CLINICAL AND VACCINE IMMUNOLOGY, Mar. 2008, p. 534–539 Vol. 15, No. 3
1556-6811/08/$08.00+0 doi:10.1128/CVI.00444-07

Evaluation of the Antigenic Relationships among Canine Parvovirus Type 2 Variants▽

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Received 7 November 2007/Returned for modification 5 December 2007/Accepted 15 December 2007

The antigenic relationships among the original canine parvovirus type 2 (CPV-2) and the variants CPV-2a, -2b, and -2c were evaluated. Cross-antigenic evaluation revealed clear differences among the CPV variants, which were more appreciable by serum neutralization (SN) than by hemagglutination inhibition. Antigenic differences were found mostly between the original CPV-2 and the variants, but they were also observed among the variants CPV-2a, -2b, and -2c. The variant CPV-2c exhibited a unique antigenic pattern, since it was poorly recognized by the sera of animals immunized with CPV-2, CPV-2a, and CPV-2b. However, animals immunized with CPV-2c exhibited higher SN titers to CPV-2b than to the homologous virus CPV-2c. The observed antigenic differences might drive selection of CPV strains by generating differential immune pressure in the canine population, which raises concerns about vaccine efficacy.

Canine parvovirus type 2 (CPV-2) is responsible for a severe, highly contagious gastroenteric disease in pups. CPV-2 was first identified in the late 1970s, when outbreaks of fatal myocarditis and hemorrhagic gastroenteritis were observed in young puppies worldwide (3, 8, 23, 24). By sequence analysis CPV-2 appeared to be closely related to feline parvovirus (FPV) and also to parvoviruses from raccoons, minks, and arctic foxes (30, 41), with the nucleotide variation from FPV being lower than 0.5%. In the 1980s the original CPV-2 was completely replaced by new antigenic variants designated CPV-2a and CPV-2b, and the original virus is no longer present in the canine population and exists only in the vaccine formulations. There are at least six or seven amino acid changes between FPV and CPV-2 and at least five or six amino acid changes between the variants CPV-2a/b and the original CPV-2 in the VP2 capsid protein (31, 32), while the variant CPV-2a differs from the variant CPV-2b only in the change 426-Asn→Asp within the major antigenic site of the capsid (Table 1) (31, 32). Soon after the appearance of the CPV-2a/b variants, a number of additional, unusual mutations affecting important residues of the capsid protein VP2 of CPV were recognized (Table 1), suggesting that CPV is still evolving (6, 22, 42). One such variant, Glu-426 (CPV-2c) appears to be widespread in Europe (15, 25) and has been detected in the Asiatic and American continents as well (20, 28, 34).

The few amino acids differences in FPV, CPV-2, and CPV-2a/b appear to have altered the antigenic features of the virus and to have modified important biological properties, such as the in vivo and vitro host ranges (36, 43, 44), the interactions with the cellular receptor, the transferrin protein (21, 29), and the virulence (9). Also, there is concern that the vaccines used currently to prevent CPV infection in dogs may fail to effectively protect pups against the new CPV antigenic variants (40). Although the original CPV-2 was completely replaced by the antigenic variants a few years after its appearance, the original CPV-2 is still used in most commercial vaccines. Several studies have demonstrated that CPV-2 vaccines are still effective to induce protection against CPV variants (9, 18, 39, 45). However, new modified live (ML) vaccines have been developed and licensed using CPV-2b strains.

Studies with antisera raised against the original CPV-2 and the variants have been performed to test the amount of neutralizing activity, particularly against the heterologous types. These studies have revealed substantial difference in the neutralization titers and have suggested that the hemagglutination (HA)-inhibiting antibodies do not correlate well with the neutralizing antibodies and may incorrectly estimate the protective immunity against the antigenic variants in pups with passively acquired antibodies against the original type of CPV (37, 40). In this study, the antigenic relationships among the original CPV-2 and the variants CPV-2a, -2b, and -2c were evaluated by HA inhibition (HI) and serum neutralization (SN) in order to acquire more conclusive data on the antigenic relationships among the various CPV-2 variants.

MATERIALS AND METHODS

Cells. Virus cultivation and SN were performed on the canine A-72 cell line grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS).

Viruses. Four CPV-2 strains were used in the study. Strain 17/80 ISS, with a titer of 3.2×10^5 50% tissue culture infectious doses ($TCID_{50}$/50 μl), was used as a representative of the original CPV-2 (5). Strain 192/98 (3.2×10^3 $TCID_{50}$/50 μl) was used as representative of the CPV-2a variant. The virus was obtained from the feces of a pup that died from CPV-induced gastroenteritis in 1998 in Bari, Italy. Strain 29/97 (3.2×10^4 $TCID_{50}$/50 μl) (4–7) and strain 136/00 (3.2×10^3 $TCID_{50}$/50 μl) (6) were used as representatives of CPV variants 2b and 2c, respectively. Titration of the viral strains was performed in microtiter plates. Tenfold virus dilutions were prepared in quadruplicates in DMEM and were added to wells with 2×10^4 A-72 cells/per well. After incubation at 37°C for 4 days in a CO_2 atmosphere, the plates were frozen and thawed three times, and the undiluted cryolysate of each well was tested by HA using 1% pig erythrocytes. The virus titer was considered the end point dilution showing HA activity in 50%

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▽ Published ahead of print on 26 December 2007.

