Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Infection, Genetics and Evolution 61 (2018) 67–73

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Molecular typing of a novel canine parvovirus type 2a mutant circulating in Italy

Francesco Mira[a,*], Giulia Dowgier[b], Giuseppa Purpari[a], Domenico Vicari[a], Santina Di Bella[a], Giusi Macaluso[a], Francesca Gucciardi[a], Vincenzo Randazzo[a], Nicola Decaro[b], Annalisa Guercio[a]

[a] Istituto Zooprofilattico Sperimentale della Sicilia, Via Gino Marinuzzi 3, 90129 Palermo, Italy
[b] Department of Veterinary Medicine, University of Bari, Strada provinciale per Casamassima Km 3, 70010 Valenzano, Bari, Italy

ARTICLE INFO

Keywords:
Dogs
Canine parvovirus
Protoparvovirus
Molecular characterization
Sequence analysis
Point mutations

ABSTRACT

Canine parvovirus (CPV) is the etiological agent of a severe viral disease of dogs. After its emergence in late 1970s, the CPV original type (CPV-2) was rapidly and totally replaced by three antigenic variants named CPV-2a, CPV-2b and CPV-2c. CPV has an evolutionary rate nearest to those of RNA viruses, with consequences on disease diagnosis and epidemiology. This paper reports the molecular characterization of eight CPV-2a strains collected from dogs in Italy in 2016–2017. Genetic analysis was conducted on a CPV genomic region encompassing both open reading frames (ORFs) encoding for nonstructural (NS1-NS2) and structural proteins (VP1-VP2). Sequence analysis indicates new and unreported sequence changes, mainly affecting the VP2 gene, which included the mutation Tyr324Leu. This study represents the first evidence of a new CPV-2a mutant (VP2 324Leu) and illustrates the importance of a continuous molecular survey in order to obtain more information on effective spread of new CPV mutants.

1. Introduction

Canine parvovirus (CPV) is a member of the *Protoparvovirus* genus (family *Parvoviridae*, subfamily *Parvovirinae*) and, together with feline panleukopenia virus (FPLV), it was recently included in the unique specie *Carnivore protoparvovirus 1* (Cotmore et al., 2014; Tijssen et al., 2011). CPV is a small, non-enveloped, linear single-stranded DNA virus. Its genome consists of an approximately 5200 nucleotide (nt) DNA molecule containing two open reading frames (ORFs), encoding respectively for two nonstructural proteins (NS1 and NS2) and for two structural proteins (VP1 and VP2) through alternative splicing of the same mRNAs (Decaro and Buonavoglia, 2012; Reed et al., 1988). VP2 is the major capsid protein, representing the major determinant of host range (Hueffer et al., 2003) and being subject to antibody mediated selection (Nelson et al., 2007). For this reason, over the years most studies were focused on the evolution of the VP2 gene, with limited studies on the nonstructural genes (Hoelzer et al., 2008). Also NS gene sequences available in GenBank are relatively few if compared to partial or total length VP2 sequences.

CPV emerged in late 1970s as host variant of FPLV or FPLV-like parvovirus (Parrish et al., 1991), causing acute gastroenteritis, leukopenia and myocarditis in dogs. Amino acids changes between CPV and FPLV affect the VP2 domain interacting with the host-cell transferrin receptor (TfR) and may have contributed to CPV ability of binding domestic dog TfR, which has been recognized as the determinant for the canine host shift (Hueffer et al., 2003; Shackelton et al., 2005). Soon after its emergence, the original type CPV-2 was rapidly and totally replaced by different CPV variants commonly named, on the basis of differences observed in the VP2 amino acid (aa) residue 426, as CPV-2a (Asn), CPV-2b (Asp) and CPV-2c (Glu) (Buonavoglia et al., 2001; Decaro et al., 2005; Parrish et al., 1985, 1988, 1991). Over the years many other changes have arisen in the VP2 gene and some of them (Ser297Ala, Thr440Ala, Tyr324Ile) were reported in several countries (Battilani et al., 2002; Decaro et al., 2009a; Geng et al., 2015; Ikeda et al., 2000; Jeoung et al., 2008; Mittal et al., 2014; Truyen, 1999; Truyen et al., 2000). Although CPV-2a and CPV-2b variants showing Ala at aa 297 were designated respectively as “new CPV-2a” and “new CPV-2b” (Decaro et al., 2009a; Ohshima et al., 2008), CPV strains with others specific changes (Thr440Ala, Tyr324Ile) did not receive any clear taxonomical or common designation, despite their presence in strains detected in large different geographical areas. For instance, the VP2 change Tyr324Ile may be considered a common change in CPV strains of Asian countries (Geng et al., 2015). Therefore, current typing of CPV variants (CPV-2a, CPV-2b, CPV-2c, new CPV-2a, new CPV-2b) reported in the literature is based mainly on antigenic and genetic features at critical residues on the VP2 sequence (aa 297 and 426). The

* Corresponding author.
E-mail address: dottoremira@gmail.com (F. Mira).

https://doi.org/10.1016/j.meegid.2018.03.010
Received 13 November 2017; Received in revised form 8 March 2018; Accepted 11 March 2018
Available online 13 March 2018

aim of this paper is the characterization of eight new CPV-2a strains collected from dogs in Italy between November 2016 and May 2017, which have shown common specific nucleotide and amino acid changes. The detection of CPV strains with unique molecular features can contribute to better understand the genetic diversity and molecular evolution of this canine virus. Moreover, the characterization of both viral ORFs provides useful data for further epidemiological and evolutionary analyses, improving the limited information on NS1 sequence of circulating CPV strains.

2. Material and methods

2.1. Clinical samples

Samples of eight dogs with clinical signs of severe haemorrhagic gastroenteritis and suspected of parvovirosis were analyzed. Samples were collected from November 2016 to May 2017 and analyzed at Istituto Zooprofilattico Sperimentale della Sicilia "A.Mirri" (Palermo, Italy) for diagnostic purposes. Dogs showed depression, fever, intense haemorrhagic vomiting, diarrhea, and severe leukopenia (from 2.2 to 0.55×10^3 cells/µl of blood). Seven dogs died within three days after the onset of clinical signs despite hospitalization and intensive cares, while the fate of the other dog was unknown. Samples collected (organs from necropsy, rectal swabs and blood sera) were submitted to virological or serological assays with suspect of parvovirosis. Details are summarized in Table 1.

2.2. DNA extraction and parvovirus PCR

Viral DNA was extracted from 200 µl of swab/organ homogenate using DNeasy Blood & Tissue Kit (Qiagen s.p.a., Milan, Italy), according to the manufacturer's instructions. All PCR assays were performed using the commercial kit GoTaq® G2 DNA Polymerase (Promega Italia s.r.l., Milan, Italy).

Presence of CPV DNA was screened using a PCR protocol amplifying a 700-bp fragment of the VP2 gene (Touihri et al., 2009) in a 50-µl reaction mix, as previously described (Mira et al., 2018). PCR products were checked after electrophoresis on a 3% agarose gel supplemented with 0,005% ethidium bromide.

