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Canine circovirus and Canine adenovirus type 1 and 2 in dogs with parvoviral enteritis

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17

18

19 **Original Article**

20

21 **Canine circovirus and Canine adenovirus type 1 and 2 in dogs with parvoviral enteritis**

22

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38 **Abstract**

39 Canine parvovirus type 2 (CPV-2) is one of the most relevant pathogens associated with enteritis in dogs and is
40 frequently reported in association with the detection of other pathogens in faeces. In this study the concomitant
41 presence of Canine circovirus (CanineCV) and Canine adenovirus (CAAdV) DNA in faecal or intestine samples of
42 95 dogs with parvovirus enteritis sampled in Italy (1995-2017) was investigated and the viruses identified were
43 genetically characterised. Potential correlations with the antigenic variant of CPV-2 and with signalment data
44 and outcome were evaluated. Twenty-eight of 95 (29.5%) CPV-2 infected dogs tested positive to other viruses:
45 7/28 were also positive to CanineCV, 1/28 to CAAdV-1, 18/28 to CAAdV-2, 1/28 to CanineCV and CAAdV-2, and
46 1/28 to CAAdV-1 and CAAdV-2. The frequency of CAAdV DNA detection and coinfections was significantly higher
47 in purebred dogs compared to mixed breed ones ($P=0.002$ and 0.009 , respectively). The presence of coinfection
48 was not associated with any other relevant data available, including CPV-2 variant and final outcome. The
49 detection of CanineCV in a dog sampled in 2009 allowed to backdating its circulation in dogs. The eight
50 CanineCV completely sequenced were phylogenetically related to the CanineCV identified in dogs, wolves and a
51 badger from Europe, USA, Argentina and China. Nine CAAdV were partially sequenced and phylogenetic
52 analysis showed a separate branch for the oldest CAAdV-2 identified (1995). From the results obtained in this
53 study population, CanineCV and CAAdV coinfections in dogs with parvoviral enteritis did not result in more
54 severe disease.

55

56 *Keywords:* Canine adenovirus, Canine circovirus, Canine parvovirus, Coinfections, Dog, Enteritis

57 **Introduction**

58 Coinfections involving viral diseases represent an increasingly reported problem in veterinary medicine, as they
59 could worsen illness severity and interfere with diagnostic and therapeutic protocols (Kumar et al. 2018a).
60 Among viral diseases, viral enteritis are widespread causes of morbidity and mortality in dogs (Cardillo et al.,
61 2020), and are commonly sustained by multiple pathogens of different virulence (da Rocha Gizzi et al. 2014;
62 Deng et al. 2018; Ortega et al. 2017). Canine parvovirus type 2 (CPV-2) is one of the most relevant pathogens
63 causing severe acute haemorrhagic enteritis in dogs, being highly contagious and lethal especially in non-
64 vaccinated puppies (Decaro and Buonavoglia 2012; Mylonakis et al. 2016). CPV-2 infection is frequently
65 associated with the detection of other pathogens in faecal samples (Alves et al. 2018; da Rocha Gizzi et al. 2014;
66 Ortega et al. 2017; Qi et al. 2020; Zobba et al., 2021). Specifically, several studies reported the coexistence of
67 CPV-2 with Canine circovirus (CanineCV) and Canine adenovirus (CAAdV) type 1 and 2 (Dowgier et al.2017;
68 Headley et al. 2013; Headley et al. 2018; Headley et al. 2019). CanineCV are non-enveloped single-stranded and
69 circular DNA viruses, belonging to the genus *Circovirus* of the family Circoviridae, associated with several
70 disease entities (Bexton et al. 2015; Kapoor et al. 2012; Piewbang et al. 2018; Zaccaria et al. 2016). CAAdV are
71 non-enveloped double-stranded and linear DNA viruses belonging to the genus *Mastadenovirus* of the family
72 Adenoviridae, responsible of a serious multisystem disease, the infectious canine hepatitis (CAAdV-1), or
73 implicated in the aetiopathogenesis of infectious tracheobronchitis (CAAdV-2) (Decaro et al. 2008; Buonavoglia
74 and Martella 2007). CAAdV-2, although associated with respiratory disease, has also been frequently identified in
75 the stool or internal organs of dogs and wildlife (Balboni et al. 2013; Balboni et al. 2014; Chaturvedi et al.2008;
76 Dowgier et al. 2018). To date, although the reported frequency of these coinfections is high in dogs with
77 diarrhoea, the effects of multiple pathogens on the disease outcomes remain unclear (da Rocha Gizzi et al. 2014;
78 Dowgier et al. 2017). Furthermore, no information is available on the frequency of multiple infections in
79 association with the different antigenic variants of CPV-2 (2a, 2b and 2c) in dogs with enteritis.

80 Aims of this study were i) to investigate the presence of coinfections sustained by CanineCV and CAAdV (type 1
81 and type 2) in dogs with parvoviral enteritis; ii) to evaluate whether coinfections were related with the antigenic
82 variant of CPV-2, signalment data and outcome, ii) to genetically characterise the viruses identified.