TABLE 1. Amino acid residues in the VP2 of FPV, mink enteritis virus, and CPVs

Virus	Origin, yr	Strain	Host	Amino acid at residue:													
				80	87	93	101	232	265	297	300	305	323	426	555	564	568
FPV	United States, 1967	FPV-b	Cat	Lys		Lys		Val					Asp			Asn	Ala
Mink enteritis virus	United States, 1975	MEV-b	Mink	Lys		Lys		Val					Asp			Asn	Ala
CPV-2	United States, 1978	CPV-b	Dog	Arg	Met	Asn	Ile	Ile	Thr	Ser	Ala	Asp	Asn	Asn	Val	Ser	Gly
		CPV-Norden	Dog														
	United States	Cornell 780916	Dog														
		154	Dog														
CPV-2a	United States, 1984	CPV-15	Dog		Leu		Thr				Gly	Tyr			Ile		
	United States, 1983	CPV-31	Dog		Leu		Thr				Gly	Tyr			Ile		
CPV-2b	United States, 1984	CPV-39	Dog		Leu		Thr				Gly	Tyr		Asp			
	United States, 1990	CPV-133	Dog		Leu		Thr				Gly	Tyr		Asp			
CPV-2c	Italy, 2000	56/00	Dog		Leu		Thr			Ala	Gly	Tyr		Glu			
Asp-300[b]	Vietnam, 2000	LCPV-V203	Leopard		Leu		Thr			Ala	Asp	Tyr		Asp			
		LCPV-V140	Leopard		Leu		Thr			Ala	Asp	Tyr					
Pro-265[b]	Italy, 2000	CPV-616	Dog		Leu		Thr		Pro		Gly	Tyr		Asp			
CPV-2	Italy, 1980	17/80 ISS[a]	Dog														
CPV-2a	Italy, 1998	192–98[a]	Dog		Leu		Thr			Ala	Gly	Tyr					
CPV-2b	Italy, 1997	29/97[a]	Dog		Leu		Thr			Ala	Gly	Tyr		Asp			
CPV-2c	Italy, 2000	136/00[a]	Dog		Leu		Thr			Ala	Gly	Tyr		Glu			

[a] Strain was used in this study.
[b] CPV-2 mutants identified sporadically and not relevant epidemiologically.

of the wells using the Karber method. The amino acid differences in the capsid proteins of the four CPV-2 strains are depicted in Table 1.

Canine sera. A total of 21 sera, taken from pet dogs of various breeds, were tested. The sera were classified into three groups. Group A included eight sera obtained from eight dogs inoculated subcutaneously with 1 ml of undiluted CPV-2 (17/80 ISS) ML virus. Blood samples were taken for antibody quantification from all the animals 30 days after vaccination (T_1). Group B included nine sera taken from nine dogs 30 days (T_1) after subcutaneous vaccination with 1 ml of undiluted CPV-2b (29/97) ML virus. Group C included four sera obtained from four unvaccinated dogs 30 days (T_1) after natural infection by CPV-2c. The sera of the dogs in groups A and B at the time of vaccination (T_0) did not possess CPV-specific antibodies (≤1:10) by HI and SN, and the feces tested virus negative by real-time PCR (13) on seven consecutive days before vaccination (T_0). It was not possible to obtain canine serum samples with antibodies raised exclusively against the CPV-2a variant.

Rabbit sera. Antisera against CPV-2, CPV-2a, CPV-2b, and CPV-2c were produced in normal adult rabbits. The antigen for rabbit hyperimmunization was prepared in A-72 cell monolayers grown in DMEM supplemented with 10% FCS. Freshly seeded A-72 cells were washed with DMEM to remove the FCS and then were infected with CPV. After virus adsorption for 30 min at 37°C, FCS-free maintenance medium was added and the cells were incubated for 4 days at 37°C. The supernatant of the infected cultures was collected, centrifuged at 5,000 × *g* for 20 min, and then titrated in 96-well plates as described above. Each viral suspension was emulsified with the adjuvant Montanide ISA 740 (Seppic, France) at a 2:3 ratio (vol/vol).

Each virus emulsion was used to immunize two New Zealand rabbits of 2.5 kg of body weight (CPV-2 in rabbits A_1 and A_2, CPV-2a in rabbits B_1 and B_2, CPV-2b in rabbits C_1 and C_2, and CPV-2c in rabbits D_1 and D_2). A total of 3 ml of emulsion per rabbit was administered by three separate subcutaneous inoculations. Rabbit immunization was repeated at 30, 50, and 70 days after the first antigen administration, using the same protocol. Serum samples were taken from rabbits to determine the antibody titers at the time of the first inoculation (T_0) and then 30 days (T_1) and 80 days (T_2) after T_0. At T_0 all the rabbits tested seronegative for CPV variants (CPV-2, CPV-2a, CPV-2b, and CPV-2c) by HI and SN. At the end of the study, all the rabbits were euthanatized.

