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Detection of respiratory viruses in shelter dogs maintained under varying environmental conditions

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ABSTRACT

Three dog shelters in Rio Grande do Sul were investigated for associations between the occurrence of respiratory viruses and shelter environmental conditions. Nasal secretions randomly collected during the cold season were tested via PCR, and this data collection was followed by nucleotide sequencing of the amplicons. In shelter #1 (poor sanitary and nutritional conditions, high animal density and constant contact between dogs), 78% (58/74) of the nasal samples were positive, 35% (26/74) of which were in single infections and 44% (32/74) of which were in coinfections. Shelters #2 and #3 had satisfactory sanitary and nutritional conditions, outdoors exercise areas (#2) and animal clustering by groups (#3). In shelter #2, 9% (3/35) of the samples were positive for *Canine parainfluenza virus* (CPIV), and 6% (2/35) were positive for *Canid herpesvirus 1* (CaHV-1). In shelter #3, 9% (7/77) of the samples were positive for *Canine adenovirus type 2* (CAdV-2), and 1% (1/77) were positive for *Canine distemper virus* (CDV). The amplicon sequences (CPIV and CDV nucleoprotein gene; CAdV-2 E3 gene; CaHV-1 glycoprotein B gene) showed 94–100% nucleotide identity with GenBank sequences. Our results demonstrate that CPIV, CAdV-2 and CDV are common in dog shelters and that their frequencies appear to be related with environmental and nutritional conditions. These results indicate the need for control/prevention measures, including vaccination and environmental management, to minimize these infections and improve dog health.

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Introduction

Canine infectious respiratory disease (CIRD) may be associated with single virus infections or with a multifactorial etiology and are assigned to infectious agents that replicate sequentially or in synergy.[1] The main viral agents involved in CIRD include *Canine distemper virus* (CDV), *Canine parainfluenza virus* (CPIV), *Canine adenovirus type 2* (CAdV-2) and *Canid herpesvirus* 1 (CaHV-1).[2]

In Brazil, CDV infection is endemic in dog populations, is associated with respiratory and/or multisystemic disease, and causes thousands of deaths each year.[3,4] Due to its impact on animal health, CDV is one of the most important infectious diseases in dogs.[2,5] Similarly to CDV, CAdV-2 has a worldwide distribution and is a major agent of canine infectious tracheobronchitis (CIT) or "kennel cough", a disease characterized by restricted infection of the respiratory system.[6] CPIV has a wide distribution in canine populations with an estimated seroprevalence ranging from 30 to 70%.[7] CPIV infection is related to high population density; the virus is highly transmissible and presents with rapid dissemination between animals.[2] CaHV-1 has a worldwide distribution and is associated with respiratory and reproductive disease.[8] Like other *Alphaherpesviruses*, CaHV-1 establishes latent infections in nerve ganglia and can periodically reactivate the infection.[9] An estimated 30–100% of domestic dogs have antibodies to CaHV-1.[10]

The transmission of respiratory viruses occurs through direct or indirect contact between animals, primarily through contaminated nasal secretions and aerosols.[1] CIRD may affect dogs of both genders and ages; puppies under 90 days old are more susceptible, as well as immunosuppressed dogs, animals without a history of vaccination; vaccination failures or maternal immunity may also contribute.[11] The disease presents a seasonal pattern with a higher incidence in cold months.[12]

The diagnosis of CIRD is largely based on the epidemiology, clinical signs and response to therapy. However, an etiologic diagnosis requires the identification of the agent or its products (proteins or nucleic acids).[4] Vaccination is largely used to prevent or control respiratory infections in dogs and helps minimize clinical disease; however, current vaccines are not always effective.[11]

In Brazil, despite the wide distribution of these infections and informal reports by veterinarians, very few reports describe viral respiratory disease in dogs.[13–18] Additionally, there is little information regarding these infections in local environments with high densities and constant animal movement such as dog shelters. The identification of the more common respiratory viruses in dogs in various epidemiological conditions is essential for developing efficient control and prevention measures.

Thus, the objective of this study was to investigate respiratory viral infections in dogs in shelters. For this, three shelters located in Rio Grande do Sul state, Brazil, presenting diverse sanitary and nutrition conditions were included in an attempt to associate the occurrence of viral infections with the conditions observed. The viruses were detected in nasal secretions via polymerase chain reaction (PCR) and focused on the main agents involved in this condition (CDV, CPIV, CAdV-2 and CaHV-1).