83

84 **Materials and methods**

85 *Study design, inclusion criteria and samples*

86 For the purposes of the study, dogs with a diagnosis of parvoviral enteritis recorded from 1994 to 2017 were
87 retrospectively selected. The final diagnosis of parvoviral enteritis was achieved if dogs had clinical signs
88 consistent with enteritis including anorexia or lethargy, foul-smelling diarrhoea which could range from mucoid
89 to purely haemorrhagic, vomiting, dehydration and fever (Mylonakis et al. 2016), and if they tested positive to
90 CPV-2 DNA using a qualitative PCR assay (Mochizuki et al. 1993) carried out on faecal or intestine samples.
91 Samples were tested and DNA extracts were stored after routine diagnostic activity at the Service of Clinical
92 Pathology (Department of Veterinary Medical Sciences – DIMEVET, University of Bologna, Italy). Only dogs
93 for which the VP2 gene of CPV-2 was partially or completely sequenced [569 nucleotides (nts) (Balboni et al.
94 2018) or 1745 nts (Battilani et al. 2019), respectively] and the antigenic variant of CPV-2 deduced from the
95 predicted amino acid residue 426 were included in this study. Signalment data (year of sampling, sex, age, breed
96 and geographical origin), vaccination status and outcome of enrolled dogs were retrieved from medical records.

97

98 *Detection of Canine circovirus and Canine adenovirus type 1 and 2 DNA*

99 Viral DNA extraction from faecal or intestine samples was performed using the NucleoSpin Tissue Kit
100 (Macherey-Nagel, Germany) according to the manufacturer's instructions. The extracted DNA was eluted in 100
101 µL of elution buffer and stored at -20 °C until use. Canine circovirus DNA screening was carried out using a
102 SYBR Green real-time PCR (qPCR) assay according to De Arcangeli and collaborators (De Arcangeli et al.
103 2020). Canine AdV-1 and 2 DNA screening was carried out using a SYBR Green qPCR assay able to
104 discriminate the two viral types on the basis of melting curve analysis developed and validated by Balboni and
105 collaborators (Balboni et al. 2015). The two qPCR assays were performed using the PowerUp SYBR Green
106 Master Mix (Thermo Fisher Scientific, Life Technologies, USA) and the StepOnePlus Real-Time PCR System
107 (Thermo Fisher Scientific, Life Technologies, USA) following the manufacturer's instructions. Reactions were
108 carried out testing in duplicate eight 10-fold dilutions of a standard plasmid (pCR4 plasmid, Invitrogen, USA)
109 containing one copy of the target sequence used as external positive standard controls for the construction of the
110 assay standard curve, the DNA extracts and a no template negative control. Samples were considered positive
111 when the fluorescence curve in the amplification plot showed an exponential increase and a specific melting
112 peak was observed.

113

114 *Genetic characterisation of the viruses identified*

115 The complete genome of CanineCV was amplified in positive samples integrating rolling circle amplification
116 (RCA) and endpoint PCR methods (De Arcangeli et al. 2020). The RCA was performed on the positive samples
117 to increase the amount of circular DNA using the TempliPhi 100 amplification kit (GE Healthcare, USA)
118 following the manufacturer's instructions. Subsequently, viral DNA was amplified by end-point PCR using two
119 couples of primers and a proofreading DNA polymerase (Phusion Hot Start II High-Fidelity DNA Polymerase,
120 Thermo Fisher Scientific, USA) (De Arcangeli et al. 2020).

121 For the CAdV identified, amplification of partial E3 gene and flanking regions, and of hexon and fiber genes
122 was carried out according to Hu et al. (Hu et al. 2001) and Balboni et al. (Balboni et al. 2017), respectively.
123 Reactions were performed using a proofreading DNA polymerase (Phusion Hot Start II High-Fidelity DNA
124 Polymerase, Thermo Fisher Scientific, USA).

125 The amplicons obtained were directly sequenced, assembled, aligned with reference sequences from GenBank
126 (see **Online Resource 1** for CanineCV and **Online Resource 2** for CAdV) and translated into amino acid
127 sequences using the ClustalW method implemented in the BioEdit 7.2.5 software. The variability of the different
128 nucleotide residues of replicase (Rep) and capsid (Cap) genes of CanineCV and of hexon and fiber genes of
129 CAdV-2 was evidenced using entropy (H(x)) plot function implemented in BioEdit 7.2.5; only the reference
130 strains, from which both Rep and Cap or hexon and fiber genes sequences were available, were used for the
131 analysis (**Online Resource 1 and 2**). The total number of polymorphic sites, the total number of mutations,
132 nucleotide diversity, average number of nucleotide differences, and the number of haplotypes were calculated on
133 the viral genes using DnaSP package version 5.10.01 (Librado and Rozas 2009) and compared from the two
134 viruses.