Serological assays. The canine and rabbit sera were tested by HI and SN to estimate the antibody titers against the four CPV variants (CPV-2, CPV-2a, CPV-2b, and CPV-2c).

HI test. HI was carried out at 4°C using 1% pig erythrocytes and 10 HA units of each CPV variant. Twofold dilutions of each serum sample in phosphate-buffered saline (pH 7.2), starting from 1:10, were tested. The HI titer was expressed as the reciprocal of the highest serum dilution that completely inhibited the HA activity.

SN test. Serial twofold dilutions in DMEM (starting from 1:10) of each serum were mixed with 50 μl of viral suspensions containing 100 $TCID_{50}$ of CPV variants (CPV-2, CPV-2a, CPV-2b, and CPV-2c). Each serum dilution was evaluated in duplicate. After 1 h of incubation at room temperature, 2×10^4 A-72 cells were added to each well. The plates were incubated at 37°C in a humidified CO_2 atmosphere for 4 days and then were frozen and thawed three times. The undiluted cryolysate of each well was tested by HA to monitor virus replication. The neutralizing antibody titer was expressed as the reciprocal of the highest serum dilution that completely neutralized the virus (absence of HA activity).

Statistical analysis. All the antibody titers were transformed into the base 2 logarithmic equivalent in order to normalize their frequency distributions prior to statistical analysis. In advance, the Shapiro-Wilk test (38) showed that the variables included in the analysis had a normal distribution (P = 99.65%). In order to verify whether any significant distortion was linked to individual animals (dogs and rabbits), we analyzed the preliminary variance using the general linear model procedure of the Statistical Analysis Systems program (SAS release 8.01; SAS Institute Inc., Cary, NC), setting the individual animals as independent variables. In this analysis, no differences were found. The data were then subjected to analysis of variance, using the general linear model procedure of the Statistical Analysis Systems program (SAS release 8.01, SAS Institute Inc., Cary, NC) with the model $y_{ij} = \mu + VAR_i + \varepsilon_{ij}$, where y_{ij} is the antibody titer, μ is the mean, VAR_i is the effect of the ith CPV variant tested (i = 1, 2, 3, or 4), and ε_{ij} is the error term.

The results are presented as the least-square means for the different CPV variants tested, and the variability of the data is expressed as the standard error of the mean. A P value of ≤0.05 was considered significant. A comparison between the homologous and heterologous HI and SN means was performed to assess the existence of statistically significant differences.

RESULTS

Canine sera. The last-square and geometric means of the HI and SN titers against the four CPV variants in the dogs immunized/infected with CPV-2, CPV-2b, and CPV-2c are reported in Table 2. In the dogs immunized with CPV-2 (group A), the homologous HI titer (geometric mean) was 3,620 and the heterologous titers were 1,810, 1,234, and 1,395 for CPV-2a, CPV-2b, and CPV-2c, respectively. Statistically significant differences were observed in the heterologous titers against CPV-2b and CPV-2c. The homologous SN titer (geometric mean) was

TABLE 2. Antibody titers in canine sera as measured by HI and SN tests 30 days (T_1) after vaccination or infection with CPV-2 or its antigenic variants

Dog group (virus)	Antibody raised	HI			SN		
		Antibody titer[a]		P value[b]	Antibody titer[a]		P value[b]
		Least-square mean	Geometric mean		Least-square mean	Geometric mean	
A (CPV-2)	CPV-2	**11.82 ± 0.39**	**3,620**		**14.20 ± 0.44**	**18,780**	
	CPV-2a	10.82 ± 0.39	1,810	NS	8.44 ± 0.45	354	<0.001***
	CPV-2b	10.48 ± 0.37	1,234	0.019*	9.54 ± 0.42	842	<0.001***
	CPV-2c	10.32 ± 0.42	1,395	0.014**	8.46 ± 0.48	348	<0.001***
B (CPV-2b)	CPV-2	10.21 ± 0.22	1,185	<0.001***	10.76 ± 0.54	1,741	NS
	CPV-2a	11.32 ± 0.22	2,593	NS	9.60 ± 0.53	766	NS
	CPV-2b	**11.37 ± 0.21**	**2,677**		**11.02 ± 0.52**	**2,282**	
	CPV-2c	11.20 ± 0.23	2,370	NS	9.38 ± 0.58	723	0.042*
C (CPV-2c)	CPV-2	11.57 ± 0.34	3,044	NS	13.82 ± 0.42	14,481	<0.001***
	CPV-2a	12.20 ± 0.35	4,764	NS	10.57 ± 0.43	1,522	NS
	CPV-2b	11.52 ± 0.31	2,560	NS	11.92 ± 0.39	5,120	0.026*
	CPV-2c	**11.32 ± 0.40**	**3,044**		**10.32 ± 0.50**	**1,280**	

[a] Homologous values are in boldface.
[b] The statistical significance of the comparison between homologous and heterologous titers is rated as follows: NS, not significant; *, significant; ** or ***, highly significant.

