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

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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
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; Ntufis 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. 1999), the sequences of all primers were 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. 1999), the sequences of all primers were shown in Table 1.

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 | CPV-2         | CCATGGAAAAACCAACCATACC AGTAAATCTCGTATCTCACCTCAA                         | Decaro et al. 2008a, b      | VP2     | 717                |
| CPV4116-R | CPV-2         | CAGGATCGAATTTGCTACA CATTTGGATAAAACGTGGT                                | Buonavoglia et al. 2001     | VP2     | 611                |
| Hfor      | CCoV Type-IIa | GGCTCTATACATAAATCAGTCTCCTAG GCTGAACACATKTCATCCCTACAC                  | Decaro et al. 2010a, b      | S        | 754                |
| Hrev      | CCoV Type-IIb | GGCTCTATACATAAATCAGTCTCCTAG CAAAATGAACCTTTGCTCTTGAT                             | Decaro et al. 2010a, b      | S        | 499                |
| 20,179    | CCoV-2        | GTGCTTCCTCTGGAAGGTACA TCGTGAGTAAATCCACGGCT                             | Pratelli et al. 2002        | M       | 239                |
| 174-268   | CCoV type I   | GTGCTTCCTCTGGAAGGTACA TCGTGAGTAAATCCACGGCT                             | Pratelli et al. 1999a, b    | RdRp    | 300                |
| CCov1a    | AsTVs         | GTACTATACCCRTGATTATATT AGACCAARGTGTCTAGT                                  | Martella et al. 2011a, b    | RdRp    | 318                |
| CCov2     | calicivirus_p290 | gtaatactcaaggggaactacag tgaatgaatcatacata                                 | Jiang et al. 1999           | RdRp    | 318                |
| AsTVs_625F-1 | calicivirus_p290 | gtaatactcaaggggaactacag tgaatgaatcatacata                                 | Jiang et al. 1999           | RdRp    | 318                |
| AsTVs_626F-1 | calicivirus_p290 | gtaatactcaaggggaactacag tgaatgaatcatacata                                 | Jiang et al. 1999           | RdRp    | 318                |
| calicivirus_p290 | calicivirus_p289 | gtaatactcaaggggaactacag tgaatgaatcatacata                                 | Jiang et al. 1999           | RdRp    | 318                |
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-Ila 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-Ila 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%).

Parovirus 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,
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 (GG121371). 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 2

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

### Table 3

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

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**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.

**References**

Alfano F, Dowgier G, Valentino MP, Galiero G, Tinelli A, Nicola D, Fusco G (2019) Identification of pantropic canine coronavirus in a wolf (*Canis lupus italicus*) in Italy. J Wildl Dis 55:504–508

Alfano F, Fusco G, Mari V, Occhiogrosso L, Miletti G, Brunetti R, Galiero G, Desario CC, Cirilli M, Decaro N (2020) Circulation of pantropic canine coronavirus in autochthonous and imported dogs, Italy. Transbound Emerg Dis 12:10

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Alves CDBT, Granados OFO, Budaszewski RDF, Streck AF, Weber MN, Cibulski SP, Pinto LD, Ikuta N, Canal CW (2018) Identification of enteric viruses circulating in a dog population with low vaccine coverage. Braz J Microbiol 49:790–794

Balboni A, Bassi F, De Arcangelis S, Zobba R, Dedola C, Alberti A, Battilani M (2018) Molecular analysis of carnivore Protoparvovirus detected in white blood cells of naturally infected cats. BMC Vet Res 14:41

Battilani M, Scagliarini A, Ciutili S, Morganti L, Prosperi S (2006) High genetic diversity of the VP2 gene of a canine parvovirus strain detected in a domestic cat. Virology 352:22–26

Battilani M, Balboni A, Ustulin M, Giunti M, Scagliarini A, Prosperi S (2011) Genetic complexity and multiple infections with more Parvovirus species in naturally infected cats. Vet Res 42:43

Battilani M, Balboni A, Giunti M, Prosperi S (2013) Co-infection with feline and canine parvovirus in a cat. Vet Ital 49:127–129

Battilani M, Modugno F, Mira F, Purpari G, Di Bella S, Guercio A, Balboni A (2019) Molecular epidemiology of canine parvovirus type 2 in Italy from 1994 to 2017: recurrence of the CPV-2b variant. BMC Vet Res 15:393

Binn LN, Norby EA, Marchwicki RH, Jarman RG, Keiser PB, Hang J (2018) Canine calcivirus of four serotypes from military and
research dogs recovered in 1963–1978 belong to two phylogenetic clades in the Vesivirus genus. Virol J 15:39

