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Detection of selected viral pathogens in dogs with canine infectious respiratory disease in Austria

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Objectives: To assess the prevalence of canine parainfluenza virus, canine adenovirus type 2, canine distemper virus, canine respiratory coronavirus and influenza virus A infections in: (1) privately-owned or, (2) kennelled dogs showing signs consistent with canine infectious respiratory disease and, (3) clinically healthy dogs in Vienna, Austria.

Materials and Methods: Prospectively, nasal and tonsillar swabs from 214 dogs affected with infectious respiratory disease, and 50 healthy control dogs were tested for nucleic acids specific to the various viral infections. Concurrent bronchoalveolar lavage fluid from 31 dogs with chronic respiratory disease was investigated for the same viral pathogens. Additionally, anti-canine respiratory coronavirus antibody concentrations were measured in paired blood samples from 30 acutely diseased dogs.

Results: Canine respiratory coronavirus (7.5%) and canine parainfluenza virus (6.5%) were the most commonly detected viruses in samples from the upper airways of dogs with respiratory infections. Serological results showed a significant seroconversion in response to coronavirus in 50% of the examined cases. None of the samples was positive for influenza virus A-specific nucleic acid. Canine coronavirus-specific nucleic acid was detected in 4.0% of healthy dogs.

Clinical Significance: Canine coronavirus should be considered as a clinically relevant cause of infectious respiratory disease in crowded dog populations. For sample collection, the nasal mucosa can be recommended as the favoured site. Analysis of paired serum samples aids verification of canine coronavirus infection in respiratory disease.

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INTRODUCTION

Canine infectious respiratory disease (CIRD), synonymous for infectious tracheobronchitis or "kennel cough," is a disease caused by single or multiple infectious agents with a high worldwide prevalence. Apart from several viral and bacterial agents, the individual health and constitution, vaccination status and environmental influences including husbandry conditions (*e.g.* crowding of animals) may have an impact on the manifestation of clinical signs. Non-complicated forms of typically self-limiting character may be distinguished from complicated forms associated with, possibly fatal, pneumonia. A severe course of disease typically develops as a consequence of coinfections (Chalker *et al.* 2003, Chvala *et al.* 2007, Schulz *et al.* 2014a). However, even

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isolated viral infections [*e.g.* canine influenza virus (CIV)] may lead to clinically relevant and sometimes lethal respiratory disease (Crawford *et al.* 2005). Commonly recognised viral causes of CIRD are canine parainfluenza virus (CPiV), canine adenovirus type 2 (CAV-2) and canine distemper virus (CDV) (Ford 2012).

However, according to more recent studies, the understanding of this disease complex has changed. New viral pathogens have been detected within the past two decades. In 2003, canine respiratory coronavirus (CRCoV) emerged as a cause of CIRD in a rehoming centre in the UK (Erles *et al.* 2003). Further studies from several countries detected CRCoV-specific nucleic acid in dogs suffering from respiratory disease (Yachi & Mochizuki 2006, Decaro *et al.* 2007, Spiss *et al.* 2012, Schulz *et al.* 2014a, Viitanen *et al.* 2015).

In association with an outbreak of respiratory disease in racing greyhounds in Florida, CIV types closely related to influenza subtype H3N8, originally detected in horses were isolated (Crawford *et al.* 2005). Subsequently, several studies from different countries detected CIV isolates in respiratory samples and concurrent anti-CIV antibodies in dogs with mild respiratory signs as well as cases of fatal respiratory disease (Yoon *et al.* 2005, Daly *et al.* 2008, Payungporn *et al.* 2008, Song *et al.* 2008, Kirkland *et al.* 2010, Li *et al.* 2010, Song *et al.* 2013). Furthermore, isolation of human-related influenza strains from dogs was successful (Lin *et al.* 2012). To date, at least seven influenza virus subtypes showing different ability of interspecies and intraspecies transmission have been isolated from dogs. These subtypes are mainly prevalent in the USA (H3N8), Eastern China and South Korea (*e.g.* H3N2), but some of them have also been reported from European countries (Sun *et al.* 2013, Xie *et al.* 2016) supporting the hypothesis that dogs may play a role in transmission and spread of influenza virus among animal species and even humans.

