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Canine Distemper Virus with Concomitant Infections Due to Canine Herpesvirus-1, Canine Parvovirus, and Canine Adenovirus in Puppies from Southern Brazil

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Abstract

The clinical, pathological, and molecular findings associated with mortalities due canine distemper virus (CDV) with concomitant infections of Canid herpesvirus type 1 (CaHV-1), Canid adenovirus type 2 (CAdV-2), and canine parvovirus type 2 (CPV-2) are described in puppies from southern Brazil. Four 3-8 months-old, German Spitz, puppies (1, female; 3, males) with clinical manifestations of abdominal pain, extreme vocalization, convulsions, and icterus died suddenly and were necropsied. Significant pathological alterations included necrohemorrhagic hepatitis, necrohemorrhagic nephritis, disseminated vasculitis, pulmonary hemorrhage with necrotizing bronchitis and/or bronchiolitis, non-suppurative myocarditis, and hemorrhagic enteritis. Polymerase chain reaction (PCR) assays amplified the desired amplicons of the CaHV-1 glycoprotein B gene, CDV N gene, CPV-2 VP2 capsid protein gene, and CAdV-2 E gene from multiple tissue samples of all puppies. Quadruple and triple viral coinfections were identified. The target tissues/organs of each pathogen demonstrated the characteristic pathological pattern(s) and contained viral DNA and/or RNA. Direct sequencing and phylogenetic analyses confirmed the PCR assays. These findings support a diagnosis of infections due to CDV with concomitant coinfections by CaHV-1, CPV-2a, and CAdV-2 in these dogs, and confirm the participation of these agents in the etiopathogenesis of the lesions herein described.

Keywords: Viral Diseases; Coinfections; Histopathology; Molecular Biology

Introduction

Canid herpesvirus type 1 (CaHV-1) is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae* [1] that produces diseases in domestic and wild canids [2]. CaHV-1 associated syndromes are age-related [2,3], and include in utero infection and systemic fatal disease in neonates [2]; and ocular [4], genital [2], respiratory [5], fatal hepatitis [6], and cutaneous manifestations [7]in older pups and adult dogs.

Serological studies have demonstrated infections due to CaHV-1 in the USA [8], UK [9], Europe [10], and South Africa [11]; there is no serological evaluation of CaHV-1 in Brazil. Although this disease is considered of worldwide distribution [2,8], CaHV-1 is not considered as an important canine infectious agent in Brazil, not included in routine vaccination protocols or available in commercial vaccines. Further, the only report of CaHV-1 infection from Brazil was diagnosed by direct immunofluorescence assay using polyclonal antibodies [12].

Alternatively, canine distemper virus (CDV; family *Paramyxoviridae*, genus *Morbillivirus*) and canine parvovirus type 2 (CPV-2; family *Parvoviridae*, genus *Parvovirus*) are widespread throughout urban canine populations in Brazil. Epidemiological studies have suggested that CPV-2c is the predominant circulating strain in Brazilian cities [13]. CDV is endemic in most urban canine populations within Brazil [14], and results in the loss of an estimated US$ 147.5 – 160.3 million annually due to the systemic effects of CDV [14]. Canine adenovirus A (family *Adenoviridae*, genus *Mastadenovirus*) type 1 (CAdV-1) is the cause of infectious canine hepatitis (ICH) that is frequently seen in dogs less than one year of age [15,16]. Canine adenovirus A type 2 (CAdV-2) is one of the pathogens associated with the clinical disease known as canine infectious respiratory disease [17]. This study describes the findings associated with mortalities due to CDV with simultaneous coinfections by CaHV-1, CPV-2, and CAdV-2 in puppies from southern Brazil

Materials and methods

Animals and clinical history

During mid-February, 2012 four (1 male, 3 females), 3-8 months-old German Spitz puppies kept in a populated kennel located in Londrina, southern Brazil died within a period of four days after the initial manifestations of discomfort. All puppies were submitted for routine necropsy soon after death. The referring veterinarian related that these dogs did not receive all recommended of a commercial vaccine, clinically demonstrated extreme vocalization, convulsions, respiratory difficulties, mucopurulent ocular secretions, icterus, and diarrhea, and that medication with anti-convulsive drugs and antibiotic therapy was unsuccessful. Parasitological analyses, with the flotation technique, done one week prior to the onset of clinical manifestations revealed negative results. The owner of the kennel related that the dogs used for breeding were imported from Argentina, Chile, Mexico, Indonesia, and the USA; three pups were born from the same dam, while one was from a different mother.