135 Phylogeny was carried out on complete genome nucleotide sequences of CanineCV and on partial E3 gene and
136 flanking regions sequences and multiple gene sequences (concatenated hexon and fiber genes sequences) of
137 CAdV using MEGA X version 10.1.7 (Kumar et al. 2018b).

138

139 *Statistical analysis*

140 The data were evaluated using descriptive statistics and reported as median and range. Categorical data were
141 analysed using the Chi-squared test, while continuous data (age) were analysed by the Mann-Whitney *U* test.
142 Statistical significance was set at $P < 0.05$. Statistical analysis was performed using a commercially available
143 statistical software (MedCalc Statistical Software version 16.8.4).

144

145 **Results**

146 *Study population*

147 A total of 95 dogs with clinical signs indicative of parvoviral enteritis tested positive to CPV-2 DNA (90 from
148 faecal samples and five from intestine samples), sampled between 1995 and 2017, were included in this study
149 (**Table 1**). 48/95 (50.5%) dogs were males, 30/95 (31.6%) were females and for 17/95 (17.9%) this data was
150 unknown. The median age of all dogs was 3 months (range 1 month - 10 years). 65/95 (68.4%) dogs were
151 purebred, 24/95 (25.3%) were mixed breed and for 6/95 (6.3%) dogs this data was not available. On the basis of
152 the amino acid residue 426 of the predicted VP2 protein, the canine parvovirus identified belonged to CPV-2a
153 variant in 49/95 (51.6%) dogs, CPV-2b in 21/95 (22.1%) dogs and CPV-2c in 25/95 (26.3%) dogs. 32/95
154 (33.7%) dogs had undergone at least one administration of a trivalent modified live vaccine against canine
155 parvovirus (original CPV-2 or CPV-2b), infectious canine hepatitis (CA_{AdV}-2) and canine distemper (canine
156 distemper virus, CDV). This group was composed by dogs that undergone a full vaccination scheme and dogs
157 that undergone an incomplete vaccination scheme because they showed gastrointestinal signs and were sampled
158 when they were too young to complete the vaccination protocol. 54/95 (56.8%) dogs did not received any dose
159 of the vaccine and for 9/95 (9.5%) dogs this data was not available. 36/95 dogs (37.9%) recovered from the
160 disease, 22/95 (23.2%) died and for 37/95 (38.9%) dogs the outcome was not available. Based on the availability
161 of DNA extracts, 85/95 (89.5%) dogs were tested for the presence of both CanineCV and CA_{AdV} DNA, whereas
162 4/95 (4.2%) and 6/95 (6.3%) dogs were tested only for the presence of CanineCV and CA_{AdV} DNA, respectively.

163

164 *Detection of Canine circovirus and Canine adenovirus type 1 and 2 DNA*

165 From the 89 dogs tested for CanineCV DNA, 8 (8.9%) were positive (**Table 1**). The median amount of viral
166 DNA detected in tested samples was 1.8×10^3 copies of target DNA / μL of extracted DNA (range $5.1 - 1.9 \times 10^5$).
167 No significant association was found between the positivity to CanineCV DNA and the variables analysed
168 (**Table 1**). The oldest sample in which CanineCV DNA was identified was collected in 2009 (lab ID 800/2009).
169 From the 91 dogs tested to CA_{AdV} DNA, 21 (23.1%) were positive (**Table 1**): 1/21 was positive to CA_{AdV}-1,
170 19/21 to CA_{AdV}-2 and 1/21 was positive to both CA_{AdV} types. The median amounts of viral DNA detected in
171 tested samples were 6.1 (range 4.5 - 7.6) and 1.1×10^2 (range 5.7 - 8.5×10^5) copies of target DNA / μL of
172 extracted DNA for CA_{AdV}-1 and CA_{AdV}-2, respectively. The frequency of CA_{AdV} DNA positivity was
173 significantly higher in purebred dogs (all positive dogs were purebred) compared to mixed breed ones. ($P=0.002$,

174 **Table 1**). No other significant association was found between the positivity to CAdV DNA and the variables
175 analysed (**Tables 1**).

176 From the 95 dogs included in the study, 28 (29.5%) were coinfecting. In 26/28 coinfecting dogs, one other viral
177 DNA were detected in addition to CPV-2: 7/28 were positive to CanineCV, 1/28 was positive to CAdV-1 and
178 18/28 were positive to CAdV-2. The remaining 2/28 coinfecting dogs showed triple infection: one was positive
179 for CPV-2, CanineCV and CAdV-2, and one was positive for CPV-2, CAdV-1 and CAdV-2. From 1994 to
180 2008, before the first detection of CanineCV DNA, 5/44 (11.4%) dogs included in the study were coinfecting by
181 CPV-2 and CAdV. From 2009 (year in which the first CanineCV was detected) to 2017, 23/51 (45.1%) dogs
182 included in the study were coinfecting. Among the coinfecting dogs, 13/28 (46.4%) were infected by CPV-2a
183 variant, 4/28 (14.3%) by CPV-2b and 11/28 (39.3%) by CPV-2c. No significant association was found between
184 the CPV-2 variant and the presence of other viruses. From the 28 coinfecting dogs, 26 were purebred and 2 were
185 mixed breed. A significant association was found between the presence of a coinfection and the purebred status
186 ($P=0.009$, **Table 1**). No other significant association was found between the state of coinfection and the
187 variables analysed (**Table 1**).

