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Canine Parvovirus Types 2c and 2b Circulating in North American Dogs in 2006 and 2007[▽]

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Parvovirus is the most common viral cause of diarrhea in young puppies. Based on the analysis of a partial VP2 sequence of 54 samples, canine parvovirus type 2c (CPV-2c) ($n = 26$), CPV-2b ($n = 25$), and CPV-2 ($n = 3$) were detected in the United States. The American CPV-2b isolates have unique codons (494 and 572) in VP2.

Canine parvovirus (CPV) remains the most significant viral cause of enteritis in puppies over the age of 2 months (1). The virus infects the intestinal epithelium, leading to crypt necrosis, crypt dilatation, and villous atrophy, which is diagnostic of CPV infection (4). CPV emerged in May 1978 worldwide (14) and was termed CPV type 2 (CPV-2) to distinguish it from CPV-1 (minute virus of canines). In 1979, CPV-2a was identified, and within 1 year it became the predominant type of CPV. CPV-2b was later recognized among canine populations. In 2001, a new antigenic type (CPV-2c) was reported in Italy (2). CPV-2c has been detected in Western Europe (5), including Italy, Spain (6), and Germany; Asia (Vietnam and Japan); and South America (15). While this article was under review, there was a report of CPV-2c in the United States (10).

Samples ($n = 54$) were collected between February 2006 and August 2007. Three types of clinical samples were examined: fecal samples, intestinal loops, and tongues. Many cases were submitted with a history of previous vaccination. The clinical symptoms and results from commercially available CPV diagnostic tests were noted when available.

For direct fluorescent-antibody (FA) tests, ~10-μm-thick sections were stained with a mouse anti-CPV monoclonal antibody conjugate labeled with fluorescein isothiocyanate (VMRD, Pullman, WA). The positive cells were fluorescent green, and negative cells were brick red due to an Evans blue counterstain. An Assure test (Synbiotics, San Diego, CA) was used to check for CPV antigen in fecal samples. Many cases were also tested for CPV antigen by the Snap test (Idexx, Bar Harbor, ME) at the referring veterinarian's office prior to laboratory submission.

For PCR, total DNA was extracted from the specimens with a Qiagen viral DNA kit. About 0.1 to 1 μg of DNA was added per reaction. The primers (forward primer, 5′-CAGGAAGA TATCCAGAAGGA-3′; reverse primer, 5′-GGTGCTAGTTG ATATGTAATAAACA-3′) were designed as reported previously (9). The PCR products (583 bp) were purified by electrophoresis on 2% agarose. The DNA was further purified by binding to glass under high-salt conditions and elution under low-salt conditions (Wizard SV gel and PCR clean-up system; Promega, Madison, WI). About 1 μg of the purified DNA was sent to the Oklahoma Medical Research Foundation, Oklahoma City, OK, for sequencing with the forward primer. The sequence was subjected to BLASTN analysis and the CLUSTALW program.

All specimens were stored at −85°C for the study. Intestinal specimens were prepared by scraping the intestinal lining with a scalpel blade. The mucosal lining was finely chopped and homogenized in a stomacher with about 1 ml of sterile phosphate-buffered saline (PBS). After clarification by centrifugation at 12,000 × *g*, the clear supernatant was filtered. For virus isolation, Crandall Ress feline kidney cells (American Type Culture Collection, Manassas, VA) were seeded at a low density (100,000 cells per ml). At 30 to 60 min after the cells were seeded, the filtered (0.45 μm pore size) CPV-positive samples were inoculated. The cell cultures were observed daily for 4 to 5 days for cytopathic effects, such as rounding and detachment of cells. The virus isolation procedure has been described in detail previously (9). To quantify the CPV by a hemagglutination test, the cell culture-propagated CPV isolates were diluted twofold in PBS (pH 7.2) in V-bottom plates. For detection of CPV, about 0.5% swine erythrocytes in PBS with 1% fetal calf serum were added. After incubation for 4 h at 8°C, the hemagglutination titers were calculated as the reciprocals of the highest dilution showing complete matting. The details of the hemagglutination test for CPV have been described previously (9).

