Original article

Phylogenetic analysis of canine parvovirus CPV-2 strains and its variants isolated in Poland

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Abstract

Canine parvovirus disease appeared in the world and in Europe during the second half of the 1970s. Over the course of 40 years the original CPV-2 strains mutated and variants 2a, 2b and 2c appeared. Their appearance is connected with specific amino acid changes, mainly in the capsid protein VP2. Strains isolated by the authors were adapted for *in vitro* cell culture. Phylogenetic analysis revealed differences between strains isolated in Poland in 1982-1985 and in 1995-2009. Strains from the 1980s were shown to belong to variant CPV-2a (11 strains) and variant 2b (2 strains), while no fundamental differences were found among the genetic profiles of the strains from 1995-2009, which were classified as belonging to variant 2c.

Key words: canine parvovirus strains, tissue culture, antigenic types, phylogenetic analysis

Introduction

Canine parvovirus (CPV-2) appeared as a new pathogen in 1978 and spread rapidly on all continents. CPV-2 is believed to be closely related to feline pan-leukopenia virus (FPV), mink enteritis virus (MEV) and raccoon parvovirus (RPV). These viruses exhibit over 98% homology at the level of nucleotides and amino acid sequences (Parrish et al. 1990, 1991, Truyen et al. 1992, 1995, 1996b). Due to the high survival rate of CPV-2 in the environment, clinical parvovirus cases are a recurring problem, particularly in young animals. Because there is no specific pharmacological treatment, and symptomatic treatment is expensive and not entirely effective, the best means of combating parvovirus is preventive vaccination. Immunoprophylaxis significantly reduces cases of the disease in pups, but even the most carefully planned and rigorously observed vaccination programme cannot entirely prevent clinical cases. This is mainly due to the lack of an adequate immune response in weakened animals or those with questionable or immature immune status.

Maternal antibody interference with the virus used for the vaccination can make it difficult to obtain adequate vaccine protection in this age group. Neutralization of the virus by the mother's antibodies leads to suppression of the active immune response, resulting in disease and even death (Macartney et al. 1988, Mockett and Stahl 1995, Larson and Schultz 1997, Doki et al. 1999).

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Nevertheless, a vaccination programme should be rigorously observed to prevent transmission of the virus. Unfortunately, in Poland particularly in rural areas, dogs are rarely vaccinated against parvovirus, which is a significant risk factor and facilitates the spread of infection in populations of sensitive animals.

In 2009 the authors examined soil samples from the central eastern Poland in order to isolate parvovirus. Material for the study came from urban areas (housing estate paths, public green spaces, lawns) and rural areas (meadows, fields, farms). The virus was isolated in 50% of the samples studied (study in preparation for print). Due to shedding of the virus by sick animals and its high resistance to unfavourable environmental conditions, the disease can spread easily.

Viruses are subject to evolutionary processes, and new variants arise as a result of genetic mutation. This process is particularly characteristic of RNA viruses. Among DNA viruses such rapid differentiation is in general rarely observed, but parvoviruses has a high genetic substitution rate (Shackelton et al. 2005).

Although for 12 years after the first isolation the degree of differentiation of CPV-2 genes responsible for encoding VP1 and VP2 proteins was shown to be 10 to 100 times lower that for the HA gene of the influenza virus, it is higher than other DNA virus. Phylogenetic analysis has shown a progressive evolution of the original CPV-2. This model of viral evolution appears to be most similar to that observed for influenza A virus (Fitch et al. 1991, Parrish et al. 1991). Due to its structure parvovirus is genetically unstable and undergoes mutation relatively easily, which can lead to the emergence of more virulent mutants that are dangerous for dogs despite vaccinations (Truyen et al. 1995, Truyen 1999, Shackelton et al. 2005).

The recently detected antigenic variant CPV-2c induces more severe symptoms in dogs than the previous variants (Decaro et al. 2005a). Experimental infection of pups with varying levels of maternal antibodies showed that even dogs with a hemagglutination inhibition (HI) titre of 160 were susceptible to infection. The CPV-2c virus proved to be highly pathogenic for naturally infected pups; they had severe symptoms, a long period of viral shedding in faeces and a long recovery time (Decaro et al. 2005b).