188

189 *Sequence data*

190 The complete genome sequences of eight CanineCV were obtained and were 2063 nts in length. The genome
191 structure was the same described elsewhere (Decaro et al. 2014; Kotsias et al. 2019; Li et al. 2013; Piewbang et
192 al. 2018; Urbani et al. 2021). Nucleotide alignment between the CanineCV sequences obtained and 110
193 reference sequences showed an overall nucleotide identity ranging from 80.8 to 100%. Entropy plot analysis
194 showed that nucleotide variation was equally distributed throughout the Rep and Cap genes (**Online Resource**
195 **3**). DnaSP analysis showed a very high and comparable nucleotide variability between the CanineCV Rep and
196 Cap genes, which are approximately the same length (**Table 2**). Furthermore, the values of nucleotide diversity,
197 average number of nucleotide differences and number of haplotypes calculated for CanineCV genes were clearly
198 higher than those calculated for CAdV-2 genes, regardless of the different number of sequences analysed. The
199 phylogenetic tree constructed with complete genome nucleotide sequences showed a well distinguishable
200 clustering of the CanineCV nucleotide sequences into five groups (**Fig. 1**), as previously reported by Urbani and
201 colleagues (Urbani et al. 2021). The CanineCV identified in this study were included in the group 1 together
202 with other CanineCV identified in dogs, wolves and a badger from Europe, USA, Argentina and China.

203 Nucleotide sequences of partial E3 gene and flanking regions were obtained for one CAdV-1 and eight CAdV-2.
204 The only one CAdV-1 E3 nucleotide sequence was 462 nts in length, while the eight CAdV-2 E3 were 870 nts in
205 length. The unrooted phylogenetic tree constructed with these nucleotide sequences and 71 reference sequences
206 identified in dogs, foxes and wolves showed a clear subdivision of CAdV sequences into two main clusters: the
207 CAdV-1 clade, including the only one CAdV-1 nucleotide sequence obtained in this study, and the CAdV-2
208 clade, including all the CAdV-2 sequenced in this study (**Online Resource 4**). The CAdV-2 618/1995 formed a
209 separated branch, while other CAdV-2 sequences obtained in this study clustered together. PCR products
210 specific for the hexon gene (2718 nts in length, corresponding to 905 amino acid residues) and the fiber gene
211 (1629 nts in length, corresponding to 542 amino acid residues) were generated from eight and seven CAdV-2,
212 respectively (GenBank ID: MT193135-MT193149). For both hexon and fiber genes, all the nucleotide sequences
213 obtained in this study showed a complete identity between themselves, except for CAdV-2 618/1995 that
214 showed for the two genes an identity of 99.7% and 99.4% with other viruses sequenced in this study. Entropy
215 plot analysis showed that nucleotide variation was equally distributed throughout the hexon gene, whereas
216 greater nucleotide variability was present in the 3' portion of the fiber gene (**Online Resource 5**). DnaSP
217 analysis showed greater nucleotide variability in the hexon gene than the fiber gene (**Table 2**), with a higher
218 number of polymorphic sites (12 and 6, respectively) and haplotypes (3 and 2, respectively). In the rooted
219 phylogenetic tree constructed from the concatenated nucleotide sequences of hexon and fiber genes obtained in
220 this study and 15 reference sequences, the CAdV-1 and CAdV-2 sequences formed two distinct clusters (**Fig.2**).
221 The CAdV-2 618/1995 formed a separated branch while other CAdV-2 sequences obtained in this study grouped
222 together with the vaccine strain Toronto A26/61.

223

224 **Discussion**

225 In this study, 28 out of 95 (29.5%) dogs infected by CPV-2 (1995-2017) were found also positive to at least a
226 different virus of those examined. In particular, 7/28 were also positive to CanineCV, 1/28 to CAdV-1, 18/28 to
227 CAdV-2, 1/28 to CanineCV and CAdV-2, and 1/28 to CAdV-1 and CAdV-2. From 2009 onwards there was an
228 increase in the frequency of viral coinfections detected: passing from 5/44 (11.4%) in 1994-2008 to 23/51
229 (45.1%) in 2009-2017. Since no CanineCV DNA was detected prior to 2009 in this study, the low frequency of
230 coinfection found can be explained by a genuine limited spread of the CanineCV in the dogs sampled before
231 2009 or by a degradation of the DNA due to prolonged storage of samples over time with reduced detection of
232 small amounts of viral DNA. No significant association was found between the CPV-2 variant and the presence