The diagnosis of CPV infection was confirmed by histopathology in all cases when tissues were submitted. There was a strong positive correlation between FA test results for small-intestine samples and those for tongue samples. A total of 209 tissues (intestine and/or tongue) were examined by the FA test and histopathology, considered the "gold standard." Of the 209 tissues, 133 were positive and 76 were negative by FA testing. There was a strong correlation between FA and histopathology results. In 48 cases, both tongue and intestinal sections were examined for CPV antigen by the FA test along with histopathology. Among the 33 cases positive for CPV based on lesions, CPV antigen was detected in all 33 of the tongue specimens and 31 of the intestinal specimens by the FA assay. For the 15 cases negative for CPV based on histopathology, both tongue

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TABLE 1. OADDL case numbers, CPV genotypes, codon usage at critical residues, breeds of dogs affected with CPV, U.S. states of origin of clinical samples, and vaccination histories

Sample	CPV type	Sequence at critical codon[b]:								Breed	State	Previous vaccination?[a]
		426	431	440	449	469	494	562	572			
1	2c	**GAA**	CTA	**GCA**	AAT	ATT	TGT	GTA	GTA	German Shepherd	TX	Y
2	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Mix	OK	NR
3	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Poodle	OK	Y
4	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Labrador	MO	Y
5	2b	GAT	**CTG**	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Yorkshire Terrier	MO	NR
6	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Chihuahua	OK	Y
7	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Scottish Terrier	OK	NR
8	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Boston Terrier	AR	NR
9	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Austrian Shepherd	OK	Y
10	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Poodle	OK	Y
11	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	**GTG**	GTA	Cocker Spaniel	OK	Y
12	2b	GAT	CTA	ACA	AAT	**ATC**	**TGC**	GTA	**GTC**	Jack Russell Terrier	OK	Y
13	2b	GAT	CTA	ACA	AAT	**ATC**	**TGC**	GTA	**GTC**	Jack Russell Terrier	OK	Y
14	2b	GAT	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Mix	GA	NR
15	2b	GAT	CTA	ACA	**AAC**	ATT	**TGC**	GTA	**GTC**	Yorkshire Terrier	OK	NR
16	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Papillon	KS	Y
17	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	NR	TX	Y
18	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Beagle	KS	Y
19	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Beagle	KS	Y
20	2b	GAT	CTA	ACA	AAT	AAT	**TGC**	GTA	**GTC**	Mix	KS	Y
21	2c	**GAA**	CTA	ACA	AAT	AAT	TGT	GTA	GTA	Mix	CA	Y
22	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Pomeranian	MO	Y
23	2	AAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Labrador/poodle	KS	Y
24	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Great Dane	GA	Y
25	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Mix	FL	Y
26	2c	**GAA**	CTA	CCA	AAT	ATT	TGT	GTA	GTA	Boston Terrier	MO	Y
27	2c	**GAA**	CTA	GCA	AAT	ATT	TGT	GTA	GTA	Schnauzer	AL	Y
28	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Labrador Retriever	MO	Y
29	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	NA	NA	Yorkshire Terrier	OK	Y
30	2c	**GAA**	CTA	**GCA**	AAT	ATT	TGT	GTA	GTA	Chihuahua	OK	Y
31	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	NR	IL	NR
32	2	AAT	CTA	ACA	AAT	ATT	TGT	GTA	GTA	NR	IL	NR
33	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	NR	IL	NR
34	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	NR	IL	NR
35	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Welsh Corgi	WA	Y
36	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Chihuahua	OK	NR
37	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Yorkshire Terrier	OK	Y
38	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Schnauzer	OK	NR
39	2b	GAT	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Labrador Retriever	AZ	Y
40	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Great Dane	OK	Y
41	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Great Dane	OK	Y
42	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Yorkshire Terrier	OK	NR
43	2c	**GAA**	CTA	**GCA**	AAT	ATT	TGT	GTA	GTA	Bulldog	OK	NR
44	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Chihuahua	OK	NR
45	2b	GAT	CTA	ACA	AAT	AAT	**TGC**	GTA	**GTC**	Mix	GA	Y
46	2b	GAT	CTA	ACA	AAT	AAT	TGT	GTA	GTA	Welsh Corgi	OH	Y
47	2	AAT	CTA	ACA	AAT	AAT	TGT	GTA	GTA	Boxer	VA	Y
48	2c	**GAA**	CTA	ACA	AAT	AAT	TGT	GTA	GTA	Chihuahua	NR	Y
49	2c	**GAA**	CTA	ACA	AAT	AAT	TGT	GTA	GTA	Bulldog	OK	Y
50	2b	GAT	CTA	ACA	AAT	ATT	**TGC**	GTA	**GTC**	Staffordshire Bull Terrier	NC	NR
51	2b	GAT	CTA	ACA	AAT	AAT	**TGC**	GTA	**GTC**	Brussels Griffon	OK	NR
52	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Schnauzer	OK	NR
53	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Dachshund	OK	Y
54	2c	**GAA**	CTA	ACA	AAT	ATT	TGT	GTA	GTA	Rottweiler	OK	Y

[a] Y, yes (reported); NR, not reported.
[b] "Third-base" changes at critical codons are boldfaced.

and intestinal sections were negative. In these CPV-negative cases, death was due to other causes. PCR followed by sequencing was performed on 54 cases (see Table 1). There was a strong correlation (about 99%) between the direct FA test and PCR results on tissue samples. Samples that were FA positive yet failed to PCR amplify on the first attempt were diluted further to reduce the effects of PCR inhibitors in feces. This dilution step resulted in successful PCR amplification for CPV genotyping. Data including CPV type, codon usage, breed of dog, and state of origin of submitting veterinarians are shown in Table 1.