In recent years canine parvovirus has also been isolated from cats (Mochizuki et al. 1996, Truyen et al. 1996a,b, Ikeda et al. 2000, Steinel et al. 2000). Phylogenetic similarity of isolates from dogs and cats indicates that the virus is transmitted between species. The combination of a few specific changes in the nucleotide sequence of genes coding capsid protein synthesis resulted in CPV-2 variants with properties similar to those of FPV or other related viruses (Truyen et al. 1996b). Further research showed that the new mu-

tants can replicate in feline cells *in vivo*, and experimental infection of these animals can induce disease (Truyen et al. 1996a,b, Nakamura et al. 2001a,b). The significance of this research for immunoprophylaxis in dogs and cats is not yet fully known. Vaccinations based on the original type of CPV-2 are thought to protect dogs from infection with new types. However, successive variants of the virus which will probably arise as the result of further mutations may prove to be more pathogenic than previous ones and the vaccines used will not ensure adequate immunity. In this case a canine parvovirus vaccine enhanced with new mutant variants may prove to be more effective. The matter is unquestionably still open and further research should be conducted on the natural evolution of CPV and its pathogenicity for dogs and cats.

The aim the present study was to compare the phylogenetic similarity between CPV strains isolated in different years based on genetic analysis of a fragment of the gene encoding the VP2 protein.

Materials and Methods

Parvovirus isolates

DNA of virus strains isolated from dogs with acute diarrhoea and confirmed parvovirus disease was used for the study. The material was derived from isolates from the first half of the 1980s (1982-1985) and from 1995-2009. The virus was isolated from faeces and purified using standard methods. Filtrates were tested for hemagglutination activity and used to inoculate a continuous cell line of CCC clone 81 (clone 81 of transformer feline kidney cells). Three passages were performed. A cytopathic effect was observed in the first passages, and after five passages the haemagglutination (HA) titre of the strains reached 400-3200 and the cell culture infectious dose $(CCID_{50})$ ml^{-1} was $10^{3.5}$-$10^{5.5}$. The presence of the virus was confirmed by electron microscopy and an ELISA test. The strains were lyophilized and used as a pool in further testing. A reference strain of CPV-2 (VR-953) was adapted for the continuous cell line of CCC clone 81 (HA titre 3200, $CCID_{50}$ $ml^{-1} - 10^6$). The lyophilized virus strains were reactivated by inoculating the cell line of CCC clone 81.

DNA extraction and amplification

Viral DNA was isolated and cellular debris was removed. A QIAmp DNA Mini Kit (Qiagen) was used for isolation. Amplification of the isolated DNA was performed using primers for two VP2 gene fragments (5'-CTACTCAGCCACCAACTAAAG-3';
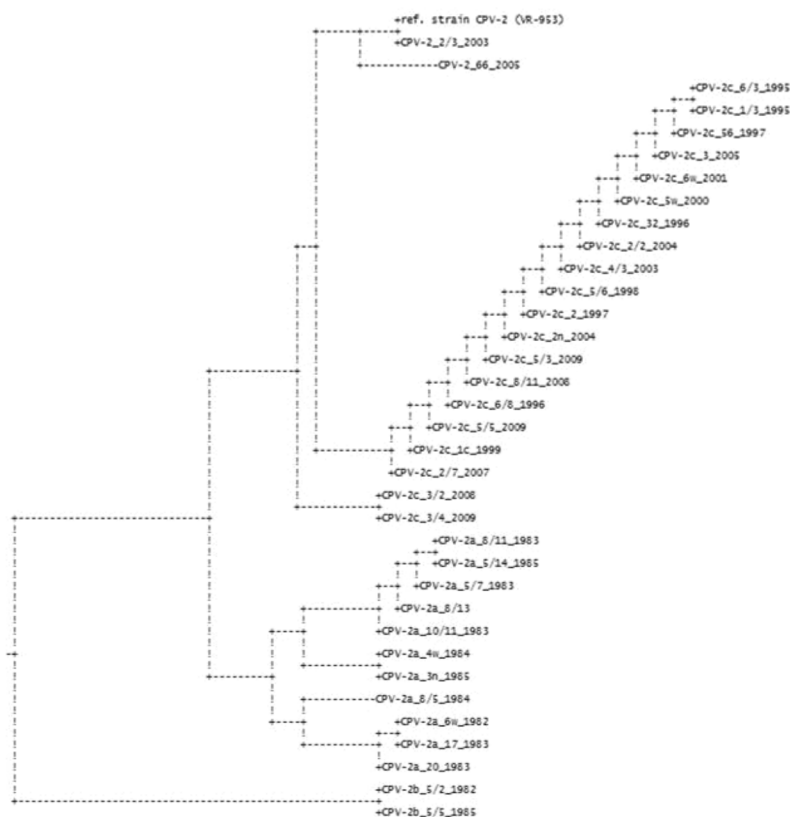
Fig. 1. Phylogenetic tree of CPV2 variants circulating in Poland. The name of each isolate is followed by the variant's strain, lab number and year of isolation.

