys	Met	Lys	Val	Val	Ser	Val	Asp	Asp	Asn	Val	Asn	Ala	
CPV-2	Arg	Met	Asn	Ala	Ile	Ser	Ala	Asp	Asn	Asn	Val	Ser	Gly	Reference
CPV-2a	Arg	Leu	Asn	Ala	Ile	Ser/Ala	Gly	Tyr	Asn	Asn	Ile	Ser	Gly	strains
CPV-2b	Arg	Leu	Asn	Ala	Ile	Ser/Ala	Gly	Tyr	Asn	Asp	Val	Ser	Gly	

reactivity against MAb B4A2 suggested that LCPV-203 was antigenically related to CPV-2b.

Sequence Analyses of the VP2 Capsid Gene. The full VP2 nucleotide sequences of the isolates were determined and phylogenetically informative amino acid sequences were summarized. As shown in Table 2, most of the isolates had the same VP2 amino acid sequences as conventional CPV-2a or CPV-2b, in spite of their isolation from cats, while only two isolates, V208 and V211, had the exact FPLV VP2 amino acid sequences. Interestingly, V142, which could replicate efficiently in canine cells (Fig. 1) and was antigenically classified as FPLV-type virus (Table 1), was shown to have almost the same VP2 amino acid sequences as conventional FPLV but possess one natural CPV-specific Asp-Asn substitution at amino acid residue 323. On the other hand, the new antigenic CPV-2c-type viruses, LCPV-V139, LCPV-V140, and LCPV-V203, were genetically similar to CPV-2a/2b-type viruses rather than to FPLV or CPV-2 and had a natural mutation of VP2 residue 300 Gly to Asp in common, which might lead to the remarkable changes in their antigenic properties. In addition, the amino acid sequences of the three isolates at residue 426, which determine the difference between CPV-2a/2b-type viruses, revealed that LCPV-V203 had CPV-2b-specific Asp, while LCPV-V139 and LCPV-V140 had CPV-2a-specific Asn. Therefore, we designate LCPV-V203, LCPV-V139, and LCPV-V140 as CPV-2c(b) and CPV-2c(a), respectively.

Phylogenetic Relationships of the Parvovirus Isolates Based on the VP2 Nucleotide Sequences. To examine the phylogenetic relationships of the recent Asian isolates with representative FPLV, MEV, and CPV isolates, phylogenetic analyses based on the full VP2 nucleotide sequences were performed. As shown in Fig. 2A, the phylogenetic tree consisted of three major groups, FPLV/MEV-1/MEV-2, CPV-2, and CPV-2a/2b/2c. Although the three Vietnamese FPLV-type isolates, V142, V208, and V211, formed a small cluster with the other MEV-type viruses, most of the Vietnamese and Taiwanese isolates from cats formed one major cluster with the other CPV-2a/2b-type isolates from dogs. Two CPV-2c(a)-type isolates, LCPV-V139 and LCPV-V140, formed a small cluster with the Vietnamese CPV-2a-type isolates, while the CPV-2c(b)-type isolate, LCPV-V203, formed a group with the Vietnamese CPV-2b-type viruses. The reasons that CPV-2c(a)- or CPV-2c(b)- and Vietnamese CPV-2a- or CPV-2b-type isolates form small clusters can be explained by the fact that they have a natural mutation at nucleotide (nt) 537 from T to C or at nt 1659 from T to C in common (Fig. 2B). These observations suggest the possibility that the CPV-2c(a)- and CPV-2c(b)-type viruses emerged from CPV-2a- and CPV-2b-type viruses, respectively.

Discussion. In the present study, we examined 18 recent parvovirus isolates from domestic and leopard cats in Vietnam and Taiwan and demonstrated that 15 of 18 isolates are essentially identical to CPV isolates from

