Veterinary Research Communications (2021) 45:31–40
https://doi.org/10.1007/s11259-020-09785-w

ORIGINAL ARTICLE

Molecular survey of parvovirus, astrovirus, coronavirus, and calicivirus in symptomatic dogs

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Received: 9 December 2020 / Revised: 9 December 2020 / Accepted: 16 December 2020 / Published online: 4 January 2021

Abstract

Gastrointestinal disorders caused by enteric viruses are frequently reported in dogs worldwide, with significant mortality rates in unvaccinated individuals. This study reports the identification and molecular characterization of Canine parvovirus (CPV-2), Canine coronavirus (CcoV), Canine astrovirus (AstV), and Canine calicivirus (CcaV) in a panel of dogs showing severe enteric clinical signs sampled in a typical Mediterranean environment (Sardinia, Italy). At least one of these viral species was detected in 92.3% samples. CPV-2 was the most frequently detected virus (87.2%), followed by AsTv (20.5%), CCoV-IIa (18%), and CCoV-I (10.3%). CCoV-IIb and CaCV were not detected in any sample. Single infection was detected in 24 samples (66.7%), mainly related to CPV-2 (91.7%). Coinfections were present in 33.3% samples with constant detection of CPV-2. Canine coronavirus was present only in coinfected animals. The VP2 sequence analysis of CPV-2 positive samples confirmed the presence of all variants, with CPV-2b most frequently detected. Phylogeny based on the CcoV-IIa spike protein (S) gene allowed to identify 2 different clades among Sardinian isolates but failed to distinguish enteric from pantropic viruses. Study on presence and prevalence of enteroviruses in dogs increase our knowledge about the circulation of these pathogens in the Mediterranean area and highlight the need for dedicated routine vaccine prophylaxis. Molecular analyses of enteric viruses are fundamental to avoid failure of vaccines caused by frequent mutations observed in these enteroviruses.

Keywords Canine parvovirus · Canine coronavirus · Canine astrovirus · Molecular characterization

Introduction

Single or multiple viral intestinal infections are common causes of canine viral enteric illness and cause high rate of mortality in unvaccinated populations Hao et al. 2019; Greene and Decaro 2012; Pollock et al. 1983). Among these, canine parvovirus type 2 (CPV-2) and canine coronavirus (CCoV) are the most common viral enteric pathogens in dogs worldwide (Alves et al. 2018). CPV-2 emerged in 1978 and became globally distributed in 2 years (Parrish et al. 1991). In 1979 and 1980 the virus's original type was replaced in several countries by a new variant called CPV-2a characterised by few changes in the amino acid sequence of VP2 (Parrish et al. 1985, 1991), the most abundant structural protein and major determinant of host range and virus-host interaction (Decaro and Buonavoglia 2012a). Between 1984 and 2000, viral variants with new changes in the VP2 amino acid sequence were detected (CPV-2b and −2c, Buonavoglia et al. 2001; Parrish et al. 1991). An additional amino acid change at

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position 297 (Ser → Ala), both in CPV-2a and CPV-2b, led to the designation of "new CPV-2a" and "new CPV-2b" (Decaro and Buonavoglia 2012a). It was found that few changes in the amino acid structure of VP2 led to alteration of the biological characteristics of the virus (Parrish and Carmichael 1986). CPV-2 infection is characterized by fever, severe diarrhoea, and vomiting, with high morbidity (Studdert et al. 1983). In unprotected hosts, the disease can rapidly evolve and lead to death within 2–3 days after the onset of symptoms (Carman and Povey 1985; Parrish 1995). Neonatal puppies between 3 and 8 weeks of age, but sometimes up to 16 weeks, show higher mortality than adult dogs caused by myocarditis (Miranda and Thompson 2016; Parrish 1995).

