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RAPID COMMUNICATION

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Introduction of Asian canine parvovirus in Europe through dog importation

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Summary

Canine parvovirus (CPV) is an important infectious agent of domestic and wild carnivores, responsible for severe and often fatal haemorrhagic gastroenteritis and leukopenia. This paper reports the genomic characterization of a CPV strain collected from a dog recently imported to Italy from Thailand. The virus was detected in all tissue samples collected. The whole genome encompassing the two open reading frames encoding for non-structural (NS1/NS2) and structural (VP1/VP2) proteins was amplified and sequenced. On the basis of genetic analysis of the VP2 gene, the isolate was characterized as CPV-2c, but it presented genetic signatures typical of Asian strains. Sequence analysis revealed the presence of amino acid changes never observed in European CPV-2c strains (NS1: Ile60Val, Tyr544Phe, Glu545Val, Leu630Pro; VP2: Ala5Gly, Phe267Tyr, Tyr324Ile, Gln370Arg). By phylogenetic analysis of full-length VP2 gene, the analysed strain clustered together with Asian viruses. Therefore, a possible introduction of the virus from Asia through the imported dog was suggested, thus confirming the important role of movement of dogs in the global spread of viruses. In addition, full-length genome analysis could help better trace the spread of canine viruses through different continents.

KEYWORDS

Asia, canine parvovirus, dogs, molecular survey, phylogeny, sequence analysis

1 | INTRODUCTION

Canine parvovirus (CPV) is a member of the genus *Protoparvovirus* (family *Parvoviridae*, subfamily *Parvovirinae*) and together with *Feline panleukopenia virus* (FPLV) is now included in a unique viral specie, *Carnivore protoparvovirus 1* (Cotmore et al., 2014). CPV is a small, non-enveloped, single-stranded DNA virus. Genome consists of an approximately 5,000-nucleotide (nt) DNA molecule containing two open reading frames (ORFs), ORF1 and ORF2, encoding for two non-structural (NS1 and NS2) and two structural (VP1 and VP2) proteins through alternative splicing of the same mRNAs (Decaro & Buonavoglia, 2012; Reed, Jones, & Miller, 1988). VP2 is the major capsid protein, represents the major determinant of parvovirus host range (Hueffer et al., 2003) and is subjected to antibody-mediated selection (Nelson, Palermo, Hafenstein, & Parrish, 2007). For this reason, most studies during the years have focused on the evolution of the VP2 gene, with limited works on the non-structural genes (Hoelzer, Shackelton, Parrish, & Holmes, 2008).

The original strain CPV-2 emerged in the late 1970s as host variant of an FPLV or FPLV-like parvovirus (Parrish et al., 1991), causing panzootic of acute gastroenteritis, leukopenia and myocarditis in dogs. After its emergence, two CPV variants (CPV-2a and CPV-2b) rapidly replaced the original type 2 (Decaro et al., 2005; Parrish, O'Connell, Evermann, & Carmichael, 1985; Parrish et al., 1988, 1991). In 2000, a new antigenic type (CPV-2c), which showed amino acid (aa) Glu at VP2 residue 426, emerged in Italy (Buonavoglia

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et al., 2001), spreading in the last 15 years to many countries worldwide (Miranda & Thompson, 2016). Additional changes (Ser297Ala, Gly300Asp, Thr324Ile) in the VP2 sequence were also observed in strains detected in last decades (Ikeda et al., 2000; Jeoung, Ahn, & Kim, 2008; Truyen, 1999). Among these, a change at residue 324, first reported in 2006 (Zhou, Zeng, Zhang, & Li, 2017), may represent a common change in CPV strains circulating in the Asian continent (Geng et al., 2015). Recently, change Thr324Ile has been observed in European CPV-2a strains (Cságola, Varga, Lőrincz, & Tuboly, 2014). The aim of this work was to report the detection and genetic characterization of a CPV strain from a dog imported from Thailand to Italy.