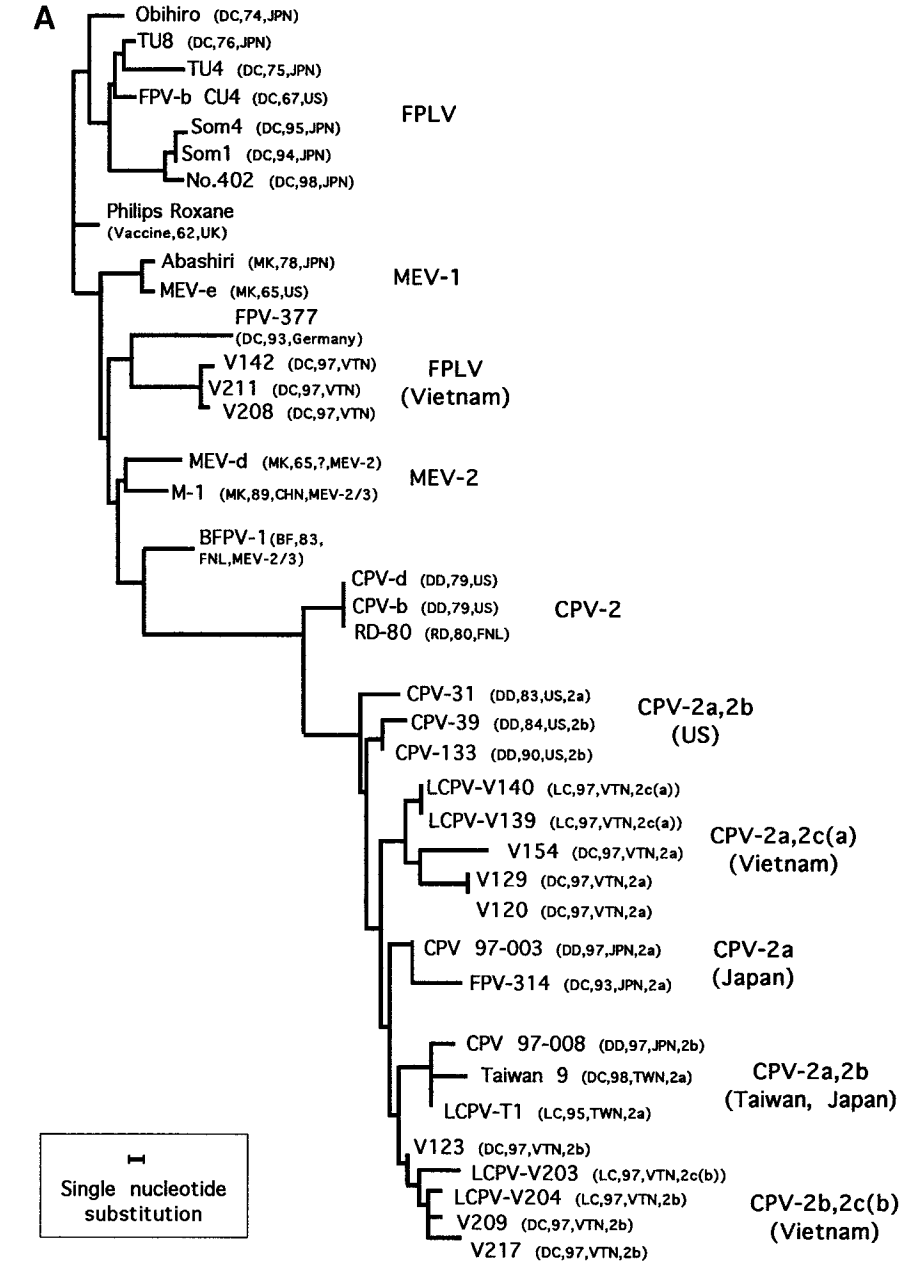

FIG. 2. (A) Phylogenetic analysis of the complete VP2 nucleotide sequences of FPV isolates. The tree was constructed by the NJ (boot-strap) program. DC, domestic cat; LC, leopard cat; DD, domestic dog; MK, mink; BF, blue fox. JPN, Japan; TWN, Taiwan; CHN, China; VTN, Vietnam; FNL, Finland. (B) Phylogenetically informative nucleotide sequences in the VP2 gene. Phylogenetically informative nucleotide sequences, which all the isolates classified as FPLV, MEV-1, FPLV (Vietnam), MEV-2, CPV-2, CPV-2a (United States), CPV-2a (Vietnam), CPV-2a (Taiwan), CPV-2c(a) (Vietnam), CPV-2b (United States), CPV-2b (Vietnam), or CPV-2c(b) (Vietnam) group in (A) have in common, are summarized. VTN, Vietnam; TWN, Taiwan.

dogs. Additionally, we reported the emergence of new antigenic types of CPVs in cats.