Canine coronavirus (CCoV) belonging to the genus *Alphacoronavirus* include two different genotypes, CCoV-I and CCoV-II, sharing up to 96% nucleotide sequence identity in the viral genome but highly divergent in the S protein gene (Pratelli et al. 2003). A recombination between transmissible gastroenteritis virus of swine and CCoV-II led to the distinction of two subtypes including the classical CCoV-IIa and the recombinant CCoV-IIb (Decaro et al. 2009c, 2010b). Dogs infected with CCoV alone are likely to have mild but highly contagious diarrhoea, whereas an increase of disease severity, particularly in puppies, can appear with coinfection with other enteric viruses such as CPV, Canine distemper virus or Canine adenovirus type 1 (Tennant et al. 1991; Pratelli et al. 1999a, 2001). A new strain of CCoV-IIa (CB/05) has been shown to be able to cause a fatal disease characterised by systemic spread of the virus (Decaro et al. 2008a), and in recent years, an increasing number of reports of infections by this new highly virulent pantropic virus (pCCoV) have also been documented in puppies and in a wolf. Infection is characterised by fatal multisystemic illness with lethargy, inappetence, vomiting, haemorrhagic diarrhoea, lymphopenia, ataxia, and seizures (Alfano et al. 2019; Haake et al. 2020; Licitra et al. 2014). However, several studies have demonstrated that this pantropic strain can also associate to subclinical infections and/or to the decrease of lymphocyte counts, rather than to severe clinical signs and death (Marinaro et al. 2010).

Canine astrovirus (CaAstV) belongs to the family *Astroviridae* and to the genus *Mamastrovirus*, whose members infect several mammals including humans (Bosch et al. 2012). CaAstV, isolated for the first time in 1980 and now globally distributed, can be detected in healthy and diarrhoeic dogs (Li et al. 2018; Williams 1980). It has been demonstrated that CaAstV can cause gastroenteritis and infect more easily dogs under one year of age, especially in coinfection with other enteroviruses (Martella et al. 2012; Takano et al. 2015; Zhou et al. 2017).

Caliciviruses, causative agents of a wide range of diseases in human and animals including gastroenteritis, are not considered relevant pathogens in dogs. Few studies have reported the recovery of feline calicivirus (Martella et al. 2002), *Norovirus* (Mesquita and Nascimento 2012; Ntafis et al. 2010; Scipioni et al. 2008), *Sapovirus* (Gabriel et al. 1996; Soma et al. 2015) and candidate canine calicivirus (Binn et al. 2018; Martella et al. 2015; Mochizuki et al. 1993) in dogs.

Aim of this study is investigating the presence of enteric viruses in stool samples from severely symptomatic unvaccinated dogs living in a typical Mediterranean environment (Sardinia, Italy). A first molecular typing of isolated strains is also provided.

Materials and methods

Study design and sampling

From 2013 to 2014 fecal samples were collected from 39 dogs with symptoms consistent with viral enteritis. All dogs came from Province of Sassari, Northern Sardinia, Italy. They were presented to the Veterinary Teaching Hospital of the University of Sassari. Feces were stored at -20 °C until use for parvovirus detection and at -80 °C with RNA*later* stabilization solution (Ambion, Italy) for corona-, calici-, and astrovirus detection. They were 20 males and 19 females.

23 dogs had an age between 2 and 4 months, 11 dogs were between 5 and 9 months, and 5 dogs were aged between 1 and 4 years. A clinical examination was performed on all patients. The vaccination status of 18 dogs was unknown, 17 dogs were not vaccinated, and 4 dogs received an uncompleted vaccination.

DNA/RNA isolation and reverse transcription (RT)

DNA was extracted from faecal samples using the DNeasy Blood and tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The extracted DNA was eluted in 100 µl of ultrapure RNasi and DNasi free water and stored at -20 °C until use. Total RNA was extracted from faecal samples using QIAamp Viral RNA Mini (Qiagen, Italia) and treated with DNase I (Sigma Aldrich, Italy). The cDNA was synthesized with SuperScript®III Reverse Transcriptase Kit (LifeTechnologies, Italy), according to the manufacturer's instructions.

