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High genetic diversity of the VP2 gene of a canine parvovirus strain detected in a domestic cat

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Abstract

This study reports the detection of co-infection by multiple CPV variants and the high genetic complexity of a CPV-2 strain detected in a domestic cat. The CPV variants selected by cloning the VP2 gene were sequenced, and genetic diversity and selection pressure were investigated. Comparison of the nucleotide sequences has evidenced 10 different viral populations, and, in the same animal, more CPV variants coexist. Our analysis excludes the possibility that the recombination events took place during infection and that negative selection acted on the VP2 gene. These findings confirm that CPV-2 shows high genetic heterogeneity resembling the quasispecies found in RNA viruses.

Keywords: Canine parvovirus; Cat; Quasispecies; Selection pressure

Introduction

Canine parvovirus type 2 (CPV-2) is a recent new pathogenic agent which causes severe hemorrhagic gastroenteritis in dogs. CPV-2 was first isolated from a dog in 1978 in the U.S. and spread rapidly all over the world. Between 1979 and 1981, the original type 2 was replaced by a new genetic and antigenic variant, type 2a (CPV-2a), and then, between 1983 and 1984, it was replaced by type 2b (CPV-2b) which differs from type 2a by only a single epitope localized in residue 426 (Parrish et al., 1991).

The new variants have gained the feline host range to cats since they are able to infect both dogs and cats, whereas the original CPV-2 does not replicate in cats (Truyen et al., 1996). Recently, CPV-2a or 2b have been isolated from domestic cats in Japan, Germany, the U.S., Taiwan, Vietnam and Italy (Truyen et al., 1996; Ikeda et al., 2000; Battilani et al., in press). The evolution of the canine parvovirus group continues. Recently, a new antigenic change has been observed in CPV-2 strains isolated from domestic dogs in Italy (Buonavoglia et al., 2001); it was then also detected in Vietnam (Nakamura et al., 2004). This new variant, also recently detected in cats (Battilani et al., in press), is characterized by the presence of glutamic acid in residue 426 of the VP2 protein, the epitope which distinguishes the 2a/2b variants, and this amino acid change causes an antigenic difference detectable using monoclonal antibodies (Nakamura et al., 2004) so the N/D426E mutant can be considered a novel antigenic variant, tentatively named type 2c. Additional antigenic variants which have emerged during recent years have been detected among CPV-2a and CPV-2b, resulting from single amino acid substitutions in the VP2 gene (Battilani et al., 2002; Ikeda et al., 2000).

As a result of the species jump of CPV from dogs to cats, several studies have been carried out in order to investigate which CPV variants circulate among the feline population and how CPV evolves in cats (Ikeda et al., 2000). Furthermore, the potential pathogenicity of CPV in domestic cats is still obscure; experimentally-caused infections suggested that CPV-2a/2b viruses have a lower pathogenicity in infected cats and could cause persistent infection, although the general condition of the cats before infection determines the seriousness of the illness.

In the study described here, we report the detection of co-infection by multiple CPV variants and the high genetic complexity of a CPV-2 strain detected in a domestic cat. The

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CPV variants selected by cloning the VP2 gene were sequenced, and genetic diversity and selection pressure were investigated. An extensive sequence diversity in the viral clones analyzed was detected with 10 different viral populations, differing in identity by 99.5 to 99.9%. Detailed analysis of the amino acid sequences of the clones indicated that multiple CPV variants were present at the same time in the infected animal. These findings showed that CPV, despite being a single-stranded DNA virus, has high genetic heterogeneity resembling the quasispecies found in RNA viruses and multiple infection with several virus types can occur *in vivo*.

Results

The nucleotide sequences of the VP2 gene inserted in the recombinant clones were 1745 bp in length. Fourteen clones were chosen for sequencing. Comparison of the nucleotide sequences has evidenced 10 different viral populations; clones 10-13-14-15 are identical, while the remaining clones differ in identity by 99.5 to 99.9%.

The deduced amino acid sequences are identical in clones 9-10-13-14-15, the other clones showed a sequence similarity which varied from 99.3 to 99.8%. Several synonymous and non-synonymous substitutions were detected by comparison of the sequences clones. Nucleotide changes and the predicted amino acid sequence substitutions are shown in Table 1. Plotting the amino acid substitutions detected in the clones in the 3D structure of CPV VP2 (PDB ID 4DPV), we observed that the major part of the mutations is located in the less accessible part of the VP2 and only three substitutions, R397G, N/D426E, T440A, are located on the exposed surface of VP2.

