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A molecular survey for selected viral enteropathogens revealed a limited role of *Canine circovirus* in the development of canine acute gastroenteritis

Giulia Dowgier, Eleonora Lorusso, Nicola Decaro*, Costantina Desario, Viviana Mari, Maria Stella Lucente, Gianvito Lanave, Canio Buonavoglia, Gabriella Elia

Department of Veterinary Medicine, University of Bari, Valenzano, Bari, Italy

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ABSTRACT

Canine circovirus (CanineCV) is a canine virus, whose pathogenetic role is still uncertain. Based on recent data suggesting its role as entheropathogen, a case-control study was conducted between 2013 and 2016 to investigate the association of CanineCV with gastroenteritis in dogs, alone or in combination with other viral pathogens, including canine parvovirus (CPV), canine coronavirus (CCoV) and canine distemper virus (CDV). A total of 219 dogs suffering from acute gastroenteritis disorders and 67 controls randomly recruited among healthy dogs or patients presenting without enteric signs were screened by a panel of real-time (RT-)PCR assays for CanineCV, CPV, CCoV and CDV. A high prevalence of viral infections was detected in dogs with gastroenteritis (77.16%), with CPV representing the most frequently detected enteropathogen, followed by CanineCV and CCoV. While CPV and CCoV infections displayed a strong association with occurrence of acute gastroenteritis ($p < 0.00001$), detection of CanineCV in control dogs (28.35%) occurred with prevalence comparable to that of clinical cases (32.42%), so that its correlation with gastrointestinal disease was not statistically supported ($p = 0.530988$). Different from the clinical cases, where co-infections were frequently observed, all positive samples from the control group contained single infections. Noteworthy, a significant association was calculated between co-infections with CanineCV and occurrence of acute gastroenteritis ($p < 0.00001$). This study supports the role of CanineCV as a co-pathogen in the development of gastrointestinal disease, mainly acting in synergism with other enteric viruses.

1. Introduction

Viral enteropathogens that are mainly reported in dogs consist of canine parvovirus (CPV) (Decaro and Buonavoglia, 2012) and coronavirus (CCoV) (Decaro and Buonavoglia, 2011), although other agents have been traditionally related to enteric disease, such as canine distemper virus (CDV) (Martella et al., 2008a), canine adenovirus type 1 (CAdV-1) (Decaro et al., 2008a), rotaviruses (Eugster and Sidwa, 1979), reoviruses (Kokubu et al., 1993), caliciviruses (Mochizuki et al., 1993), including noroviruses (Martella et al., 2008b) and sapoviruses (Li et al., 2011), astroviruses (Martella et al., 2012) and kobuviruses (Li et al., 2011; Di Martino et al., 2013). More recently, dog circovirus (CanineCV) has been reported in diarrheal dogs from several countries (Li et al., 2013; Decaro et al., 2014; Hsu et al., 2016; Thaiwong et al., 2016). Circoviruses (family *Circoviridae*, genus *Circovirus*) are non-enveloped spherical viruses with a small monomeric single-stranded circular DNA of approximately 2 kb in length (Kapoor et al., 2012). Currently, the genus *Circovirus* consists of a number of species detected in domestic and wild birds, and some mammalian species, including two swine viruses, *Porcine circovirus 1* (PCV-1) and *Porcine circovirus 2* (PCV-2). Infections with either porcine or avian circoviruses are characterised by clinical courses that may vary from asymptomatic infections to lethal disease. In pigs, PCV-1 completely lacks any pathogenic role and single infections with PCV-2 rarely conduct to severe clinical disease. However, concurrent infections with other viruses or bacteria have been demonstrated to enhance PCV-2 replication in target tissues, increasing the severity of the induced lesions and the clinical course (Opriessnig and Halbur, 2012). CanineCV has been firstly reported in serum samples from dogs with no clinical history (Kapoor et al., 2012). Subsequent reports suggest that CanineCV is circulating in dogs, causing haemorrhages (Li et al., 2013) or severe gastroenteritis (Decaro et al., 2014; Taiwong et al., 2016; Zaccaria et al., 2016). However, the exact role of this virus in the development of clinical disease is still unclear. Therefore, the aim of this study was to investigate the association of CanineCV with gastroenteritis in dogs, alone or with other viral pathogens.