18,780, whereas the heterologous SN titers were 354, 842, and 348 for CPV-2a, CPV-2b, and CPV-2c, respectively. All the differences were statistically significant ($P < 0.001$).

In dogs immunized with CPV-2b (group B), the homologous HI titer (geometric mean) was 2,677 and the heterologous HI titers were 1,185, 2,593, and 2,370 for CPV-2 CPV-2a, and CPV-2c, respectively, with a significant difference against CPV-2 ($P < 0.001$). By SN, the homologous titer was 2,282 and the heterologous titers were 1,741, 766, and 723 for CPV-2, CPV-2a, and CPV-2c, respectively. There was a statistically significant difference ($P = 0.042$) in the heterologous titer against CPV-2c.

In dogs naturally infected by CPV-2c (group C) there was no statistically significant difference between the homologous HI titer (3,044) and the heterologous titers against CPV-2 (3,044), CPV-2a (4,764), and CPV-2b (2,560). By SN, the homologous titer was 1,280 and the heterologous titers against CPV-2, CPV-2a, and CPV-2b were 14,481, 1,522, and 5,120, respectively. Statistically significant differences were observed against CPV-2 ($P < 0.001$) and CPV-2b ($P = 0.026$).

Rabbit sera. The last-square and geometric means of the HI and SN titers against the four CPV variants in the T_1 (30 days after immunization) and T_2 (80 days after immunization) sera are reported in Table 3 and Table 4, respectively.

In the sera taken from rabbits A_1 and A_2 inoculated with CPV-2, there were no significant differences between the homologous HI titer and the heterologous HI titers against the variants. A significant difference was observed only in the T_2 serum against the CPV-2b variant. Conversely, there were differences between the homologous and heterologous SN titers at both T_1 and T_2, and these differences tended to be statistically significant.

In the sera of the rabbits inoculated with CPV-2a (B_1 and B_2), differences were observed only in the HI titers against the original CPV-2 at both T_1 and T_2. Conversely, in SN marked differences were observed in both the T_1 and T_2 sera against the CPV-2c variant.

In the T_1 and T_2 sera of the rabbits inoculated with CPV-2b (C_1 and C_2), by HI there was a statistically significant difference only against the original CPV-2. Conversely, by SN the differences against the original type and against the variants CPV-2a and CPV-2c as well were marked.

In the sera from the rabbits inoculated with CPV-2c (D_1 and D_2), the only statistically significant difference in the T_1 and T_2 HI titers was found against the original CPV-2. Intriguingly, by SN the T_1 and T_2 titers against the homologous virus were significantly lower than the titers against the variant CPV-2b. Differences were also observed in the T_2 titer against the original type.

DISCUSSION

The antigenic relationships among the original CPV-2 and the variants CPV-2a, CPV-2b, and CPV-2c were evaluated by HI and SN using the sera of immune dogs and rabbits. Inoculation of rabbits with the various CPV-2 strains was done in order to obtain a monospecific serological response, as rabbits, unlike dogs, are not a natural host of CPV-2 infection and therefore may not experience previous "priming" by CPV. Cross-antigenic evaluation of the CPV-2 variants revealed clear differences, which were more appreciable by SN than by HI. These findings confirm preliminary observations (37) and deserve particular attention, as HI is the gold standard test used in diagnostic laboratories for evaluation of humoral immunity to CPV-2. Accordingly, the results obtained with HI may tend to overrate the real immune status of the animals.

As previously observed (37), the greatest antigenic differences were found between the original CPV-2, which is still largely employed in vaccine formulations, and the variants. This finding was not unexpected, since the original CPV-2 differs in at least five or six amino acid changes from the recent CPV-2 variants (31). However, it was also possible to observe antigenic differences among the CPV-2a, CPV-2b, and CPV-2c variants, which may differ from each other even by a single

TABLE 3. Antibody titers in rabbit sera as measured by HI and SN tests 30 days (T_1) after inoculation with CPV-2 or its antigenic variants

Rabbit group (virus)	Antibody raised	HI			SN		
		Antibody titer[a]		*P* value[b]	Antibody titer[a]		*P* value[b]
		Least-square mean	Geometric mean		Least-square mean	Geometric mean	
A_1A_2 (CPV-2)	CPV-2	**8.82 ± 0.50**	**452**		**10.82 ± 0.40**	**1,810**	
	CPV-2a	7.82 ± 0.50	226	NS	7.82 ± 0.40	226	0.003**
	CPV-2b	7.82 ± 0.50	226	NS	8.82 ± 0.40	452	0.022*
	CPV-2c	7.82 ± 0.50	226	NS	7.82 ± 0.40	226	0.003**
B_1B_2 (CPV-2a)	CPV-2	8.32 ± 0.47	320	0.053*	10.32 ± 0.13	1,280	NS
	CPV-2a	**9.82 ± 0.47**	**905**		**10.32 ± 0.13**	**1,280**	
	CPV-2b	9.82 ± 0.47	905	NS	11.07 ± 0.13	2,217	0.003**
	CPV-2c	9.82 ± 0.47	905	NS	9.32 ± 0.13	640	<0.001***
C_1C_2 (CPV-2b)	CPV-2	7.82 ± 0.47	226	0.005**	8.82 ± 0.57	452	<0.001***
	CPV-2a	10.82 ± 0.47	1,810	NS	10.82 ± 0.57	1,810	0.017*
	CPV-2b	**10.32 ± 0.47**	**1,357**		**12.82 ± 0.57**	**7,240**	
	CPV-2c	9.82 ± 0.47	905	NS	8.82 ± 0.57	452	<0.001***
D_1D_2 (CPV-2c)	CPV-2	9.82 ± 0.35	905	0.004**	11.32 ± 0.10	2,560	NS
	CPV-2a	10.82 ± 0.35	1,810	NS	10.32 ± 0.10	1,280	<0.001***
	CPV-2b	11.82 ± 0.35	3,620	NS	14.32 ± 0.10	20,480	<0.001***
	CPV-2c	**11.82 ± 0.35**	**3,620**		**11.32 ± 0.10**	**2,560**	

