Detection of canine parvovirus types 2b and 2c in canine faecal samples contaminating urban thoroughfares in Brazil.

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Title: Detection of canine parvovirus types 2b and 2c in canine faecal samples contaminating urban thoroughfares in Brazil.

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Abstract

Canine parvovirus type 2 (CPV-2) is a highly contagious virus that causes acute gastroenteritis in dogs all over the world. Due to its stability in the environment, CPV-2 can remain infective for long time, especially if protected in organic matter. To demonstrate the CPV-2 potential as an environmental hazard for non-immunized susceptible hosts, we investigated 50 fecal samples collected from public areas in a municipality of Paraná state, Brazil. Seven samples tested positive for CPV by a PCR assay targeting the partial VP2 gene, being three strains confirmed as CPV-2b and one as CPV-2c variant by sequence analysis. These findings were supported by a phylogenetic analysis and the species identity of fecal samples source was confirmed by canine mitochondrial DNA amplification and sequencing. Our results demonstrate the presence of CPV in canine faeces contaminating urban thoroughfares and reinforce the importance of environmental control to reduce the potential exposure risks to susceptible hosts.

Keywords: canine parvovirus, environment, mitochondrial DNA, faecal contamination, VP2 gene.
Introduction

Canine parvovirus type 2 (CPV-2) (family Paroviridae, subfamily Parovirinae, genus Protoparvovirus, specie Carnivore protoparvovirus 1) is a highly contagious virus that causes acute hemorrhagic gastroenteritis in young dogs with high morbidity and mortality rates (Nandi and Kumar 2010; Cotmore et al. 2019).

CPV-2 is a small, non-enveloped virus with a single-strand DNA genome of about 5 Kb. The CPV-2 genome is surrounded by an icosahedral capsid of approximately 25 nm in diameter, composed of structural proteins VP1 and VP2. VP2 is the most abundant structural protein and plays a major role in virus-host interaction regarding viral antigenicity and cell tropism (Decaro and Buonavoglia 2012). Like many non-enveloped viruses, CPV-2 is extremely resistant to adverse environmental conditions as well as physical and chemical agents (Lamm and Rezabek 2008).

Unlike most DNA viruses, CPV-2 presents a high substitution rate in its genome, similar to RNA viruses (Shackelton et al. 2005). Three major closely related genetic and antigenic variants have been reported for CPV-2. Indeed, few years later its emergence as a new pathogen of dogs in 1978, a new variant known as CPV-2a globally replaced the original CPV-2. This variant was characterized by amino acid substitutions at VP2 amino acids (aa) 87 (Met → Leu), 300 (Ala → Gly), 305 (Asp → Tyr) and 555 (Val → Ile) (Parrish et al. 1985).

In 1984, CPV-2b variant was first recognized in the USA, differing from CPV-2a at residues 426 (Asn → Asp) and 555 (Ile → Val) (Parrish et al. 1991). CPV-2c, the third variant, was identified in 2000 in Italy, with an important substitution at VP2 amino acid (aa residue 426 (Asp → Glu) (Buonavoglia et al. 2001). Therefore, antigenic differences among the three variants are associated to divergences at residue 426 (Asn in 2a, Asp in 2b and Glu in 2c) in the VP2 protein (Miranda and Thompson 2016).

The infection is generally transmitted through the fecal-oral route (Carmichael 2005). Viral shedding has been detected from 4 to 50 days post infection and decreasing levels from the peak were observed around the second week (Decaro et al. 2005a; Decaro and Buonavoglia 2012). Virus excretion with faeces begins at least four days before the onset of symptoms and continues for several weeks after clinical recovery. Within this period, infected hosts can shed billions of infectious viral particles per gram of feces (Decaro et al. 2005b). To limit the spread and to protect the dogs, modified live virus vaccines (MLV) have been recommended to provide protection against canine parvovirus individually and international guidelines prescribe a minimum of 75% immunization coverage of individuals to provide herd immunization and prevent epidemic outbreaks (Day et al. 2016).
In Brazil, the occurrence of canine parovirus outbreaks is still very common and may be associated with low vaccine coverage (Alves et al. 2018). Observed prevalence of CPV-2 infection in different regions of Brazil often exceed 50%. In a wide study conducted on six Brazilian states, 29.2% of positive samples was observed, including dogs with and without clinical signs (Pinto et al. 2012). Another research with only symptomatic dogs reported 54% of positive faecal samples (Fontana et al. 2013). A work conducted with healthy free-roaming dogs, found viral shedding in 67% of them (Vieira et al. 2017). When 325 dog rectal samples from eight different Brazilian states were analyzed, 54.3% were positive for CPV-2, including healthy and symptomatic animals (Alves et al. 2018). In addition, stray and free-roaming dogs associated with poor environmental management, increase the risk of CPV-2 infection in susceptible hosts (Curi et al. 2016).