233 of the other viruses examined. Thus, it seems that the antigenic CPV-2 variant causing parvoviral enteritis is not
234 a predisposing factor for the onset of coinfections. Furthermore, no significant association was found between
235 the state of coinfection and all the variables analysed, with the exception of purebred status ($P=0.009$). Indeed,
236 26/28 coinfecting dogs were purebred, with all dogs testing positive to CAdV DNA that were purebred
237 ($P=0.002$). This result suggests a possible predisposition of purebred dogs infected by CPV-2 to be coinfecting
238 with CAdV. A previous study that investigated the presence of CAdV DNA in dogs referred to a veterinary
239 hospital in Italy did not detect this association (Balboni et al. 2014), but the study did not investigate the
240 presence of other infectious agents and the dogs were not enrolled on the basis of clinical signs related to
241 gastroenteritis. In the absence of epidemiological data to support this finding, the potential association found
242 between purebred dogs infected by CPV-2 and co-infection with CAdV should be considered with caution.
243 Indeed, this result could be a mere representation of the dogs included in the study, since the origins of most of
244 the included dogs were from one geographical location. In light of this, further studies are needed to confirm this
245 possible predisposition and clarify which factors can determine it. The mortality of CPV-2 infected dogs
246 appeared not increased if they were coinfecting with CanineCV or CAdV. Our findings agree with da Rocha
247 Gizzi and colleagues (da Rocha Gizzi et al. 2014) who did not report increased mortality in dogs coinfecting by
248 CPV-2 and other pathogens. In contrast, Anderson and colleagues (Anderson et al. 2017) reported a significantly
249 higher mortality rate of dogs coinfecting by CPV-2 and CanineCV. Different case series compositions and
250 variable epidemiological features of the considered viruses might explain such different results.
251 CanineCV DNA was detected in faecal samples of 8/89 (8.9%) dogs with parvoviral enteritis. Several studies
252 reported a higher frequency of CanineCV infection in diarrhoeic dogs (Dowgier et al. 2017; Hsu et al. 2016; Niu
253 et al. 2020). The low frequency of CanineCV infection found in our study might recognise a genuine limited
254 spread of the CanineCV in the dogs sampled before 2009 or a degradation of the DNA due to prolonged storage
255 of samples over time as discussed above for coinfections. CanineCV DNA was detected in a dog sampled in
256 2009 (lab ID 800/2009), two years before the first report of CanineCV infection in dogs (Kapoor et al. 2012).
257 This data, together with the identification of CanineCV DNA in arctic foxes (*Vulpes lagopus*) in 1996-2001 from
258 Svalbard archipelago (Urbani et al. 2021), supports the hypothesis that CanineCV has been circulating in canids
259 for much longer than previously assumed. All the complete CanineCV nucleotide sequences analysed showed an
260 overall identity $\geq 80.8\%$. According to the species demarcation threshold of 80% genome-wide nucleotide
261 sequence identity for members of the family Circoviridae (Breitbart et al. 2017; Rosario et al. 2017), this result
262 confirms the existence of a unique canine circovirus species, including the viruses detected in this study.

263 Phylogeny reconstruction evidenced that the CanineCV identified in this study were included in the group 1 of
264 five clusters (Niu et al. 2020; Urbani et al. 2021), together with other viruses identified in dogs, wolves and a
265 badger from Europe, USA, Argentina and China, supporting the hypothesis of a possible transmission of
266 CanineCV from dogs to wild carnivores and vice versa (Balboni et al., 2021; De Arcangeli et al. 2020).

267 CAdV DNA was detected in faecal samples of 21/91 (23.1%) dogs with parvoviral enteritis, the majority of
268 which belonged to type 2 (20/21, one of which tested positive to both CAdV-1 and CAdV-2). Ji and
269 collaborators (Ji et al. 2020) reported 19/224 (8.5%) dogs with diarrhoea tested positive for CAdV-2, five of
270 which were concomitantly infected with CPV-2, but no association between the infection with CAdV-2 and
271 clinical signs was demonstrated. A frequent detection of CAdV-2 DNA in faecal samples (58.8%) has already
272 been reported in a study performed on dogs not enrolled in relation to gastrointestinal signs and showing various
273 pathological conditions or no clinical symptoms (Balboni et al. 2014). . Further studies should be carried out to
274 investigate whether CAdV-2 may actually play a pathogenic role in gastrointestinal diseases. CAdV-2 sequences
275 analysis evidenced a clear distinction of the oldest virus detected in this study, identified in a dog sampled in
276 1995 (lab ID 618/1995), showing that CAdV-2 accumulated mutations in the following years. The other CAdV-
277 2 identified in this study grouped phylogenetically with the vaccine strain Toronto A26/61, suggesting that the
278 vaccine currently adopted should not exhibit reductions in efficacy.