PCRs profile, sequencing, and phylogenetic analyses

PCR and RT-PCR were performed with the Taq DNA Polymerase kit (Qiagen, Italy), according to vendor recommendations. The CPV-2 PCR protocol aimed to amplify 717 bp of the VP2 gene with the primers CPV3381-F/CPV4116-R (Decaro et al. 2008b). All positive samples were tested with a conventional PCR as described by Buonavoglia et al. 2001 to amplify a 611 bp fragment of hypervariable

region of VP2 gene with primers Hfor/Hrev. Amplicons were purified using the DNA Clean & concentrator Kit (Zymoresearch, CA, USA) according to the manufacturer's protocol, and directly sequenced by BMR Genomics, Padova, Italy. Chromatograms were edited with Chromas 2.2 (Technelysium, Helensvale, Australia), and aligned with CLUSTALX to assign sequences to unique sequence types (Larkin et al. 2007). Sequence types were checked against the GenBank database by BLASTN (http://blast.ncbi.nlm.nih.gov/, Altschul et al. 1990) in order to identify the different CPV-2 strains circulating in Northern Sardinia. Sequence translation was done by using the software ORF-finder (https://www.ncbi.nlm.nih.gov/orffinder/).

For CCoV detection and genotyping, three RT-PCR were performed. Separate RT-PCRs with primers 20,179/INS-R-dg or 20,179/174–268 were conducted (Decaro et al. 2010b) to amplify a fragment of 754 bp of CCoV-IIa and 499 bp of CCoV-IIb respectively of the S gene, while RT-PCR with primers CCov1a/CCov2 was used to amplify a fragment of 239 bp of the transmembrane protein gene (M gene) of CcoV-I (Pratelli et al. 1999b, 2002). For Astrovirus detection, primers AsTVs_626F-1/AsTVs_626F-1 were used to amplify a 300 bp fragment of the RNA dependent-RNA polymerase (RdRp) located in ORF1b (Martella et al. 2011a). At last, for CaCV detection, primers p290/p289 were used to amplify a 318 pb of the RdRp region of the polymerase complex (Jiang et al. 1999). The sequences of all primers are shown in Table 1. Positive samples for CcoV-IIa, CcoV-I, and AstV were sequenced, and sequences were edited as described above. By using ClustalX (Larkin et al. 2007), CcoV-IIa sequences were aligned among them and with a set of 38 sequences belonging to CcoV-II subtypes representative of different geographic areas. Genbank accession numbers of the 38 sequences are reported in Fig. 1. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018) by using the Maximum Likelihood method based on Tamura 3-parameter (Tamura 1992), identified as the best model with the same software. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+ *G*, parameter = 0.6444)). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 43 nucleotide sequences. There was a total of 423 positions in the final dataset. The robustness of tree was evaluated by bootstrapping over 500 reiterations (Felsenstein 1985).

DNA preparations of CPV-2 and RNA preparations of CCoV and AsTVs isolated in Sardinia were used as positive controls. The commercial vaccine Felocell 4 (Zoetis, USA) was used as a positive control for *Calicivirus*. Sampling was approved by the Ethical Committee of the University of Sassari.

Results

Viral DNA/cDNA was amplified from 36 out of 39 samples examined (92.3%). Thirty-four (87.2%) samples tested positive for CPV-2, 8 (20.5%) were positive for AsTv, 7 (18%) were positive for CCoV-IIa, and 4 (10.3%) were positive for CCoV-I. CCoV-IIb and CaCV were not detected in any

Table 1 Primers used for PCR amplification and molecular characterization of Canine parvovirus, Canine coronavirus, Canine astrovirus, and Canine calicivirus

Primers	Virus	Sequence (5' to 3')	Reference	Gene	Amplicon size (bp)
CPV3381-F CPV4116-R	CPV-2	CCATGGAAACCAACCATACC AGTTAATTCCTGTTTTACCTCCAA	Decaro et al. 2008a, b	VP2	717
Hfor Hrev	CPV-2	CAGGTGATGAATTTGCTACA CATTTGGATAAACTGGTGGT	Buonavoglia et al. 2001	VP2	611
20,179 INS-R-dg	CCoV Type-IIa	GGCTCTATCACATAACTCAGTCCTAG GCTGTAACATAKTCRTCATTCCAC	Decaro et al. 2010a, b	S	754
20,179 174–268	CCoV Type-IIb	GGCTCTATCACATAACTCAGTCCTAG CAACATGTAACCTTTGTCTGTGATCT GC	Decaro et al. 2010a, b	S	499
CCov1a CCov2	CCoV type I	GTGCTTCCTCTTGAAGGTACA TCTGTTGAGTAATCACCAGCT	Pratelli et al. 2002 Pratelli et al. 1999a, b	M	239
AsTVs_625F-1 AsTVs_626R-1	AstVs	GTACTATACCRTCTGATTTAATT AGACCAARGTGTCATAGTTCAG	Martella et al. 2011a, b	RdRp	300
calicivirus_p290 calicivirus_p289	CaCV	gattactccaagtgggactccac tgacaatgtaatcatcaccata	Jiang et al. 1999	RdRp	318