Detection of further viral pathogens (*e.g.* canine herpesvirus, canine reovirus, canine pneumovirus (CnPnV), pantropic canine coronavirus, canine hepacivirus and canine bocavirus) has been associated with respiratory disease in dogs (Buonavoglia & Martella 2007, Decaro & Buonavoglia 2008, Kawakami *et al.* 2010, Renshaw *et al.* 2010, Decaro & Buonavoglia 2011, Kapoor *et al.* 2011, Kapoor *et al.* 2012, Mitchell *et al.* 2013b, Priestnall *et al.* 2014). However, these viruses are uncommonly detected in dogs with CIRD or their possible role as causative agents is not yet completely determined.

The aim of this study was to assess the prevalence of common CIRD-associated viruses (CPiV, CAV-2, CDV) in dogs in and around Vienna, Austria. Although there may be environmental factors specific to this location, our findings are likely to generalise to other locations within Western Europe and possibly further afield. It was further investigated whether emerging viruses (CRCoV and CIV) have a significantly higher prevalence in dogs with CIRD compared to dogs without respiratory disease.

MATERIALS AND METHODS

Animals

Samples were collected from 214 dogs with signs of CIRD between April 2013 and October 2015. The main criterion for inclusion was coughing. A complete history was obtained, and a thorough physical examination was followed by further diagnostic evaluation (*e.g.* complete blood count, blood chemistry, thoracic radiographs) as indicated to exclude non-infectious causes of respiratory disorders (*e.g.* congestive heart failure, airway foreign bodies, airway-compressive neoplasia). All dogs included in this prospective study were either presented at an academic or a private veterinary clinic, or recruited from animal shelters located in the following Austrian regions: Vienna, Lower Austria, Burgenland and Styria, Austria.

Dogs meeting the inclusion criteria were separated into two groups according to different forms of husbandry: group A consisted of 173 privately owned dogs and group B included 41 dogs from animal shelters. Fifty clinically healthy privately owned dogs sampled during routine examinations and procedures (annual vaccination, castration) served as controls (group C). In order to compare the results within the population, the dogs were additionally divided into three subgroups of age: puppies (younger than 6 months), adolescent dogs (from 6 to 18 months) and adults (older than 18 months). According to the period of time from onset of clinical signs until presentation and sample generation the dogs were also classified into two subgroups: acute (within 14 days of onset), and chronic (after 14 days of onset).

Sampling and sample preparation

Nasal and tonsillar swabs (sterile cotton swabs) were collected from all 264 dogs (groups A, B, C) in this study. In 31 animals with progressive respiratory disorders that had been resistant to previous treatment, bronchoalveolar lavage fluid (BALF) samples were additionally collected during tracheobronchoscopy. From 30 acutely diseased dogs, paired blood samples were retrieved for serological examinations.

Swab samples were transferred into tubes containing 1 mL of diethyl pyrocarbonate-treated water and vortexed for 10 seconds. BALF obtained from right and left lung was pooled in equivalent amounts. Swab and BALF samples were frozen at –80°C until further analysis.

Serum was prepared by centrifugation (10 minutes at 3000*g*), was subsequently lifted from the blood samples, and frozen at –20°C.

PCR

For nucleic acid extraction from swabs and BALF samples, QIAamp Viral RNA mini kit (QIAGEN) was used according to the manufacturer's instructions. Despite its name, this kit allows extraction of RNA as well as DNA from different kinds of samples. If not immediately processed, nucleic acid extracts were stored at –80°C.

For detection of virus-specific nucleic acids of CAV-1/CAV-2, CDV, CPiV, CRCoV and influenza virus A, different PCR-techniques and protocols were followed. Published protocols were adapted to commercially available PCR kits quoted below (see also Table 1).

The PCR protocol for CAV-1/CAV-2 published by Nell *et al.* (2000) was carried out using the Fast Cycling PCR Kit (QIAGEN). After an initial step of 95°C for 5 minutes, the PCR was run for 45 cycles of denaturation at 96°C for 5 seconds, primer annealing at 60°C for 5 seconds and primer extension at 68°C for 21 seconds. PCR was finalised by an ultimate extension of 1 minute at 72°C.

CDV-reverse transcription (RT)-PCR described by Frisk *et al.* (1999) was carried out using QIAGEN's OneStep RT-PCR Kit.