There is a limited number of studies concerning the effect on infectiveness of long-term environmental exposure of CPV-2; nevertheless, for most authors it is safe to affirm that the virus can remain infective in faeces for more than five or six months (Pollock 1982; Gordon and Angrick 1986; McGaving 1987; Nandi and Kumar 2010; Day et al. 2016) or at least one year (Day et al. 2016).

Since CPV-2 is highly contagious and resistant in the environment, infections can result also from the exposure of susceptible dogs to contaminated environment as well. In order to identify and confirm a potential source of infection from the environment, we investigated the presence and determined the subtypes of CPV-2 in fecal samples collected from public urban areas.

### Materials and Methods

A total of 50 fecal samples were collected from September 2015 to March 2017 from public areas such as playgrounds, squares and sidewalks in a municipality of Palotina, 31,564 inhabitants, located in western region of Paraná State, southern Brazil (24°20'41" S 053°49'44"W).

Samples were stored at 4°C-8°C for a maximum of 24h and subjected to DNA extraction using a homemade protocol, based on a combination of phenol/chloroform/isoamyl alcohol and silica/guanidine isothiocyanate methods (Alfieri et al. 2006). A conventional polymerase chain reaction (PCR) assay was carried out to amplify the 583 base pair (bp) fragment of the VP2 encoding gene. The homemade reaction mixture (50 μL) consisted of 5 μL of template DNA and 45 μL of PCR-MIX composed of PCR buffer 1x (Thermo Fischer Scientific, USA) (20 mM Tris HCl pH 8.4 and 50 mM KCl), 20 pmol of each primer 555 for and 555 rev (Buonavoglia et al. 2001), 1.5 mM of MgCl2, 1.6 μM of each dNTPs, 2.5 U of TaqPlatinum® DNA Polymerase (Thermo Fischer Scientific, USA) and diethyl pyrocarbonate treated water. The following thermal conditions...
were used for amplification: initial denaturation step at 94°C for 10 min; 40 cycles of: denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min; a final extension step of 7 min at 72°C. The amplified products were detected by electrophoresis through a 2% agarose gel in TBE buffer pH 8.4 (89 mM Tris, 89 mM boric acid, 2 mM EDTA), under constant voltage (100 V) for approximately 60 minutes and visualization under UV light after bromide ethidium staining (solution 0.5 μg/mL).

In order to confirm the canine origin of faeces, all positive samples were further analyzed by conventional PCR assay using species-specific mitochondrial DNA marker, originally designed for a qPCR assay. Primers "dog forward" (5´-GGCATGCGCTTTCCCTACGGATTC-3´) and “dog reverse” (5´-GGGATGTGGCAACGAGTGTAATTATG-3´) were used to amplify a 102 bp fragment of the NADH dehydrogenase subunit 5 (ND5) gene (Caldwell and Levine 2009). The homemade reaction mixture (25 µL) consisted of 2.5 µL of template DNA and 22.5 µL of PCR-MIX composed of 1x PCR buffer (Thermo Fischer Scientific, USA) (20 mM Tris HCl, pH 8.4 and 50 mM KCl), 20 pmol of each primer, 1.5 mM of MgCl₂, 1.6 µM of each dNTP, 2.5 U of TaqPlatinum® DNA Polymerase (Thermo Fischer Scientific, USA) and diethylpyrocarbonate-treated water. The following three-step cycle program was used for amplification: initial denaturation at 94°C for 10 min; 40 cycles of: denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min; and a final extension step of 7 min at 72°C.