Analyzing the amino acid positions which distinguish the antigenic types of CPV, it was seen that, in the same animal, two CPV variants coexist: the prevalent antigenic type in the viral population is 2a, but clone 5 belongs to the new variant N/D426E.

Statistical analyses were used to test nucleotide diversity within the group of 116_05 clones, and the results were compared to the sequence data of Italian CPV-2 strains.

A total of 19 polymorphic sites were found scattered within the sequence data of sample 116_05. At the amino acid level, we found 12 polymorphic sites. Summaries of sample sequence variability are presented in Table 2. Sequence variability within Italian CPV-2 strains was greater than variability within the 116_05 clones, as was seen by analysis of the value of parameters k and π. These results were explainable by the fact that the sequence data of Italian CPV include different strains isolated in a temporal fraction of 10 years; therefore, during this time, natural selection produced different variants which have generally reflected an elevated background mutation rate typical of the carnivore parvovirus group (Shackelton et al., 2005). The nucleotide diversity of non-synonymous sites π (a) was greater for the clone population for Italian CPV, indicating a clear prevalence of the number of non-synonymous mutations in sample 116_05. In fact, in the clone population, there were 12 non-synonymous mutations out of a total of 19 mutations; in the

Table 1
Variable nucleotides in the sequences of the VP2 genes analyzed in this study; differing nucleotides are indicated by a letter; the nucleotides identical to clone 1 are indicated as dashes while the nucleotides differing from clone 1 are written

Nt. complete genome	(2955)	(3099)	(3172)	(3211)	(3513)	(3533)	(3534)	(3662)	(3680)	(3746)	(3767)	(3975)	(3983)	(4062)	(4064)	(4104)	(4388)	(4348)	(4471)
Nt. VP2 gene	169	313	386	425	727	747	748	876	894	960	981	1189	1197	1276	1278	1318	1380	1562	1685
Clone 1	A	A	C	A	T	C	G	T	A	A	A	A	A	A	T	A	G	C	T
Clone 2	G	–	T	–	–	T	–	C	–	–	–	G	G	–	–	–	A	T	–
Clone 3	G	–	T	G	–	T	–	–	–	–	–	–	–	–	–	–	A	T	–
Clone 4	G	–	T	–	C	T	–	–	–	–	–	–	–	–	–	–	A	T	–
Clone 5	G	–	T	–	–	T	–	–	–	–	–	–	–	G	A	–	A	T	–
Clone 6	G	–	T	–	–	T	–	–	–	–	–	–	–	–	–	G	A	T	–
Clone 7	G	–	T	–	–	T	A	–	–	G	–	–	–	–	–	–	A	T	–
Clone 9	G	–	T	–	–	T	–	–	G	–	G	–	–	–	–	–	A	T	–
Clone 10	G	–	T	–	–	T	–	–	–	–	–	–	–	–	–	–	A	T	–
Clone 11	G	G	T	–	–	T	–	–	–	–	–	–	–	–	–	–	A	T	–
Clone 12	G	–	T	–	–	T	–	–	–	–	–	–	–	–	–	–	A	T	C
Clone 13	G	–	T	–	–	T	–	–	–	–	–	–	–	–	–	–	A	T	–
Clone 14	G	–	T	–	–	T	–	–	–	–	–	–	–	–	–	–	A	T	–
Clone 15	G	–	T	–	–	T	–	–	–	–	–	–	–	–	–	–	A	T	–
VP2		R57 → G	I105 → V	T129 → I	E142 → G	F243 → L	V250 → M					R397 → G		N426 → E		T440 → A		T521 → I	V562 → A

Nucleotide changes which result in amino acid substitutions are indicated with the residue position of the VP2 protein and deduced amino acid changes.

Table 2
Summaries of sample sequence variability

Sample	S[a]	π[b]	π (s)[c]	π (a)[d]	θw[e]	k[f]
116_05 (n = 14)[g]	19	0.00156 SE 0.00036	0.00248	0.00128	0.00342 SE 0.0000021	2.714
Italian CPV (n = 19)	28	0.00295 SE 0.00027	0.00950	0.00099	0.00459 SE 0.00176	5.146

[a] S, number of variable sites.
[b] π, nucleotide diversity.
[c] π (s), nucleotide diversity of synonymous sites.
[d] π (a), nucleotide diversity of non-synonymous sites.
[e] θw, Watterson's estimator (per site).
[f] k, average number of nucleotide differences.
[g] n, sample size.