Table 1. PCRs, RT-PCRs and real-time PCRs used in this study

Pathogen	PCR-type	Primer/probe sequences	Annealing temperature (°C)	Reference
CRCoV	Real-time RT-PCR	Primer-F 5'-ACGTGGTGTTCCTGTTGTTATAGG-3' Primer-R 5'-AACATCTTTAATAAGGCGACGTAACAT-3' Probe: FAM-5'-CCACTAAATTTTATGGCGGCTGGGATG-3'-TAMRA	60	Spiss *et al*. 2012
CPiV	RT-PCR	Primer-F: 5'-AGTTTGGGCAATTTTTCGTCC-3' Primer-R: 5'-TGCAGGAGATATCTCGGGTTG-3'	55	Erles *et al*. 2004 (modified)
CAV-1/CAV-2	PCR	Primer-F: 5'-GCCACTACTCTCCTGTTGAT-3' Primer-R: 5'-GAAGAAGAAGTCCGAGACAC-3'	60	Nell *et al*. 2000
CDV	RT-PCR	Primer-F: 5'-ACAGGATTGCTGAGGACCTAT-3' Primer-R: 5'-CAAGATAACCATGTACGGTGC-3'	60	Frisk *et al*. 1999
CDV	Real-time RT-PCR	Primer-F: 5'-AGCTAGTTTCATCTTAACTATCAAATT –3' Primer-R: 5'-TTAACTCTCCAGAAAACTCATGC-3' Probe: FAM-5'-ACCCAAGAGCCGGATACATAGTTTCAATGC-3'-TAMRA	48	Elia *et al*. 2006
Influenza A	Real-time RT-PCR	FLUAM-1F: 5'-AAGACCAATCCTGTCACCTCTGA –3' FLUAM-1R: 5'-CAAAGCGTCTACGCTGCAGTCC -3' FLUAM-1P: 5'-FAM- TTTGTGTTCACGCTCACCGT-TAMRA-3'	60	http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf (modified)

RT-PCR Reverse transcription PCR, CRCoV Canine respiratory coronavirus, CPiV Canine parainfluenza virus, CAV-1/2 Canine adenovirus type-1/2, CDV Canine distemper virus

After two pre-steps (50°C for 30 minutes and 95°C for 15 minutes), the PCR was run for 45 cycles of denaturation at 94°C for 30 seconds, primer annealing at 60°C for 30 seconds and primer extension at 72°C for 1 minute. Final extension was at 72°C for 10 minutes. Owing to practical reasons, the protocol was changed using a comparable real-time RT-PCR assay published by Elia *et al.* (2006) during the study. Overall, 18.9% of the samples from diseased dogs, and 34.0% of the samples from control dogs were examined using the Real time RT-PCR assay. Samples showing positive results after conventional PCR were reassessed via Real time RT-PCR assay.

A nested RT-PCR protocol for detection of CPiV-specific nucleic acids (Erles *et al.* 2004) was optimised by using OneStep RT-PCR Kit (QIAGEN) and was found to give better results if only the outer primer pair was used.

Real time RT-PCRs for CRCoV (Spiss *et al.* 2012), Influenza A viruses (WHO 2009) and CDV (Elia *et al.* 2006) were carried out on an Applied Biosystems 7300 Real-Time PCR System in a reaction volume of 25 µL using the SuperScript III platinum OneStep q-RT-PCR Kit (Invitrogen) following the manufacturer's guidelines.

Positive controls and blanks were run with all PCRs. For the CRCoV q-RT-PCR a betacoronavirus (BCoV L9 strain), for the Influenza A q-RT-PCR an EIV 639/69 strain, and for the other PCRs a modified live vaccine (Virbagen canis SHPPi, Virbac) served as positive controls. Blanks consisted of sample-free extracts produced simultaneously to each extraction process.

PCR products of conventional PCRs/RT-PCRs were visualised by performing gel electrophoresis on an 1.2% agarose gel at 100 Volt for 80 minutes. In case of positivity of CAV-1/CAV-2 PCR sequence analysis (Microsynth) was performed to distinguish between CAV type-1 and CAV type-2.