Selected tissue samples (brain, lungs, liver, kidneys, palatine tonsils, heart, urinary bladder, intestine, spleen, and lymph nodes) of all dogs were fixed by immersion in 10% buffered formalin solution and routinely processed for histopathological evaluation. Fresh tissue fragments from all dogs, as well as the urine samples from the dams of the German Spitz pups, were collected, and stored at -80ºC until processed for molecular biology.

PCR amplification of viral pathogens

Viral DNA/RNA was extracted from selected tissue fragments of all dogs and from the urine of the two dams by using the phenol/chloroform and silica/guanidinium-isothiocyanate techniques [18]. These samples were used in PCR assays designed to amplify the 450 base pair (bp) fragment of the CaHV-1 glycoprotein B (gB) gene [19]; the 583 bp fragment of the CPV-2 VP-2 gene [20]; the 287 bp of CDV N gene [21], and the 508 bp and 1030 bp of the CAdV-1 and -2 E gene, respectively [22].

Positive control was not included in the CaHV-1 PCR assay; however, viral RNA [21] and DNA [23] from previous studies served as positive controls for the CDV, CPV-2, CAdV-1, and CAdV-2 PCR assays. Nuclease-free water (Invitrogen Corporation, Carlsbad, CA, USA) was used as negative controls in all PCR assays. All PCR products were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide, and examined under ultra-violet light.

Sequencing and phylogenetic analyses

The PCR products obtained from the CaHV-1 and CPV-2 assays were purified (illustra GFX PCR DNA and Gel Band Purification Kit; GE Healthcare, Buckinghamshire, UK) and submitted for direct sequencing using the forward and reverse primers of each assay; the obtained sequences were deposited in GenBank.

The partial nucleotide sequences obtained were initially compared with those deposited in GenBank by using the Blast (http://www.ncbi.nlm.nih.gov/BLAST) program. Phylogenetic trees and sequence alignments were created using MEGA 6[36], constructed by the Neighbor Joining method, based on 1,000 bootstrapped data sets. The GenBank accession numbers used are given in Figures 3-5.

Results

Pathological alterations

The principal gross and histopathological findings were subjectively graded for comparison [21] and are resumed in Tables 1 and 2, respectively. All puppies demonstrated bilateral renal hemorrhages (Fig. 1A), pulmonary edema and hemorrhage, subcutaneous edema, purulent ocular discharge, and accumulation of serous fluid within body cavities (thoracic, cardiac, and abdominal). Further, congestion of the meningeal vessels (Fig. 1B), prominence of liver pattern (Fig. 1C), enlarged tonsils, and lymphadenopathy occurred in all puppies. The intestinal content of all pups was mixed with blood (Fig. 1D) and/or contained watery content. However, icterus of mucus membranes, subcutaneous tissue, and abdominal fat was observed in puppy # 3.

Table 1. Principal gross findings and signalment of dogs coinfected with several viral agents.

Gross findings	Dog 1	Dog 2	Dog 3	Dog 4
	German Spitz, male, 6 months	German Spitz, female, 3 months	German Spitz, female, 8 months	German Spitz, female, 3 months
Bilateral renal hemorrhage	+++	+++	+++	+++
Blood mixed/watery intestinal content	+	+	+	+
Cavitary edema	+++	+	++	+
Congested meningeal vessels	+	++	++	+
Depletion/necrosis of Peyer patches	+++	++	+++	+
Enlarged and/or hemorrhagic tonsils	+++	+++	++	+
Icterus	0	0	++	0
Intestinal hemorrhage	0	0	++	0
Lymph node enlargement	++	++	++	++
Ocular discharge	+	+	+	+
Prominent lobular pattern of liver	++	++	++	+
Pulmonary edema and/or hemorrhage	++	++	++	0
Serosal petechial hemorrhages	0	0	++	0
Subcutaneous edema	+++	++	+	+

0, absent; +, discrete; ++, moderate; +++, severe.

Table 2. Histopathological findings in dogs coinfected with several viral agents.