Italian CPV data, there were 7 non-synonymous mutations out of a total of 28 mutations. In the data of both sequences, the parameter θw was higher than nucleotide diversity π, suggesting that there exists a deviation from the assumption $\theta = \pi$ which takes place if there is no natural selection in a large population of constant size. Summaries of sample sequence variability are presented in Table 2. To evaluate the selection pressure which acts on the VP2 gene, the ratio of synonymous (dS) and non-synonymous substitutions (dN) per site was calculated for the entire gene; the value of the dN/dS ratio was estimated as 0.40 for clones 116_05 and as 0.08 for Italian CPV data set, indicating that the ratio dN/dS for the clones 116_05 is fivefold higher than that for the Italian CPV. The difference between the estimates of the dN/dS values for the two groups was statistically significant as estimated by a t test ($P < 0.05$). The higher dN/dS ratio for sample 116_05 suggests that the amino acids encoded by the VP2 gene underwent substitutions at a relatively accelerated rate during adaptation in the animal. For this reason, a maximum likelihood approach was used for the identification of sites under selection in the viral clones. All the methods used in the Datamonkey web interface were run, and comparative analysis integration was performed. The SLAC method does not detect sites subject to negative or positive selection, whereas FEL and REL analysis found 7 negatively selected sites (codons 249, 292, 298, 320, 327, 399, 460). The only REL analysis detected 11 positively selected sites when the value of the Bayes factor was 50 (codons 57, 105, 129, 142, 243, 250, 397, 426, 440, 521, 562), providing strong evidence for the hypothesis, but when the Bayes factor value was 100, no selection was found at the sites. As Bayes methods are prone to false positives above all when the data sets are small, as in our case, we hypothesized that no positive selection was involved in these sites (Kosakovsky Pond and Frost, 2005).

No potential recombination events were detected in the data set of the viral clones.

Discussion

Recent findings pointed out the presence of genetic instability among DNA viruses with higher mutation and substitution rates for ssDNA as compared to dsDNA (López-Bueno et al., 2006). Among the ssDNA viruses, the parvoviruses of carnivores provide an important evolutionary model to explain how the emergence of a new pathogen can take place by multiple amino acid changes in the capsid protein. The high substitution rates observed during the emergence and evolution of the CPV were similar to those seen in rapidly evolving RNA viruses. A high degree of genetic variability was also observed for other autonomous parvoviruses such as the Aleutian mink disease virus (AMDV) (Gottschalck et al., 1994) and the minute virus of mice (MVM) (Rubio et al., 2005; Lopez-Bueno and Mateu, 2003). Among the subgroups of feline parvoviruses, FPV and CPV show a different evolution pattern in their respective hosts. FPV is in evolutionary stasis in cats and it evolves with a random genetic drift, while CPV evolves in dogs with an high mutation rate much closer to that of RNA viruses, with strong positive selection which acts on specific residues of VP2, resulting in the emergence of new variants (Shackelton et al., 2005). It is unknown how CPV evolves in cats. Since CPV is a new pathogen for cats, it was hypothesized that host-specific factors could accelerate the evolution of CPV, operating as a driving force (Ikeda et al., 2002); our findings seem to confirm this hypothesis. We reported on one domestic cat carrying multiple strains of CPV with a variability detected in the VP2 region which ranged from 0.1 to 0.5 at the DNA level and from 0.2 to 0.7 at the protein level. In the same animal, we detected co-infection with several CPV variants, type 2a and N/D426E. Similar findings were detected also by Url et al. (2003) who reported the coexistence of CPV-2 and FPV in one cat, confirming that the infection of one animal with several parvoviruses may occur; however, in our report, the genetic complexity of 116_05 strain, with ten different viral clones, is surprising. The simultaneous presence of multiple viral variants in the same subject has already been reported for other parvoviruses such as the AMDV (Gottschalck et al., 1991) but has never been detected *in vivo* in CPV. A preceding study regarding the evolution of CPV *in vitro* during viral attenuation in tissue culture reported a high genetic heterogeneity in the viral genome with 9 clones which have unique sequences at passage 80 of the virus (Badgett et al., 2002). The authors hypothesized that the cause of this extensive polymorphism may be due to recombination events which have allowed the simultaneous ascent of many mutations. Our analysis seems to exclude the fact that recombination events happened during the infection and it is possible that the animal was infected with many variants simultaneously; subsequently, the high rates of nucleotide substitution together with elevated population growth allowed the adaptation and the appearance of CPV variants detected in the animal.