Serology

For detection of antibodies against betacoronaviruses, 30 acute serum samples as well as the corresponding sera (obtained 2 to 3 weeks later) of the convalescent dogs were examined by an indirect immunofluorescence test. Madin-Darby bovine kidney cells were disseminated on 96-well microtitre plates (100 µL/well) and then incubated at 37°C in a humid 5% CO_2-atmosphere overnight. After washing the plates with phosphate-buffered saline (PBS) solution, the adherent cells were infected with BCoV strain 15317/82 and incubated at 37°C for 48 hours. Subsequently cells were washed with PBS again and fixed with 100 mL of 96% ethanol.

The sera underwent twofold serial dilutions from 1:20 to 1:5120 with PBS and immunofluorescence test was performed as follows:

Ethanol was discharged, and the 96-well microtitre plates were washed three times with PBS; 50 µL of the previously diluted sera *per* well were added and incubated at 37°C for 30 minutes. Thereafter, the plates were washed three times with PBS and 50 µL of 1:40 diluted fluorescein isothiocyanate (FITC)-conjugate (anti-dog IgG, Jackson) was added to each well. After incubation at 37°C for another 30 minutes and three washing cycles with PBS, 50 µL/well Eriochrome black T indicator (diluted 1:200 with PBS) was filled in each well of the 96-well microtitre plate to reduce background fluorescence. Plates incubated for 5 minutes at room temperature before cells were washed three times with PBS once more. Finally, wells were filled with 50 µL/well of glycerine buffer solution to prevent the cells from drying.

For evaluation of the microtitre plates an inverse ultraviolet microscope was used. The highest dilution with a clear cytoplasmatic fluorescence was equivalent to the specific antibody titre of each serum sample. Samples that showed no fluorescence in dilution 1:20 were regarded as negative (no antibodies present). Each assay included a positive and a negative control serum.

Statistical analysis

The obtained data are initially presented in a descriptive way and a 95% confidence interval was calculated. Analysis was performed using IBM SPSS.

RESULTS

Study population

The dog population (n=264) predominantly comprised purebred dogs (66.7%) including 66 different breeds. Among these, the most common were Rottweilers (6.1%), Chihuahuas (5.7%),

Labrador retrievers and Australian shepherds (both 3.0%). By age, the population consisted of 31 puppies (11.7%), 59 adolescent dogs (22.3%) and 174 adult dogs (65.9%).

Of 214 dogs with respiratory signs (group A and B), 94 dogs were male and 120 were female. Their median age was 3.53 years (min 0.08; max 15.0). Of these, 140 dogs were presented with acute onset of signs (65.4%), and 56 were chronically diseased dogs (26.2%). For the rest of the study population these data were not available. Of the investigated diseased dogs, more than two-thirds (72.0%) were adequately core vaccinated (against CAV-2, CDV) and almost half of them (45.8%) additionally vaccinated against CPiV.

The control group C consisted of 18 males and 32 females. Their median age was 1.33 years (min 0.17; max 13.3). In this group, 66.0% of the dogs were vaccinated against CAV-2 and CDV and, apart from two exceptions, also against CPiV.

PCR results

Focussing on upper respiratory tract samples (nasal and tonsillar swabs), viral nucleic acids were detected in 31 of 214 diseased dogs (14.5%). Sixteen dogs tested positive for CRCoV (7.5%), 14 dogs for CPiV (6.5%) and one of these dogs additionally for CAV-2-specific nucleic acid (0.5%). One single dog tested positive for CDV-specific nucleic acid (0.5%). In none of the obtained samples from the upper respiratory tract was CIV-specific nucleic acid detected. Of those 31 positive dogs, 21 were privately owned (group A), and 10 kept in shelters (group B). They consisted of five puppies, 12 adolescent dogs and 14 adult dogs. Twenty-seven of the 31 positive dogs (87.1%) showed acute onset of signs, three suffered from chronic disease (9.7%) and for one diseased dog this information was not available (Table 2).

Furthermore, upper respiratory tract samples from two dogs (4.0%) of the clinically healthy control group C tested positive for CRCoV-specific nucleic acid (Table 2).

Nine dogs from group A (5.2%) and seven dogs out of group B (17.0%) tested positive for CRCoV in either nasal, tonsillar or both samples at one time. One of these dogs belonged to the subgroup of puppies; nine dogs were from the adolescent subgroup and six animals from the subgroup of adult dogs. With one exception, all these animals showed acute onset of CIRD (93.7%).