Principal histopathological diagnoses	Dog 1	Dog 2	Dog 3	Dog 4
Depletion of splenic germinating centers	++	+	++	+
Necrohemorrhagic hepatitis	++	++	++	+
Necrotizing bronchitis and/or bronchiolitis	++	+	++	0
Non-suppurative demyelinating meningoencephalitis	+	+	++	+
Non-suppurative interstitial nephritis	0	0	0	0
Non-suppurative myocarditis	++	++	++	0
Parvoviral enteritis	++	++	+	0
Pulmonary hemorrhage and/or edema	++	+++	++	+
Renal tubular necrosis and/or hemorrhage	+++	+++	+++	+++
Widespread necrotizing vasculitis [a]	++	+	++	++

0, absent; +, discrete; ++, moderate; +++, severe. aLesions observed in multiple tissues/organs

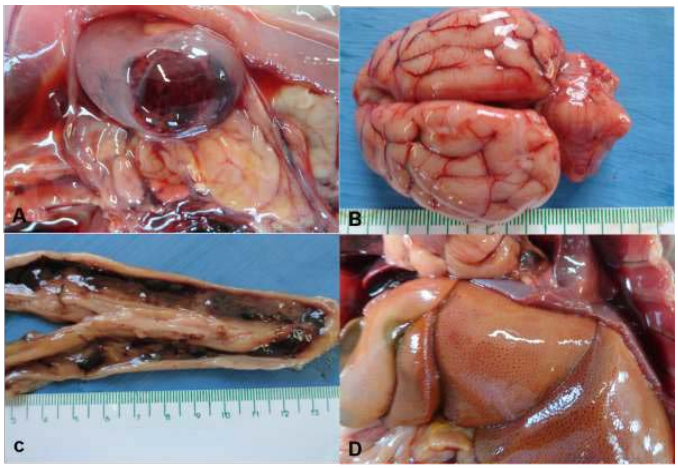

Figure 1. Gross images of dogs infected with multiple viral agents. There is hemorrhage and edema of the right kidney of dog # 1 (A). Observe congestion of the meningeal vessels of dog # 2 (B). The intestinal content is admixed with blood (C), with increase of the hepatic pattern of dog #3 (D). Scale in centimeters.

Significant histopathological alterations included renal tubular necrosis (Fig. 2A) with dystrophic mineralization and intratubular bacterial accumulations, white matter non-suppurative demyelinating encephalitis associated with few intranuclear eosinophilic inclusion bodies in astrocytes, pulmonary hemorrhage and/or edema; and random necrohemorrhagic hepatitis associated with intranuclear inclusion bodies within hepatocytes (Fig. 2B).

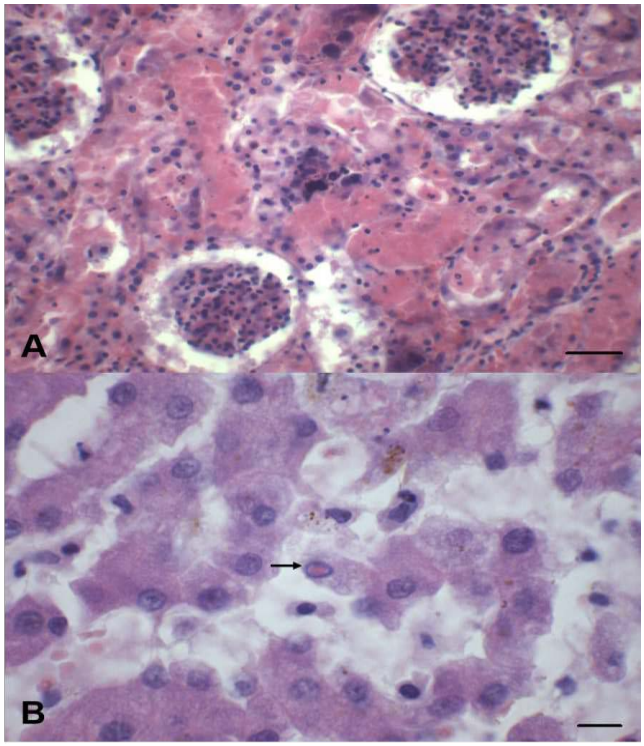

Figure 2. Histopathological features of dogs coinfected by several viral agents. There is necrosis of tubular epithelial cells with foci of dystrophic calcification within the kidney (A), and an intranuclear eosinophilic herpesvirus inclusion body (arrow) within a hepatocyte (B). (Hematoxylin and Eosin stain, Bar; A 30 µm; B, 10 µm).