Material and methods

Dogs from three shelters located in Rio Grande do Sul state (RS), Brazil, were included in this study. Two shelters (#1 and #2) are located in Cachoeira do Sul (30° 02′ 21″ S and 52° 53′ 38″ W), and one shelter (#3) is located in Passo Fundo (28° 15′ 46″ S and 52° 24′ 24″ W). The sample collection was performed in 2014 in months of low temperatures (July and August). Fig. 1 illustrates the environmental conditions observed in these shelters. Shelter selection was performed to include a variety of shelter conditions, including those with low temperature seasons, varying population densities (to take into account the appearance and frequency of cleaning of the premises), and the nutritional states of the dogs (taking into consideration the type of food and the frequency of feeding according to healthy/unhealthy animal appearance). The selection of the animals within each shelter was performed randomly.

Shelter #1 hosts stray dogs and cats of both genders, of all ages and primarily crossbred animals. The animal population of the shelter was 150 dogs and 30 cats on the date of sampling. The young dogs (six months up to two years old) and adults (more than two years old) were maintained in individual cages/small barns held by leashes in an open space, and most animals had direct and indirect contact with each other. Small, medium and large dogs had individual cages/houses shelters within the same area (approximately 1 m^2). Puppies were maintained in collective cages indoors without direct contact with adult animals. Shelter #1 was visited in the cold season when the temperatures ranged of 5–10 °C. At the visit, several animals presented with nasal discharge, indicating respiratory infection. Sanitary and nutritional conditions were inadequate, and the locality had not recently been cleaned. The animals did not receive good quality food and were not fed sufficient amounts.

Shelter #2 hosts stray dogs of both genders independent of age and primarily crossbred animals. On the day of the visit, the number of animals was 70. During the day, the animals remained outdoors in fenced areas or held by leashes and were grouped according to gender and age. During the night, the animals were allocated indoors, in collective cages, with approximately 10 animals/cage. The dogs had a wide area in which to run and exercise during the day and had contact with each other. Shelter #2 was visited when the outdoor temperatures were between 10 and 15 °C. The sanitary and nutritional conditions were fair to good; the animals were fed once per day, and the cages were cleaned three times per week.

Shelter #3 hosts stray dogs and cats of both genders, varying ages and primarily crossbred animals. At the visit, 180 dogs and 20 cats were present in the shelter. The dogs were allocated according to gender and age in collective fenced barns that had at least one dog house per animal. The animals had constant direct contact with other animals from the same cage/barn. The individual area was approximately 3 m^2/animal. The shelter was visited when the ambient temperatures ranged from 5 to 10 °C. The sanitary and nutritional conditions were good, the cages were cleaned once per day, the animals had clean water ad libitum and were fed three times per day.

Fig. 1 – Conditions of the shelters included in this study. (A) Shelter #1. (B) Shelter #2. (C) Shelter #3.

Nasal swabs of 74, 35 and 77 dogs in shelters #1, #2 and #3, respectively, were randomly collected; approximately 50% of the dogs in each shelter were sampled. After sample collection, the swabs were maintained in RNAlater solution (Life Technologies, Carlsbad, CA, USA), and the samples remained in dry ice during transport to the laboratory where they were stored at −80 °C. All proceedings involving animal manipulation were performed under the supervision of a veterinarian and according to the recommendations of the Brazilian Committee of Animal Experimentation (Comitê Brasileiro de Experimentação Animal – COBEA, law #6.638, May 8, 1979). This research was approved by the institutional Ethics and Animal Welfare Committee (UFSM, Comitê de Ética e Experimentação Animal: approval number 080/2014).

RNA and DNA extraction from nasal swabs were performed using an RTP DNA/RNA virus extraction kit (Invitek, Hayward, CA, USA) according to the manufacturer's instructions. After RNA extraction, complementary DNA (cDNA) was synthesized using an enzyme Super Script III Reverse Transcriptase Kit (Life Technologies, Carlsbad, CA, USA). The PCR reactions were initially standardized to optimize the concentration of each reagent. Viruses obtained from two commercial vaccines were used as controls for CDV, CPIV and CAdV-2. For CaHV-1, nucleic acid extracted from the liver of a puppy naturally infected with CaHV-1 was used as a control.[17] Ultrapure water was used as a negative control in all reactions. The primers used in all reactions are described in Table 1. All reactions were performed using a total volume of 25 μL with 2 μL of total DNA (100–200 ng) according to the PCR conditions described for each virus. Primers to CPIV were obtained using the Clone Manager 7 program (http://www.scied.com), and the sequences are shown in Table 1. PCR reaction to CPIV was performed in a 25 μL volume using 100–200 ng of template DNA, 12.5 μM of each primer, 2.5 mM $MgCl_2$, 10 mM of dNTPs, 1× reaction buffer and 0.75 units of Taq polymerase (Invitrogen, Carlsbad, CA, USA). The PCR condition was one step at 95 °C for 7 min, followed by 35 cycles of denaturation (95 °C, 45 s), primer annealing (50 °C, 1 min), extension (72 °C, 45 s), and a final extension of 7 min at 72 °C. The PCR products were resolved in 1.5% agarose gel stained with Gel Red® (Biotium, Hayward, CA, USA) and visualized under UV light after electrophoresis (60 V, 40 min).