* Corresponding author at: Department of Veterinary Medicine, University of Bari, Strada per Casamassima Km. 3, 70010 Valenzano, Bari, Italy.
E-mail address: nicola.decaro@uniba.it (N. Decaro).

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2. Materials and methods

2.1. Study design

A case-control study was conducted over two subsets of dogs, selected on the basis of the presence of acute gastroenteric signs for clinical cases and the absence of gastroenteritis for controls. Samples were collected between 2013 and 2016 from dogs presenting at the Department of Veterinary Medicine of the University of Bari, Italy, as well as from diagnostic laboratories, private practitioners, animal shelters, commercial dog brokers and breeding kennels. A total of 219 patients suffering from gastrointestinal disorders were enrolled, considering as inclusion criteria the presence of mild to severe disease. Control subjects ($n = 67$) were randomly recruited among both healthy dogs or patients presenting without clinical signs of gastroenteritis and matching with cases for age and living conditions, in order to avoid statistically significant differences between the two subsets of animals. Dogs of 1 year of age or older were classified as adults, accordingly to pet food industry standard categorisation ($n = 37$ for cases, 16.9%; $n = 11$ for controls, 16.4%), whereas dogs younger than 1 year were considered young or puppies, including in the study only dogs older than 1 month ($n = 182$ for cases, 83.1%; $n = 56$ for controls, 83.6%). The sampled dogs were client-owned ($n = 159$ for cases, 72.6%; $n = 52$ for controls, 77.6%) or shelter dogs ($n = 60$ for cases, 27.4%; $n = 15$ for controls, 22.4%), representing different living conditions. Ages of the selected animals, along with clinical data and vaccination records, were collected in order to correctly support the inclusion criteria adopted and provide elements for further analyses. The presence of CanineCV, CPV, CCoV and CDV was investigated in all samples by molecular assays and further characterisation of the positive samples was carried out for each pathogen, as subsequently described. Finally, statistical analysis was performed in order to investigate the possible interaction among the viruses detected.

2.2. Sample processing

Faecal samples and/or rectal swabs were collected from all cases and controls and submitted to our lab for virological investigations and molecular analysis. Collected swabs were immersed in 1 ml of viral transport medium consisting of Dulbecco's modified Eagle's medium (DMEM), whereas faeces were homogenised (10% w/v) in DMEM and subsequently clarified by centrifuging at 2500*g* for 10 min. DNA and RNA were extracted from 200 µl of viral suspension by using the QIAamp *Cador* Pathogen Mini Kit (Qiagen S.p.A., Milan, Italy), following the manufacturer's instructions. Each sample was eluted in 100 µl of AE buffer (elution buffer) and stored at −70 °C until use.

2.3. Molecular analyses

All the nucleic acid extracts were screened for CanineCV (Li et al., 2013), CPV (Decaro et al., 2005a, 2006a), CCoV (Decaro et al., 2004) and CDV (Elia et al., 2006) by a panel of real-time PCR assays based on the TaqMan or minor groove binder (MGB) probe technology preceded by a reverse transcription step when appropriate. TaqMan and MGB probe assays were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Srl) with iTaq Universal Probes Supermix (Bio-Rad Laboratories Srl, Milan, Italy). Samples were considered positive if the amplification curves were higher than the threshold line generated by the software on the basis of the background fluorescence. Briefly, specific detection of CanineCV was carried out following the method proposed by Li et al. (2013), with minor modifications. Detection of CPV was primarily carried out by a generic TaqMan assay able to detect all carnivore protoparvoviruses (Decaro et al., 2005a). Samples that tested positive were further characterised by means of a panel of MGB probe assays able to discriminate between CPV/feline panleukopenia virus, CPV-2a/2b, CPV-2b/2c and CPV vaccine/field viruses (Decaro et al., 2006a,b, 2008b). The specificity and sensitivity of all molecular assays used in the study had been previously calculated (Decaro et al., 2005a, 2006a,b, 2008b). Similarly, screening of all samples for CCoV and CDV was performed through previously established TaqMan-based real-time RT-PCR assays (Decaro et al., 2004; Elia et al., 2006). For CDV, samples resulted positive were characterised using a discriminative hemi-nested PCR (Martella et al., 2007). RT-PCR and PCR assays were performed using Superscript™ One-Step RT-PCR for Long Templates (Life Technologies, Monza, Italy) and LA PCR Kit Ver 2.1 (Takara Bio Inc., Shiga, Japan), respectively. Samples were considered positive if amplicons of the expected size were visualised after gel electrophoresis on an imaging system (Gel Doc™ EZ System with Image Lab software, Bio-Rad Laboratories).