Most puppies had necrotizing bronchiolitis and/or bronchitis, non-suppurative myocarditis with vasculitis and lesions suggestive of canine parvoviral enteritis (atrophy and fusion of intestinal villi with cryptal necrosis, regeneration of enterocytes, and lymphoid depletion). Disseminated vasculitis with tissue hemorrhage and necrosis occurred in all dogs. Other lesions included epithelial necrosis with depletion of lymphoid tissue of the tonsils; interstitial pneumonia; depletion of splenic lymphoid tissue and germinating centers of lymph nodes.

Molecular demonstration of viral agents

The results of the PCR assays are resumed in Table 3. All puppies were infected by CDV and CaHV-1; three of these were infected by CPV-2 (puppies # 1, 2, and 3), two by CAdV-2 (# 2 and 4), and none by CAdV-1. Concomitant infections were identified in all puppies: being one quadruple infection (puppy # 2; CDV, CaHV-1, CPV-2, and CAdV2) and three triple infections. Two puppies (1 and 3) were infected by CDV, CaHV-1, and CPV-2; puppy # 4 was infected by CDV, CaHV-1 and CAdV-2. Single viral infections were not demonstrated in any of the puppies evaluated. Additionally, only the urine sample from one dam contained viral CDV RNA; all other PCR assays were negative for these two animals.

Sequencing and phylogenetic analyses

The desired amplicons of CaHV-1 gB (liver and spleen; dogs # 1 and 4) and CPV-2 VP-2 (intestine, myocardium, and mesenteric lymph node; dogs # 1 and 2) genes were sequenced. BLAST analyses revealed that the CaHV-1 gB sequences demonstrated 99-100% identity with similar sequences deposited in GenBank. Although there are few sequences of this gene, two distinct clusters were formed when all sequences were aligned (Fig. 3); the sequences from this study were grouped with strains from Australia and Taiwan, while those from the UK and China clustered with another Taiwanese strain. BLAST analyses revealed that the CPV-2 sequences demonstrated 98-100% identity with CPV-2a strains; this was confirmed when these were aligned with selected strains of CPV-2a, -2b, and -2c (Fig. 4). The desired amplicons of the CDV N gene was obtained from all RT-PCR assays; blast analyses revealed that the sequences derived from this study were similar to other isolates of CDV from different geographical locations (Fig. 5).

Table 3. PCR demonstration of viral agents in dogs from southern Brazil.

Dog numbers*	Organs/samples evaluated									
	Urine	Kidney	Lung	Intestine	Mesenteric lymph node	Myocardium	Liver	Spleen	Palatine tonsils	Cerebellum
# 1										
CaHV-1	ND	+	0	ND	+	ND	+	+	+	ND
CDV	+	0	+	+	0	+	0	0	+	+
CPV-2	0	ND	ND	+	+	+	+	+	+	ND
CAdV-1	0	ND	0	ND	0	ND	0	0	0	ND
CAdV-2	0	ND	0	ND	0	ND	0	0	0	ND
# 2										
CaHV-1	0	0	0	ND	+	ND	0	0	NC	ND
CDV	+	0	+	0	0	0	0	+	NC	0
CPV-2	0	ND	ND	+	+	+	+	0	NC	ND
CAdV-1	ND	0	0	ND	0	ND	0	0	NC	ND
CAdV-2	ND	+	0	ND	+	ND	0	0	NC	ND
# 3										
CaHV-1	NC	+	+	ND	+	ND	0	+	+	ND
CDV	NC	0	0	0	+	0	0	0	0	0
CPV-2	NC	ND	ND	+	+	+	+	0	+	ND
CAdV-1	NC	0	0	ND	ND	ND	0	0	0	ND
CAdV-2	NC	0	0	ND	ND	ND	0	0	0	ND
# 4										
CaHV-1	0	0	0	ND	+	+	ND	+	NC	ND
CDV	0	+	0	ND	ND	ND	ND	0	NC	0
CPV-2	0	ND	ND	0	0	0	ND	ND	NC	ND
CAdV-1	0	0	0	ND	0	0	0	0	NC	ND
CAdV-2	0	0	0	ND	0	+	0	0	NC	ND

CaHV-1, Canid herpesvirus-1; CDV, canine distemper virus; CPV-2, canine parvovirus-2; CAdV-1, canine adenovirus-1; CAdV-2, canine adenovirus-2. PCR reaction: +, positive; 0, negative; NC, not collected; ND, not determined.