Fourteen diseased dogs (6.5%) tested positive for CPiV. From those, 11 belonged to group A and three to group B. They all harboured CPiV-specific nucleic acid in sample material from the nose and one dog concurrently from the tonsils. Four of these dogs were classified as puppies; three dogs were from the adolescent subgroup and seven dogs were adults. Twelve out of these 14 animals showed acute onset of clinical signs (85.7%), one dog was chronically ill, and for another dog this information was not available. Seven dogs (50.0%) were regularly vaccinated-including against CPiV.

In one of these 14 CPiV-positive dogs, CAV-specific nucleic acid was detected concurrently. This dog was privately owned (group A) and tested positive for CAV in both nasal and tonsillar swabs and CAV-2 strain (Toronto) was confirmed by DNA sequencing. Belonging to the subgroup of adults this dog had been irregularly vaccinated and received its latest vaccine 45 days before sample collection. It presented with a several week history of clinical signs including severe coughing, nasal and ocular discharge, dyspnoea and fever. Apart from that case, in no other dog was viral nucleic acid of two or more different viruses detected. In addition, no other proband of the study tested positive for CAV.

One dog from group A tested positive for CDV-specific nucleic acid in a sample retrieved from the tonsils. RNA sequencing enabled the identification of a CDV vaccine strain (Onderstepoort). The dog was an adult and presented with chronic respiratory disease but no other signs consistent with CDV infection. The vaccination status of this dog was unknown.

Additional information regarding all PCR-positive dogs is summarised in Table 3.

All BALF samples collected from 31 chronically ill dogs revealed negative PCR results.

Serologic examination

From 30 available paired serum samples 17 (56.7%) were obtained from privately-owned dogs (group A). Five of these 17 samples (29.4%) showed a significant increase in anti-CRCoV antibody titres. The antibody titres of two dogs increased more than 128-fold. CRCoV-specific nucleic acid from nasal or tonsillar swabs was detected in these two dogs.

Another 13 paired serum samples (43.3%) were collected from a population of 15 kennelled working dogs with an acute episode of CIRD (group B). As two of the 15 dogs were non-compliant with blood sampling, in these cases no serum samples were obtained. In 10 of the 13 paired serum samples (76.9%), a significant increase in anti-CRCoV antibodies was found. Six dogs revealed a 16- to 128-fold antibody titre increase and concurrent evidence of CRCoV RNA in sample material from the nose or tonsils. No further causative viral agent was detected in these cases (Table 4).

Table 2. Detection rates of viral nucleic acid in samples taken from the upper airways of dogs with and without CIRD

Pathogen	Group	Total diseased dogs (n=214)	A (n=173)	B (n=41)	C (n=50)
CRCoV	n (% dogs) 95% CI	16 (7.5) 4.0 - 11.0	9 (5.2) 1.9 - 8.5	7 (17.0) 5.6 - 28.6	2 (4.0) 0.0 - 9.4
CPiV	n (% dogs) 95% CI	14 (6.5) 3.2 to 9.9	11 (6.4) 2.7 to 10.0	3 (7.3) 0.0 to 15.3	0 (0.0)
CAV-2	n (% dogs) 95% CI	1 (0.5) 0.0 to 1.4	1 (0.6) 0.0 to 1.7	0 (0.0)	0 (0.0)
CDV	n (% dogs) 95% CI	1 (0.5) 0.0 to 1.4	1 (0.6) 0.0 to 1.7	0 (0.0)	0 (0.0)
CIV	n (% dogs)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

CIRD Canine infectious respiratory disease, CRCoV Canine respiratory coronavirus, CPiV Canine parainfluenza virus, CAV-2 Canine adenovirus type-2, CDV Canine distemper virus, CIV Canine influenza virus, Group A Privately owned diseased dogs, Group B Kennelled diseased dogs, Group C Healthy control group, CI Confidence interval
Comparing group A and group B, significantly more CRCoV positives were found within shelters (P<0.009)

Table 3. Additional information about the dogs with positive PCR results concerning their subgroup of age, source of positive sample material and onset of signs