[a] Homologous values are in boldface.
[b] The statistical significance of the comparison between homologous and heterologous titers is rated as follows: NS, not significant; *, significant; ** or ***, highly significant.

amino acid change (27). In the animals immunized with CPV-2, the SN titers to the antigenic variants CPV-2a, CPV-2b, and CPV-2c were significantly lower than the homologous titers (raised to the original type). It is improbable that these differences may account for decreased protection against the variants in dogs that are protected by a strong active immune response, since after repeated immunizations the antibody titers in young dogs appear to be markedly higher than the minimum levels required for protection against disease and infection. However, it is possible that these differences may allow escape from the limited antibody repertoire of maternal origin in young, unvaccinated pups. Severe parvovirus outbreaks have been observed in pups with HI titers of maternally derived antibodies above the threshold (1:80) related to protection against disease and infection (C. Buonavoglia, unpublished data). Likewise, experimental infection by virulent

TABLE 4. Antibody titers in rabbit sera as measured by HI and SN tests 80 days (T_2) after inoculation with CPV-2 or its antigenic variants

Rabbit group (virus)	Antibody raised	HI			SN		
		Antibody titer[a]		*P* value[b]	Antibody titer[a]		*P* value[b]
		Least-square mean	Geometric mean		Least-square mean	Geometric mean	
A_1A_2 (CPV-2)	CPV-2	**9.82 ± 0.50**	**905**		**11.82 ± 0.40**	**3,620**	
	CPV-2a	8.82 ± 0.50	452	NS	8.82 ± 0.40	452	0.003**
	CPV-2b	7.82 ± 0.50	226	0.022*	10.82 ± 0.40	1,810	NS
	CPV-2c	8.82 ± 0.50	452	NS	9.82 ± 0.40	905	0.022*
B_1B_2 (CPV-2a)	CPV-2	9.32 ± 0.47	640	NS	11.32 ± 0.13	2,560	0.003**
	CPV-2a	**11.32 ± 0.47**	**2,560**		**12.07 ± 0.13**	**4,434**	
	CPV-2b	10.32 ± 0.47	1,280	NS	12.32 ± 0.13	5,120	NS
	CPV-2c	11.32 ± 0.47	2,560	NS	9.32 ± 0.13	640	<0.001***
C_1C_2 (CPV-2b)	CPV-2	8.82 ± 0.47	452	<0.017*	10.82 ± 0.57	1,810	<0.001***
	CPV-2a	11.82 ± 0.47	3,620	NS	12.82 ± 0.57	3,620	0.005**
	CPV-2b	**10.82 ± 0.47**	**2,715**		**14.32 ± 0.57**	**20,480**	
	CPV-2c	10.82 ± 0.47	1,810	NS	9.82 ± 0.57	905	<0.001***
D_1D_2 (CPV-2c)	CPV-2	11.32 ± 0.35	2,560	0.004**	13.32 ± 0.10	10,240	<0.001***
	CPV-2a	12.32 ± 0.35	5,120	NS	12.32 ± 0.10	5,120	NS
	CPV-2b	13.32 ± 0.35	10,240	NS	14.32 ± 0.10	20,480	<0.001***
	CPV-2c	**13.32 ± 0.35**	**10,240**		**12.32 ± 0.10**	**5,120**	

[a] Homologous values are in boldface.
[b] The statistical significance of the comparison between homologous and heterologous titers is rated as follows: NS, not significant; *, significant; ** or ***, highly significant.

CPV-2b strains of unvaccinated pups with high maternally derived antibody HI titers (≥80), which are usually expected to prevent CPV infection and disease, resulted in clinical signs, virus shedding, and an antibody response (14, 16).