2.4. Data analysis

Sample sizes and characteristics were selected setting a statistical significance to $p < 0.05$ with an absolute precision of 0.11 based on an estimated prevalence, in order to allow a reliable comparative analysis between the control group and the cases. For each pathogen, statistical analysis was performed to evaluate the association with the clinical status, specifically with gastrointestinal disease. Comparison between cases and controls was carried out examining the data with a Chi-square test or Fisher's exact test when appropriate, considering significant values of $p < 0.05$, calculated by the statistical software R (R version 3.3.0; http://www.r-project.org/). In addition, results were evaluated in reference to age and living conditions of the sampled dogs in order to assess whether these could represent risk factors. Odds ratio and 95% confidence interval CI (95%) were calculated based on the analysis provided by the online tool Medcalc® (https://www.medcalc.org/calc/odds_ratio.php). Logistic regression was used to identify possible association among different pathogens detected in the same sample and to evaluate the role of co-infections in the development of disease.

3. Results

3.1. Detection of viral agents and association with enteric disease

The results of the molecular detection of the selected viral pathogens are listed in Tables 1–3. Viral RNAs/DNAs from CanineCV, CPV, CCoV and CDV, alone or in co-infections, were detected in 190 dogs out of 286 faecal samples tested for entheropathogens using molecular assays (66.43%; 95% CI: 61–71.98%). Of the 219 animals enrolled as cases, 169 were infected by at least one viral agent (77.16%; 95% CI: 71.6–82.72%), whereas among the 67 dogs taken as controls, viral RNA/DNA was found in 21 samples (31.35%; 95% CI: 20.25–42.45%) (Table 1). An extremely significant association was observed between molecular detection of viral RNA/DNA and occurrence of diarrhoea in dogs (OR 7.40, 95% CI: 4.04–13.55, $p < 0.0001$), thus confirming the important role played by viral agents in the development of gastrointestinal disease.

Not surprisingly, CPV was the pathogen most frequently detected in clinical cases, with a 57.99% prevalence (127/219, 95% CI: 51.46–64.52%) and the majority of cases being infected by the variant 2a (57/219, 26.02%) (Table 2). Consequently, association between CPV and gastrointestinal disease was fully supported by statistical analysis ($p = 0.0001$) and a statistical association was also evident with regard to age, since puppies were more frequently infected than adults (OR 3.73, 95% CI: 1.78–7.84, $p = 0.0005$). Three CPV positive faeces from diarrhoeic dogs (1.36%) and one sample from the control group (1.49%) were proven to contain CPV-2 (vaccinal strain), which was supported by an anamnesis of recent vaccination.

CCoV prevalence was 24.65% (54/219; 95% CI: 19.97–29.33%) for cases and 1.49% (1/67; 95% CI: 0–4.39%) for controls, with no association with age ($p = 0.191287$). Association with enteric disease was strongly supported by statistical analysis ($p < 0.0001$).

Table 1
Enteric disease associated to single or multiple infections.

Results	Cases (%) (n = 219)	95% CI	Controls (%) (n = 67)	95% CI	OR (95% CI)	p-value
Total	169 (77.16)	71.6–82.72%	21 (31.35)	20.25–42.45%	7.40 (4.04–13.55)	< **0.00001**[b]
Single infections	94[a] (42.92)	36.37–49.47%	20 (29.85)	18.9–40.8%	4.32 (2.30–8.09)	< **0.00001**[b]
Co-infections	75 (34.24)	27.96–40.52%	1[a] (1.49)	0–6.65%	ND	< **0.00001**[b]
Dual infections	67 (30.59)	24.49–36.69%	1[a] (1.49)	0–6.65%	ND	< **0.00001**[b]
Triple infections	7[a] (3.19)	0.87–5.51%	0	ND	ND	ND
Quadruple infections	1[a] (0.45)	0–3.21%	0	ND	ND	ND

ND, not determined.
[a] Sample containing a CPV vaccinal strain.
[b] Bold numbers indicate statistically significant p values ($p < 0.05$).

Table 2
Prevalence of selected viral agents in dogs with and without acute gastroenteritis.