279 From the comparison between CanineCV and CAdV genes, a greater nucleotide variability for CanineCV than
280 CAdV-2 emerged. Since viruses with small and circular genomes tend to mutate faster than viruses with large
281 and linear genome (Sanjuán and Domingo-Calap 2016; Shackelton et al. 2005), this result was expected from the
282 genome characteristics of the two viruses analysed: small single-stranded and circular DNA for CanineCV and
283 medium-large double-stranded and linear DNA for CAdV-2. In particular, CanineCV showed higher nucleotide
284 variability than the one reported for CPV-2, which has a similarly sized but linear genome and for which
285 genomic substitution rate comparable to those of RNA viruses was reported (Battilani et al. 2019; Shackelton et
286 al. 2005). This result highlights a greater propensity of viruses with circular genomes to accumulate mutations.

287 From the analysis of hexon and fiber genes sequences of CAdV-2, it was shown the presence of some nucleotide
288 mutations equally distributed throughout the hexon gene, and preferentially localized in the 3' portion of the
289 fiber gene. The 3' portion of the fiber gene codify for the head region (also known as the knob) of the fiber
290 protein, that is responsible to receptor binding and antigenic property (King et al. 2011). A large number of
291 mutations in this region of the fiber gene have already been reported for CAdV-2 in China by Ji and colleagues
292 (Ji et al. 2020) and for CAdV-1 in Italy by Balboni and colleagues (Balboni et al. 2019), highlighting that this

293 genetic region undergoes rapid evolution in all canine mastadenoviruses. Contrary to CAdV-1 (Balboni et al.
294 2019), a higher nucleotide variability was found in the CAdV-2 hexon gene compared to the fiber gene.
295 The present study has some limitations. First of all, a small number of dogs were included in a very long period
296 of time, limiting the representativeness of the results obtained. Secondly, only DNA viruses were screened in our
297 population due to the availability of stored DNA extracts in our lab. Thus, coinfections with RNA viruses (such
298 as canine coronavirus, canine distemper virus, canine calicivirus, canine astrovirus, etc.) were not investigated.
299 Moreover, for the purposes of the study, the frequency of coinfections was evaluated in dogs with parvoviral
300 enteritis; the inclusion of dogs with gastroenteritis of different origin as well as of healthy controls would have
301 better clarified the pathogenetic role of such coinfections. Finally, due to the retrospective nature of the study,
302 clinical data indicative of disease severity and days of hospital stay were not available, and data regarding
303 outcome were lacking for some patients. Hence, prospective studies focused on the clinical course of dogs with
304 parvoviral enteritis and eventually coinfecting by these viruses are needed to better understand the impact of such
305 coinfections in clinical practice.

306

307 **Conclusions**

308 In this study, we report new data on the concomitant presence of CanineCV and CAdV in dogs with parvoviral
309 enteritis. From the results obtained, the presence of CanineCV and CAdV coinfection was not associated with
310 the antigenic variant of canine parvovirus causing enteritis and coinfections do not seem to worsen the outcome
311 of enrolled dogs. The genetic characterisation of the identified viruses allowed clarifying new aspects concerning
312 spread and evolution of CanineCV and CAdV in the canine population. The detection of CanineCV in a dog
313 sampled in 2009 allowed to backdating its circulation in the domestic dog population and supports the
314 hypothesis that CanineCV circulates in canids for much longer than previously assumed.

315 **Declarations**

316 **Funding:** Not applicable

317 **Conflicts of interest/Competing interests:** The authors declare no conflict of interest.

318 **Availability of data and material:** The datasets generated and analysed during the current study are available in
319 the International Nucleotide Sequence Database Collaboration (INSDC) repository (<http://www.insdc.org/>; ID:
320 MT193135-MT193166).

321 **Code availability:** Not applicable.

322 **Authors' contributions:** Conceptualization: [Andrea Balboni, Massimo Giunti, Mara Battilani]; Methodology:
323 [Andrea Balboni, Alessia Terrusi, Lorenza Urbani, Silvia A M Stefanelli, Roberta Troia]; Formal analysis and
324 investigation: [Andrea Balboni, Alessia Terrusi, Lorenza Urbani, Silvia A M Stefanelli, Roberta Troia]; Writing
325 - original draft preparation: [Andrea Balboni, Alessia Terrusi, Lorenza Urbani]; Writing - review and editing:
326 [Massimo Giunti, Mara Battilani]; Resources: [Massimo Giunti, Mara Battilani]; Supervision: [Mara Battilani].

327 **Ethics approval:** The study was carried out using stored DNA extracts of faecal samples which had been
328 collected with the agreement of the dog owners for clinical and diagnostic purposes independent of the study. As
329 stored DNA extracts of faecal samples were used, no separate ethical approval was required for the study. All
330 efforts were made to minimise the discomfort of the animals during sampling.

331 **Consent to participate:** All authors participated voluntarily in the research.