Pathogen	Group	Total diseased dogs (n=214)			A (n=173)			B (n=41)			C (n=50)		
CRCoV	Dogs (n)		16			9			7			2	
	Puppies/adolescent/adult (n)	1	9	6	0	4	5	1	5	1	0	1	1
	Nose/tonsils/both (n)	9	2	5	5	1	3	4	1	2	1	0	1
	Acute/chronic (n)	15		1	8		1	7		0	–		–
CPiV	Dogs (n)		14			11			3			0	
	Puppies/adolescent/adult (n)	4	3	7	4	3	4	0	0	3			
	Nose/tonsils/both (n)	13	0	1	10	0	1	3	0	0			
	Acute/chronic (n)*	12		1	9		1	3		0			
CAV-2	Dogs (n)		1			1			0			0	
	Puppies/adolescent/adult (n)	0	0	1	0	0	1						
	Nose/tonsils/both (n)	0	0	1	0	0	1						
	Acute/chronic (n)	0		1	0		1						
CDV	Dogs (n)		1			1			0			0	
	Puppies/adolescent/adult (n)	0	0	1	0	0	1						
	Nose/tonsils/both (n)	0	1	0	0	1	0						
	Acute/chronic (n)	0		1	0		1						
CIV	Dogs (n)		0			0			0			0	

CIRD Canine infectious respiratory disease, CRCoV Canine respiratory coronavirus, CPiV Canine parainfluenza virus, CAV-2 Canine adenovirus type-2, CDV Canine distemper virus, CIV Canine influenza virus, Group A Privately owned diseased dogs, Group B Kennelled diseased dogs, Group C Healthy control group
Significantly more acutely diseased dogs ($P<0.039$) and dogs from the adolescent subgroup ($P<0.002$) tested positive for CRCoV. Samples derived from the nose provided detection of viral nucleic acid in 90.6% of the positive dogs
*Information not available for one dog of group A

Table 4. Detection of anti-CRCoV antibody titres in paired serum samples (acute; convalescent) from dogs of group A and B

	Dogs	Acute	2 to 3 weeks later	Titre increase	CRCoV (qPCR)
Group A	1	<1:20	1:2560	>128-fold	Positive
	2	<1:20	1:2560	>128-fold	Positive
	3	1:40	1:1280	32-fold	Negative
	4	<1:20	1:320	>16-fold	Negative
	5	1:80	1:640	Eightfold	Negative
	6	1:640	1:1280	Twofold	Negative
	7	1:320	1:640	Twofold	Negative
	8	1:160	1:320	Twofold	Negative
	9	1:320	1:320	0	Negative
	10	1:320	1:320	0	Negative
	11	<1:20	<1:20	0	Negative
	12	1:160	1:160	0	Negative
	13	1:640	1:640	0	Negative
	14	1:1280	1:1280	0	Negative
	15	1:2560	1:2560	0	Negative
	16	<1:20	<1:20	0	Negative
	17	1:640	1:640	0	Negative
Group B	1	<1:20	1:2560	>128-fold	Positive
	2	<1:20	1:1280	>64-fold	Positive
	3	<1:20	1:1280	>64-fold	Positive
	4	<1:20	1:1280	>64-fold	Positive
	5	1:80	1:1280	16-fold	Positive
	6	1:80	1:1280	16-fold	Positive
	7	<1:20	1:2560	>128-fold	Negative
	8	1:80	1:2560	32-fold	Negative
	9	1:320	1:1280	Fourfold	Negative
	10	1:640	1:2560	Fourfold	Negative
	11	1:640	1:1280	Twofold	Negative
	12	1:2560	1:2560	0	Negative
	13	≥1:5120	1:2560	0	Negative
	14	n.a.	n.a.	n.a.	Positive
	15	n.a.	n.a.	n.a.	Negative

CRCoV Canine respiratory coronavirus, qPCR Quantitative PCR, n.a. Not available, Group A Privately owned diseased dogs, Group B Kennelled diseased dogs

DISCUSSION

Various studies from Europe (Erles *et al.* 2004, Schulz *et al.* 2014b, Viitanen *et al.* 2015, Decaro *et al.* 2016), Japan (Mochizuki *et al.* 2008) and the USA (Lavan & Knesl 2015) have reported detection rates for different viral pathogens causing CIRD in dogs. Influenced by their geographic origin, these studies show quite divergent results indicating a dynamic process of virus spread. Frequent surveillance is key for assessing the emergence and spread of novel, potentially zoonotic or vaccine resistant viral diseases. The present study was initiated to investigate the status quo of viral CIRD-associated pathogens in and around Vienna, Austria, with a population of almost 1.9 million citizens and 55,705 registered dogs (Magistrat Wien 2017).