For nucleotide sequencing, 90 μL of each PCR product was purified using a PureLink® Quick Gel Extraction and PCR purification Combo Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Positive samples were randomly selected and sequenced in quadruplicates in an automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were analyzed using the Staden Package for consensus sequences achievement.[22] The matrix of sample identity with sequences deposited in GenBank was performed using the BioEdit

Table 1 – Primers used for the detection of canine respiratory viruses via PCR in dog samples from shelters in the Rio Grande do Sul state.

Virus	Amplified gene	Primers (5′–3′)	Amplicon length	Reference
CDV	Nucleoprotein	TTCTGAGGCAGATGAGTTCTTC CTTGGATGCTATTTCTGACACT	829 pb	19
CPIV	Nucleoprotein	GAGGCTCGACGAATAATC GTTCGGCTTGAGTTAGACC	532 pb	This reference
CAdV-2	E3	CGCGCTGAACATTACTACCTTGTC CCTAGAGCACTTCGTGTCCGCTT	1030 pb	20
CaHV-1	Glycoprotein B	CCTAAACCTACTTCGGATGA GGCTTTAAATGAACTTCTCTGG	450 pb	21

CDV, canine distemper virus; CPIV, canine parainfluenza virus; CAdV-2, canine adenovirus type 2 and CaHV-1, canid herpesvirus 1.

Table 2 – Infections and coinfections of respiratory viruses in dog shelters in Rio Grande do Sul state.

Shelter	+/total (%)	Single infection – *n* (%)				Coinfection – *n* (%)			
		CPIV	CAdV-2	CaHV-1	CDV	CDV CAdV-2	CPIV CAdV-2	CPIV CDV	CPIV CAdV-2 CDV
#1	58/74 (78)	22 (30)	4 (5)	*nd*	*nd*	2 (3)	17 (23)	3 (4)	10 (14)
#2	5/35 (14)	3 (9)	*nd*	2 (6)	*nd*	*nd*	*nd*	*nd*	*nd*
#3	7/77 (9)	*nd*	6 (8)	*nd*	1 (1)	*nd*	*nd*	*nd*	*nd*

nd, not detected.

Sequence Alignment Editor Software suite, version 7.0.5.3 (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Results and discussion

The present study investigated the presence of respiratory viruses in dogs of three shelters in Rio Grande do Sul state, Brazil, through virus detection in nasal secretions via PCR. Considering the previous serological studies on canine respiratory viruses in Brazil,[14,15] the primary difference of the present study was the direct demonstration and identification of the viruses involved in CIRD.

Our results showed the occurrence of the main canine respiratory viruses in these shelters with varying frequencies and combinations of single and mixed infections. In shelter #1, 78% of the 74 samples were positive for at least one virus; CPIV was the most frequent agent (71% of the samples). CPIV was detected in single (30%) or in mixed infections and was associated with CAdV-2 (23%), CDV (4%), or both (14%). CDV and CAdV-2 were found in a high percentage of animals, especially in coinfections (Table 2). In shelters #2 and #3, unlike shelter #1, a small percentage of samples were positive for the virus and only in single infections. In shelter #2, CPIV was detected in 9% of the samples and CaHV-1 was detected in 6%. In shelter #3, 9% of the samples were positive for CAdV-2 and 1% for CDV (Table 2). The varying sanitary and nutritional conditions and the dog crowding/density of the respective shelters may explain the important differences in the rates of positive animals.

Shelter #1 had precarious nutritional and sanitary conditions, poor infrastructure and poor food quality (Fig. 1A). In shelter #2, the animals had a wide outdoors area in which to play and exercise; however, the dogs of varying ages had direct contact (Fig. 1B). Fig. 1C shows shelter #3 with individual dog houses and cages with a low population density and good sanitary conditions (approximately six dogs/cage). Factors associated with animal overcrowding, such as excessive noise, poor air quality and diet, in addition to bad kennel cleaning, may cause stress, which may in turn promote the establishment and dissemination of viral infections.[23–25] Thus, the poor sanitary and nutritional conditions of shelter #1 may have favored the high rate of respiratory viruses.