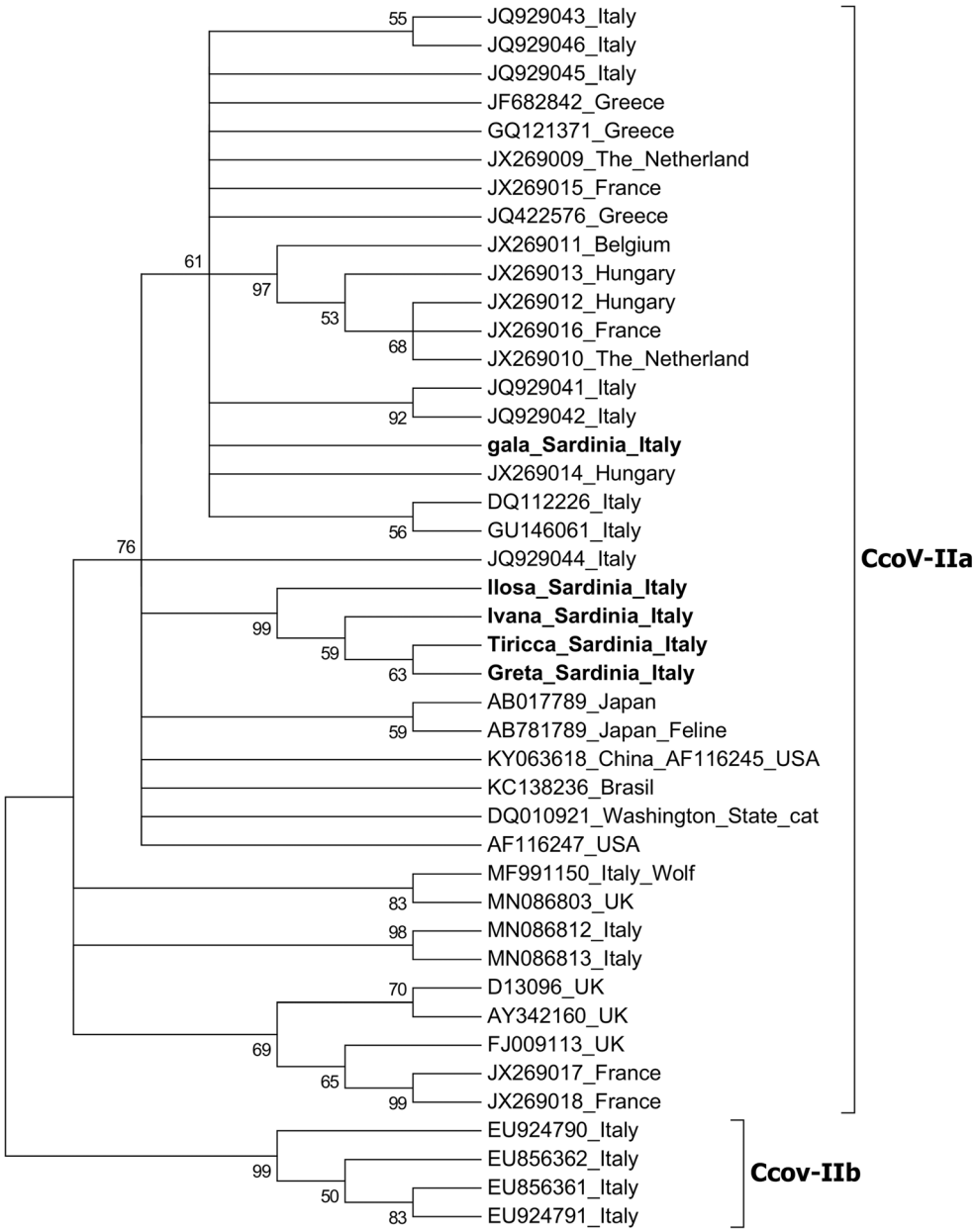

Fig. 1 Phylogenetic tree of the 5 CcoV-IIa strains of this study based on a 423 bp fragment of the S gene sequence. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