2.3. Sequence analysis

Analyses were conducted amplifying a long genome sequence, encompassing both major ORFs, using primers pairs described by Pérez et al. (2014). For this purpose, two separate reactions with the same reaction mix and thermal conditions were performed in 50 µl, using primer pairs NS-Fext/NS-Rext and 2161F/4835R, respectively. After electrophoresis on agarose gel supplemented with ethidium bromide, one positive amplicon of each dog from both screening and sequencing PCR analyses were purified with Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Amersham, Buckinghamshire, UK) and submitted to BMR Genomics srl (Padova, Italy) for direct Sanger sequencing with same primers used for amplification and also with additional internal primers, as previously described (Mira et al., 2018).

According to an overlapping strategy, sequences were assembled using BioEdit ver 7.2.5 software (Hall, 1999) and two sequences for each sample (partial VP2 sequence and complete genome sequence) were obtained. Assembled nt sequences were submitted to nBLAST program (Zhang et al., 2000) to search related sequences into public domain databases (additional data files: Supplementary Dataset 1, 2 and 3). Phylogenetic relationships among the 324-VP2 mutants detected and the CPV reference sequences were evaluated in order to elucidate the relationships with previous available sequences. Due to the few available CPV complete genomes and single NS1 genes in comparison with the more abundant VP2 sequences and the higher variability of the VP2 gene, phylogeny was restricted to this gene. Phylogenetic analysis was performed using the best-fit model of nt substitution with MEGA7 software (Kumar et al., 2016) using ML method according to the Tamura 3-parameters model with discrete Gamma distribution (bootstrap 1000 replicates). Complete genome sequences data were submitted to the DDBJ/EMBL/GenBank databases under accession numbers reported in Table 1.

2.4. Virus isolation

In order to have viral isolates of the CPV mutants for future studies, four samples (rectal swabs of dogs PA43847/2017 and PA5610/2017; intestines of dogs PA48686/2016 and PA3213/2017) (Table 1) were processed by homogenization (10% w/v) in Minimum Essential Medium Eagle (EMEM; Lonza, BioWhittaker, VWR, UK) supplemented with antibiotics and antimycotic solution (1000 U/ml penicillin G sodium salt, 1 mg/ml streptomycin sulfate, 2,5 µg/ml amphotericin B; PAA Laboratories GmbH, Austria). Then, sample homogenates were centrifuged at low speed (1500 x g for 15 min at +4 °C) and supernatants were collected, decontaminated at +37 °C for 1 h and finally stored at −80 °C until use. Supernatants were inoculated in 80% confluent cell monolayers (A-72, CRFK), cultivated in Minimum Essential Medium (MEM) added with 5% of bovine fetal serum, 1% of sodium pyruvate (A-72) and 0.1% of lactalbumin. Inoculated cells were monitored daily for maximum five days and viral growth was evaluated by detection of cytopathic effect (CPE) and PCR. A total of five passages were carried out before considering virus isolation as unsuccessful.

2.5. CPV serology

In order to evaluate the immune status of the infected animals, the serum antibody titers for CPV were investigated in collected blood sera (Table 1) using the haemagglutination inhibition (HI) test. HI tests were carried out as previously described (Elia et al., 2005) at 4 °C using 1% pig erythrocytes and 10 haemagglutinating (HA) units of CPV-2b antigen. Serial two-fold serum dilutions were made in phosphate-buffered saline (pH 7.2), starting from a 1:10 dilution. Titers were expressed as the reciprocal of the highest serum dilution completely inhibiting viral haemagglutination.

Table 1
Details of dogs and collected/tested samples.

Dog id	Date of sampling	Age	Breed	Origin	Vaccination	Exitus	Samples	Acc. nr.
PA43847/2016	21-Nov-16	8 m	Rottweiler	Private	Complete	Dead	Rectal swab[a], blood serum	MG434738
PA48686/2016	21-Dec-16	Adult	Mixed breed	Animal shelter	Incomplete	Dead	Intestine[a], spleen, brain, lung, heart, kidney, liver	MG434739
PA3213/2017	09-Feb-17	Adult	Mixed breed	Animal shelter	Incomplete	Dead	Intestine[a], spleen, brain, lung, heart, kidney, liver	MG434740
PA5610/2017	03-Mar-17	3 yo	English setter	Private	Complete	Unknown	Rectal swab[a], blood serum	MG434741
PA10388/2017	11-Apr-17	2 m	Mixed breed	Stray dog	Unknown	Dead	Intestine, spleen[a], lung, heart, kidney, liver	MG434742
PA13577/2017	15-May-17	3 m	Mixed breed	Stray dog	Unknown	Dead	Intestine, spleen[a], brain, lung, heart	MG434743
PA13579id90/2017	15-May-17	2 m	Mixed breed	Stray dog	Unknown	Dead	Intestine[a], spleen, brain, lung, heart	MG434744
PA13579id93/2017	15-May-17	2 m	Mixed breed	Stray dog	Unknown	Dead	Intestine, spleen[a], brain, lung, heart	MG434745

[a] Samples used for virus isolation and CPV DNA sequencing: accession number are referred to sequences of strains isolated from these samples.

2.6. Other virological tests

RNAs were extracted from samples using QIAamp Viral RNA Mini Kit (Qiagen S.p.A.), according to the manufacturer's instructions. Extracted DNAs/RNAs were amplified using a set of real-time (RT-)PCR assays for detection of canine distemper virus (CDV) (Elia et al., 2006), canine adenovirus (CAdV) type 1 and type 2 (Dowgier et al., 2016), and canine coronavirus (CCoV) (Decaro et al., 2004).

3. Results

3.1. Detection and characterization of CPV

All samples tested negative for CDV, CAdVs and CCoV by real-time (RT-)PCR assays. A single band of the expected size (700 bp) referred to a fragment of CPV VP2 gene was observed for all samples.

One amplicon from each dog, obtained both from PCR and sequencing analyses, was sequenced. According to aa residues 297 (Ala) and 426 (Asn) in VP2 gene sequence, all strains were characterized as new CPV-2a.

Comparing with sequences available in GenBank database, the highest identity rates were obtained with new CPV-2a strains B-2004 (99.56–99.50%), CPV-LZ1 (99.52–99.48%), CPV_IZSSI_29451_09 (99.52–99.46%) and CPV/Coyote/C67/NL_2014 (99.50–99.44%). Strain CPV_IZSSI_29451_09 was collected in the same Italian region of the present study. Analysis of NS1 and VP2 genes showed different results as a consequence of differences among available sequences for the two genes. NS1 genes of all strains showed the highest identities with new CPV-2b strain 412 (99.90–99.85%) and with CPV-2a isolate CPV-31 1983 and new CPV-2b strain CPV-402 (99.80–99.75%). The VP2 gene of all strains gave quite different results, displaying the highest identities to new CPV-2a isolate CPV/BJ018/07 (99.82–99.77%) collected in 2007 from China and to 19 new CPV-2a strains (99.77–99.71%) collected both from dogs in Asia in 2004–2014 and from a coyote (*Canis latrans*) in Canada in 2014 (isolate CPV/Coyote/C67/NL_2014). All these strains showed Ile at aa residue 324 and Thr at residue 440.