Despite the seasonal influenza epidemics in humans, in none of the obtained canine samples were influenza A virus RNA detected. This finding is in line with the results of other studies from Europe that also found no evidence of influenza A viruses within investigated dog populations (Schulz *et al.* 2014b, Viitanen *et al.* 2015, Decaro *et al.* 2016, Mitchell *et al.* 2017).

In our study, one dog each tested positive for CDV- and CAV-2-specific nucleic acid in samples from the upper respiratory tract. In both cases, sequence analysis confirmed the detection of a vaccine-strain. This finding might be explained by the shedding of viral nucleic acid derived from recently administered live vaccines in these dogs (European Medicines Agency 2014, Wilkes *et al.* 2014). CDV and CAV-2 field strains were not detected in this study. This is consistent with other studies reporting low detection rates of these viruses. In contrast, a study from Japan detected CDV in 1.5% and CAV-2 in 2.9% of the examined household dogs (Mochizuki *et al.* 2008), and studies

from Germany and Italy (Schulz *et al.* 2014a, Decaro *et al.* 2016) detected no CDV- or CAV-2-specific nucleic acid at all. However, CDV- and CAV-2-specific nucleic acid has been detected in 7.4 and 12.5% of asymptomatic shelter dogs in the USA (Lavan & Knesl 2015). This discrepancy most likely reflects consequent vaccination regimens for privately owned and kennelled dogs in Central Europe and indicates that vaccines against CDV and CAV are providing good protection.

Interestingly, only solitary viral infections were detected within the examined population. This finding is in contrast to a study from Germany that reported 12.1% of viral coinfections in clinically ill and healthy dogs (Erles *et al.* 2004). However, it is in accordance with the results of three other studies in which the detection rate of viral coinfections was 2.9, 3.3 and 1.4%, respectively (Mochizuki *et al.* 2008, Schulz *et al.* 2014a, Decaro *et al.* 2016).

Overall, viral nucleic acid from samples of the upper respiratory tract was detected in 31 of 214 diseased dogs (14.5%). A much higher detection rate of viruses was found in the examined shelter animals (24.4%, group B) than in the group of privately owned dogs (12.1%, group A). This finding meets the general opinion that CIRD predominates in crowded dog populations because of higher infection pressure in shelters (Erles & Brownlie 2008, Ford 2012). The most commonly detected pathogens in and around Vienna were CRCoV (7.5%) and CPiV (6.5%). Recent studies from other European countries revealed higher prevalence rates for CPiV, namely 37.7% (Schulz *et al.* 2014a), 35.0% (Viitanen *et al.* 2015) and 13.0% (Decaro *et al.* 2016), respectively. In the present study, the comparably low detection rate of CPiV (6.5%) may be related to a generally high vaccination rate in the population under investigation, especially dogs in group B that were almost all vaccinated against CPiV (92.9%). Interestingly, 50% of the dogs that tested positive for CPiV were regularly vaccinated against it. As the time between vaccination and onset of signs was documented in most of the positive cases, positive results due to recent vaccination were unlikely. Detecting CPiV-specific nucleic acid in dogs despite a current vaccination status might be explained by the fact that the vaccine is not preventing CPiV infection but is rather diminishing the severity of disease caused by CPiV (Day *et al.* 2016).

The different detection rates may also be explained by different study designs. Whereas Schulz *et al.* (2014a) solely included acutely diseased dogs presenting with multiple signs of infectious respiratory disease, Viitanen *et al.* (2015) focused on the detection of viral coinfections in bacterial pneumonia or tracheobronchitis caused by *Bordetella bronchiseptica*. Both studies investigated severely diseased dogs. In contrast, the current study included all dogs presenting for clinical signs compatible with CIRD without regard to onset, extent or duration (excluding non-infectious causes). Leading to a broader spectrum of individuals, this should generalise our findings better into general veterinary practice.