sample. Single infections were observed in most of the positive samples (72.7%, 24/33), where CPV-2 was the most frequently detected (66.7%, 22/33), followed by AsTv (6.1%, 2/33). Coronavirus (CCoV-IIa and CcoV-I) were only detected in coinfections. Coinfections were observed in 36.4% (12/33) of the positive samples. Among them, double infections were the most common (66.7%, 8/12), although triple (25%, 3/12) and quadruple (8.3%, 1/12) infections were also found (Table 2).

CPV-2 was the most frequent virus involved in co-infections (12/12; 100%), while CCoV-IIa was involved in 7 (7/12; 58.3%) co-infections, AsTv in 6 co-infections (6/12; 50%), and CcoV-I in 4 co-infections (4/12; 33.3%).

Parvovirus sequences obtained with primers Hfor/Hrev were submitted to the NCBI database (NCBI; Bethesda, MD) using the BankIt v3.0 submission tool (http://www3.ncbi.nlm.nih.gov/BankIt/) under accession numbers MW051532 to MW051561 (Table 3).

The analysis of CPV-2 sequences allowed to identify 7 different sequence types/strains (Table 3), named seqtype1–7. Seqtype1 and seqtype2 respectively showed 99.81% and 100% similarity with CPV-2b strains already isolated in Sardinia (Dei Giudici et al. 2017). Seqtype3 and seqtype4 showed 100% identity with strains belonging to the CPV-2a variant respectively isolated in Italy/Hungary, and worldwide. Finally,

Table 2 Single infection and co-infection rates in dogs with positive PCR detection of Canine parvovirus, Canine astrovirus, and Canine coronavirus

Virus	Positive samples
CPV-2	22/36
AsTv	2/36
CCoV-IIa	0/36
CCoV-I	0/36
CaCV	0/36
CPV-2 + CCoV-IIa	4/36
CPV-2 + AsTv	4/36
CPV-2 + CCoV-IIa + CCoV-I	2/36
CPV-2 + AsTv + CCoV-I	1/36
CPV-2 + AsTv + CCoV-IIa + CCoV-I	1/36
TOTAL	36/36

seqtype5, seqtype6 and seqtype7 were 100% similar to CPV-2c strains respectively isolated in USA/Uruguay, Sardinia, and other geographical regions of Italy.

At the amino acid level, seqtype3 and seqtype4 showed Ala and Asn at position 297 and 426, as observed in strains belonging to the new CPV-2a variant, while Seqtype1 and seqtype2 showed Ala and Asp at the same positions, similarly to strains belonging the new CPV-2b variant. Seqtype5, seqtype6 and seqtype7 showed Glu at position 426, as observed in strains belonging to CPV-2c variant. All CPV-2b strains identified in this study had Gly at position 371, Thr at position 418, Asn at position 321, and Thr at position 440. All CPV-2a strains had Ile at position 324. Strains belonging to the CPV-2a or CPV-2b variants never presented Asp instead of Gly at position 300.

Five CcoV-IIa sequence types were obtained and submitted to the NCBI under accession numbers MW147006-8 and MW186832-3. Four of these sequence types (MW147006, MW147007, MW186832, and MW186833) shared 89.65–96.91% similarity with a pCcoV isolated in Italy (JQ929044), while one sequence (MW147008) shared 90.04% similarity with a strain isolated in Greece (GQ121371). Sequence types were named based on host name and geographical origin (Gala Sardinia Italy, Losa Sardinia Italy, Ivana Sardinia Italy, Tiricca Sardinia Italy, and Greta Sardinia Italy).