Although animals immunized correctly with CPV-2 vaccines are fully protected clinically (2, 18), there is evidence that the active immunity elicited by the vaccines may sometimes fail to protect adult dogs, and the reasons for this may rely on a physiological decline of the protective immunity or on the increased virulence/tropism inherent to some CPV strains. Infection of adult dogs by CPV-2 is uncommon, as CPV-2 usually causes enteritis in young pups (1, 35). However, sporadic cases of CPV-2c infection in adult dogs (>1 year) have been diagnosed in our laboratories (6, 10; Buonavoglia, unpublished data). More recently, we observed a large outbreak of disease caused by CPV-2c in adult dogs housed in a breeding kennel. All the dogs had been immunized three times with a vaccine containing the original CPV-2, followed by a yearly booster vaccination (12). In this case, decreased levels of immunity in the adult dogs, coupled with mechanisms of antigenic escape and/or modified age-related tropism by the CPV-2c variant, are possible reasons that may have contributed to facilitate the virus spread and the onset of the disease in this animal group. These findings raise doubts about the real duration and level of immunity induced by CPV-2 vaccines in dogs, notably in view of the new guidelines for vaccine prophylaxis in dogs, which suggest booster vaccinations at 3-year intervals (33).

Interestingly, it was also possible to observe differences among the antigenic variants CPV-2a, CPV-2b, and CPV-2c. Based on the fact that the original CPV-2 does not exist any longer in the field and on the proposition that the antigenic differences may somehow decrease the effectiveness of the vaccines (40), new ML vaccines using CPV-2b strains have been developed and licensed. In our study, marked antigenic differences were observed by SN in the sera of dogs and rabbits immunized with the CPV-2b vaccine, as the heterologous SN titers (versus CPV-2a and -2c) were significantly lower than the homologous SN titer (versus CPV-2b).

Even more interestingly, evaluation of the antigenic features of CPV-2c by cross-neutralization revealed a unique pattern for the variant CPV-2c. This variant was first identified in 2000 in Italy and became predominant in a few years (25, 27). Subsequently, it has been identified in other European countries and in the Asiatic and American continents (11, 28, 20, 34). The spread of such a CPV-2 mutant may be accounted for by changes in biological properties, such as improved adaptation to the canine host and/or stabilization of the capsid structure, or by mechanisms of antigenic escape triggered by the change Asn/Asp-426→Glu (26). In this study, the CPV-2c variant was less effectively recognized in SN by the sera of dogs and rabbits inoculated with the heterologous (CPV-2, -2a, and -2b) viruses. Conversely, in dogs and rabbits infected/inoculated with CPV-2c, the homologous (versus CPV-2c) titers tended to be lower than the heterologous titers, notably versus CPV-2b. To a lesser extent, a similar inconsistent pattern was observed in rabbits inoculated with the variant CPV-2a, as the homologous (versus CPV-2a) titers tended to be lower than the heterologous titers to CPV-2b. A similar antigenic paradox has been observed by analysis of porcine parvovirus (PPV) strains. By SN using immune porcine and rabbit sera, the highly virulent strain PPV 27a displayed homologous titers 100 to 1,000-fold lower than the heterologous titers raised against other PPV strains (46).

That the antigenic paradox exhibited by CPV-2c may generate a different selective pressure in the dog population and may have contributed to the spread of the variant CPV-2c is an intriguing hypothesis. Also, these findings warrant studies to evaluate the opportunity to develop ML vaccines based on the CPV-2c variant.

In conclusion, the findings of this study indicate discrepancies between the HI and SN titers, suggesting that HI is not adequate to evaluate the real protective immunity of dogs, in particular against the antigenic variants. Also, by SN we observed significant differences in the homologous and heterologous antibody titers. These differences were more marked between the original CPV-2 and the recent variants CPV-2a, CPV-2b, and CPV-2c. However, significant differences were also observed among the CPV-2 variants. Like the human influenza virus and human rotavirus vaccines (17, 19), vaccines containing strains matching the antigenic features of the field strains circulating in the local canine population, or polyvalent vaccines, could represent an alternative strategy to improve the effectiveness of prophylaxis for CPV-2.