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

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474 **Table 1** Descriptive statistics and frequency of infection among the positive CanineCV, CA₂V type 1 and 2, and coinfecting dogs included in this study

Variables	Total	CanineCV	<i>P</i> value	CA ₂ V (type 1 and 2)	<i>P</i> value	Coinfections	<i>P</i> value
Number of dogs	95	89 (93.7)		91 (95.8)		95	
Positive dogs		8 (8.9)		21 (23.1)		28 (29.5)	
Sex			0.765		0.205		0,217
Male	48 (50.5)	4 (4.5)		9 (9.9)		13 (13.7)	
Female	30 (31.6)	4 (4.5)		10 (11)		13 (13.7)	
NA ^a	17 (17.9)	-		2 (2.2)		2 (2)	
Breed			0.749		0.002		0,009
Mixed breed	24 (25.3)	2 (2.2)		-		2 (2)	
Purebred	65 (68.4)	6 (6.7)		21 (23.1)		26 (27.4)	
NA ^a	6 (6.3)	-		-		-	
Geographical origin			0.999		0.663		0,576
Emilia Romagna	79 (83)	8 (8.9)		20 (22)		27 (28.4)	
Tuscany	2 (2)	-		-		-	
Campania	1 (1.1)	-		1 (1.1)		1 (1.1)	
Veneto	4 (4.2)	-		-		-	
Marche	1 (1.1)	-		-		-	
Lazio	1 (1.1)	-		-		-	
Abruzzi	1 (1.1)	-		-		-	
Basilicata	1 (1.1)	-		-		-	
Friuli Venezia Giulia	1 (1.1)	-		-		-	
Piedmont	1 (1.1)	-		-		-	
Apulia	1 (1.1)	-		-		-	
NA ^a	2 (2)	-		-		-	
CPV-2 variant			0.247		0.164		0,147
2a	49 (51.6)	2 (2.2)		11 (12.1)		13 (13.7)	
2b	21 (22.1)	2 (2.2)		2 (2.2)		4 (4.2)	
2c	25 (26.3)	4 (4.5)		8 (8.8)		11 (11.6)	
Vaccine administration			0.475		0.089		0,063
Yes ^b	32 (33.7)	4 (4.5)		11 (12.1)		14 (14.7)	
No ^c	54 (56.8)	3 (3.3)		9 (9.9)		12 (12.6)	
NA ^a	9 (9.5)	1 (1.1)		1 (1.1)		2 (2)	

Exitus			0.836		0.729		0,498
Survivors	36 (37.9)	5 (5.6)		12 (13.2)		16 (16.8)	
Dead	22 (23.2)	2 (2.2)		5 (5.5)		7 (7.4)	
NA ^a	37 (38.9)	1 (1.1)		4 (4.4)		5 (5.3)	
Age (months) ^d	3 [1-120]	3.5 [2-11]	0.542	3.5 [1-11]	0.773	3 [1-11]	0,721

475

476 The chi-squared test and the Mann-Whitney *U* test (age) were carried out on the positive and negative CanineCV, CAdV type 1 and 2, and coinfecting and non-coinfecting
477 dogs. Data are reported as n (%). ^a Not available data was excluded to statistical analysis. ^b Dogs undergone at least one administration of a trivalent modified live vaccine
478 against canine parvovirus (original CPV-2 or CPV-2b), infectious canine hepatitis (CAdV-2) and canine distemper (canine distemper virus, CDV); this group was composed
479 by dogs that undergone a full vaccination scheme or dogs that undergone an incomplete vaccination scheme because they showed gastrointestinal signs and were sampled
480 when they were too young to complete the vaccination protocol) ^c Dogs did not received any dose of the vaccine. ^d Data are reported as median [range]. Values in bold
481 indicate statistical significance. NA, not available; CPV-2, canine parvovirus type 2; CanineCV, canine circovirus; CAdV, canine adenovirus

482 **Table 2** Summaries of sequence variability of CanineCV and CAdV-2 genes

Sequences	<i>No. of sequences</i>	<i>Total no. of sites</i>	<i>S</i>	η	π	<i>k</i>	<i>h</i>
CanineCV - Rep gene	109	909	386	580	0.11975 SD 0.00430	109.04961	97
CanineCV - Cap gene	109	810	374	567	0.14514 SD 0.00256	117.99762	93
CAdV-2 - hexon gene	8	2715	12	12	0.00110 SD 0.00060	3.0	3
CAdV-2 - fiber gene	8	1626	6	6	0.00092 SD 0.00066	1.5	2