Comparison of the obtained sequences revealed a high nt identity (99.97–99.95%) between the detected strains. In the common tract of the obtained sequences (4329 nt), differences were observed at five nucleotide residues: at nt residue 716, 1189 and 1608 of the NS1 gene (Table 2) and at nt residues 876 and 1731 of the VP2 gene (Table 3).

3.2. Sequence analysis of NS1 and VP2 genes

Comparison with 193 NS1 and 250 VP2 sequences (additional data file: Supplementary Dataset 1 and 2) retrieved from the GenBank database and with 156 VP2 sequences (additional data file: Supplementary Dataset 3) from CPV strains collected in Italy from 1994 to 2017, showed 17 nt changes resulting in 11 synonymous and 6 non-synonymous substitutions (Tables 2 and 3).

NS1 sequence analysis evidenced four synonymous and five non-synonymous substitutions (Table 2). Changes at nt residues 633, 716, 1189 and 1608 were observed only in our strains. Other three changes at nt residues 96, 1164 and 1790 had been previously observed in CPV strains collected in Canada from coyotes in 2014 (CPV/Coyote/C16/NL_2014 and CPV/Coyote/C55/NL_2014), from dogs in Uruguay in 2010 (CPV-2c-UY235) and in USA in 1998 (strains CPV-412 and CPV-402) and in 2003 (strains CPV-435 and CPV-436). Change at nt residue 1048 was common to 9 CPV and 2 MEV strains, while change at residue 1631 was present in 38 CPV strains collected from dogs and 2 CPV strains collected from coyotes (CPV/Coyote/C16/NL_2014 and CPV/Coyote/C55/NL_2014). None of these changes were observed in FPLV NS1 sequences (Supplementary Dataset 1).

Four synonymous and one non-synonymous substitutions (T970C, A971T → Tyr324Leu or A970C → Ile324Leu) were observed in the VP2 sequences (Table 3). Nucleotide changes at residues 876 and 1731 were shared only by our strains. Change T1482C was observed only in 22 new CPV-2b strains collected in Italy, France, Turkey, South Korea and mainly in USA. Change A1710G was observed only in 30 new CPV-2a strains collected mainly in Europe (Italy, France, Germany, Portugal, Hungary) as well as in Turkey, Vietnam and China. The main difference was observed at VP2 aa residue 324 where our sequences showed aa Leu in place of Tyr, as for the original type CPV-2 and for all Italian CPV strains, or of Ile, as reported for the most recent Asian CPV strains. The non-synonimous substitution Tyr324Leu resulted from two nt changes at the first and second codon positions (TAT → CTT), while change Ile324Leu was due to a first codon nt mutation (ATT → CTT) (Supplementary Dataset 2,3). The aa change at VP2 residue 324 was recently reported in CPV-2b strains from Brazil (Silva et al., 2017), but the codon analysis showed a difference at the nt level (TTA and CTT in Brazilian and Italian sequences, respectively). Moreover, by sequence analysis of both ORFs, additional nt differences were encountered in the NS1 (twenty-four synonymous and ten non-synonymous substitutions) and in the VP2 sequence (sixteen synonymous and one non-synonymous substitutions). At key positions of the VP2 protein all our sequences showed aa Ala at residue 297 and Thr at residue 440.

VP2 sequence analysis of previous collected Italian CPV type 2a/2b/2c strains (Supplementary Dataset 3) showed mainly five synonymous changes at nt residues 36, 303, 537, 1482 and 1710 (Table 3). Comparison with 52 Italian CPV-2a sequences evidenced that change T1482C was unique to our sequences while other changes were already present in Italian CPV-2a strains from 1999 (accession number KF373570), 1996 (AF306445) and 1994 (AF306446) with low frequency rate (15–31%), except for residue 1710 (68%). While changes at aa positions 537 and 1710 were observed also in CPV-2b and CPV-2c Italian sequences, changes at positions 36, 303 and 1482 were not observed in these variants with exception of two CPV-2b sequences (accession numbers KF373599 and FJ005265) (Supplementary Dataset 3).

3.3. Phylogeny

The phylogenetic tree inferred from VP2 sequences (Fig. 1) shows that the eight novel sequences detected in this study cluster in a distinct clade located between two main clusters including almost all European and Asian strains, respectively. The only exceptions in these clusters are VP2 sequences of strains Ku5_08 (Thailand, 2008; VP2 324Tyr), located within the European cluster, and of strains H-Erd, H-Illatos, H-Halmai, H-41 (Hungary, 2012; VP2 324Ile), located within the Asian cluster. Sequences cluster also according to variations at aa residue 324: in the phylogenetic tree, sequences displaying Tyr (Y) or Ile (I) at residue 324 cluster separately from our sequences presenting Leu (L). However, due to the few differences among available sequences and the low bootstrap values, the tree topology could not reflect the real clustering of the two 324-VP2 mutants.

3.4. Virus isolation

All four inoculated samples induced cytopathic effect (CPE) at the second/third passage in A-72 cells, whereas two samples showed CPE at the fourth passage in CRFK cells. CPE consisted of surrounding of cells and lysis of the inoculated monolayers. No alterations were observed in CRFK cells at least up to the fifth passage for others two samples (PA43847/2016 and PA3213/2017). CPE included typical cell rounding and detachment of the monolayer from the flask surface. A PCR assay confirmed the presence of CPV DNA in cell cultures showing CPE.

3.5. CPV serology

The results of HI test carried out on the sera of dogs PA43847/2016

Table 2
NS1 amino acid and nucleotide (in brackets) described features of new CPV-2a 324-Leu variants.