Table 3 Amino acid mutations in the VP2 sequence, GenBank accession numbers, sequence type, and CPV2 variants observed in this study

Isolate	Amino acid position								Accession number	Sequence type and nucleotide blast analysis	CPV variant
	297	300	321	324	371	418	426	440			
Tiricca	Ala	Gly	Asn	Tyr	Gly	Thr	Asp	Thr	MW051532	seqtype1; 100% new CPV-2b (Sardinia, Italy)	New CPV-2b
Charlie	.	.	.	.	.	.	.	.	MW051534		New CPV-2b
Marilù	.	.	.	.	.	.	.	.	MW051535		New CPV-2b
Greta	.	.	.	.	.	.	.	.	MW051536		New CPV-2b
Lucky	.	.	.	.	.	.	.	.	MW051538		New CPV-2b
Thoir	.	.	.	.	.	.	.	.	MW051540		New CPV-2b
Thessa	.	.	.	.	.	.	.	.	MW051541		New CPV-2b
Voldemort	.	.	.	.	.	.	.	.	MW051542		New CPV-2b
Senzanome	.	.	.	.	.	.	.	.	MW051543		New CPV-2b
Snoopy	.	.	.	.	.	.	.	.	MW051544		New CPV-2b
Principessa	.	.	.	.	.	.	.	.	MW051545		New CPV-2b
Asia	.	.	.	.	.	.	.	.	MW051547		New CPV-2b
Llosa	.	.	.	.	.	.	.	.	MW051549		New CPV-2b
Milo	.	.	.	.	.	.	.	.	MW051550		New CPV-2b
Tea	.	.	.	.	.	.	.	.	MW051551		New CPV-2b
Kelly	.	.	.	.	.	.	.	.	MW051552		New CPV-2b
topo	.	.	.	.	.	.	.	.	MW051553		New CPV-2b
Horus	.	.	.	.	.	.	.	.	MW051554		New CPV-2b
Gala	.	.	.	.	.	.	.	.	MW051555		New CPV-2b
Leo	.	.	.	.	.	.	.	.	MW051556		New CPV-2b
Cannella	.	.	.	.	.	.	.	.	MW051557		New CPV-2b
Billy	.	.	.	.	.	.	.	.	MW051560		New CPV-2b
Astrid	.	.	.	.	.	.	.	.	MW051561		New CPV-2b
Callmebaby	.	.	.	.	.	.	.	.	MW051539	Seqtype2; 99.81% new CPV-2b (Sardinia, Italy)	New CPV-2b
Cris	.	.	.	Ile	Ala	Ile	Asn	.	MW051533	Seqtype3; 100% new CPV-2a (Italy, Hungary)	New CPV-2a
Hulk	.	.	.	Ile	Ala	Ile	Asn	.	MW051548		New CPV-2a
Ivana	.	.	.	Ile	Ala	Ile	Asn	.	MW051537	Seqtype4; 100% new CPV-2a (worldwide)	New CPV-2a
Dora	.	.	.	.	Ala	Ile	Glu	.	MW051546	Seqtype5; 100% CPV-2c (USA/Uruguay)	CPV-2c
Walf	.	.	.	.	Ala	Ile	Glu	.	MW051558	Seqtype6; 100% CPV-2c (Sardinia, Italy)	CPV-2c
Celestina	.	.	.	.	Ala	Ile	Glu	.	MW051559	Seqtype7; 100% CPV-2c (Italy)	CPV-2c

Phylogenetic analysis allowed to assign sequence types to 2 distinct clades (Fig. 1). One sequence type (Gala Sardinia Italy) clustered in a monophyletic clade together with CcoV-IIa sequences obtained in different regions of Europe, while the other 4 sequence types (Losa Sardinia Italy, Ivana Sardinia Italy, Tiricca Sardinia Italy, and Greta Sardinia Italy) formed an independent cluster strongly supported by bootstrap.

Four CcoV-I M sequences types (173 bp fragment) and five AstV sequence belonging to 4 types (198 pb fragment) were obtained. CcoV-I and AstV Sequences were submitted to GenBank under accession numbers MW186839-MW186842 and MW186834- MW186838, respectively.