5'-ATTTTCTAGGTGTAGTTGAGA-3') and (5'-CAGGAAGATATCCAGAAGGA-3'; 5'-GGTGCTAGTTGATATGTAATAAACA-3'). DNA was amplified using *Taq*DNA polymerase. PCR was carried out in a 20 µl reaction volume containing 4 µl of processed sample as a source of template DNA and 16 µl reaction mix (Taq PCR Core Kit-Qiagen). The PCR conditions were as follows: initial denaturation at 94ºC for 5 min, denaturation at 94ºC for 60 s, annealing at 58ºC for 60 s, extension at 72ºC 120 s for 30 cycles (the cyclic condition was repeated 30 times), and a final extension for 10 min. Amplicons were characterized by means of restriction endonuclease analysis using *AluI, RsaI, BsuRI (HaeIII)* and *MboII.* Following restriction endonuclease digestion electrophoresis was performed on 2% agarose gel containing ethidium bromide to a final concentration of 1 µg/ml. It was then loaded into the well and run together with O'GeneRuler 100bp DNA Leader in 1x TBE electrophoresis buffer at 8 volts/cm. The progress of mobility was monitored by following the migration of the dye. After electrophoresis, the gel was visualized under a UV transilluminator using the computer program Scion Image v. 1.0.

Digestion by the restriction enzyme MboII was performed to identify the variant CPV-2c. To differentiate the remaining types of the virus, sequencing was carried out using primers (5'-CAG-GAAGATATCCAGAAGGA-3'; 5'-GGTGCTAG-TTGATATGTAATAAACA-3'). The PCR products were purified and then sequenced directly using an ABI PRISM 3100 Avant sequencer and a Big-Dye® Terminator v.3.1 Sequencing Kit, according to the manufacturer's recommendations. In this way CPV-2 and its variants 2a and 2b were identified by comparing them with the reference strain and sequence data from the NCBI GenBank.

Analyses

A dendrogram tree was constructed using the program POPGEN32 according to Nei's coefficient. Then the UPGMA algorithm (Unweighted Pair-Group Method Using Arithmetic Averages) was chosen for hierarchical clustering analysis. Bootstrap values were estimated using 1000 replicates.