Bosch A, Guix S, Krishna NK, Méndez E, Monroe SS, Pantin-Jackwood M, Schultz-Cherry S (2012) Astrovirus. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds) Virus taxonomy: classification and nomenclature of viruses: ninth report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego, pp 953–959

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

Carman PS, Povey RC (1985) Pathogenesis of canine parvovirus-2 in dogs: haematology, serology, and virus recovery. Res Vet Sci 38:134–140

Chen S, Liu D, Tian J, Kang H, Guo D, Jiang Q, Liu J, Li Z, Hu X, Qu L (2019) Molecular characterization of HLV-073, a recombinant canine coronavirus strain from China with an ORF3abc deletion. Arch Virol 164:2159–2164

Costa EM, de Castro TX, Bottino Fde O, Garcia, Rde C (2014) Molecular characterization of canine coronavirus strains circulating in Brazil. Vet Microbiol 168:8–15

De Benedictis P, Schultz-Cherry S, Burnham A, Cattoli G (2011) Astrovirus infections in humans and animals - molecular biology, genetic diversity, and interspecies transmissions. Infect Genet Evol 11:1529–1544

Decaro N, Buonavoglia C (2012a) Canine parvovirus a review of epidemiological and diagnostic aspects with emphasis on type 2c. Vet Microbiol 155:1–12

Decaro N, Camero M, Greco G, Zizzo N, Elia G, Campolo M, Pratelli A, Buonavoglia C (2004) Canine distemper and related diseases: report of a severe outbreak in a kennel. N Microbiol 27:177–181

Decaro N, Martella V, Ricci D, Elia G, Desario C, Campolo M, Cavaliere N, Di Trani L, Tempesta M, Buonavoglia C (2005) Genotype-specific fluorogenic RT-PCR assays for the detection and quantitation of canine coronavirus type I and type II RNA in faecal samples of dogs. J Virol Methods 130:72–78

Decaro N, Campolo M, Elia G, Buonavoglia D, Colaianni ML, Lorusso A, Mari V, Buonavoglia C (2007) Infectious canine hepatitis: an “old” disease reemerging in Italy. Res Vet Sci 83:269–273

Decaro N, Campolo M, Lorusso A, Desario C, Mari V, Colaianni ML, Elia G, Martella V, Buonavoglia C (2008a) Experimental infection of dogs with a novel strain of canine coronavirus causing systemic disease and lymphopenia. Vet Microbiol 128:253–260

Decaro N, Desario C, Miccolupo A, Campolo M, Parisi A, Martella V, Amorisco F, Lucente MS, Cavalli A, Buonavoglia C (2008b) Genetic analysis of feline panleukopenia viruses from cats with gastrointestinal syndrome. J Gen Virol 89:2290–2298

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

Decaro N, Desario C, Parisi A, Martella V, Lorusso A, Miccolupo A, Mari V, Colaianni ML, Cavalli A, Di Trani L, Buonavoglia C (2009b) Genetic analysis of canine parvovirus type 2c. Virology 385:5–10

Decaro N, Mari V, Campolo M, Lorusso A, Camero M, Elia G, Martella V, Cordioli P, Enjuanes L, Buonavoglia C (2009c) Recombinant canine coronavirus strains related to transmissible gastroenteritis virus of swine are circulating in dogs. J Virol 83:1532–1537

Decaro N, Buonavoglia D, Desario C, Amorisco F, Colaianni ML, Parisi A, Terio V, Elia G, Lucente MS, Cavalli A, Martella V, Buonavoglia C (2010a) Characterisation of canine parvovirus strains isolated from cats with feline panleukopenia. Res Vet Sci 89:275–278

Decaro N, Mari V, Elia G, Addie DD, Camero M, Lucente MS, Martella V, Buonavoglia C (2010b) Recombinant canine parvovirus strains in dogs. Europe. Emerg Infect Dis 16:41–47

Decaro N, Desario C, Amorisco F, Losurdo M, Colaianni ML, Greco MF, Buonavoglia C (2011) Canine parvovirus type 2c infection in a kitten associated with intracranial abscesses and convulsions. J Feline Med Surg 13:231–236

Decaro N, Mari V, von Reitzenstein M, Lucente MS, Cirone F, Elia G, Martella V, King VL, Di Bello A, Varello K, Zhang S, Caramelli M, Buonavoglia C (2012a) A pantropic canine coronavirus genetically related to the prototype isolate CB/05. Vet Microbiol 159:239–244