Amplified products were purified from TBE agarose gel using the PureLink® Quick Gel Extraction Kit (Thermo Fischer Scientific, USA), and DNA quantification was performed using Qubit™ fluorometer (Thermo Fischer Scientific, USA). DNA sequencing reaction was performed in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®) according to the manufacturer's instructions, in the ABI 3500 automatic gene analyzer (Applied Biosystems®).

The quality of the 583 bp and 102 bp sequences obtained was assessed by the Phred Electropherogram Quality Analysis software (http://asparagin.cenargen.embrapa.br/phph/) and the adopted criteria to cut-off score was equal or greater than 20, which means a base call accuracy of 99% (Patton 2006). Consensus sequences were aligned by the ClustalW software and the identity of the products was compared with canine parvovirus sequences retrieved from public databases (GenBank) which were select based on Query and E-value, using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The maximum-likelihood phylogenetic tree of the 583 bp sequences was inferred based on Tamura 3-parameter model as implemented in MEGA (version 7.0) (Saitou and Nei 1987; Tamura et al. 2013; Kumar et al. 2016). The sequence of feline parvovirus (Genbank accession number AJ249556) was used as outgroup for the
583 bp phylogenetic tree and it was evaluated by running 1,000 replicates in the bootstrap test (Felsenstein 1985). The sequences were submitted to GenBank (accession numbers MG560134-7).

Results

Out of 50 fecal samples tested, seven (14%) tested positive for CPV-2. However, after DNA sequencing, only four of these seven samples had enough quality for molecular characterization. The sequences analysis of four amplicons showed the amino acid Asp at the VP2-426 residue in three sequences and amino acid Glu in one sequence, which are consistent with the CPV-2b and CPV-2c variants, respectively. The characteristics of the faeces at the time of collection of the positive samples, the determined CPV-2 subtype and GenBank accession numbers are specified in Table 1. Phylogenetic analysis revealed the clustering according to the CPV-2 variants (Fig.1). The dog mitochondrial DNA fragment was successfully amplificated and sequenced from all positive samples, confirming the host source of the collected fecal sample.

Discussion

The direct fecal-oral contact allows rapidly dissemination of the CPV-2 (Goddard and Leisewitz 2010), while indirect spread is also important to virus maintenance in a population (Hoelzer and Parrish 2010). These two routes could acts as pathways to introduce or disseminate the virus to new geographic areas or populations such as wild carnivores, notably from sub-urban areas or fragmented forest (Steinel et al. 2001; Furtado et al. 2016; Vieira et al. 2017; Mira et al. 2018, 2019).

The aim of this study was to demonstrate that the presence of contaminated faeces in urban surfaces, such as dirt from public areas, could represent a potential source of CPV-2 infection through an indirect transmission route. Such environmental contamination is possible through high viral excretion rates by infected hosts and high viral particle resistance for long time, especially in organic matter (Nandi and Kumar 2010; Day et al. 2016; Cavalli et al. 2018). In this manner, the virus can be potentially found anywhere animal groups are also present, including parks and playgrounds (Nandi and Kumar 2010).

The present study aimed to identify CPV-2 in fecal samples collected in urban areas. The rate of positive results found (14%) was low but could represent an underestimation of the potential risk to susceptible populations; therefore, a larger study, based on a wider area and a higher number of faecal samples, is necessary to better clarify the real impact of contaminated faeces as risk for susceptible animals. Indeed, in studies conducted in several regions of Brazil, including individuals with and without diarrhea, 29.2% (Pinto et al. 2012)
and 54.3% (Alves et al. 2018) of samples tested positive for CPV-2 by molecular techniques. In animals with enteritis, the occurrence ranged from 34.6% (Gizzi et al. 2014) to 46% (Castro et al. 2007). In another research, in Midwestern Brazil, the authors found 54% of positive samples through direct diagnostic method (Fontana et al. 2013) and, in Southeastern Brazil, 67% of tested dogs were shedding the virus even without clinical manifestations (Vieira et al. 2017). To an overview of exposure measure, scientists found 68.7% of seropositive animals in a city located in south of Brazil (Dezengrini et al. 2007) and 97% in Southeastern Brazil (Curi et al. 2016).