	Clinical cases (n = 219)		Controls (n = 67)		
Virus	Positive (%)	95% CI	Positive (%)	95% CI	p-value
CanineCV	71 (32.42)	26.23–38.61%	19 (28.35)	17.56–39.14%	0.530,988
CPV	127[a] (57.99)	51.46–64.52%	1[a] (1.49)	0–4.39%	< **0.00001**[d]
2a	57 (26.02)	20.21–31.83%	0	–	–
2b	39 (17.80)	12.74–22.86%	0	–	–
2c	28 (12.78)	8.36–17.2%	0	–	–
2[b]	3 (1.36)	0–2.89%	1 (1.49)	0–4.39%	–
CCoV	54 (24.65)	19.97–29.33%	1 (1.49)	0–4.39%	**0.000026**[d]
CDV	1[c] (0.45)	0–1.33%	1[c] (1.49)	0–4.39%	0.373,243

[a] Samples containing a CPV vaccinal strain are included.
[b] Vaccinal strain.
[c] The detected CDV was a vaccinal strain.
[d] Bold numbers indicate statistically significant p values ($p < 0.05$).

Finally, CDV was detected in single samples from clinical cases (0.45%; 95% CI: 0–1.33%) and controls (1.49%; 95% CI: 0–4.39%), but the virus was characterised as vaccinal strain in both animals. Therefore, CDV was excluded from statistical analysis, since the sporadic detection of vaccinal strains did not provide any meaningful data about its association with disease.

A total of 90 samples tested positive for CanineCV, including 71/219 cases (32.42%; 95% CI: 26.23–38.61%) and 19/67 controls (28.35%; 95% CI: 17.56–39.14%) (Table 2). Correlation of single CanineCV infections with gastrointestinal disease was not statistically supported using Chi-square calculations ($p = 0.530988$). CanineCV loads were generally low, ranging from 3.57×10^1 to 8.37×10^8 (mean of 1.03×10^3) and from 8.60×10^1 to 5.38×10^5 (mean of 2.45×10^2) viral DNA copies μl^{-1} of template for clinical cases and control animals, respectively.

Table 3
Co-infections with CanineCV and other viruses.

Viruses	Clinical cases (%) (n = 71)	95% CI	Controls (%) (n = 19)	95% CI
CanineCV	13 (18.30)	9.31–27.29%	19 (100)	ND
Co-infections	58 (81.69)	72.70–90.68%	0	ND
CanineCV + CPV	41[a] (57.74)	46.25–69.22%	0	ND
CanineCV + CCoV	9 (12.67)	4.94–20.40%	0	ND
CanineCV + CPV + CCoV	7[a] (9.85)	2.92–16.78%	0	ND
CanineCV + CPV + CCoV + CDV	1[a,b] (1.40)	0–4.13%	0	ND

ND, not determined.
[a] Including one sample containing a CPV vaccinal strain.
[b] The detected CDV was a vaccinal strain.

3.2. Co-infections and association with enteric disease

As shown in Table 1, 94 ill dogs had single infections (42.92%; 95% CI: 36.37–49.47%), 67 had dual infections (30.59%; 95% CI: 24.49–36.69%) and 7 had triple infections (3.19%; 95% CI: 0.87–5.51%). In a single sample (0.45%; 95% CI: 0–3.21%), all the four viruses investigated in this study were detected but, noteworthy, the CDV strain was characterised as vaccinal virus. Twenty out of 21 control dogs had single infections (29.85%; 95% CI: 18.9–40.8%) and one sample contained vaccinal strains of both CPV and CDV, as expected by recent vaccination (1.49%; 95% CI: 0–6.65%). Noteworthy, no cases of natural co-infections were detected in controls. Gastroenteric disease resulted as a dependent variable with regard to viral detection in single infections (OR 4.32, 95% CI: 2.30–8.09, $p < 0.0001$) and more significantly in co-infections ($p < 0.0001$), as also supported by logistic regression analysis.

Co-infections with CCoV/CPV (17/219 dogs, 7.76%; 95% CI: 6.02–9.5%) (data not shown) and CCoV/CanineCV (9/219 dogs, 4.10%, 95% CI: 1.48–7.72%) were observed only among clinical cases, as were the 7 triple CPV/CCoV/CanineCV infections (3.19%, 95% CI: 0.89–5.19%) (Table 3). However, the most frequent co-infection in clinical cases was caused by CPV and CanineCV (41/219; 18.72%, 95% CI: 13.56–23.88%). Different from dogs with single CanineCV infections, a significant association was calculated between co-infections with CanineCV and occurrence of acute gastroenteritis (OR 28.25, 95% CI: 7.26–109.88, $p < 0.00001$).