Results

The phylogenetic analysis revealed several clades which made it possible to distinguish separate antigenic variants. In the strains isolated from the faeces of sick dogs in 1982-1985 and adapted for *in vitro* cell
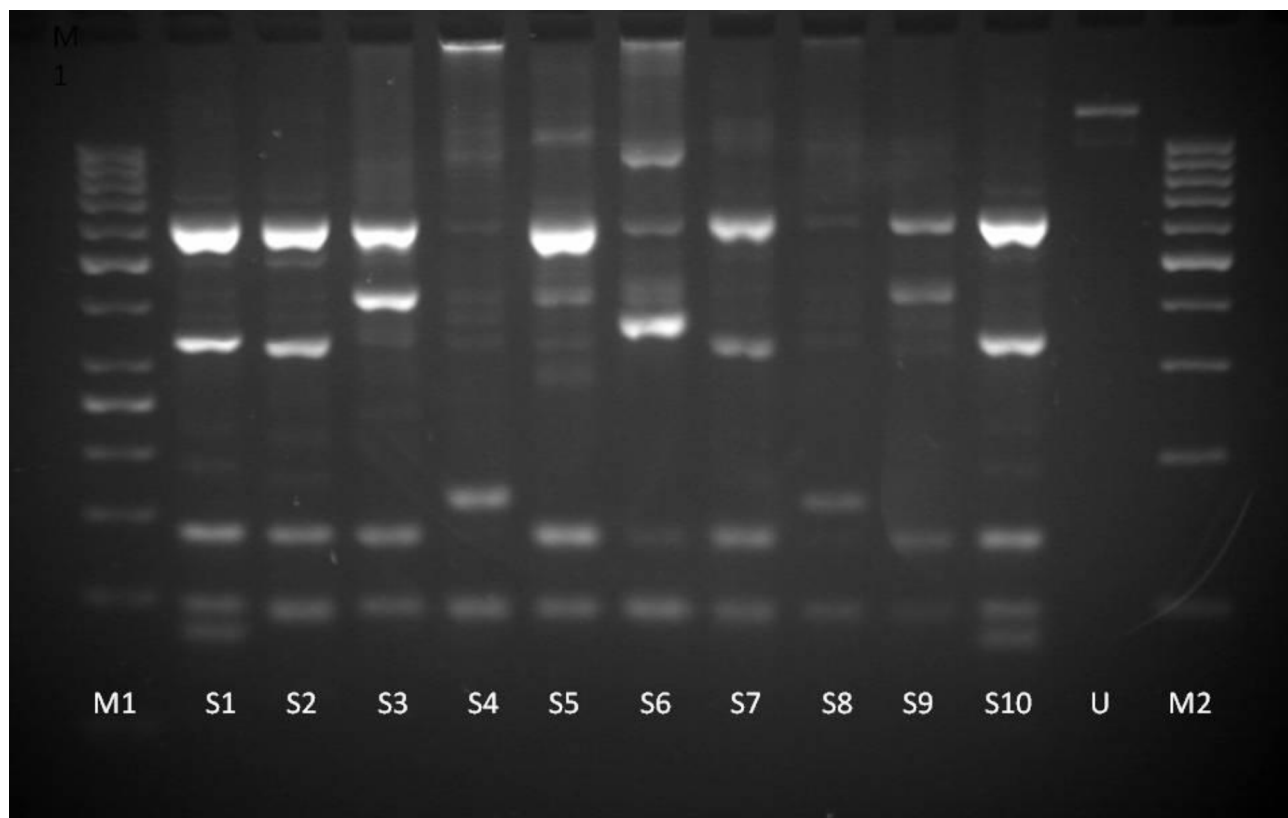
Fig. 2. Restriction fragment length polymorphism (RFLP) of PCR products. Lanes M1 – molecular weight marker (100 bp ladder), U – uncleaved PCR product, S1-10 – product of samples digested by AluI.

cultures, 2 groups with different genetic profiles can be found (Fig. 1).

Most of them (11 strains) belong to the variant CPV-2a, while two within the same clade have a different profile and form a second group (variant CPV2b). Within variant 2a substantial diversity of strains is observed, but this does not affect their belonging to this variant (Fig. 2). No isolates were found whose genetic profiles were identical to that of the original strain of CPV-2.

The 21 virus strains from 1995-2009 constitute a separate clade differing from the one comprising the strains isolated in the first half of the 1980s. The genetic profiles of most of them (19 strains) did not differ from one another, and they belong to variant 2c (Fig. 1). Although 2 strains were noted with a few nucleotides that differed from those of the others, indicating a mutation, this does not alter the fact that they belong to variant 2c (Fig. 1). Among the strains studied not even one was found with a genetic profile matching that of the strains isolated in 1982-1985. However, two were distinguished with profiles identical to those of the original CPV2 (Fig. 1).

The genetic similarity observed between the strains studied (variants 2a, 2b, 2c) and the reference strain (CPV-2) was 0.448-0.891. The reference strain was genetically markedly different from its variants, particularly variant 2b (0.448). In variants CPV-2a and 2c diversity is observed that indicates a certain heterogeneity among the strains studied. In case of CPV-2a, their values were in the range of 0.557-0.773, and for 2c they were 0.782-0.891 (Fig. 1). The results confirm those obtained by other authors regarding SNP polymorphism, but this does not alter the phenotype within the variants.

Discussion

Genetic research has shown that the feline panleukopenia virus (FPV) may have given rise to the feline parvovirus group, which along with mink enteritis virus (MEV), raccoon parvovirus (RPV) and blue fox parvovirus (BFPV) also includes canine parvovirus (CPV). It is believed that these viruses could be variants of FPV which arose due to evolutionary changes in adapting to the infection of successive hosts.