There is a long-standing controversy over the question of the origin of CPV-2, and the most widely accepted hypothesis is that the virus arose from FPLV of domestic cats or from FPV retained by closely related wildlife animals (*4*). As shown in Fig. 1 and Table 2, although V142 strain is antigenically classified as FPLV or MEV-1, the isolate has a mutation of VP2 residue 323 Asp to CPV-specific Asn, and this mutation may be ascribed to its relatively high titers in a canine cell line. This observation confirms previous reports that amino acid residues at 93, 103, and 323 in VP2 protein determine canine host range (*12*). These observations suggest the possibility that FPLV can gain a canine host range by natural mutation and support the hypothesis of the emergence of CPV-2 from FPLV, although the phylogenetic analysis indicates that V142 is unlikely to be a direct ancestor of CPV-2 (Fig. 2). In addition, since the two very similar viruses, FPLV and CPV-2a/2b, are circulating in cats at the same time, it remains possible that this single amino acid substitution has occurred by recombination between CPV and FPLV *in vivo.*

Parrish *et al.* (*1*) reported the antigenic differences between FPLV/MEV-1 and MEV-2, which resulted from a mutation of VP2 amino acid residue 300 Ala to Val. Similarly, remarkable antigenic changes of the new-type CPVs were shown to be the result of a natural mutation of VP2 residue 300 Gly to Asp (Tables 1 and 2). CPV-2c-type viruses were isolated only from leopard cats (Table 1) and CPV-2a- and CPV-2b-type viruses were likely to evolve to CPV-2c(a)- and CPV-2c(b)-type viruses, respectively (Figs. 2A and 2B). Therefore, it is probable that the mutation at the residue 300 Gly to Asp is ascribed to the adaptation of CPV-2a/2b-type viruses to leopard cats. Interestingly, the same mutation was previously reported in a mutant of CPV derived by 102 passages in primary dog kidney cells and 10 passages in NLFK cells, which has antigenic properties and *in vitro* host range that are different from those of its parental strain (*13*). Moreover, a study using a CPV mutant containing the residue 300 Asp mutation clearly demonstrated that the mutation stabilizes the capsid (*14*). Therefore, it is possible that the natural mutation at residue 300 found in LCPVs results in greater stability and has some advantages over conventional feline parvoviruses under natural conditions.

Although we and others previously reported on infection with CPV-like viruses in domestic cats in Japan and the United States, the proportions of CPV-like viruses from cats were not high in these countries (*9–11*). However, as shown in Table 2, more than 80% of the FPV isolates from cats in Vietnam and Taiwan were essentially identical to the CPVs from dogs. Recently, Steinel *et al.* (*15*) reported the detection of CPV-2a/2b-related sequences from African large cats, suggesting that CPV-2a/2b-type viruses are spreading in cat populations worldwide. At present, the precise mechanism of the predominance of CPV-2a/2b-type viruses in cats in these countries remains obscure. However, since FPVs are likely to spread freely and rapidly in the environment where few cats and dogs are vaccinated against FPV, it is indicated that CPV-2a/2b-type viruses have more advantages over conventional FPLV in cats under natural conditions. Therefore, it is possible that CPV-2a/2b-type viruses will replace FPLV-type viruses in domestic cats in the future, even in developed countries where FPV vaccines are commonly used.

As we reported previously, all the parvovirus isolates in this study were isolated from the peripheral blood mononuclear cells (PBMCs) of cats with high virus-neutralizing antibodies against FPV and most of the cats showed no clinical sign (*16, 17*), suggesting the possibility that the CPV-type viruses have lower virulence in the infected cats and can infect cats persistently. Our preliminary *in vivo* experiments using two Vietnamese CPV-2a- and CPV-2c-type isolates from cats clearly demonstrated that the CPV-2a-type viruses were less virulent than FPLV in domestic cats, whereas CPV-2c virus showed intermediate virulence in cats (*18*). In terms of viral evolution, it is notable that CPV-2a/2b-type viruses have comparatively lower virulence in spite of their predominance in cat population under natural conditions (*18, 19*), since CPV-type viruses could act as a natural vaccine against virulent FPLV-type viruses in cats. On the other hand, in addition to the relatively high virulence of CPV-2c, we found that the virus neutralizing antibody titers in the cats experimentally infected with FPLV were much lower against CPV-2c than against FPLV (160:2560 [$n = 2$, 4 weeks after infection]) (*18*), suggesting that CPV-2c-type viruses have some advantages over conventional FPLV in cats vaccinated with a commercial FPLV vaccine. Therefore, it will be necessary to monitor the spread of CPV-2c-type viruses to prevent a possible outbreak of the new-type viruses.