2 | MATERIALS AND METHODS

2.1 | Clinical case

A four-month-old Pomeranian purebred pup displayed clinical signs of parvovirosis 2 days after its importation to Italy from Bangkok (Thailand). After 3 days of gastroenteric signs (anorexia, diarrhoea and fever), despite hospitalization and intensive care, the pup died and was subjected to necropsy. Tissue samples (spleen, liver, intestine, heart, lungs, kidney) were collected for virological investigations.

2.2 | DNA/RNA extraction

DNA and RNA were extracted from samples using the DNeasy Blood & Tissue Kit (Qiagen S.p.A, Milan, Italy) and QIAamp Viral RNA Mini Kit (Qiagen S.p.A.), respectively, according to the manufacturer's instructions, and stored at −80°C until processed.

2.3 | Parvovirus PCR and RFLP

CPV DNA was amplified using a PCR protocol recently developed (Touihri et al., 2009). PCR was carried out using the GoTaq G2 DNA Polymerase (Promega Italia s.r.l., Milan, Italy) in a 50-μl reaction mix consisting of 5× GoTaq® Reaction Buffer 1×, $MgCl_2$ 0.5 mM, dNTP mix 0,2 mM, 0.5 μM of each primer VP2-850-Forward (5′-GAGCATT GGGCTTACCA-3′) and VP2-1550-Reverse (5′-GCAAGATGCATCAG GATC-3′), GoTaq® G2 DNA Polymerase 1.25 U and 5 μl of DNA extract. Amplification was conducted under the following thermal conditions: 94°C for 2 min to activate TaqPol followed by 40 cycles of 94°C for 30 s, 55°C for 60 s, 72°C for 60 s and a final extension of 72°C for 10 min. RFLP was carried out on the amplicons of 700 bp. Ten microlitres of each amplicon was digested with 10U of restriction endonuclease *Mbo*II (Thermo Scientific, Thermo Fisher Scientific, Monza, Italy) and the profile determined by electrophoresis on a 3% agarose gel supplemented with 0,005% ethidium bromide.

2.4 | Sequence and phylogenetic analyses

Sequencing of the complete genome, encompassing both NS and VP genes, was carried out using primers pairs NS-Fext (5′-GACCGTTACTGACATTCGCTTC-3′)/NS-Rext (5′-GAAGGGTTAGT TGGTTCTCC-3′) and 2161F (5′-TTGGCGTTACTCACAAAGACGTGC-3′)/4835R (5′-ACCAACCACCCACACCATAACAAC-3′) (Pérez et al., 2014), using two separate reactions and the same reaction mix and thermal conditions described above. Amplicons 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 additional internal primers: NS-Fint (5′-GTTGAAACCACAGTGACGACAG-3′) for the PCR product amplified with primers pair NS-Fext/NS-Rext, R2 (5′-TTTTGAATCCAATCTCCTTCTGGAT-3′) and 3475R (5′-GTTGGTGTG CCACTAGTTCCAGTA-3′) for the product amplified with primers pair 2161F/4853R.

According to an overlapping strategy, sequences were assembled and analysed using BioEdit ver 7.2.5 software (Hall, 1999). Assembled nucleotide sequences were submitted to the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search related sequences. Sequences were also compared with those of two CPV-2c reference strains (CPV_IZSSI_25835_09, acc.n. KU508407; CPV_IZSSI_41113_c1_16, acc.n. MF510158) collected from the same Italian area and previously sequenced. Phylogenetic analysis based on the full-length VP2 gene sequences was performed using the best-fit model of nucleotide substitution with MEGA5 software (Tamura et al., 2011) using ML method according to the Tamura 3-parameter model (bootstrap 1,000 replicates). These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number MF510157.

2.5 | Other virological tests

Extracted DNA/RNA were amplified using a set of PCR useful for the detection of canine distemper virus (CDV) (Elia et al., 2006), canine adenovirus (CAdV) type 1 and type 2 (Dowgier et al., 2016), canine herpesvirus (CaHV-1) (Decaro et al., 2010) and canine coronavirus (CCoV) (Decaro et al., 2004).