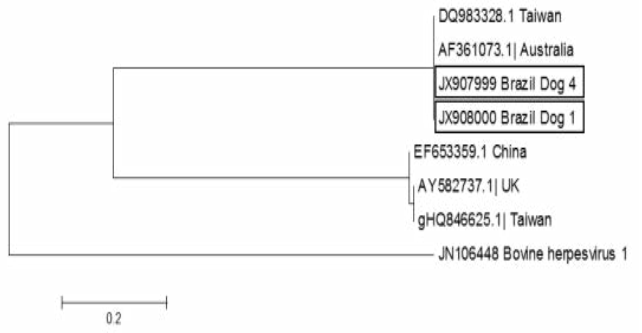

Figure 3. The phylogenetic relationship based on strains of the CaHV-1 glycoprotein B gene generated by MEGA 6. The GenBank accession numbers and the country of origin of the sequences used are given. The sequence derived from this study is highlighted within the boxes. Bovine herpesvirus was used as the out-group.

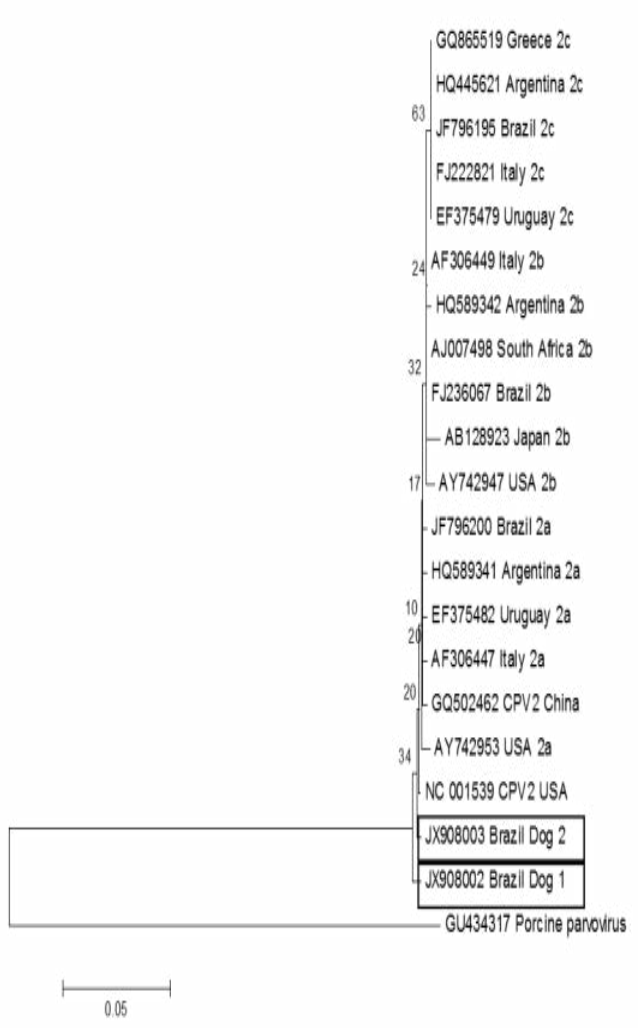

Figure 4. The phylogenetic relationship of selected strains of the CPV-2 VP2 gene generated by MEGA 6. The GenBank accession numbers and the country of origin of the sequences used are given. The sequences derived from this study are highlighted within the boxes. Porcine parvovirus was used as the out-group.

