Analysis of canine parvovirus sequences from wolves and dogs isolated in Italy

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The VP2 genes of Italian canine parvovirus (CPV) type 2 strains isolated from dogs and wolves were sequenced and a three-dimensional model of the VP2 capsid protein was constructed. Two mutations were detected in the VP2 sequences of the Italian strains: one at residue 297 and one at residue 265. Variant 297 is the predominant CPV isolate in Europe, whereas variant 265 has never been detected before. The mutation at residue 265 causes a disruption in a G strand of the β-barrel in the VP2 protein. Data on strains isolated from wolves demonstrated that the same strain of CPV can circulate among domestic and wild canids; therefore, this result leads us to exclude the possibility that a separate parvovirus pool exists in wild populations.

Canine parvovirus type 2 (CPV-2) is an important pathogen in domestic dogs and several wild carnivore species. It was first identified in USA in 1978 (Appel et al., 1979) and was found later to have spread worldwide in domestic and wild canid populations.

After its initial appearance, it was shown that antigenic drift continuously changes the antigenicity of CPV: the original CPV-2 strain has been completely replaced by the newer antigenic types CPV-2a and CPV-2b (Parrish et al., 1991), which have also extended their host range to include cats (Mochizuki et al., 1996). The new types of CPV differ from the original type 2 strain in that there are some nucleotide changes (positions 3045, 3685, 3699, 4062 and 4449) in the gene encoding the VP2 coat protein (Parrish et al., 1991; Truyen et al., 1995). Sequences important for the determination of antigenic type and for the control of host range are located in the VP2 capsid protein (Parrish, 1991; Chang et al., 1992).

Several hypotheses have been proposed to explain the sudden emergence of CPV. The most probable of which suggests that CPV arose from wild carnivores that harboured the original CPV ancestor (Truyen, 1999).

The presence of CPV-2 in the wolf population in USA was confirmed through serological analyses (Goyal et al., 1986; Mech et al., 1986) and virus isolation from faeces (Muneer et al., 1988). Serological evidence of CPV-2 in wolf sera in Italy has been reported previously (Fico et al., 1996); CPV has also been isolated from wolf faeces collected in the north-central Apennine mountains (Martinello et al., 1997). As the CPV subtype circulating in the wolf population has not been identified, the aim of our research was to characterize several wolf strains by analysing the VP2 gene sequences and to compare these sequences with those from isolates originating from Italian dogs.

In this study, and as described previously, four Italian CPV strains isolated from samples of wolf faeces (Martinello et al., 1997) were analysed. Eight faecal samples from dogs showing clinical signs of haemorrhagic gastroenteritis were also examined. The specimens were first examined using the Canine Parvovirus Antigen Test kit (IDEEX). Viruses were propagated in feline embryonic fibroblast (FEA) cells as described by Mochizuki & Hashimoto (1986). Cells were cultured for three blind passages and the supernatants were monitored for virus growth by using the haemagglutination (HA) test, as described by Carmichael et al. (1980). The viruses examined are listed in Table 1(a).

Viruses were typed for antigenicity by using the haemagglutination inhibition (HI) test with specific monoclonal antibodies (MAbs), as described previously (Table 1b) (Parrish et al., 1982; Parrish & Carmichael, 1983); the American reference strains CPV-d (type 2), CPV-15 (type 2a) and CPV-39 (type 2b) were used as the comparison strains. The American reference strains and the MAbs were kindly supplied by Colin Parrish (Cornell University, Ithaca, NY, USA).

During passage in cell culture, virus-induced CPE was detected for each sample. Virus growth was expressed as an...
increase in the HA titre of the infected supernatant and resulted in a titre greater than 1:4000. Viral DNA was extracted from the cryo-lysate of the third passage supernatant from infected FEA cells using the QIAamp DNA mini kit (QIAGEN), according to the manufacturer’s instructions. The VP2 capsid protein gene was then amplified by PCR in four sections with four sets of primers, namely P1 and P2, N1 and N2, P3 and P4, and VPM and VPR. The sequences of the primers were selected from the conserved regions of the VP2 genes (Reed et al., 1988; Rhode, 1985) and are as follows: P1, 5′ ATGATGGAGACGTTC 3′ (nt 2788–2807); P2, 5′ TCATCTAAAGCCATGTTTC 3′ (nt 3068–3087, complementary); N1 and N2 (Senda et al., 1995); P3, 5′ CCATTCTAAATTCTTTG 3′ (nt 3752–3770); P4, 5′ AAGTCAGTATCAATTCTTT 3′ (nt 4094–4113); VPR, 5′ TGGAGGTAAAACAGGAATT 3′ (Mochizuki et al., 1995).

PCR was carried out using Phi DNA polymerase (Stratagene), essentially as described by Mochizuki et al. (1995).

Nucleotide sequences of the PCR products were determined with an automated DNA sequencer (ABI PRISM 310, Perkin Elmer) and the sequences obtained were submitted to GenBank under the accession numbers AF306444–AF306450.

The sequences were then compared to those available in GenBank and aligned with the MegAlign program of the DNASTAR multiple program package (Lasergene) using the Clustal method (Higgins et al., 1992).

Phylogenetic analysis was performed using the MEGA program (Kumar et al., 1994): pairwise genetic distances were calculated by using the Jukes–Cantor method and phylogenetic trees were constructed by using the neighbour-joining method. A bootstrap analysis with 500 replicates was carried out to assess the confidence level of each branch pattern: a bootstrap value of > 70% was considered to be significant (Hills & Bull, 1993).

A three-dimensional (3D) model of the VP2 protein was constructed using the Modeller program (Šali & Blundell, 1993). The template structure used was that of the full capsid protein (PDB code 4DPV) at a resolution of 2.9 Å (Xie & Chapman, 1996).