CPV has a worldwide distribution. In Europe, CPV-2a,

CPV-2b, and CPV-2c have been reported from Italy (2). In South America, Uruguay has reported that CPV-2c is the predominant type (15). In Asia, India has reported the prevalence of CPV-2a and CPV-2c (3); Vietnam has reported CPV-2a, CPV-2b, and CPV-2c (12); and Japan has reported CPV-2c in cats (11). There are no reports of CPV-2c from Africa or Australia. CPV-2c is now widespread in several states ($n = 9$) of the United States, based on this study. While this article was under review, another group reported occurrences of CPV-2c in American dogs in several states ($n = 5$), including Arizona (10). There has been an exponential increase in case submissions to the Oklahoma Animal Disease Diagnostic Laboratory (OADDL) for CPV diagnosis because of increased awareness of these CPV variants among kennel breeders.

CPV spreads easily because it is stable in the environment and has a wide host range: Mustelidae (weasels, ferrets, minks, and badgers), Canidae (dogs, foxes, and wolves), Procyonidae (raccoons), and Felidae (cats, lions, tigers, and cheetahs). The high prevalence of anti-parvovirus antibodies in wildlife sanctuaries indicates that cross-infections can occur and may be responsible for interspecies transmission of canine and feline parvoviruses (18). CPV-2c also has a potential to spread to cats (11).

In the American CPV-2c cases, many dogs had either mucoid yellow diarrhea or hemorrhagic diarrhea. We have observed no specific breed predilection for the current CPV types circulating in the United States. However, 5 of the 54 cases were seen in the Yorkshire terrier breed. Severe symptoms of CPV-2c have been reported in Uruguay along with failure of vaccination. In Italy, CPV-2c has been responsible for mucoid diarrhea, leukopenia, and lymphopenia in puppies (6). Increased mortality among puppies in kennels was the main reason for the submission of specimens to the OADDL. In another recent study on CPV-2c, the authors have recommended that monoclonal antibody and nucleic acid-based tests for CPV should be monitored for sensitivity (10). We did not use minor grove binder (MGB) probe technology, which has been validated in Italy, because we have observed three CPV-2c isolates that differ from Italian CPV-2c isolates at codon 440. Thus, application of MGB probe technology for American CPV isolates will require further validation by study of the effects of mismatches near critical codon 426 (10). The molecular basis of CPV vaccine failure is complex and may be related to the emergence of novel CPV types or due to noncompliance with vaccine administration.

A few critical amino acid substitutions determine the host ranges of members of the feline parvovirus genus. CPV isolates are about 99% genetically identical. The new CPV variants that emerge differ from previous strains by only a few base changes. In addition to the codon change at amino acid 426, we observed third-base changes at amino acids 494 and 572 (Table 1). These nucleotide changes have been observed only in the American CPV-2b isolates and served as "markers" of the American CPV-2b isolates. In addition, several other point mutations (third-base changes) were observed among individual CPV isolates. We were able to propagate the American CPV types (CPV-2b and CPV-2c) to high titers (about 80,000 hemagglutinating units per ml) in the CRFK cell line, which could be used for CPV-2c vaccine production. The efficiency of virus isolation was about 45% after one round of cell culture propagation. Virus isolation is not considered a sensitive assay for most enteric viruses, including CPV (10).

To provide protection, induction of antibodies against the VP2 protein of CPV is preferred, because that protein constitutes 90% of the virus. The original CPV-2-based vaccines have been shown to provide protection against the Italian isolate of CPV-2c (17). Individual animal responses to a polyvalent vaccine are quite variable (16). In field settings, vaccination protocols and reductions in maternal antibody levels are variable, and these influence appropriate response to the vaccine. This variability could lead to partial protection of animals in kennel situations. It is important to continually monitor for the emergence of CPV types, because the virus appears to allow some infectivity in the presence of low levels of antibodies (13). The amount of maternal antibody that can prevent infection with a heterologous CPV type has not been studied (19). It is possible that the evolution of CPV-2c will allow new types to break through the antibody barrier. Most of the cases in this study had a history of previous vaccination, and CPV-2c appears to have potential for further spread in American canine populations.

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