In this shelter, CDV, CPIV and CAdV-2 were detected in single or mixed infections, corresponding to 78% of the positive dogs. CPIV was detected in 71% of the samples, of which 30% were single infections and 41% were associated with CAdV-2 and/or CDV. CPIV is considered the primary virus involved in respiratory disease in dogs,[2,7,26–29] and has been most frequently reported in conditions of high animal density.[2] CPIV infection produces pathology in the tracheal epithelium[15] and favors secondary respiratory infections by other pathogens such as CAdV-2.[6]

In shelter #1, CDV was detected only associated with CAdV-2 and/or CPIV, corresponding to 21% of the positive samples. CDV replication occurs in epithelial cells and macrophages of the upper respiratory system, pharynx and tonsils, followed by lymph node infection and systemic dissemination that can evolve to multisystem disease and immunosuppression.[30] For this reason, bacterial secondary infections are often detected in dogs with distemper in addition to coinfection by other viruses, such as CAdV-2 and CaHV-1.[5,31,32]

CAdV-2 detection in 45% of the samples from shelter #1 may have been influenced by the high CPIV and CDV infections in the kennel because CPIV promotes primary lesions in the tracheal epithelium[15] and CDV induces immunosuppression.[30] Additionally, adenoviruses are highly resistant in environmental conditions and remain viable in the environment for an extended duration, thereby favoring dissemination among animals.[6] Notably, a high prevalence of CAdV in dog populations has been reported in shelters without a history of vaccination.[33,34]

An investigation of respiratory viruses in dogs in Germany analyzed 58 samples of shelter animals with and without respiratory signs and detected 22.4% (13/58) to be positive for CPIV and one positive for CAdV-2 and CPIV.[29] A similar study performed in Germany examined 68 nasal swabs of domestic dogs[28]; in this study, 7.4% (5/68) of the samples were positive for CPIV, 2.9% (2/68) for CAdV-2 and 1.5% (1/68) for CDV. Despite varying frequencies, these studies reported CPIV to be the most frequent respiratory virus in dogs, followed by CAdV-2 and CDV.

There are few reports of direct diagnosis of respiratory viruses in dogs; however, some serological studies have been performed in Brazil.[14,15] In southern Brazil, a serological investigation of 817 domestic dogs without a vaccination history showed that 43% of the animals were seropositive to CAdV and 27.3% to CDV.[14] A similar study was conducted in a population of 173 dogs in shelters, also from the RS state, in which antibodies to CPIV and CDV were detected in 51.4% and 4.1–9.3% of the samples, respectively.[15] These studies showed that respiratory viruses circulate among domestic and shelter dogs in southern Brazil in varying combinations and prevalences that likely reflect environmental and epidemiological differences between regions and dog populations.

In shelters #2 and #3, the respiratory viruses were detected only in single infections with 14% of infections caused by CPIV

or CaHV-1 (shelter #2) and 9% by CDV or CAdV-2 (shelter #3). CaHV-1 was detected in samples collected only from the dogs of shelter #2, corresponding to 6% of the collected samples. Although CaHV-1 may cause respiratory disease, the infection has also been associated with other clinical outcomes, including reproductive disease.[35,36] Due to the ability of CaHV-1 to remain latent in the host, its diagnosis in dog populations should preferentially be performed via serological testing.[37–40] In this sense, we detected positive serology for CaHV-1 in 7 out of 8 dogs in shelter #1 (not shown). A two-year longitudinal investigation in a shelter in the United States involving 211 necropsied dogs showed CaHV-1 involvement in 12.8% and 9.6% of trachea and lung samples, respectively, reinforcing the involvement of CaHV-1 in respiratory disease in dogs.[2]

The identity of the sequenced matrix of the shelter samples with sequences deposited in GenBank revealed 96 to 99% identity (KU341102, KU341103, KU341104 and KU341105) with the N gene (AJ009656.1, JF965338.1, KC590511.1, AY386315, JF965338.1, KF856711.1, KP738610.1 and JN381190.1) of CDV, 98 to 99% identity (KU341106, KU341107, KU341108, KU341109) with the N gene (EF543648.1, EF546391.1 and AY581307.1) of CPIV, 97–100% identity (KU315333, KU315334, KU315335, KU315336 and KU315337) with the E3 gene (KF676978.1, JX416842.1, JX416842.1, U77082.1 and GQ915311.1) of CAdV-2, and 99–100% identity (KU315338 and KU315339) with the gB gene (KJ946357.1, KJ676506.1, JX908000.1, HQ846625.1, AF361073.1 and AY582737.1) of CaHV-1.

Thus, the results obtained in this research showed that respiratory virus infections (CPIV, CDV, CAdV-2 and CaHV-1) are common in dogs housed in public shelters. The frequency and dissemination of these viral infections appear to be related to a high population density and poor sanitary and nutritional conditions. These results also indicate the need for control/prevention measures, such as vaccination and good environmental conditions, to minimize these infections in shelter dogs. CPIV infection appears to play an especially predominant role in winter respiratory infections in dog shelters and warrants further preventive measures.

Conflicts of interest

The authors declare no conflicts of interest.

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