In this study, none of the collected feces was diarrheic. The viral shedding begins before the onset of clinical signs and may persist for up to 6 weeks after its cessation (Decaro et al. 2005a; Lamm and Rezabek 2008). Moreover, subclinical infections and mild forms of the disease may warrant the presence of the viral genome in normal faeces (Freisl et al. 2017).

In Brazil, the currently available commercial vaccines are produced from the original CPV-2 or CPV-2b. While wild strains encode the lysine (AAA) amino acid at residue 570, the vaccine strains encode glutamic acid (GAA) (Pinto et al. 2012). The three samples identified as CPV-2b in this study encoded the amino acid lysine, so the possibility of samples being infected due to vaccine virus excretion was discarded.

The CPV variants co-circulate worldwide with different relative rates according to geographical area (Miranda and Thompson 2016). In Latin America, CPV-2c has been the main subtype detected in the canine population (Zhou et al. 2017). In Brazil, CPV-2a was the most frequent strain from 1980 to 1986, which was substantially replaced by CPV-2b during the first half of the 1990s (Pereira et al. 2000; Costa et al. 2005). This intercalated inversion between CPV-2a and CPV-2b remained in the subsequent period (Costa et al. 2005; Pinto et al. 2012), until CPV-2c strain emergence, first detected in the country in 2008 (Streck et al. 2009), becoming predominant until 2010 (Pinto et al. 2012). The most recent studies in Brazil demonstrated co-circulation of the three CPV-2 variants, with predominance of CPV-2b (Silva et al. 2017).

Although the vaccination is the main way to prevent clinical manifestations (Hoelzer and Parrish 2010), best practices on public hygiene could greatly contribute to prevent outbreak risk from contaminated environment, as a main factor to CPV-2, through disinfecting strategic areas by aspersion with sodium hypochlorite 0.75% (Cavalli et al. 2018), and public politics on environmental education, with an emphasis on responsible dog ownership for example.

To the best of our knowledge, this study innovates using mitochondrial DNA research methodology to verify the canine origin of CPV-2 positive faecal samples, successfully adapting, for the first time, the original
protocol developed for qPCR assay (Caldwell and Levine 2009). The interest in specific mitochondrial DNA
detection was to confirm the canine-origin of the fecal matter as the CPV-2a, 2b and 2c variants are also able to
infect and replicate in feline host (Clegg et al. 2012).

In conclusion, these results demonstrate the CPV-2 presence in faeces collected in public thoroughfares
of high circulation of people or animals and, therefore, these faeces could potentially act as indirect source of
infection for susceptible animals. Although the chosen methodology of this study does not allow conclusions to
be drawn about the infectivity of the virion, the relevant and long-term viral shedding associated with the
recognized environmental resistance of CPV-2 support the risks connected with the virus dissipation in urban
environments through this indirect route. In this manner, more attention should be given to unassisted dogs that
roam freely as they have a greater chance of exposure to CPV-2. Moreover, an effective immunization program
should be reinforced to warrant high vaccination coverage to increase protection in these contaminated areas.

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doi:10.1099/0022-1317-82-12-3021.


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Table 1: Fecal sample characteristics, dates of collection, CPV-2 variants and GenBank accession numbers detected in this study.

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<td>-</td>
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<td>-</td>
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*ND = not determined
Fig. 1 Phylogenetic analysis of Canine parvovirus type 2 (CPV-2) based on a partial fragment of the VP2 gene. The CPV-2 sequences obtained in this study are indicated with the symbol “▲”. The phylogenetic tree was constructed using the maximum-Likelihood method based on the Tamura 3-parameter model. Bootstrapping was statistically supported with 1,000 replicates. The sequence of feline parvovirus (Genbank AJ249556.1) was used as outgroup.