The relevance of CRCoV as a primary cause of CIRD remains unclear as the virus can be found in clinically healthy dogs although a moderate to high prevalence of CRCoV infection has been found in dogs with respiratory disease (Yachi & Mochizuki 2006, Schulz *et al.* 2014a, Viitanen *et al.* 2015). It has further been demonstrated that dogs being experimentally infected with five geographically-unrelated CRCoV isolates showed seroconversion and mild respiratory signs (Mitchell *et al.* 2013a). In the present study, 16 diseased dogs (7.5%) tested positive for CRCoV-specific nucleic acid. These dogs were lacking concurrent proof of other viral pathogens. A recent European multicentre study that focused on emerging pathogens in CIRD found a similar detection rate for CRCoV (7.7%) in the examined dog population but also indicated that the odds of CnPnV infection is doubled in CRCoV positive dogs (Mitchell *et al.* 2017). As not all potential CIRD-associated viral agents have been evaluated in this study (*e.g.* CnPnV) viral coinfection cannot truly be excluded.

The detection rate of CRCoV in group B (17.0%) was much higher than in group A (5.2%). Conversely, there was little difference in the detection rate of CRCoV between group A (5.2%) and the healthy control group C (4.0%). As seroconversion of asymptomatic home-raised working dogs after transferal to training centres at the age of 1 year has been demonstrated (Erles & Brownlie 2005) the existence of subclinical CRCoV infections in certain dog populations has already been proven and could be an explanation for positive PCR results in some dogs. Nevertheless, the complementary serological examinations of the obtained paired serum samples in this study basically reveal good concordance to the PCR results for CRCoV, mostly confirming acute infection. This is especially true for 13 kennelled working dogs that showed seroconversion (fourfold increase in antibody titre or higher) in 76.9% of the cases during an outbreak of CIRD. Concurrent CRCoV-specific nucleic acid was detected in six of these dogs. As viral shedding has been shown to occur in the first days after infection, starting at day 2 and ceasing at day 6 in most cases (Mitchell *et al.* 2013a), collection of swab samples too early or too late in the course of disease might be an explanation for negative PCR results in the remaining dogs. However, the results of the present study further contribute to the assumption that CRCoV can be a primary cause of CIRD especially in kennelled dog populations and emphasise the supportive value of obtaining paired serum samples to verify viruses as an underlying cause of disease.

In the present study, only dogs with progressive, therapy-resistant signs attributed to CIRD underwent tracheobronchoscopy. As detection of viral pathogens in samples from the lower respiratory tract of chronically diseased dogs is thought unlikely (Ford 2012), it is not surprising that none of the obtained BALF samples revealed a positive viral PCR result.

Other viral agents (*e.g.* canine herpesvirus, CnPnV, canine bocavirus) have been detected in dogs with respiratory disease (Buonavoglia & Martella 2007, Kapoor *et al.* 2012, Mitchell *et al.* 2013b). They are discussed as secondary contributing factors probably causing more severe CIRD. However, it cannot be excluded that some are actually opportunistic agents that benefit from the weakened immune system in affected dogs. In this study, these viruses were not investigated and so we cannot exclude them as causes or contributors to the clinical presentation in the evaluated dog population.

In conclusion, CRCoV should be considered as a clinically-relevant cause of CIRD in crowded dog populations. Acutely diseased dogs of younger age are predisposed. For sample collection, the nasal mucosa can be recommended as the favoured site. Especially in larger dog shelters, supplementary use of paired serum samples is indicated to confirm the causal relevance of a certain virus in dogs with CIRD in order to implement adequate hygiene measures. Failure to detect CAV-2 and CDV infection in this study indicates good immunisation protection in the examined dog population and emphasises the relevance of regular core vaccinations. WSAVA guidelines for the vaccination of dogs consider vaccines against CPiV as non-core vaccines (Day *et al*. 2016). This recommendation may be generally applicable for dogs in Austria unless they are exposed to possibly non-vaccinated dog populations (*e.g.* dog exhibitions, animal shelters). More studies are needed to discover the true relevance of CRCoV but also other emerging viral pathogens in the context of CIRD and to assess the need for new vaccines.

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Conflict of interest

No conflicts of interest have been declared.

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