REFERENCES

1. **Appel, M. J. G., and C. R. Parrish.** 1987. Canine parvovirus type 2, p. 69–92. *In* M. J. G. Appel (ed.), Virus infections of carnivores. Elsevier, Amsterdam, The Netherlands.
2. **Appel, M. J. G., and L. E. Carmichael.** 1987. Can a commercial vaccine protect pups against a recent isolate of canine parvovirus? Vet. Med. Small Anim. Clin. **87:**1091–1094.
3. **Appel, M. J. G., W. F. Scott, and L. E. Carmichael.** 1979. Isolation and immunization studies of canine parvo-like virus from dogs with haemorrhagic enteritis. Vet. Rec. **105:**156–159.
4. **Buonavoglia, C., A. Pratelli, M. Tempesta, V. Martella, and G. Normanno.** 1998. Valutazione delle caratteristiche di innocuità e immunogenicità di una variante 2b di parvovirus del cane (CPV2b). Veterinaria **12:**55–58.
5. **Buonavoglia, C., M. Compagnucci, and Z. Orfei.** 1983. Dog response to plaque variant of canine parvovirus. J. Vet. Med. B **30:**526–531.
6. **Buonavoglia, C., V. Martella, A. Pratelli, M. Tempesta, A. Cavalli, D. Buonavoglia, G. Bozzo, G. Elia, N. Decaro, and L. E. Carmichael.** 2001. Evidence for evolution of canine parvovirus type 2 in Italy. J. Gen. Virol. **82:**3021–3025.
7. **Buonavoglia, D., A. Cavalli, A. Pratelli, V. Martella, G. Greco, M. Tempesta, and C. Buonavoglia.** 2000. Antigenic analysis of canine parvovirus strains isolated in Italy. Microbiologica **23:**93–96.
8. **Burtonboy, G., F. Coignoul, P. P. Pastoret, and N. Delferriere.** 1979. Canine hemorrhagic enteritis detection of viral particles by electron microscopy. Arch. Virol. **61:**1–11.
9. **Carmichael, L. E.** 1994. Canine parvovirus type-2, an evolving pathogen of dogs. Ann. Med. Vet. **138:**459–464.
10. **Cavalli, A., G. Bozzo, N. Decaro, A. Tinelli, A. Aliberti, and D. Buonavoglia.** 2001. Characterization of a canine parvovirus strain isolated from an adult dog. Microbiologica **24:**239–242.
11. **Decaro, N., C. Desario, D. D. Addie, V. Martella, M. J. Vieira, G. Elia, A. Zipola, C. Davis, G. Thompson, E. Thiry, U. Truyen, and C. Buonavoglia.** 2007. The molecular epidemiology of canine parvovirus, Europe. Emerg. Infect. Dis. **13:**1222–1224.
12. **Decaro, N., C. Desario, G. Elia, V. Martella, V. Mari, A. Lavazza, M. Nardi, and C. Buonavoglia.** 2008. Evidence for immunisation failure in vaccinated adult dogs infected with canine parvovirus type 2c. New Microbiol. **31:**137–143.
13. **Decaro, N., G. Elia, V. Martella, C. Desario, M. Campolo, L. Di Trani, E. Tarsitano, M. Tempesta, and C. Buonavoglia.** 2005. A real-time PCR assay for rapid detection and quantization of canine parvovirus type 2 in the faeces of dogs. Vet. Microbiol. **105:**19–28.
14. **Decaro, N., M. Campolo, C. Desario, G. Elia, V. Martella, E. Lorusso, and C. Buonavoglia.** 2005. Maternally-derived antibodies in pups and protection from canine parvovirus infection. Biologicals **33:**261–267.
15. **Decaro, N., V. Martella, C. Desario, A. L. Bellacicco, M. Camero, L. Manna D. d'Aloja, and C. Buonavoglia.** 2006. First detection of canine parvovirus type 2c in pups with haemorrhagic enteritis in Spain. J. Vet. Med. B **53:**468–472.