Upon BlastN analysis, CcoV-I sequences were 97.69% − 98.84% similar to CcoV-I strains isolated worldwide, while 4 AstV sequences were 98.48% − 100% similar to AstV strains isolated in UK, and one 97.98% to an AstV strain isolated in China.

Discussion

This study reports the occurrence of 3 enteroviruses (Coronavirus, Astrovirus, Parvovirus) and the absence of *Calicivirus* with symptoms consistent with enteritis. CPV-2 was the prevalent viral species. CPV-2 and CCoV are the most commonly reported viral enteric pathogens in dogs worldwide, even if CPV-2 is considered more pathogenic than CCoV (Alves et al. 2018). Since its first isolation in 1978, CPV-2 still represents a significant cause of dog disease and death worldwide (Miranda and Thompson 2016). The lack of vaccination and the neutralization of vaccines by maternal antibodies are factors predisposing to severe infections (Greene and Decaro 2012), even if sometimes severe clinical cases in vaccinated adult dogs have also been reported (Decaro et al. 2009a). Data obtained on parvoviruses indicate CPV-2b as the prevalent variant, and CPV-2a and CPV-2c both present but with a much lower frequency. The greater prevalence of CPV-2b (24 out of 30 positive samples; 80%) compared to CPV-2a (3 out of 30; 10%) and CPV-2c (3 out of 30; 10%) in Sardinia is in contrast with results obtained by previous reports about distribution of different strains in continental Italy. More specifically in 2007, Decaro et al. (2007) reported CPV-2c as the most prevalent variant (53%), followed by CPV-2a (40.2), and low frequency of CPV-2b (2.8%) and CPV-2 (3.7%). More recent data show CPV-2a as the prevalent variant in continental Italy, followed by CPV-2b and CPV-2c (Tucciarone et al. 2018; Battilani et al. 2019). However, data obtained in this study on parvovirus are consistent with what observed by Dei Giudici et al. (2017).

Amino acid sequence analysis showed that Sardinian CPV-2a and CPV-2b sequence types have Ala instead of Ser at position 297, and that is characteristic of the new CPV-2a and new CPV-2b variants (Decaro and Buonavoglia 2012a). This confirms what already observed by Dei Giudici et al. (2017). It has been hypothesized that substitutions at aa position 297 may be responsible for changes in antigenicity of CPV variants (Truyen 2006).

We never observed substitution of Gly with Asp at position 300. This mutation is typical of Asp-300 CPVs strains, which are CPV-2a and CPV-2b mutants more adapted to the feline host (Ikeda et al. 2000). Similarly, substitution Thr (T) with Ala (A) at position 440 was also never observed even if this mutation was reported by other authors in Italy and in other part of the world.

In contrast with what observed by Dei Giudici et al. (2017) we never observed variability at position 321. Moreover, we confirm the presence of amino acid Gly at position 371 and Thr at position 418 in all new CPV-2b in contrast with results reported by Battilani et al. (2019) in other Italian regions. CPV-2a sequences showed Ile at position 324, as reported in the most recent Asian CPV strains, instead of Tyr or Leu as described in the Italian strains (Mira et al. 2018). Mutation Y324I was observed only once in one Italian strain by Battilani et al. (2019).

CPV-2b seqtype1 was 100% identical with 12 sequences previously described in the same region (Dei Giudici et al. 2017). Moreover, CPV-2b seqtype2 share 99.81% similarity with the same sequences. These results are indicative of regional genetic variation of CPV-2b strains, which are closely related to a strain (KF373599) reported in Italy by Battilani et al. (2019).

The evolution of the old CPV-2 into the new variants CPV-2a/2b/2c, associated with specific changes in VP2 amino acids, led to an increase of disease severity, a lower incubation period, and a recrudescence of cat's susceptibility to infection (Decaro and Buonavoglia 2012a).