3 | RESULTS AND DISCUSSION

All tissue samples tested positive for CPV by conventional PCR assay and negative for CDV, CAdVs, CaHV-1 and CCoV by real-time (RT)-PCR assays. RFLP carried out on the CPV PCR product generated three fragments of 56, 275 and 369 bp, respectively, which are typical of CPV-2c. By subsequent PCR amplifications, a sequence of 4450 nt was obtained. On the basis of aa residue Glu at residue 426 of the VP2 protein, the strain (CPV_IZSSI_2743_17) was characterized as currently named CPV-2c, confirming the RFLP result. NS1 and VP2 sequences showed 100% nucleotide identities with Asian strains CPV/CN/LN1/2014 (China, 2014) for NS1 sequence and BJ14-9 and BJ14-8 (China, 2014) for VP2 sequence. Sequence analysis revealed the presence of aa changes never observed in European CPV strains (NS1: Ile60Val, Tyr544Phe, Glu545Val, Leu630Pro; VP2: Ala5Gly, Phe267Tyr, Tyr324Ile, Gln370Arg) with exception for

change Tyr324Ile, which has been described in CPV-2a isolates in Hungary (Cságola et al., 2014) (Table 1). Phylogenetic analysis indicated that strain CPV_IZSSI_2743_17 is more related to Asian than to European CPV strains, clustering in a separate clade (Figure 1).

The detection of a CPV strain displaying genetic signatures typical of Asia viruses suggests a possible introduction of the virus from Asia through the imported dog. Whereas new CPV-2a/2b are prevalent strains circulating in Asia (Yi, Tong, Cheng, Song, & Cheng, 2016), CPV-2c has been sporadically detected in this continent. This variant has been detected in India in 2006 (Nandi, Chidri, Kumar, & Chauhan, 2010), in China from 2009 to 2015 (Geng et al., 2015; Wang et al., 2016; Zhang et al., 2010) and in Taiwan between 2014 and 2016 (Chiang, Wu, Chiou, Chang, & Lin, 2016). Our strain showed 100% nucleotide identity with complete VP2 sequence of 10 CPV strains detected in China in 2014 (Wang et al., 2016) and with partial VP2 sequence of 14 strains detected in China in years 2014-2015 (Geng et al., 2015) and with partial VP2 sequence of 6 viruses circulating in Vientiane, Laos, in 2016 (Vannamahaxay et al., 2017). Laos is located between China and Thailand, the latter being the country of origin of the infected dog. In the same country, a high prevalence of CPV strains closely related to Chinese isolates has been recently reported (Vannamahaxay et al., 2017). After the first report of CPV in Thailand (Tingpalapong et al., 1982), only CPV-2a/2b types have been proven to circulate in this country, with no evidence for the presence of the variant 2c (Phromnoi, Sirinarumitr, & Sirinarumitr, 2010). The findings of the present study suggest that CPV-2c is currently circulating in more Asian countries than previously reported, which deserves further extensive surveillance.

Most recent Asian CPV strains exhibited four non-synonymous nt changes that are responsible for aa substitutions Ala5Gly, Phe267Tyr, Tyr324Ile and Gln370Arg, and six synonymous nt changes (T135C, A207G, T101A, A732C, G1509A and A1659C) in the VP2 sequence (Table 1). Change Tyr324Ile is predominant in recent Asian CPV strains (Yi et al., 2016; Geng et al., 2015; Zhao et al., 2017). In contrast, other changes have been observed only between 2013 and 2015 (Geng et al., 2015; Wang et al., 2016), and change Gln370Arg has been detected only in China in CPV strains from dogs and a strain from a giant panda (Guo et al., 2013).

Amino acid changes into VP2 sequence of CPV strains of Asian origin involve residues exposed or not on the capsid surface (Xie & Chapman, 1996; Yi et al., 2014; Wang et al., 2016) but, apart from few reports (Cságola et al., 2014; Hoelzer et al., 2008; Hueffer et al., 2003; Wang et al., 2016), immunological implication and biological relevance of these changes need to be assessed in further studies.