Strain	NS1								
	32 (94–96)	211 (631–633)	239 (715–717)	350 (1048–1050)	388 (1162–1164)	397 (1189–1191)	536 (1606–1608)	544 (1630–1632)	597 (1789–1791)
CPV isolate CPV-15[a]	Cys (TGT)	Tyr (TAT)	Asn (AAC)	Asp (GAT)	Arg (AGA)	Leu (CTT)	Cys (TGT)	Tyr (TAT)	Leu (CTA)
CPV_IZSSI_29451_09[b]	Cys (TGT)	Tyr (TAT)	Asn (AAC)	Asp (GAT)	Arg (AGA)	Leu (CTT)	Cys (TGT)	Tyr (TAT)	Leu (CTA)
CPV_IZSSI_987_10[b]	Cys (TGT)	Tyr (TAT)	Asn (AAC)	Asn (AAT)	Arg (AGA)	Leu (CTT)	Cys (TGT)	Phe (TTT)	Leu (CTA)
CPV_IZSSI_PA43847/2016	– (TGC)	– (TAC)	Thr (ACC)	Asn (AAT)	– (AGG)	–	–	Phe (TTT)	Pro (CCA)
CPV_IZSSI_PA48686/2016	– (TGC)	– (TAC)	–	Asn (AAT)	– (AGG)	–	–	Phe (TTT)	Pro (CCA)
CPV_IZSSI_PA3213/2017	– (TGC)	– (TAC)	–	Asn (AAT)	– (AGG)	Phe (TTT)	–	Phe (TTT)	Pro (CCA)
CPV_IZSSI_PA5610/2017	– (TGC)	– (TAC)	–	Asn (AAT)	– (AGG)	–	–	Phe (TTT)	Pro (CCA)
CPV_IZSSI_PA10388/2017	– (TGC)	– (TAC)	–	Asn (AAT)	– (AGG)	–	Cys (TGC)	Phe (TTT)	Pro (CCA)
CPV_IZSSI_PA13577/2017	– (TGC)	– (TAC)	–	Asn (AAT)	– (AGG)	–	–	Phe (TTT)	Pro (CCA)
CPV_IZSSI_PA13579id90/2017	– (TGC)	– (TAC)	–	Asn (AAT)	– (AGG)	–	–	Phe (TTT)	Pro (CCA)
CPV_IZSSI_PA13579id93/2017	– (TGC)	– (TAC)	–	Asn (AAT)	– (AGG)	–	–	Phe (TTT)	Pro (CCA)

[a] Prototype CPV type 2a: isolate CPV-15 (USA – 1984; NS1 accession n.: AY787926, VP2 accession n.: M24003).
[b] Reference strains CPV type 2a: strain CPV_IZSSI_29451_09 (Italy – 2009; accession n.: KX434454), strain CPV_IZSSI_987_10 (Italy – 2010; accession n.: KX434457).

and PA5610/2017 were respectively 1:1280 and 1:640, accounting either for active CPV infection or recent vaccination.

4. Discussion and conclusions

Canine parvovirus still remains one of the most important enteric pathogens of dogs in all continents, despite the widespread use of vaccines (Parrish et al., 1991; Miranda and Thompson, 2016). Therefore, a continuous epidemiological surveillance is needed to detect new CPV variants (Decaro and Buonavoglia, 2012). The aim of this study was to analyze and describe unreported molecular features of CPV strains recovered from dogs in southern Italy. The genetic analysis was carried out on both CPV open reading frames, thus representing the first study on the complete genome of Italian strains. The present study led to the identification of a novel CPV-2a mutant, displaying previously unreported molecular features. Considering the established role

Table 3
VP2 amino acid and nucleotide (in brackets) described features of new CPV-2a 324-Leu variants.

Strain	VP2							
	12 (34–36)	101 (301 – 303)	179 (535–537)	292 (874–876)	324 (970–972)	494 (1480–1482)	570 (1708–1710)	577 (1729–1731)
CPV isolate CPV-15[a]	Gln (CAA)	Thr (ACT)	Ser (AGT)	Asn (AAT)	Tyr (TAT)	Cys (TGT)	Lys (AAA)	Gln (CAA)
CPV_IZSSI_29451_09[b]	Gln (CAG)	Thr (ACC)	Ser (AGT)	Asn (AAT)	Tyr (TAT)	Cys (TGT)	Lys (AAA)	Gln (CAA)
CPV_IZSSI_987_10[b]	Gln (CAG)	Thr (ACT)	Ser (AGC)	Asn (AAT)	Tyr (TAT)	Cys (TGT)	Lys (AAG)	Gln (CAA)
CPV_IZSSI_PA43847/2016	– (CAG)	– (ACC)	– (AGT)	–	Leu (CTT)	– (TGC)	– (AAG)	–
CPV_IZSSI_PA48686/2016	– (CAG)	– (ACC)	– (AGT)	– (AAC)	Leu (CTT)	– (TGC)	– (AAG)	–
CPV_IZSSI_PA3213/2017	– (CAG)	– (ACC)	– (AGT)	– (AAC)	Leu (CTT)	– (TGC)	– (AAG)	–
CPV_IZSSI_PA5610/2017	– (CAG)	– (ACC)	– (AGT)	–	Leu (CTT)	– (TGC)	– (AAG)	Gln (CAG)
CPV_IZSSI_PA10388/2017	– (CAG)	– (ACC)	– (AGT)	–	Leu (CTT)	– (TGC)	– (AAG)	–
CPV_IZSSI_PA13577/2017	– (CAG)	– (ACC)	– (AGT)	–	Leu (CTT)	– (TGC)	– (AAG)	–
CPV_IZSSI_PA13579id90/2017	– (CAG)	– (ACC)	– (AGT)	–	Leu (CTT)	– (TGC)	– (AAG)	–
CPV_IZSSI_PA13579id93/2017	– (CAG)	– (ACC)	– (AGT)	–	Leu (CTT)	– (TGC)	– (AAG)	–

[a] Prototype CPV type 2a: isolate CPV-15 (USA – 1984; NS1 accession n.: AY787926, VP2 accession n.: M24003).
[b] Reference strains CPV type 2a: strain CPV_IZSSI_29451_09 (Italy – 2009; accession n.: KX434454), strain CPV_IZSSI_987_10 (Italy – 2010; accession n.: KX434457).