Since new variants of CPV could persist in infected cats in spite of the presence of the neutralizing antibodies (Ikeda et al., 2002), it has been hypothesized that, also in our clinical case, there was a persistent infection. A higher diversity in persistent infection rather than in acute infection has also been observed for other ssDNA viruses, and mixed infections by multiple variants might also be an important multiplier of genetic diversity.

Analysis of selection pressure acting on the VP2 gene has shown that a purifying selection is prevalent on the VP2 protein and no sites are subject to positive selection. The major part of

amino acid changes is situated on the inner surface of the capsid, the most conserved region of the protein, while, for other parvoviruses, a correlation between polymorphic residues and their accessibility on the protein surface was observed (Martins Soares et al., 2003). Therefore, these mutations are not a direct result of immune selection but are probably the consequence of the intrinsic high rate of mutation in CPV (Shackelton et al., 2005). Furthermore, these residues are not implicated in molecular interactions or biological functions of the viruses so this region can accommodate high levels of non-synonymous mutations without interfering with the fundamental function of the protein.

In conclusion, these findings display the remarkable genetic heterogeneity and evolutionary capacity of CPV-2 which reassembles the quasispecies distribution found in the RNA virus population. Furthermore, this is the first report of the detection of multiple CPV variants in the same animal which confirms how multiple infection can also occur for CPV as is the case for other parvoviruses.

Materials and methods

The small intestine was removed from a domestic cat which died exhibiting enteric symptomatology (reference number 116_05). A parvoviral infection was detected by an examination of the intestinal contents using a parvoviral antigen detection kit (SNAP PARVO, IDEEX).

The whole VP2 gene was amplified with the primers P1 (5′-ATGAGTGATGGAGCAGTTC-3′) and VPR (5′-TTCT-AGGTGCTAGTTGAG-3′) using the Klen Taq LA Polymerase Mix (BD Biosciences, Clontech, USA) which contains Klen Taq-1 DNA polymerase as the primary polymerase and a minor amount of a 3′ → 5′ proofreading polymerase for high fidelity PCR. Direct sequencing of PCR products showed an unusually high number of ambiguities, although a proofreading Taq was used; this result suggested mixed viral populations so the amplification products of 1745 bp were cloned into the PCR 4/TOPO vector using the TOPO cloning kit (Invitrogen, Netherlands) and were transformed into *Escherichia coli* DH5α-competent cells according to the manufacturer's protocol. Fourteen recombinant clones were purified with Turbo Kit (QBIOgene, UK) and were sequenced using standard dideoxy sequencing with fluorescence-labeled nucleotides (Perkin-Elmer Applied Biosystem).

In order to determine how many substitutions were associated with *Taq* DNA polymerase-induced error, the VP2 gene was amplified using a clone previously sequenced. The resulting amplicon was sequenced directly and shown to be 100% identical to the sequence of the recombinant plasmid (data not presented), suggesting that any contribution by Taq to the observed substitution rates was low.

The nucleotide sequences obtained were compared to sequences available from the GenBank using the CLUSTAL W web interface (http://www.ebi.ac.uk/clustalw/). The degree of similarity among the sequences at both the nucleotide and the amino acid level was determined by the MEGALIGN program (DNASTAR software package, Lasergene Inc., USA).

A variety of statistical analyses regarding nucleotide diversity and sequence variability were carried out on the sequence data set using the versatile program DNASP version 4.10 (Rozas et al., 2003) which has a user-friendly interface. The following parameters were considered: the number of polymorphic sites S, the Watterson's parameter θw which estimates the level of population variation on the basis of the number of segregating sites in the sample, the mean pairwise diversity Pi (π) calculated from the total number of sites and its standard error and the average number of nucleotide differences k. In the same statistical analysis of genetic diversity, an additional sequence data set of Italian CPV-2 strains (n = 19) was also computed (Battilani et al., 2002).

To examine the evolutionary pressures which contributed to a genetic variation in the 116_05 strain, the ratio of non-synonymous substitutions per non-synonymous site (dN) to synonymous substitutions per synonymous site (dS) was estimated using the Datamonkey web interface (http://www.datamonkey.org), a maximum-likelihood-based tool for the identification of sites subject to positive or negative selection. Datamonkey uses three complementary methods: single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL) and random effects likelihood (REL).

Recombination was examined using the RDP2 package (Martin et al., 2005) which permits the detection of recombination events using 10 published recombination detection methods.

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