3.3. Influence of environment and age on the detection of viral agents

Analysing the living conditions, pathogen-positive dogs included 32.10% of kennelled (61/190; 95% CI: 25.47–38.73%) and 67.89% of owned dogs (129/190; 95% CI: 61.26–74.52%), compared with the

negative dogs that were represented by 14.58% of kennelled dogs (14/96; 95% CI: 7.52–21.64%), and 85.41% of owned dogs (82/96; 95% CI: 78.35–92.47%).

For clinical cases, kennelled and client-owned dogs were infected with at least one pathogen in 85% (51/60; 95% CI: 76–94%) and 74.21% (118/159; 95% CI: 67.41–81.01%), respectively. Therefore, diarrhoeic dogs housed in kennels resulted more likely positive to any of the pathogens tested with respect to owned animals (OR 3.24, 95% CI: 1.71–6.13, $p = 0.001466$). Among controls, positive samples were found in 66.7% (10/15; 95% CI: 42.85–90.55%) of dogs housed in kennels and only in 26.83% (11/41; 95% CI: 13.27–40.39%) of client-owned animals. Chi-square analysis from cases and controls conducted independently confirmed that control dogs housed in kennels were at higher risk to be infected by at least one pathogen compared to clinical cases (OR 7.45, 95% CI: 2.10–26.36, $p = 0.0018$ for controls; OR 1.96, 95% CI: 0.89–4.35, $p = 0.0939$ for cases).

As for age, all but 20 faecal samples that tested positive to at least one virus were collected from pups (170/286). However, albeit randomly collected, 182 out of the 219 samples from clinical cases had been collected from pups (83.1%) and dogs sampled as control groups were selected based on the proportion of the different ages among the clinical cases. Therefore, occurrence of pathogens was more likely reported in young dogs than in adults (OR 3.50, 95% CI: 1.84–6.63, $p < 0.0001$), but no statistical association was evident in the control group (OR 0.48, 95% CI: 0.12–1.79, $p = 0.2761$) compared to clinical cases (OR 8.06, 95% CI: 3.73–17.42. $p < 0.0001$).

Accordingly, infection with CanineCV was associated with age only for the clinical cases, where pups resulted more prone to be infected with this virus than adults (OR 4.80, 95% CI: 1.63–14.16, $p = 0.002067$).

4. Discussion

CanineCV is a canine virus, whose pathogenicity and association with clinical disease are still uncertain. With the aim to investigate the pathogenetic potential of CanineCV in the development of enteric signs, faecal specimens, collected from dogs with acute gastroenteritis and from control animals, were analysed for selected viral pathogens, i.e., CPV, CCoV, CDV and CanineCV. The obtained findings showed a high prevalence of viral infections in dogs with gastroenteritis, thus supporting the need to include the selected viral agents in the diagnostic flowchart of canine diarrhoea. However, 22.83% of the diarrhoeic dogs tested negative for any viruses investigated in this study, which may account for the presence of other, less common viral agents, bacteria or parasites.

The role of CPV, alone or in association with other viruses, in inducing severe gastroenteritis is well known (Decaro and Buonavoglia, 2012) and was confirmed by this study that accounted for a strong correlation between CPV infection and development of diarrhoea. As for CCoV, there is no agreement in the scientific community about the pathogenetic potential of this virus (Decaro and Buonavoglia, 2011; Gizzi et al., 2014; Schulz et al., 2008). Several studies have demonstrated the presence of CCoV in the faeces of dogs with diarrhoea in different countries, usually related to mild, self-limiting gastroenteritis (Decaro et al., 2005b, 2011; Stavisky et al., 2012; Cavalli et al., 2014; Costa et al., 2014; Licitra et al., 2014), although occasional detection of a hyper-virulent CCoV variant, referred to as pantropic CCoV, has been reported in severe, often fatal outbreaks (Buonavoglia et al., 2006; Decaro et al., 2013; Ntafis et al., 2012; Zicola et al., 2012; Pinto et al., 2014). The role of CCoV as enteropathogen has been recently reconsidered by Duijvestijn et al. (2016) that reported a strong association between diarrhoea and CCoV infection ($p = 0.001$) and results from our study also support this relation ($p < 0.0001$).