Materials and Methods. Viruses and cell culture. One Taiwanese strain (Taiwan 9) and 10 Vietnamese strains (V120, V123, V129, V142, V154, V204, V209, V211, V217, and V220) were isolated from the PBMCs of domestic cats in Taipei and Hanoi, respectively, as described previously (*17*). One FPV-like agent from a leopard cat (*Felis bengalensis*) in Taiwan, designated as LCPV T1 strain, and five isolates from Vietnamese leopard cats, LCPV-V139, LCPV-V140, LCPV-V203, LCPV-V204, and LCPV-V205, were reported previously (*16*). FPLV TU-1 (*10*), FPLV No402 (isolated from a domestic cat in Japan in 1998), MEV Abashiri (MEV-1, GenBank D00765), MEV M-1 (MEV-2, isolated from a mink in China in 1989), CPV-2 Cp49 (*10*) (GenBank D26081), CPV-b (CPV-2, GenBank M38345), CPV-31 (CPV-2a, GenBank M24000), CPV 97-003 (CPV-2a, isolated from a dog in Japan in 1997), CPV

97-008 (CPV-2b, isolated from a dog in Japan in 1997), and CPV-2b CPV-39 (GenBank M74849) strains were used as reference strains. Sequences of the previously published isolates were obtained from GenBank and the accession numbers were Obihiro (AB000056, FPLV), TU4 (AB000068, FPLV), TU8 (AB000070, FPLV), FPV-b CU4 (M24004, FPLV), Som1 (AB000059, FPLV), Som4 (AB000061, FPLV), Philips Roxane (M24002, FPLV), FPV-377 (U22188, FPLV), Abashiri (D00765, MEV-1), MEV-e (U22191, MEV-1), MEV-d Johnson (M24001, MEV-2), BFPV-1 (U22185, MEV-2/3), CPV-d (M23255, CPV-2), RD-80 (U22192, CPV-2), CPV-133 (M74852), and FPV-314 (M78585, CPV-2a). Crandell feline kidney (CRFK) cells (ATCC No. CCL94) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics. Feline and canine T-lymphoblastoid cell lines, FL74 and CL-1 cells, respectively, were maintained in RPMI 1640 growth medium supplemented with 10% FCS and antibiotics (*20, 21*).

Titration of Parvovirus Isolates in Feline and Canine Cell Lines. To estimate the growth properties of FPVs in feline and canine cells, parvovirus isolates with less than three passages in feline cells were titrated in both FL74 and CL-1 cells. The virus infection was judged by FPV-induced cytopathic effects in the infected cells as reported previously (*7, 22*). The $TCID_{50}$ were calculated by the method of Behrens–Kärber as reported previously (*20*).

HI Assay and Virus Antigenic Analysis. Antigenic analysis was performed by the HI assay with the panel of 13 MAbs, as described previously (*10*). HI titers were determined as the inverse of the highest antibody dilution completely inhibiting viral HA. MAbs A3B10 (MAb8), B6D5 (MAbF), B2F12 (MAbG), B2G11 (MAbH), B4E1 (MAbD), A4E3 (MAb14), C1D1 (1D1), C7D6 (7D6), and B4A2 (MAbI) were previously reported elsewhere (*23*). P1-215, 2D9, 3G5, and 4G1 were reported previously (*10, 24*).

Sequencing and Phylogenetic Analysis of the VP2 Gene of the Isolates. For nucleotide sequencing analysis, six synthetic oligonucleotide primers were used. The sequences are 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 by PCR, a primer pair, 51 and R3, was used and the amplified fragments were directly subjected to sequence analysis. Sequencing reactions were performed by the dideoxynucleotide chain termination method with the six primers using Big Dye Terminator cycle sequencing kit (Applied Biosystems [ABI], Foster City, CA) according to the manufacturer's instructions. The samples were resolved on an automated DNA sequencer (ABI Model 377A). The phylogenetic analysis was carried out using NJ (boot-strap) program of GENETIC-MAC ver. 9.0 (Software Development Co., Tokyo, Japan).

ACKNOWLEDGMENTS

We thank Dr. H. Tsujimoto (University of Tokyo, Japan) for providing CL-1 cells; Dr. C. R. Parrish (College of Veterinary Medicine, Cornell University, Ithaca, NY) for providing MAbs A3B10(8), B6D5(F), B2F12(G), B2G11(H), B4E1(D), A4E3(14), C1D1(1D1), C7D6(7D6), and B4A2(I); and Dr. R. A. Weiss (University College London, UK) for advice on the manuscript. This study was partly supported by grants from the Ministry of Education, Science, Sports, and Culture, and from the Ministry of Health and Welfare of Japan.

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