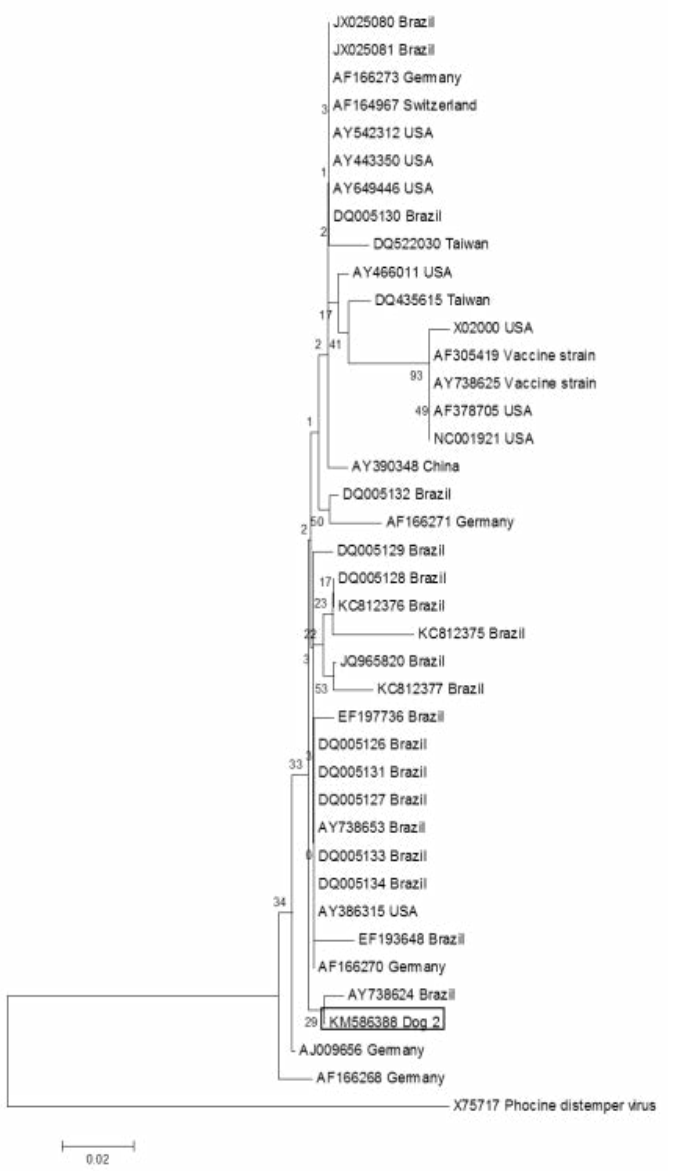

Figure 5. The phylogenetic relationship of selected strains of the canine distemper virus N gene generated by MEGA 6. The GenBank accession numbers and the country of origin of the sequences used are given. The sequences derived from this study are highlighted within the box. Phocine distemper virus was used as the out-group.

Discussion

The clinical, pathological, and molecular biology findings confirm the active and systemic participation of CDV and CaHV-1 in the etiopathogenesis of the lesions in these dogs that were concomitantly coinfected by CPV-2a and CAdV-2. Further, the lesions observed in the tissues/organs of these dogs are consistent with those associated with these infections disease pathogens [2,3,16,24,25], while all dogs demonstrated the characteristic morphological pattern(s) of each infectious agent identified in target tissues by PCR assays. Additionally, these results represent the first molecular characterization of CaHV-1 in dogs from

Brazil, definitely extends the geographical distribution of this important canine disease, and reinforce the findings of a previous study [12]. Since there is no vaccination against CaHV-1 in Brazil, the molecular demonstration of this pathogen in several tissues of unvaccinated dogs [11], definitely confirms the dissemination of this disease within these geographical locations. Although, bacterial diseases were not the focus of this study and hence not investigated, the participation of bacterial agents cannot be totally excluded as contributing to the pathological lesions herein described.

The absence of positive controls within the CaHV-1 PCR assays excludes the risk of contamination, while direct sequencing validated the results of the PCR assays. Positive controls were not included in the initial PCR assays since this pathogen is not present in commercial local vaccines, while attempts to obtain reliable CaHV-1 DNA were frustrating.