CanineCV was strongly associated to the occurrence of enteric disease only in co-infections with other, well-recognised pathogens (CPV or CCoV). While previous reports accounted for a primary role of CanineCV in the development of clinical disease (Li et al., 2013; Decaro et al., 2014), subsequent studies suggested that this virus can act as entheropathogen mainly when associated to other pathogens (Hsu et al., 2016; Thaiwong et al., 2016; Zaccaria et al., 2016). Our study has shown a high frequency of detection of CanineCV in diarrheal dogs (32.42%), similar to that reported in a recent study from China (28%) (Hsu et al., 2016) but greater with respect to a previous survey conducted in USA (11.3%) (Li et al., 2013) and to two very recent studies conducted in Germany (20.1%, Gentil et al., 2017; 3.64%, Anderson et al., 2017). However, in one of these German surveys (Anderson et al., 2017), the target canine population was quite different from that included in the present study, since it was represented by dogs displaying only haemorrhagic diarrhoea, while in our study non-haemorrhagic gastroenteritis was also considered. The CanineCV prevelance was higher in CPV-infected dogs (12.96%), thus confirming the role of this emerging virus as co-pathogen in the occurrence of acute gastroenteritis. In our study, CanineCV was also detected in dogs with no history of gastrointestinal signs, which displayed a virus prevalence comparable to that observed in the cases (28.35%). Consequently, no significant association was evident between single CanineCV infections and occurrence of acute gastroenteritis (p = 0.530988).

This finding was in agreement with a similar study conducted in the USA (Li et al., 2013), but not with Hsu et al. (2016) that reported a strong correlation between CanineCV and onset of diarrhoea. Noteworthy, the rate of co-infections with CanineCV and other pathogens in diarrhoeic dogs was high, accounting for 77.33% of the total number of co-infections. Overall, 81.69% of the CanineCV-positive samples tested positive for another pathogen. Compared with the controls, co-infections were remarkably related with disease ($p < 0.00001$), which was in agreement with the results reported in previous studies (Gizzi et al., 2014; Li et al., 2013; Zaccaria et al., 2016).

CanineCV loads in the faeces were generally low, although slightly higher in clinical cases than in controls (1.03×10^3 against 2.45×10^2 mean viral DNA copies μl^{-1} of template). This accounts either for a low viral replication in the intestinal mucosa, as a consequence of long-term infections, or for virus passage in the gut lumen without active replication. Accordingly, previous studies detected higher viral titres in internal organs than in the faeces of CanineCV infected animals (Zaccaria et al., 2014). Therefore, the potential role of CanineCV in the occurrence of extra-intestinal disease deserves to be investigated in future studies. A neurologic tropism has been suggested for circoviruses in foxes (Bexton et al., 2015), while the ability of PCV-2 to spread systemically has been reported in swine (Opriessnig et al., 2007). CPV and CCoV interact at the gut level enhancing their pathogenicity (Decaro and Buonavoglia, 2011) and the prolonged depletion of CD^{4+} T lymphocytes induced by the pantropic CCoV variant impairs the immune response of infected pups (Marinaro et al., 2010). Analogous to CPV/CCoV co-infections, a possible synergism between CPV and CanineCV may be hypothesised, so that CanineCV may exacerbate the clinical course of concurrent enteric infections through replication in the enteric epithelium, impairment of the immune response or both.

In the present study, CanineCV was detected with higher frequency in young than in adult dogs, which was in agreement with previous studies (Decaro et al., 2014; Thaiwong et al., 2016; Zaccaria et al., 2016). Quite obviously, a decrease in CanineCV prevalence related with age was observed, which may be due to the development of a specific immune response induced by previous infections with a progressive clearance of the infectious agent. Thus, an extensive serological survey in different age populations would provide interesting data corroborating this hypothesis.

Finally, the virus was more frequently detected in kennelled than in client-owned dogs, thus revealing its ability to spread in a restricted and high-density environment, as occurs for other viral pathogens. Similarly, the chance of PCV-2 transmission increases at the shortening

of the distance between infectious and susceptible animals (Rose et al., 2012).

Although all attempts to adapt CanineCV to grow in cell cultures were unsuccessful, only experimental infections would provide definitive data about virus pathobiology, including the identification of primary sites for CanineCV replication and its interaction with the host immune system.

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