Compared to European CPV strains, specific changes affecting NS1 sequence (Ile60Val, Tyr544Phe, Glu545Val, Leu630Pro) were observed only in CPV strains isolated in China between 2013 and 2014 (Wang et al., 2016). Some non-synonymous (encoding for aa changes Ile60Val and Leu630Pro) and synonymous (A51G, C159T, G1752A, A1800G) substitutions between European and Asian strains are located in the NS2-encoding sequence (Table 1). To our best knowledge, there is no full-length genomic sequence available for Asian CPV-2c strains, so that our strain CPV_IZSSI_2743_17 is the

TABLE 1 Nucleotide and deduced amino acid (in brackets) variations in NS1/VP2 sequences in analysed CPV strains

				NS1 nucleotide (amino acid) position																		
Strain/Isolate	Country, year	Accession nr	Type	51[a]	159[a]	178 (60)[a]	411	726	1059	1062	1098	1377	1464	1623	1631 (544)	1634 (545)	1662	1752[a]	1800[a]	1889 (630)[a]	1975
CPV_IZSSI_25835_09	Italy, 2009	KU508407	CPV-2c	A	C	A (Ile)	G	G	A	G	G	T	G	A	A (Tyr)	A (Glu)	T	G	G	T (Leu)	C
CPV_IZSSI_41113_c1_16	Italy, 2016	MF510158	CPV-2c	A	T	A (Ile)	A	G	A	G	G	T	A	A	A (Tyr)	A (Glu)	T	G	A	T (Leu)	C
CPV_IZSSI_2743_17	**Italy, 2017**	**MF510157**	**CPV-2c**	**G**	**T**	**G (Val)**	**A**	**A**	**G**	**A**	**A**	**C**	**A**	**G**	**T (Phe)**	**T (Val)**	**C**	**A**	**G**	**C (Pro)**	**T**
CPV/CN/LN1/2014	China, 2014	KR002800	CPV-2a	G	T	G (Val)	A	A	G	A	A	C	A	G	T (Phe)	T (Val)	C	A	G	C (Pro)	T
				VP2 nucleotide (amino acid) position																		
Strain/Isolate	Country, year	Accession nr	Type	14 (5)	60	135	207	415 (139)	732	760	800 (267)	963	970 (324)	971 (324)	1109 (370)	1509	1659	1710	1716	1746		
CPV_IZSSI_25835_09	Italy, 2009	KU508407	CPV-2c	C (Ala)	A	C	A	G (Val)	T	C	T (Phe)	T	T (Tyr)	A (Tyr)	A (Gln)	G	T	A	A	A		
CPV_IZSSI_41113_c1_16	Italy, 2016	MF510158	CPV-2c	C (Ala)	A	C	A	A (Ile)	T	T	T (Phe)	T	T (Tyr)	A (Tyr)	A (Gln)	G	T	G	T	G		
CPV_IZSSI_2743_17	**Italy, 2017**	**MF510157**	**CPV-2c**	**G (Gly)**	**T**	**T**	**G**	**G (Val)**	**C**	**T**	**A (Tyr)**	**C**	**A (Ile)**	**T (Ile)**	**G (Arg)**	**A**	**C**	**A**	**A**	**A**		
BJ14-9	China, 2014	KP260509	CPV-2c	G (Gly)	T	T	G	G (Val)	C	T	A (Tyr)	C	A (Ile)	T (Ile)	G (Arg)	A	C	A	A	A		

The main sequence object of the study are indicated in bold.
[a]Variations located also in the NS2-encoding sequence.