Of paramount interest in these cases was the confirmation of triple and quadruple viral infections in all dogs. Previous descriptions of viral coinfections in dogs have included dual infections of CDV with CAdV-1 [26], CAdV-2 [27,28], and the simultaneous infections of CPV-2 with canine coronavirus [29]. Further, triple viral infections of CDV, canine parainfluenza virus, and CAdV-2 [29] and CDV, CPV-2 and reoviruses [30] have occurred. Additionally, quadruple viral (CDV, CPV-2, CAdV-1, and CAdV-2) infections with concomitant *Toxoplasma gondii* were described in a 43-days old immunosuppressive pup [23], while there has been cases of coinfections due to CDV, *T. gondii*, and *Ehrlichia* sp. [31], and the concomitant occurrence of CDV, *Clostridium piliforme*, and *Isospora* sp. [32]. These findings suggest that coinfections in dogs are probably more frequent than previously mentioned and might be underdiagnosed. We theorize that concomitant infections in dogs are common, but since there is overlapping of the clinical/pathological manifestations associated with these different agents, the first pathogen diagnosed, that fits a particular clinical and/or pathological syndrome/entity is coined as the "cause of infection", and additional laboratory examinations are not done. Alternatively, the clinical manifestations of these diseases are not always distinct which might induce inadequate or a misdiagnosis. Therefore, it is recommended that detailed laboratory evaluations be made in an attempt to characterize the possible participation of multiple pathogens in ailing dogs.

The phylogenetic analyses of CaHV-1 nucleotide sequences based on the gB gene might suggest that two distinct strains of this pathogen are circulating worldwide. However, more sequences and a detailed phylogenetic investigation are needed to confirm this theory. Alignment of the CPV-2 VP2 sequences derived from this study demonstrated that the dogs, from these geographical locations of southern Brazil contained the CPV-2a strain; these findings are different from a study that suggested that the CPV-2c strain is the principal variant circulating throughout Brazil [13]. Further, only the -2a and -2b variants of canine parvovirus were identified in puppies from Rio de Janeiro [33]; and there has been a recent description of -2c in a puppy from northern Paraná coinfected by several infectious agents [23]. Hence the variant of CPV-2 circulating in Brazilian cities might not be restricted to the -2c subtype as suggested [13], since the -2a and –2b variants seem also to be frequent. Additionally, the -2a subtype circulating in urban Brazilian cities has been associated with vaccination failures, due to point mutation, in some pups that were completely immunized[33]. Alternatively, CPV-2 vaccination failures have been attributed to infection by wild-type field strains that occurred before or after immunization [30]. The CDV sequences derived from this study grouped with similar isolates already identified in dogs from Brazil. However, several distinct groups were observed within the strains of CDV circulating in canine populations of Brazil, suggesting that distinct clades might be present in different geographical regions of the country. Studies done with the Hemagglutinin gene of CDV have already suggested that distinct clades of CDV might be circulating in urban populations of Brazil [34].

The persistent vocalization (crying) and abdominal pain observed in these puppies is a frequent manifestation of CaHV-1 [3,16]. Although the source of infection remains obscure, it is speculated that the German Spitz pups were infected at birth or neonatally, since these were born to dams that were imported from countries where CaHV-1 is a recognized entity, and that the dams probably arrived in Brazil with latent CaHV-1 infection, which is the hallmark of herpesvirus [3]. The stress associated with parturition and concomitant CDV coinfection (at least in one dam) might have revived the latent disease resulting in consequent viral dissemination to the pups due to the ingestion and/or inhalation of contaminated material of the infected birth canal and/or nasal secretions [3,16]. Reactivation of latent infections has been associated with stress induced conditions, such as pregnancy, parturition, populated kennels, and immunosuppressive therapy [2]; the German Spitz pups were kept in densely populated shelters. Additionally, the identification of CAdV in these pups demonstrate that dogs might be subclinically infected by this pathogen [8], and highlights the importance of the diagnosis of multiple infections in pups.

All dogs in this study were coinfected by CDV, an immunosuppressive agent due to the deleterious effects on signaling lymphocytic activation molecule (SLAM)-producing cells [35], which might have been the trigger necessary to develop the systemic viral infections observed in these dogs. The ability of CDV to potentiate concomitant infections was recently demonstrated in a pup that was coinfected by CDV, CPV-2, CAdV-1, and -2, and systemic toxoplasmosis [23]; this was also seen in simultaneous

infections of CDV with CAdV-1 [26], CAdV-2 [27,28], CPV-2 [30], CPIV [29], *T. gondii*, *Ehrlichia* sp. [31], *Clostridium piliforme* and *Isospora* sp. [32]. In cases of concomitant infections involving multiple organs/system, as occurred in these dogs, death can be attributed to multiple organ failure [23], and might be the case of the death of the dogs herein described. Consequently, we believe that immunosuppression by CDV might have been the trigger responsible for the occurrence these simultaneous viral infections in these susceptible puppies.

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