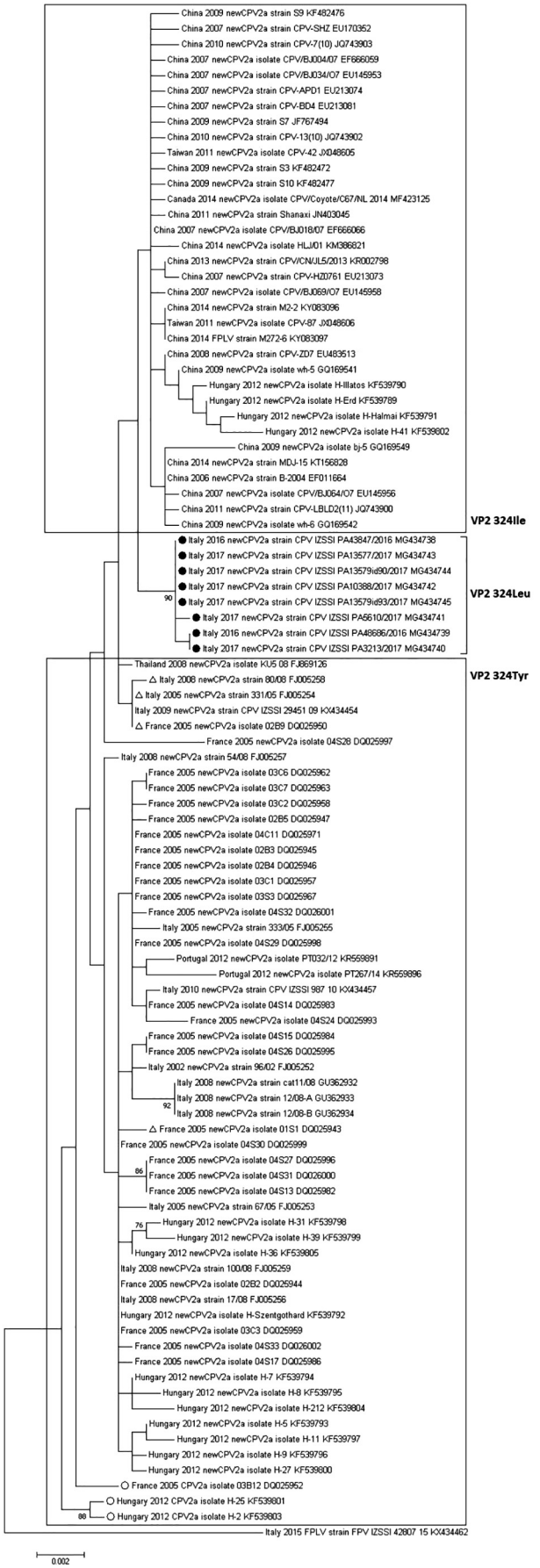

Fig. 1. Maximum Likelihood tree based on the full-length VP2 gene sequences (1755 nucleotides) of canine parvoviruses displaying the genetic relationships between analyzed strains and 90 CPV-2a strains obtained from GenBank (bootstrap 1000 replicates; bootstrap values shown greater than 70%). Black dots markings (●) indicate CPV strains analyzed in this study, white dot markings (o) indicate original CPV-2a strains (297Ser), white triangle markings (Δ) indicate CPV-2a strains with change Thr440Ala. All sequences were indicated with: country and year of collection/submission, type, isolate/strain and accession number.

of key residues of the VP2 protein, the main finding was observed at the critical amino acid residue 324 (Tyr324Leu). This finding provides new insight into the evolution of the VP2 protein gene, although further studies are needed to fully understand the biological and immunological role of this amino acid change. The full-length genomes provided in the present study will help further characterization of circulating strains, with particular regards to the analysis of the NS1 gene for which only few sequences are currently available.

Previous field studies indicate CPV-2a and CPV-2c as the most prevalent types in Italy, with CPV-2c having very rapidly replaced CPV-2b and displaying a higher prevalence in the last years (Decaro and Buonavoglia, 2012; Decaro et al., 2006). Epidemiological investigations, carried out in the same geographic area of the samples of the present study, detected CPV-2c as the most prevalent type with a minimal circulation of other CPV-2 types (including CPV-2a) from 2009 to 2015 (Purpari et al., 2018). However, those CPV-2a strains did not show a relevant sequence variability. Analogously, sequence analysis of the VP2 gene from 52 recent CPV-2a strains collected in Italy (Supplementary Dataset 3) showed a relatively low level of variability during the last years (20 aa changes) and among the detected changes only the aa residue 440 showed multiple (Thr/Ala/Ser) and frequent (84.6%/13.5%/1.9%) substitutions.

The strains detected in the present study were typed as new CPV-2a according to 297Ala and 426Asn aa residues of the VP2 gene. While aa Asn at VP2 residue 426 is critical for CPV-2/CPV-2a typing, aa Ala at residue 297 is commonly used as marker of the new CPV-2a/2b variants (Geng et al., 2015), in order to differ them from old strains. This mutation is predominant among CPV-2a/2b strains since 1990 (Zhou et al., 2017).

The detected CPV-2a strains displayed a non-synonymous change at aa residue 324 of the VP2 protein. This critical residue is located into the VP2 region that forms the GH loop located between the βG and βH strands and, along with aa residues 297 and 440, it is comprised into the VP2 region 2 (gene positions from nt 883 to 1332) that forms the 22 Å long spike of the capsid surface on the threefold axes (Horiuchi et al., 1998). Hoelzer et al. (2008) predicted that residue 324 is under positive selection, so that mutation in this site arise independently in different geographic locations. This residue is adjacent to aa residue 323, which affects canine transferrin receptor (TfR) binding (Hafenstein et al., 2007; Hueffer et al., 2003; Mittal et al., 2014), contributing to viral replication into canine cells and determining the pH dependence of haemagglutination (Chang et al., 1992). This residue, together with aa residue 93 of VP2, has been observed to play a crucial role in controlling CPV host range and in determining a specific antigenic site on the viral capsid (Govindasamy et al., 2003). Residue 324 is also likely to have the same effect as residue 323 on CPV host range (Mittal et al., 2014).

Although the introduction of CPV strains from other countries/continents was previously observed (Mira et al., 2018), the nt differences at the codon level with CPV sequences with the same VP2 324Leu mutation described by Silva et al. (2017) seem to rule out a southern America origin of the Italian mutant strains. Instead, a convergent evolution could be speculated that resulted in the same aa substitution even with different nt mutations. Moreover, the lack of related CPV sequences from the same area prevented any further speculation about the origin of the mutant. Therefore, only extensive and retrospective sequence analyses could clarify whether these mutant emerged from

CPV strains previously circulating in the same geographic area or was imported from different countries. Another change affecting the same residue (Tyr324Ile) was observed since 2006 (Jeoung et al., 2008) and it became predominant in Asia in 2014 (Zhou et al., 2017). This substitution was observed in 100% of recently analyzed Asian strains (Geng et al., 2015; Guo et al., 2013; Xu et al., 2015) and can result in a stronger receptor binding (Cságola et al., 2014). Further studies are required to better address any potential biological effect of this change and to evaluate the spread of this CPV mutant.

A high mutation substitution rate, similar to those of RNA viruses (about 10^{-4} substitution/site/year), was evidenced for CPV (Shackelton et al., 2005), thus accounting for its rapid evolution and occurrence of new variants. The region comprised between aa residues 267 and 498 of the VP2 protein, due to its exposure on the capsid surface, is affected by the greatest variability in parvoviruses (Decaro et al., 2009a) and this antigenic region represents the target for neutralizing antibodies (Agbandje et al., 1993; Tsao et al., 1991). Moreover, analysis of the CPV codon usage and evolutionary process suggested that VP2 aa residue 324 is one of the most affected substitution site, potentially able to alter relevant biological characteristics of the virus and to contribute to virus immune escape via antigenic drift and consequent vaccine failure (Li et al., 2017). According to this evolutionary scenario, after substitution of original Tyr by Ile at VP2 amino acid residue 324, our findings account for the occurrence of a second aa change at the same residue.

Evidence for a new CPV-2a mutant increases the need of a revision of current CPV typing. Due to the presence of a single aa mutation (position 426 of the VP2 protein) among types CPV-2a, 2b and 2c types, Organtini et al. (2015) suggested to consider these types as mutants of the CPV-2a clade rather than distinct subtypes. Indeed, the presence of additional aa changes in the VP2 protein (residues 297, 324, 440) requires a clearer taxonomical designation or a common naming, even if they can be considered as markers of the worldwide distribution of circulating CPV strains.