The appearance of the CPV virus was connected to specific changes in amino acids in the capsid region, which is characterized by high antigenicity and is the site of binding of neutralizing antibodies (Parrish 1990, Truyen et al. 1995, Mochizuki et al. 1996). The original CPV did not replicate in feline tissues and the virus infected only dogs (Truyen and Parrish 1992,

Hueffer and Parrish 2003). As a result of point mutations and strong positive selection associated with adaptation to new hosts, successive variants of CPV-2 began to emerge with increased capacity to infect canine cells. Circulating among canine populations they led to severe epizootics, and began to infect cats as well (Mochizuki et al. 1996, Truyen et al. 1996a,b, Ikeda et al. 2000, Steinel et al. 2000, Martella et al. 2004). The CPV virus is characterized by high genetic heterogeneity. When Shackelton et al. (2005) compared isolates from 10 years they found that different variants arose via natural selection, reflecting the mutation typical for the virus.

The CPV-2 virus was isolated from dogs mainly in 1978-1980, and later only in sporadic cases. According to Truyen et al. (1996b) variants CPV2a and 2b completely replaced the original virus in the canine population. Variant CPV-2a was predominant in 1981-1984, and type CPV-2b cases reached their peak in 1988-1990.

Our study showed that strains isolated from the first half of the 1980s (1982-1985) were markedly different genetically from strains from later years. Phylogenetic analysis showed that these strains form 2 clades – variants CPV-2a and -2b.

There were 11 strains classified as belonging to CPV-2a and 2 to CPV-2b. When Mizak and Plocienniczak (1995) examined Polish isolates from 1982-1993, they found only type 2a. When Rypuła et al. (2002) later analysed the sequence of fragments of the VP1/VP2 gene of isolates collected before the year 2000, they demonstrated that dogs were probably mainly infected with CPV-2a, but also with another type. Our study shows that at this time variant CPV-2b had also appeared in Poland.

In 1996 another antigenic variant, CPV-2c, was isolated in Germany. It also appeared in Italy and other European countries (Buonavoglia et al. 2001, Martella et al. 2004, Decaro et al. 2006a,b). Phylogenetic analysis of our strains from 1995-2009 revealed the presence of 2 isolates belonging to the original CPV-2. The remaining 19 strains were genetically homogeneous and their genetic profile was markedly different from that of viruses from 1982-1985. Further examination using the enzyme MboII enabled them to be classified as CPV-2c. Comparison of the results of our study with those obtained by other authors shows that this variant appeared in Poland even somewhat earlier than in other European countries.

Salwa et al. (2006) believe that three different antigenic biotypes of parvovirus, types 2a, 2b and 2c, are currently circulating in Poland. It is difficult to say which variant is predominant, but the results of our study suggest that variant CPV-2c currently dominates in canine populations in Poland.

When Decaro et al. (Decaro et al. 2006a,b, 2007) examined Italian strains isolated from 1995-2005 and 2005-2006, they also observed the presence of all the antigenic variants and demonstrated that CPV-2c was spreading among dogs with increasing frequency. This is also the case in other European countries, where all the antigenic types of the virus occur in varying rates depending on the country (Battilani et al. 2002. Martella et al. 2005). Antigenic variants of CPV-2 are also present in Asia (Nakamura et al. 2004, Wang et al. 2005, Chinchkar et al. 2006, Doki et al. 2006), North America (Hong et al. 2007), South America (Costa et al. 2005, Perez et al. 2007) and Northern Africa (Touihri et al. 2009).

According to Batilliani et al. (2006) there is high genetic diversity among strains isolated from cats as well as dogs. The authors demonstrated coinfection of cats with many CPV variants. They detected 10 different populations of the virus with similarity in the range of 99.5-99.9%, and analysis of the amino acid sequences of these clones showed that they were present in infected animals at the same time.

In case of parvoviruses, even slight mutations can alter the basic biological traits of the virus, which is subject to continual change, as was demonstrated by our study. For this reason these processes must be monitored and the results of this study should be treated as material contributing to our knowledge of the evolution of viruses.

The results of the present study confirm high genetic similarity among the isolated strains (variants -2a, -2b, -2c) as well as their substantial differentiation from the original strains (Fig.1). Moreover, they contribute to the map of the geographical spread of antigenic variants of canine parvovirus in Europe by expanding it to include Poland.

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