All CPV strains isolated from wolves and two strains isolated from dogs (CPV-616 and -637) were antigenically and genetically identified as type 2b strains, while the other six strains isolated from dogs were found to be type 2a (Table 1b). These data were confirmed by sequence analyses.

Comparison of the VP2 gene sequences showed 100% nucleotide identity between wolf isolates and CPV-616 as well as between CPV-618, -660 and -687. The other Italian strains differed by 0.5%. Sequence alignment analyses showed that there were different silent mutations and few coding changes in the VP2 gene. In particular, a coding or non-synonymous mutation was detected at nt 3579 in only the wolf strains and the dog strain CPV-616; this mutation results in residue 265 of the VP2 protein changing from a threonine to a proline residue.

Another coding mutation at nt 3675 was observed in all of the type 2a strains, resulting in residue 297 of the VP2 protein changing from a serine to an alanine residue (Fig. 1a). Nucleotide changes and the predicted amino acid sequence substitutions are shown in Table 1c.

To investigate the effect of the threonine to proline mutation (VP2 residue 265), a 3D model of the VP2 capsid protein was constructed to simulate the structure of the mutated sequence. In both CPV-616 and the wolf sequences,
Table 1 (cont.)
(c) Variable nucleotides in the VP2 gene sequences analysed in this study

Nucleotides differing from the CPV-584 isolate are indicated by a letter, whereas nucleotides identical to CPV-584 are indicated by dashes.

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Amino acid mutation

* Deduced amino acid substitutions resulting from nucleotide changes are indicated, with the residue position of the VP2 protein in parentheses.

T → P (265)*  S → A (297)*  N → D (426)*
Fig. 1. (a) Predicted amino acid sequences of VP2. Alignment of amino acids 241–320. Residues 265 and 297 are indicated.
(b) Phylogenetic tree constructed from the VP2 gene nucleotide sequences of CPV strains isolated in Italy and other parts of the world.

Our results show that the new CPV antigenic types 2a and 2b have replaced the old type 2 in wolf populations, as is the case in dog populations.

In Italy, the prevalent antigenic type in canine populations is type 2a (Sagazio et al., 1998; Buonavoglia et al., 2000). Our results seem to confirm these data, even though they show that both antigenic types 2a and 2b co-exist in the Italian canid populations.
Sequence analysis of Italian CPV isolates

population: it is impossible to demonstrate which type is predominant in a small sample, as neither of them exhibits an evolutionary advantage (Truyen et al., 2000). The wolf isolates were all type 2b, but it is impossible to conclude that type 2b is predominant strain in the Italian wolf population because our data are limited to an exiguous number of samples and we do not have data regarding CPV-2 dog strains from the area where the wolf samples were collected. The complete sequence identity of the VP2 gene between the wolf strains and CPV-616 leads us to exclude the possibility that a separate CPV pool could exist in wolf populations.

The coding change at nt 3579 (VP2 residue 265, threonine to proline) is very interesting because it has not been detected previously in any other strain. This mutation cannot be referred to exclusively as antigenic type 2b because the same change was also found in several type 2a strains isolated in Italy (M. Battilani, unpublished data). The mutation at residue 265 was also unexpected, as this residue is located in the \( \beta \)-barrel motif where residues are significantly more conserved compared with residues in the loops. This barrel region is not exposed at the virion surface and, therefore, is not subjected to the selective pressure of neutralizing antibodies of the host immune systems (Chapman & Rossmann, 1993). However, our data showed that variant 265 is viable, as it is able to replicate in cell culture and gives an increasing HA titre with each passage. Furthermore, it is not a defective variant, as we were able to amplify the complete VP1/VP2 region at an expected PCR product of 2200 bp (data not shown). The 265 mutation was observed in both types 2a and 2b. As is the case for domestic and wild canids, this mutation is not selected for in the population, but may have arisen independently from various backgrounds.

Sequence analysis demonstrated a non-synonymous change at nt 3675, found only in type 2a: retrospective analysis revealed that this antigenic type first appeared in the USA in 1989 and in Germany around 1993, but it is now the predominant CPV antigenic type in Europe. Our results confirm that this variant is predominant among our isolates, especially in the type 2a isolates. Current studies are investigating if this change has any biological consequence (Truyen, 1999).

The VP2 gene sequence of CPV-632 showed two peculiar silent mutations at nts 4388 and 4448 which had not been reported previously in types 2a or 2b. Furthermore, two other mutations (nts 3323 and 4496) were found in only CPV-632, type 2b and wolf strains. In fact, phylogenetic analysis showed that CPV-632 does not belong to the Italian type 2a cluster but forms a separate virus lineage (Fig. 1b).

To analyse the phylogenetic relationships of the Italian isolates with other CPV strains isolated in various parts of the world, we constructed a neighbour-joining phylogenetic tree. A representative minimal tree for the VP2 gene is shown in Fig. 1(b). The phylogenetic tree shows three branches with high bootstrap values of > 90%. One of the three groups consists of recent parvoviruses isolated from species other than the dog, the second group consists of type 2 and the third group consists of types 2a and 2b and includes our wolf and dog isolates. The CPV isolates were clearly subdivided between type 2 and types 2a and 2b, as described previously (Parrish et al., 1991); no evidence of obvious grouping was observed with respect to the geographical origin of the isolate.

This report contributes to the study of the continuing evolution of CPV and is the first study to deal with the sequence analysis of CPV strains isolated in Italy. Some
interesting results emerged, in particular, the mutation at residue 265 of the VP2 viral protein, which results in a change in the VP2 3D model. This mutation has not been detected before and further investigations will determine the biological consequences of this mutation.

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References


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