483

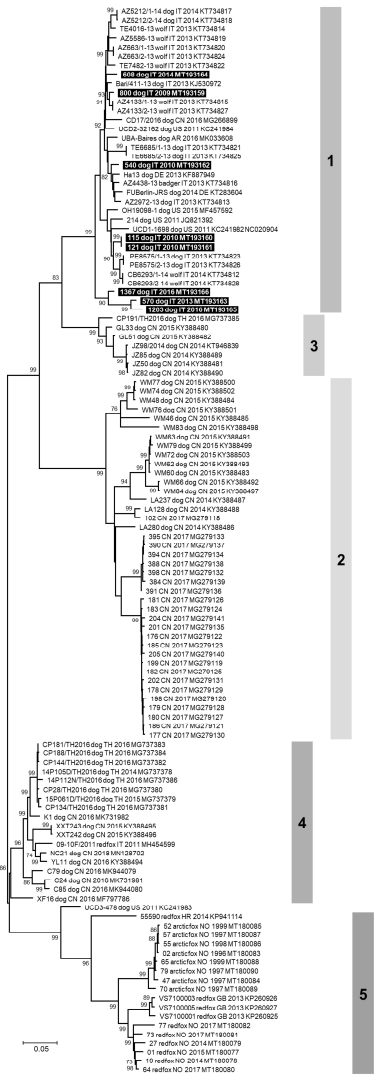
484 CanineCV, canine circovirus; CAdV-2, canine adenovirus type 2; *S*, total number of polymorphic sites; η , total

485 number of mutation; π , nucleotide diversity (average number of nucleotide differences per site) and standard

486 deviation; *k*, average number of nucleotide differences; *h*, number of haplotypes

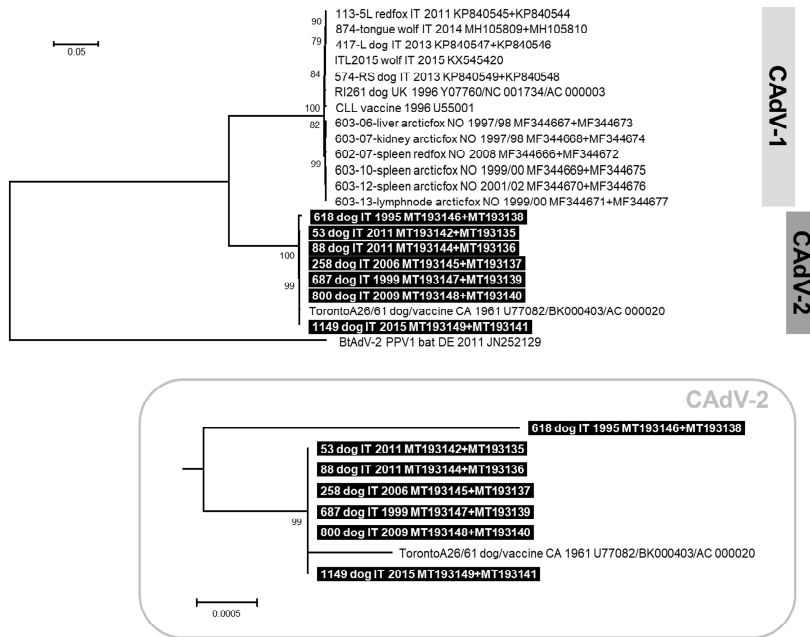
487 **Figure legends**

488



489

490 **Fig. 1** Unrooted phylogenetic tree based on the complete genome of canine circovirus (CanineCV) obtained in
 491 this study and 110 reference strains retrieved from the GenBank database (**Online Resource 1**). Phylogeny was
 492 carried out using the software MEGA X version 10.1.7 (Kumar et al. 2018b) and the Maximum Likelihood
 493 method. The best-fit model of nucleotide substitution was determined using the Find Best DNA/Protein Model
 494 function implemented in MEGA X. General Time Reversible (GTR) model with gamma distribution and
 495 invariable sites resulted optimal for the sequence data. Statistical support was provided by bootstrapping with
 496 1000 replicates. Bootstrap values greater than 70% are indicated on the respective branches. The scale bars
 497 indicate the estimated numbers of nucleotide substitutions. Highlighted in black: Sequences generated in this
 498 study. Numbers in grey are the groups evidenced in this study and from 1 to 4 correspond to genotypes proposed
 499 by Niu et al. (Niu et al. 2020)



500

501 **Fig. 2** Rooted phylogenetic tree constructed with nucleotide sequences of concatenated nucleotide sequences of

502 hexon and fiber genes obtained in this study and 15 reference strains retrieved from the GenBank database

503 (**Online Resource 2**). Phylogeny was carried out using the software MEGA X version 10.1.7 (Kumar et al.

504 2018b) and the Maximum Likelihood method. The best-fit model of nucleotide substitution was determined

505 using the Find Best DNA/Protein Model function implemented in MEGA X. The Hasegawa-Kishino-Yano

506 (HKY) model with gamma distribution and invariable sites resulted optimal for the sequence data. Statistical

507 support was provided by bootstrapping with 1000 replicates. Bootstrap values greater than 70% are indicated on

508 the respective branches. The scale bars indicate the estimated numbers of nucleotide substitutions. Highlighted in

509 black: Sequences generated in this study. On the bottom of the figure, a portion of the obtained tree is enlarged

510 to better visualise the phylogenetic relationships existing between the CAdV-2 nucleotide sequences