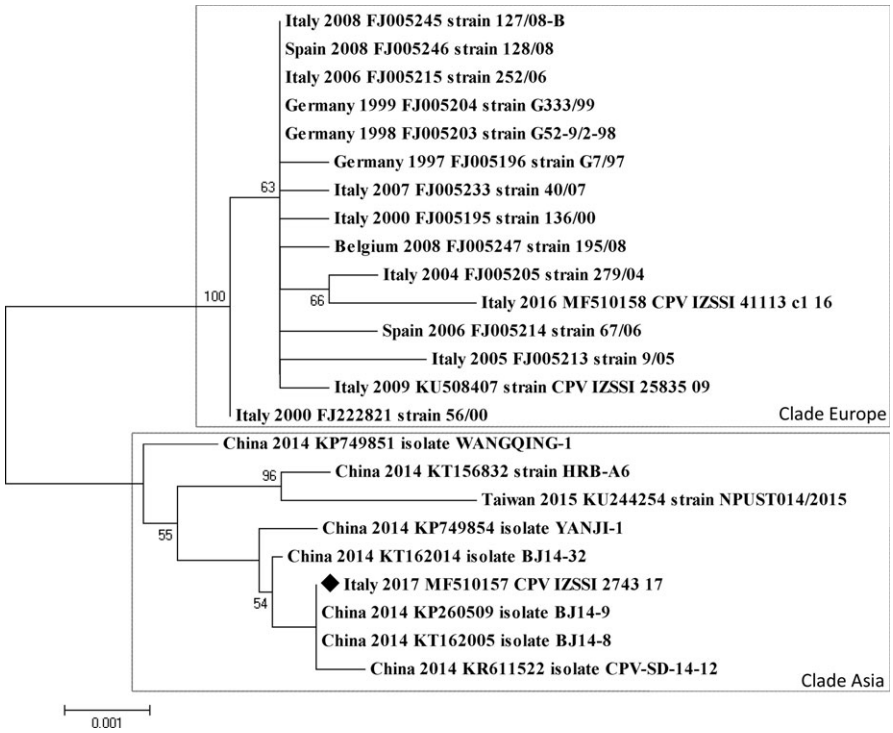

FIGURE 1 ML tree based on the full-length VP2 sequences. Each sequence was indicated with country and year of collection, accession number, strain/isolate name. Sequences are divided into two clades named "clade Asia" and "clade Europe"

first having a NS1 sequence with these specific signatures. CPV full-length genomes are needed to better understand virus epidemiology and global spreading. As already observed (Hoelzer et al., 2008), NS1 sequences of currently circulating CPV strains are scarce in comparison with VP2 sequences, particularly for CPV-2c variant.

Among CPV strains used as references (Table 1), which had been collected from the same Italian area where the Asian strain was found, the following nt differences were observed: NS1—C159T, G411A, G1464A, G1800A; VP2—G415A, C760T, A1710G, A1716T, A1746G. While others were synonymous substitutions, the non-synonymous change G415A results in the aa change Val139Ile, due to a transition in the first position of the codon. This aa residue is located inside the capsid, forming part of the beta barrel of the viral capsid (Clegg et al., 2011). Comparison with VP2 sequences available in the GenBank database shows that this change occurred into CPV-2a/CPV-2b sequences from Asia (Chinchkar et al., 2006; Wu et al., 2015), Brazil (Pereira, Leal, & Durigon, 2007) and Europe (Cságola et al., 2014), including Italy (isolate CPV-2a/151/212—accession n. KF373603). The same mutation was also found in CPV-2c strains collected in 2011-2012 in Uruguay (Pérez et al., 2014) and Ecuador (Aldaz et al., 2013).

The presence of different nt and aa changes between European and Asian CPV strains and the presence of high genetic identities among Asian strains support the Asian origin of the CPV strain detected in the imported pup, thus representing the first evidence in Europe of a CPV-2c strain with Asian signatures. In addition, determining the nearly complete genome of this Asian-like CPV contributes to gain new information about these Asian CPV strains for further studies. Trading and transport of dogs between countries and continents play an important role in introduction or re-introduction of novel or re-emerging viral strains, as already observed for other canine viruses (Decaro et al., 2007; Martella et al., 2006). Thus, this report represents the evidence for transcontinental viral spread. Future epidemiological survey and determination of complete genome sequences will allow to better trace the possible spread of CPV variants among countries and continents.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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