It is important to remember that the original CPV, from which CPV-2 strains derived, emerged as a host variant of feline panleukopenia virus (Decaro and Buonavoglia 2012a). Cases of asymptomatic infection or disease caused by CPV-2a, CPV-2b, and CPV-2c in domestic cats have been reported in different parts of the world, including Italy Balboni et al. 2018; Battilani et al. 2006, 2011, 2013; Decaro et al. 2010a, 2011; Decaro and Buonavoglia 2012a). Compared to the feline virus, continuous evolution of CPV-2 strains develops much more rapidly with a genomic substitution rates like those of RNA virus (Shackelton et al. 2005). The high frequency of amino acids mutation in the VP2 sequence and the ability to infect and cause disease in feline hosts highlight the urgency of protecting both dog and cat pets with vaccination, to reevaluate and tune the viral strains used in the production of vaccines, and to constantly monitor this virus to detect new CPV variants by molecular surveillance, also to develop dedicated molecular tests effective on viruses which could potentially escape traditional detection methods.

CcoV was constantly detected in samples together with CPV-2 strains, confirming that these viruses are often present as coinfections (Alfano et al. 2019; Decaro et al. 2012b, 2013; Hao et al. 2019; Ntafis et al. 2012; Pinto et al. 2014; Zicola et al. 2012). Also coinfections with the different genotypes CcoV-I and CcoV-IIa observed in this study were previously reported by several authors (Table 2) Alves et al. 2018; Costa et al. 2014; Decaro et al. 2005, 2009c; Erles and Brownlie 2009; Ntafis et al. 2013; Pratelli et al. 2004, Pratelli 2011; Soma et al. 2011). It was reported that dogs infected with CCoV alone are likely to have mild self-limiting diarrhoea, whereas the pathogenicity of the disease can increase when co-infections with CPV-2 strains or other intestinal viruses, such as canine adenovirus type 2 or canine distemper virus, occur (Alves et al. 2018; Decaro et al. 2004, 2007; Pratelli et al. 1999a, 2001; Tennant et al. 1991). However more virulent strains of CCoV, able to cause a more severe form of enteritis in the absence of coinfection, have occasionally been reported by Evermann et al. (2005). Moreover, co-infection with different CcoV genotypes, as observed in our study, favour recombination and mutations within the S gene with subsequent emergence of novel strains with new distinct pathogenic properties (Licitra et al. 2014).

In this study it was not possible to establish if positive dogs were affected by the pantropic or the enteric form of CcoV-IIa, for extra-intestinal tissues were not available from these patients, and sequence analysis of the S gene has not proved effective in discriminating pantropic from enteric forms (Alfano et al. 2019, 2020; Chen et al. 2019). Also, no diagnostic tools are currently available to differentiate pCCoV from enteric CCoV strains (Decaro et al. 2013).

The short AstV sequence length, the recombination reported for AstV by other authors (Li et al. 2018), and the evidence that canine AstVs are genetically and antigenically heterogeneous (Martella et al. 2012) hampered any post sequencing analyses but allowed to confirm PCR results. Reports on canine AstVs in Italy are scarce and human AstVs are considered the second or third most common cause of viral diarrhoea in children (Martella et al. 2011a, b, 2012; Mendez and Arias 2007; Toffan et al. 2009). Since interspecies transmission events have been suggested (De Benedectis et al. 2011; Japhet et al. 2019; Martella et al. 2011a; Mihalov-Kovács et al. 2017; Ulloa and Gutierrez 2010; van Hemert et al. 2007; Xiao et al. 2013), new studies are needed to confirm the zoonotic potential of canine AstV strains.

We confirm that canine parvovirus still remains one of the most important enteric viral pathogens of dogs in Italy, despite a widespread use of vaccines, and we recommend a continuous epidemiological surveillance to detect novel CPV variants which will appear in the future.

Concluding, other enteric viruses, such as canine CcoV and AstV, should not be overlooked, as they can be present as coinfectants in the same animal, increasing the severity of the disease and, in some cases, representing a zoonotic risk for humans.

Author contributions Research planning (Zobba R, Pinna Parpaglia ML, Visco S), Laboratory activities (Zobba R, Sotgiu F), clinical activity (Visco S, Pinna Parpaglia ML), writing – review and editing (Zobba R, Alberti A, pittau M), Supervision (Alberti A, Pittau M).

Data availability All data and materials are are available for publication.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent to participate All authors participated voluntarily in the research.

Consent for publication All authors read and approved the final manuscript.

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