To date, a little information is available for changes in the NS1 protein and for their potential role. NS1 is a multifunctional nuclear phosphoprotein responsible for viral replication, cellular transcription and cell death inducing apoptosis (Gupta et al., 2016; Saxena et al., 2013). Only recently some of CPV NS1 amino acids were associated to specific functions (Niskanen et al., 2010, 2013). In order to contribute to future studies on this gene, we provided a complete genome NS1 sequencing for a number of CPV strains.

Two dogs tested in this study (PA43847/2016 and PA5610/2017) had been vaccinated with at least four doses of CPV-2 or CPV-2b based vaccines according to the guideline of the Vaccination Guidelines Group (VGG) of the World Small Animal Veterinary Association (WSAVA) (Day et al., 2016) before displaying CPV clinical signs and shedding. Previous reports accounted for the occurrence of CPV infection in vaccinated dogs (Cavalli et al., 2001; Decaro et al., 2007, 2008, 2009b; Miranda and Thompson, 2016; Mittal et al., 2014; Parker et al., 2017), whereas other studies demonstrated a high prevalence of non-responders especially in some breeds (Houston et al., 1996; Kennedy et al., 2007).

The detection of intermediate HI antibody titers in the two CPV-affected cannot be useful to assess whether the multiple vaccine administrations resulted in the development of non-protective immunity or they were completely unsuccessful because dogs were not responders or had high maternally-derived antibody titers. Serological tests should be routinely carried out after CPV vaccination to assess the seroconversion of vaccinated dogs, especially in putative “high-risk” dog breeds that include most low-responders and non-responders. At the same time, further studies are required to evaluate any additional potential mechanisms of antigenic escape of the VP2 324Leu mutants.

In conclusion, this study is the first evidence of the active circulation of a novel *Carnivore protoparvovirus 1* type 2a mutant with specific molecular signatures in a canine population with high prevalence of CPV-2c. Further studies are required to better elucidate the prevalence and the potential spread of this mutant in other geographic areas. Moreover, this field study could contribute to evaluate new vaccinations strategies based on the molecular epidemiological evidences.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.03.010.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interests

The authors have declared that no competing interests exist.

Conflict of interest

None

References

Agbandje, M., McKenna, R., Rossmann, M.G., Strassheim, M.L., Parrish, C.R., 1993. Structure determination of feline panleukopenia virus empty particles. Proteins 16, 155–171. http://dx.doi.org/10.1002/prot.340160204.

Battilani, M., Ciulli, S., Tisato, E., Prosperi, S., 2002. Genetic analysis of canine parvovirus isolates (CPV-2) from dogs in Italy. Virus Res. 83, 149–157.

Buonavoglia, C., Martella, V., Pratelli, A., Tempesta, M., Cavalli, A., Buonavoglia, D., Bozzo, G., Elia, G., Decaro, N., Carmichael, L., 2001. Evidence for evolution of canine parvovirus type 2 in Italy. J. Gen. Virol. 82, 3021–3025. http://dx.doi.org/10.1099/0022-1317-82-12-3021.

Cavalli, A., Bozzo, G., Decaro, N., Tinelli, A., Aliberti, A., Buonavoglia, D., 2001. Characterization of a canine parvovirus strain isolated from an adult dog. New Microbiol. 24, 239–242.

Chang, S.F., Sgro, J.Y., Parrish, C.R., 1992. Multiple amino acids in the capsid structure of canine parvovirus coordinately determine the canine host range and specific antigenic and hemagglutination properties. J. Virol. 66, 6858–6867.

Cotmore, S.F., Agbandje-McKenna, M., Chiorini, J.A., Mukha, D.V., Pintel, D.J., Qiu, J., Soderlund-Venermo, M., Tattersall, P., Tijssen, P., Gatherer, D., Davison, A.J., 2014. The family Parvoviridae. Arch. Virol. 159, 1239–1247. http://dx.doi.org/10.1007/s00705-013-1914-1.

Cságola, A., Varga, S., Lőrincz, M., Tuboly, T., 2014. Analysis of the full-length VP2 protein of canine parvoviruses circulating in Hungary. Arch. Virol. 159, 2441–2444. http://dx.doi.org/10.1007/s00705-014-2068-5.

Day, M.J., Horzinek, M.C., Schultz, R.D., Squires, R.A., Vaccination Guidelines Group (VGG) of the World Small Animal Veterinary Association (WSAVA), 2016. WSAVA guidelines for the vaccination of dogs and cats. J. Small Anim. Pract. 57, E1–E45. http://dx.doi.org/10.1111/jsap.2_12431.

Decaro, N., Buonavoglia, C., 2012. Canine parvovirus—a review of epidemiological and diagnostic aspects, with emphasis on type 2c. Vet. Microbiol. 155, 1–12. http://dx.doi.org/10.1016/j.vetmic.2011.09.007.

Decaro, N., Pratelli, A., Campolo, M., Elia, G., Martella, V., Tempesta, M., Buonavoglia, C., 2004. Quantitation of canine coronavirus RNA in the faeces of dogs by TaqMan RT-PCR. J. Virol. Methods 119, 145–150.

Decaro, N., Elia, G., Campolo, M., Desario, C., Lucente, M.S., Bellacicco, A.L., Buonavoglia, C., 2005. New approaches for the molecular characterization of canine parvovirus type 2 strains. J. Vet. Med. B Infect. Dis Vet. Public Health 52, 316–319. http://dx.doi.org/10.1111/j.1439-0450.2005.00869.x.

Decaro, N., Elia, G., Martella, V., Campolo, M., Desario, C., Camero, M., Cirone, F., Lorusso, E., Lucente, M.S., Narcisi, D., Scalia, P., Buonavoglia, C., 2006. Characterisation of the canine parvovirus type 2 variants using minor groove binder probe technology. J. Virol. Methods 133, 92–99. http://dx.doi.org/10.1016/j.jviromet.2005.10.026.

Decaro, N., Desario, C., Elia, G., Campolo, M., Lorusso, A., Mari, V., Martella, V., Buonavoglia, C., 2007. Occurrence of severe gastroenteritis in pups after canine parvovirus vaccine administration: a clinical and laboratory diagnostic dilemma. Vaccine 25, 1161–1166. http://dx.doi.org/10.1016/j.vaccine.2006.10.020.

Decaro, N., Desario, C., Elia, G., Martella, V., Mari, V., Lavazza, A., Nardi, M., Buonavoglia, C., 2008. Evidence for immunisation failure in vaccinated adult dogs infected with canine parvovirus type 2c. New Microbiol. 31, 125–130.

Decaro, N., Desario, C., Parisi, A., Martella, V., Lorusso, A., Miccolupo, A., Mari, V., Colaianni, M.L., Cavalli, A., Di Trani, L., Buonavoglia, C., 2009a. Genetic analysis of canine parvovirus type 2c. Virology 385, 5–10. http://dx.doi.org/10.1016/j.virol.2008.12.016.

Decaro, N., Cirone, F., Desario, C., Elia, G., Lorusso, E., Colaianni, M.L., Martella, V., Buonavoglia, C., 2009b. Severe parvovirus in a 12-year-old dog that had been repeatedly vaccinated. Vet. Rec. 164, 593–595.

