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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

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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.

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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 ($p < 0.0001$).