Decaro N, Cordonnier N, Demeter Z, Egberink H, Elia G, Grelet A, Le Poder S, Mari V, Martella V, Ntafis V, von Reitzenstein M, Rottier PJ, Rusvai M, Shields S, Xylouri E, Xu Z, Buonavoglia C (2013) European surveillance for pantropic canine coronavirus. J Clin Microbiol 51:83–88

Dei Giudici S, Cubbedu T, Giagu A, Sanna G, Rocca S, Oggiano A (2017) First molecular characterization of canine parvovirus strains in Sardinia Italy. Arch Virol 62:3481–3486

Erles K, Brownlie J (2009) Sequence analysis of divergent canine parvovirus strains present in a UK dog population. Virus Res 141:21–25

Evermann JF, Abbott JR, Han S (2005) Canine coronavirus-associated puppy mortality without evidence of concurrent canine parvovirus infection. J Vet Diagn Invest 17:610–614

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Gabriel SS, Tohya Y, Mochizuki M (1996) Isolation of a calicivirus antigenically related to feline caliciviruses from feces of a dog with diarrhea. J Vet Med Sci 58:1041–1043

Greene CE, Decaro N (2012) Canine viral enteritis. In: Greene CE (ed) Infectious diseases of the dog and cat. Elsevier, New York, pp 67–75

Haake C, Cook S, Pusterla N, Murphy B (2020) Coronavirus infections in companion animals: virology, epidemiology, clinical and pathological features. Viruses 12:1023

Hao X, Liu R, He Y, Xiao X, Xiao W, Zheng Q, Lin X, Tao P, Zhou P, Li S (2019) Multiplex PCR methods for detection of several viruses associated with canine respiratory and enteric diseases. PLoS One 14(3):e0213295

Ikeda Y, Mochizuki M, Naito R, Nakamura K, Miyazawa T, Mikami T, Takahashi E (2000) Pseudomonad of canine parvovirus (CPV) in unvaccinated cat populations and emergence of new antigenic types of CPVs in cats. Virusology 278:13–19

Japhet MO, Faramurewa O, Adesina OA, Opaleye OO, Wang B, Höhne M, Bock CT, Mas Marques A, Niendorf S (2019) Viral gastroenteritis among children of 0–5 years in Nigeria: Characterization of the first Nigerian avichivirus recombinant noroviruses and detection of a zoo- notic astrovirus. J Clin Virol 111:4–11

Jiang X, Huang PW, Zhong WM, Farkas T, Cubitt DW, Matson DO (1999) Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. J Virol (1999) Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. J Virol Methods 83:145–154

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol 35:1547–1549

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal X and Clustal X version 20. Bioinformatics 23:2947–2948

Li M, Yan N, Ji C, Wang M, Zhang B, Yue H, Tang C (2018) Prevalence and genome characteristics of canine astrovirus in southwest China. J Gen Virol 99:880–889

Licitra BN, Duhamel GE, Whittaker GR (2014) Canine enteric coronaviruses: emerging viral pathogens with distinct recombinant spike proteins. Viruses 6:3363–3376
Ulloa JC, Gutiérrez MF (2010) Genomic analysis of two ORF2 segments of new porcine astrovirus isolates and their close relationship with human astroviruses. Can J Microbiol 56:569–577
van Hemert FJ, Berkhout B, Lukashov VV (2007) Host-related nucleotide composition and codon usage as driving forces in the recent evolution of the Astroviridae. Virology 361:447–454
Williams FP (1980) Astrovirus-like coronavirus-like and parvovirus-like particles detected in the diarrheal stools of beagle pups. Arch Virol 66:215–226
Xiao CT, Giménez-Lirola LG, Gerber PF, Jiang YH, Halbur PG, Opriessnig T (2013) Identification and characterization of novel porcine astroviruses (PAstVs) with high prevalence and frequent co-infection of individual pigs with multiple PAstV types. J Gen Virol 94:570–582
Zhou H, Liu L, Li R, Qin Y, Fang Q, Balasubramaniam VR, Wang G, Wei Z, Ouyang K, Huang W, Chen Y (2017) Detection and genetic characterization of canine astroviruses in pet dogs in Guangxi China. Virol J 14:156
Zicola A, Jolly S, Mathijs E, Ziant D, Decaro N, Mari V, Thiry E (2012) Fatal outbreaks in dogs associated with pantropic canine coronavirus in France and Belgium. J Small Anim Pract 53:297–300

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