Dowgier, G., Mari, V., Losurdo, M., Larocca, V., Colaianni, M.L., Cirone, F., Lucente, M.S., Martella, V., Buonavoglia, C., Decaro, N., 2016. A duplex real-time PCR assay based

on TaqMan technology for simultaneous detection and differentiation of canine adenovirus types 1 and 2. J. Virol. Methods 234, 1–6. http://dx.doi.org/10.1016/j.jviromet.2016.03.011.
Elia, G., Cavalli, A., Cirone, F., Lorusso, E., Camero, M., Buonavoglia, D., Tempesta, M., 2005. Antibody levels and protection to canine parvovirus type 2. J. Vet. Med. B Infect. Dis Vet. Public Health 52, 320–322. http://dx.doi.org/10.1111/j.1439-0450.2005.00870.x.
Elia, G., Decaro, N., Martella, V., Cirone, F., Lucente, M.S., Lorusso, E., Di Trani, L., Buonavoglia, C., 2006. Detection of canine distemper virus in dogs by real-time RT-PCR. J. Virol. Methods 136, 171–176. http://dx.doi.org/10.1016/j.jviromet.2006.05.004.
Geng, Y., Guo, D., Li, C., Wang, E., Wei, S., Wang, Z., Yao, S., Zhao, X., Su, M., Wang, X., Wang, J., Wu, R., Feng, L., Sun, D., 2015. Co-circulation of the rare CPV-2c with unique Gln370Arg substitution, new CPV-2b with unique Thr440Ala substitution, and new CPV-2a with high prevalence and variation in Heilongjiang Province, Northeast China. PLoS One 10, e0137288. http://dx.doi.org/10.1371/journal.pone.0137288.
Govindasamy, L., Hueffer, K., Parrish, C.R., Agbandje-McKenna, M., 2003. Structures of host range-controlling regions of the capsids of canine and feline parvoviruses and mutants. J. Virol. 77, 12211–12221.
Guo, L., Yang, S., Chen, S., Zhang, Z., Wang, C., Hou, R., Ren, Y., Wen, X., Cao, S., Guo, W., Hao, Z., Quan, Z., Zhang, M., Yan, Q., 2013. Identification of canine parvovirus with the Q370R point mutation in the VP2 gene from a giant panda (Ailuropoda melanoleuca). Virol. J. 10, 163. http://dx.doi.org/10.1186/1743-422X-10-163.
Gupta, S.K., Sahoo, A.P., Rosh, N., Gandham, R.K., Saxena, L., Singh, A.K., Harish, D.R., Tiwari, A.K., 2016. Canine parvovirus NS1 induced apoptosis involves mitochondria, accumulation of reactive oxygen species and activation of caspases. Virus Res. 213, 46–61. http://dx.doi.org/10.1016/j.virusres.2015.10.019.
Hafenstein, S., Palermo, L.M., Kostyuchenko, V.A., Xiao, C., Morais, M.C., Nelson, C.D.S., Bowman, V.D., Battisti, A.J., Chipman, P.R., Parrish, C.R., Rossmann, M.G., 2007. Asymmetric binding of transferrin receptor to parvovirus capsids. Proc. Natl. Acad. Sci. U. S. A. 104, 6585–6589. http://dx.doi.org/10.1073/pnas.0701574104.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program fo windows 95/98/NT. Nucl. Acids. Symp. Serket 41, 95–98.
Hoelzer, K., Shackelton, L.A., Parrish, C.R., Holmes, E.C., 2008. Phylogenetic analysis reveals the emergence, evolution and dispersal of carnivore parvoviruses. J. Gen. Virol. 89, 2280–2289. http://dx.doi.org/10.1099/vir.0.2008/002055-0.
Horiuchi, M., Yamaguchi, Y., Gojobori, T., Mochizuki, M., Nagasawa, H., Toyoda, Y., Ishiguro, N., Shinagawa, M., 1998. Differences in the evolutionary pattern of feline panleukopenia virus and canine parvovirus. Virology 249, 440–452. http://dx.doi.org/10.1006/viro.1998.9335.
Houston, D.M., Ribble, C.S., Head, L.L., 1996. Risk factors associated with parvovirus enteritis in dogs: 283 cases (1982–1991). J. Am. Vet. Med. Assoc. 208, 542–546.
Hueffer, K., Parker, J.S.L., Weichert, W.S., Geisel, R.E., Sgro, J.-Y., Parrish, C.R., 2003. The natural host range shift and subsequent evolution of canine parvovirus resulted from virus-specific binding to the canine transferrin receptor. J. Virol. 77, 1718–1726.
Ikeda, Y., Mochizuki, M., Naito, R., Nakamura, K., Miyazawa, T., Mikami, T., Takahashi, E., 2000. Predominance of canine parvovirus (CPV) in unvaccinated cat populations and emergence of new antigenic types of CPVs in cats. Virology 278, 13–19. http://dx.doi.org/10.1006/viro.2000.0653.
Jeoung, S.-Y., Ahn, S.-J., Kim, D., 2008. Genetic analysis of VP2 gene of canine parvovirus isolates in Korea. J. Vet. Med. Sci. 70, 719–722.
Kennedy, L.J., Lunt, M., Barnes, A., McElhinney, L., Fooks, A.R., Baxter, D.N., Ollier, W.E.R., 2007. Factors influencing the antibody response of dogs vaccinated against rabies. Vaccine 25, 8500–8507. http://dx.doi.org/10.1016/j.vaccine.2007.10.015.
Kumar, S., Stecher, G., Tamura, K., 2016 Jul. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33 (7), 1870–1874. http://dx.doi.org/10.1093/molbev/msw054.
Li, G., Ji, S., Zhai, X., Zhang, Y., Liu, J., Zhu, M., Zhou, J., Su, S., 2017. Evolutionary and genetic analysis of the VP2 gene of canine parvovirus. BMC Genomics 18 (534). http://dx.doi.org/10.1186/s12864-017-3935-8.
Mira, F., Purpari, G., Lorusso, E., Di Bella, S., Gucciardi, F., Desario, C., Macaluso, G., Decaro, N., Guercio, A., 2018. Introduction of Asian canine parvovirus in Europe through dog importation. Transbound. Emerg. Dis. 65 (1), 16–21. http://dx.doi.org/10.1111/tbed.12747. (Feb).
Miranda, C., Thompson, G., 2016. Canine parvovirus in vaccinated dogs: a field study. Vet. Rec. 178, 397. http://dx.doi.org/10.1136/vr.103508.
Mittal, M., Chakravarti, S., Mohapatra, J.K., Chug, P.K., Dubey, R., Upmanuyu, V., Narwal, P.S., Kumar, A., Churamani, C.P., Kanwar, N.S., 2014. Molecular typing of canine parvovirus strains circulating from 2008 to 2012 in an organized kennel in India reveals the possibility of vaccination failure. Infect. Genet. Evol. 23, 1–6. http://dx.doi.org/10.1016/j.meegid.2014.01.015.