16. **Elia, G., A. Cavalli, F. Cirone, E. Lorusso, M. Camero, D. Buonavoglia, and M. Tempesta.** 2005. Antibody levels and protection to canine parvovirus type 2. J. Vet. Med. B **52:**320–322.
17. **Estes, M. K.** 2001. Rotaviruses and their replication, p. 1747–1785. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
18. **Greenwood, N. M., W. S. K. Chalmers, W. Baxendale, and H. Thompson.** 1995. Comparison of isolates of canine parvovirus by restriction enzyme analysis, and vaccine efficacy against field strains. Vet. Rec. **136:**63–67.
19. **Hay, A. J., V. Gregory, A. R. Douglas, and Y. P. Lin.** 2001. The evolution of human influenza viruses. Philos. Trans. R. Soc. London B **356:**1861–1870.
20. **Hong, C., N. Decaro, C. Desario, P. Tanner, M. C. Pardo, S. Sanchez, C. Buonavoglia, and J. T. Saliki.** 2007. Occurrance of canine parvovirus type 2c in the United States. J. Vet. Diagn. Invest. **19:**535–539.
21. **Hueffer, K., and C. R. Parrish.** 2003. Parvovirus host range, cell tropism and evolution. Curr. Opin. Microbiol. **6:**392–398.
22. **Ikeda, Y., M. Mochizuki, R. Naito, K. Nakamura, T. Miyazawa, T. Mikami, and E. Takahashi.** 2000. Predominance of canine parvovirus (CPV) in unvaccinated cat populations and emergence of new antigenic types of CPVs in cats. Virology **278:**13–19.
23. **Johnson, R. H., and P. B. Spradbrow.** 1979. Isolation from dogs with severe enteritis of a parvovirus related to feline panleukopaenia virus. Aust. Vet. J. **55:**151.
24. **Kelly, W. R.** 1978. An enteric disease of dogs resembling feline panleucopaenia virus. Aust. Vet. J. **54:**593.
25. **Martella, V., A. Cavalli, A. Pratelli, G. Bozzo, M. Camero, D. Buonavoglia, D. Narcisi, M. Tempesta, and C. Buonavoglia.** 2004. A canine parvovirus mutant is spreading in Italy. J. Clin. Microbiol. **42:**1333–1336.
26. **Martella, V., N. Decaro, and C. Buonavoglia.** 2006. Evolution of CPV-2 and implication for antigenic/genetic characterization. Virus Genes **33:**11–13.
27. **Martella, V., N. Decaro, G. Elia, and C. Buonavoglia.** 2005. Surveillance activity for canine parvovirus in Italy. J. Vet. Med. B **52:**312–315.
28. **Nakamura, M., Y. Tohya, T. Miyazawa, M. Mochizuki, H. T. Phung, N. H. Nguyen, L. M. Huynh, L. T. Nguyen, P. N. Nguyen, P. V. Nguyen, N. P. Nguyen, and H. Akashi.** 2004. A novel antigenic variant of canine parvovirus from a Vietnamese dog. Arch. Virol. **149:**2261–2269.
29. **Parker, J. S., W. J. Murphy, D. Wang, S. J. O'Brien, and C. R. Parrish.** 2001. Canine and feline parvoviruses can use human or feline transferrin receptors to bind, enter and infect cells. J. Virol. **75:**3896–3902.
30. **Parrish, C. R.** 1999. Host range relationships and the evolution of canine parvovirus. Vet. Microbiol. **69:**29–40.
31. **Parrish, C. R., C. F. Aquadro, M. L. Strassheim, J. F. Evermann, J.-Y. Sgro, and H. O. Mohammed.** 1991. Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. J. Virol. **65:**6544–6552.
32. **Parrish, C. R., P. H. O'connel, J. F. Evermann, and L. E. Carmichael.** 1988. Global spread and replacement of canine parvovirus strains. J. Gen. Virol. **69:**1111–1116.
33. **Paul, M. A., L. E. Carmichael, H. Childers, S. Cotter, A. Davidson, R. Ford, K. F. Hurley, J. A. Roth, R. D. Schultz, E. Thacker, and L. Welborn.** 2006. AAHA canine vaccine guidelines—report of the American Animal Hospital Association (AAHA) Canine Vaccine Task Force. J. Am. Anim. Hosp. Assoc. **42:**80–89.
34. **Pérez, R., L. Francia, V. Romero, L. Maya, I. López, and M. Hernández.** 2007. First detection of canine parvovirus type 2c in South America. Vet. Microbiol. **124:**147–152.
35. **Pollock, R. V. H.** 1984. The parvoviruses. II. Canine parvovirus. Compend. Contin. Educ. Pract. Vet. **6:**653–665.
36. **Pollock, R. V., and L. E. Carmichael.** 1983. Use of modified live feline panleukopenia virus vaccine to immunize dogs against canine parvovirus. Am. J. Vet. Res. **44:**169–175.
37. **Pratelli, A., A. Cavalli, V. Martella, M. Tempesta, N. Decaro, L. E. Carmichael, and C. Buonavoglia.** 2001. Canine parvovirus (CPV) vaccination: comparison of neutralizing antibody response in pups after inoculation with CPV2 or CPV2b modified live virus vaccine. Clin. Diagn. Lab. Immunol. **8:**612–615.
38. **Shapiro, S. S., and M. Wilk.** 1965. An analysis of variance test for normality. Biometrika **52:**591–601.
39. **Spibey, N., I. Greenwood, S. Tarpey, D. Chalmers, and A. Sutton.** 2006. Canine parvovirus type 2 vaccine protects dogs following challenge with a recent type 2c strain, p. 885–886. *In* Proceedings of the 31st World Small Animal Veterinary Congress, 11 to 14 October 2006, Prague, Czech Republic.
40. **Truyen, U.** 2006. Evolution of canine parvovirus—a need for new vaccines? Vet. Microbiol. **117:**9–13.
41. **Truyen, U., A. Gruneberg, S. F. Chang, B. Obermaier, P. Veijalainen, and C. R. Parrish.** 1995. Evolution of the feline subgroup of parvoviruses and the control of canine host range in vivo. J. Virol. **69:**4702–4710.
42. **Truyen, U., A. Steinel, L. Bruckner, H. Lutz, and K. Mostl.** 2000. Distribution of antigen types of canine parvovirus in Switzerland, Austria and Germany. Schweiz Arch. Tierheilkd. **142:**115–119.
43. **Truyen, U., and C. R. Parrish.** 1992. Canine and feline host ranges of canine parvovirus and feline panleukopenia virus: distinct host cell tropisms of each virus in vitro and in vivo. J. Virol. **66:**5399–5408.
44. **Truyen, U., G. Platzer, C. R. Parrish, J. F. Evermann, E. Viller, and C. R. Parrish.** 1996. Evolution of canine parvovirus involved loss and gain of feline host range. Virology **215:**186–189.
45. **Yule, T. D., M. B. Roth, K. Dreier, A. F. Johnson, M. Palmer-Densmore, K. Simmons, and R. Fanton.** 1997. Canine parvovirus vaccine elicits protection from the inflammatory and clinical consequences of the disease. Vaccine **15:**720–729.
46. **Zeeuw, E. J., N. Leinecker, V. Herwig, H. J. Selbitz, and U. Truyen.** 2007. Study of the virulence and cross-neutralization capability of recent porcine parvovirus field isolates and vaccine viruses in experimentally infected pregnant gilts. J. Gen. Virol. **88:**420–427.