Nelson, C.D.S., Palermo, L.M., Hafenstein, S.L., Parrish, C.R., 2007. Different mechanisms of antibody-mediated neutralization of parvoviruses revealed using the fab fragments of monoclonal antibodies. Virology 361, 283–293. http://dx.doi.org/10.1016/j.virol.2006.11.032.
Niskanen, E.A., Ihalainen, T.O., Kalliolinna, O., Häkkinen, M.M., Vihinen-Ranta, M., 2010. Effect of ATP binding and hydrolysis on dynamics of canine parvovirus NS1. J. Virol. 84, 5391–5403. http://dx.doi.org/10.1128/JVI.02221-09.
Niskanen, E.A., Kalliolinna, O., Ihalainen, T.O., Häkkinen, M., Vihinen-Ranta, M., 2013. Mutations in DNA binding and transactivation domains affect the dynamics of parvovirus NS1 protein. J. Virol. 87, 11762–11774. http://dx.doi.org/10.1128/JVI.01678-13.
Ohshima, T., Hisaka, M., Kawakami, K., Kishi, M., Tohya, Y., Mochizuki, M., 2008. Chronological analysis of canine parvovirus type 2 isolates in Japan. J. Vet. Med. Sci. 70, 769–775.
Organtini, L.J., Allison, A.B., Lukk, T., Parrish, C.R., Hafenstein, S., 2015. Global displacement of canine parvovirus by a host-adapted variant: structural comparison between pandemic viruses with distinct host ranges. J. Virol. 89, 1909–1912. http://dx.doi.org/10.1128/JVI.02611-14.
Parker, J., Murphy, M., Hueffer, K., Chen, J., 2017. Investigation of a canine parvovirus outbreak using next generation sequencing. Sci. Rep. 7 (9633). http://dx.doi.org/10.1038/s41598-017-10254-9.
Parrish, C.R., O'Connell, P.H., Evermann, J.F., Carmichael, L.E., 1985. Natural variation of canine parvovirus. Science 230, 1046–1048.
Parrish, C.R., Have, P., Foreyt, W.J., Evermann, J.F., Senda, M., Carmichael, L.E., 1988. The global spread and replacement of canine parvovirus strains. J. Gen. Virol. 69 (Pt 5), 1111–1116. http://dx.doi.org/10.1099/0022-1317-69-5-1111.
Parrish, C.R., Aquadro, C.F., Strassheim, M.L., Evermann, J.F., Sgro, J.Y., Mohammed, H.O., 1991. Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. J. Virol. 65, 6544–6552.
Pérez, R., Calleros, L., Marandino, A., Sarute, N., Iraola, G., Grecco, S., Blanc, H., Vignuzzi, M., Isakov, O., Shomron, N., Carrau, L., Hernández, M., Francia, L., Sosa, K., Tomás, G., Panzera, Y., 2014. Phylogenetic and genome-wide deep-sequencing analyses of canine parvovirus reveal co-infection with field variants and emergence of a recent recombinant strain. PLoS One 9, e111779. http://dx.doi.org/10.1371/journal.pone.0111779.
Purpari, G., Mira, F., Di Bella, S., Di Pietro, S., Giudice, E., Guercio, A., 2018. Investigation on *canine parvovirus* circulation in dogs from Sicily (Italy) by biomolecular assay. Acta Vet. (Beograd) 68 (1), 80–94.
Reed, A.P., Jones, E.V., Miller, T.J., 1988. Nucleotide sequence and genome organization of canine parvovirus. J. Virol. 62, 266–276.
Saxena, L., Kumar, G.R., Saxena, S., Chaturvedi, U., Sahoo, A.P., Singh, L.V., Santra, L., Palia, S.K., Desai, G.S., Tiwari, A.K., 2013. Apoptosis induced by NS1 gene of canine Parvovirus-2 is caspase dependent and p53 independent. Virus Res. 173, 426–430. http://dx.doi.org/10.1016/j.virusres.2013.01.020.
Shackelton, L.A., Parrish, C.R., Truyen, U., Holmes, E.C., 2005. High rate of viral evolution associated with the emergence of carnivore parvovirus. Proc. Natl. Acad. Sci. U. S. A. 102, 379–384. http://dx.doi.org/10.1073/pnas.0406765102.
Silva, S.P., Silva, L.N.P.P., Rodrigues, E.D.L., Cardoso, J.F., Tavares, F.N., Souza, W.M., Santos, C.M.P., Martins, F.M.S., Jesus, I.S., Brito, T.C., Moura, T.P.C., Nunes, M.R.T., Casseb, L.M.N., Silva Filho, E., Casseb, A.R., 2017. Full-length genomic and molecular characterization of Canine parvovirus in dogs from North of Brazil. Genet. Mol. Res. 16 (3). http://dx.doi.org/10.4238/gmr16039719. (Sep 21).
Tijssen, P., Agbandje-McKenna, M., Almendral, J.M., Bergoin, M., Flegel, T.W., Hedman, K., Kleinschmidt, J., Li, Y., Pintel, D.J., Tattersall, P., 2011. The family *Parvoviridae*. In: AMQ, King, Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Virus taxonomy—Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London, pp. 405–425.
Touihri, L., Bouzid, I., Daoud, R., Desario, C., El Goulli, A.F., Decaro, N., Ghorbel, A., Buonavoglia, C., Bahloul, C., 2009. Molecular characterization of canine parvovirus-2 variants circulating in Tunisia. Virus Genes 38, 249–258. http://dx.doi.org/10.1007/s11262-008-0314-1.
Truyen, U., 1999. Emergence and recent evolution of canine parvovirus. Vet. Microbiol. 69, 47–50.
Truyen, U., Steinel, A., Bruckner, L., Lutz, H., Möstl, K., 2000. Distribution of antigen types of canine parvovirus in Switzerland, Austria and Germany. Schweiz. Arch. Tierheilkd. 142, 115–119.
Tsao, J., Chapman, M.S., Agbandje, M., Keller, W., Smith, K., Wu, H., Luo, M., Smith, T.J., Rossmann, M.G., Compans, R.W., 1991. The three-dimensional structure of canine parvovirus and its functional implications. Science 251, 1456–1464.
Xu, J., Guo, H.-C., Wei, Y.-Q., Shu, L., Wang, J., Li, J.-S., Cao, S.-Z., Sun, S.-Q., 2015. Phylogenetic analysis of canine parvovirus isolates from Sichuan and Gansu provinces of China in 2011. Transbound. Emerg. Dis. 62, 91–95. http://dx.doi.org/10.1111/tbed.12078.
Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 7, 203–214. http://dx.doi.org/10.1089/10665270050081478.
Zhou, P., Zeng, W., Zhang, X., Li, S., 2017. The genetic evolution of canine parvovirus - a new perspective. PLoS One 12, e0175035. http://dx.doi.org/10.1371/journal.pone.0175035.