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Detection of respiratory viruses and *Bordetella bronchiseptica* in dogs with acute respiratory tract infections

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ABSTRACT

Canine infectious respiratory disease (CIRD) is an acute, highly contagious disease complex caused by a variety of infectious agents. At present, the role of viral and bacterial components as primary or secondary pathogens in CIRD is not fully understood. The aim of this study was to investigate the prevalence of canine parainfluenza virus (CPIV), canine adenovirus type 2 (CAV-2), canine influenza virus (CIV), canine respiratory coronavirus (CRCoV), canine herpes virus-1 (CHV-1), canine distemper virus (CDV) and *Bordetella bronchiseptica* in dogs with CIRD and to compare the data with findings in healthy dogs. Sixty-one dogs with CIRD and 90 clinically healthy dogs from Southern Germany were prospectively enrolled in this study. Nasal and pharyngeal swabs were collected from all dogs and were analysed for CPIV, CAV-2, CIV, CRCoV, CHV-1, CDV, and *B. bronchiseptica* by real-time PCR.

In dogs with acute respiratory signs, 37.7% tested positive for CPIV, 9.8% for CRCoV and 78.7% for *B. bronchiseptica*. Co-infections with more than one agent were detected in 47.9% of *B. bronchiseptica*-positive, 82.6% of CPIV-positive, and 100% of CRCoV-positive dogs. In clinically healthy dogs, 1.1% tested positive for CAV-2, 7.8% for CPIV and 45.6% for *B. bronchiseptica*. CPIV and *B. bronchiseptica* were detected significantly more often in dogs with CIRD than in clinically healthy dogs ($P < 0.001$ for each pathogen) and were the most common infectious agents in dogs with CIRD in Southern Germany. Mixed infections with several pathogens were common. In conclusion, clinically healthy dogs can carry respiratory pathogens and could act as sources of infection for susceptible dogs.

Introduction

Canine infectious respiratory disease (CIRD), otherwise known as kennel cough or infectious tracheobronchitis, is one of the most common infectious diseases in dogs and has a worldwide distribution (Buonavoglia and Martella, 2007). CIRD describes an infection of the upper respiratory tract caused by single or multiple agents (Appel and Binn, 1987; Mochizuki et al., 2008). It has been postulated that the pathogenesis of CIRD is multi-factorial, since dogs experimentally infected with a single respiratory pathogen often showed only mild clinical signs, while severe clinical disease, such as that commonly documented in natural outbreaks, could not be reproduced (Karpas et al., 1968; Appel and Percy, 1970; Bemis et al., 1977).

CIRD is characterised by an acute onset of mild to severe episodes of a paroxysmal dry cough and nasal discharge (Buonavoglia and Martella, 2007; Mochizuki et al., 2008). Due to the highly contagious nature of the disease, dogs living in crowded conditions, such as shelters and day care centres, are especially susceptible to infection (Appel and Binn, 1987; Buonavoglia and Martella, 2007). Most dogs with CIRD recover spontaneously within days to weeks unless complicating factors occur, such as lower respiratory tract involvement or severe secondary infections (Buonavoglia and Martella, 2007). However, CIRD can become enzootic in certain environments, because of the repeated introduction of novel infectious agents into dog populations (Mochizuki et al., 2008).

A variety of viral and bacterial agents have been detected in dogs with CIRD. Canine distemper virus (CDV), canine adenovirus type 2 (CAV-2), canine parainfluenza virus (CPIV), canine herpesvirus-1 (CHV) and *Bordetella bronchiseptica* are commonly reported pathogens in this disease complex (Erles et al., 2004; Buonavoglia and Martella, 2007), but recent studies have reported the involvement of new viral agents (Priestnall and Smith, 2012). Canine respiratory coronavirus (CRCoV) was first isolated in 2003 from the airways of dogs with acute respiratory signs (Erles et al., 2003) and is highly prevalent among dogs with clinical signs of respiratory disease in many different countries (Yachi and Mochizuki, 2006; Erles et al., 2004; Mochizuki et al., 2008; An et al., 2010a). In 2004, canine influenza virus (CIV) was detected in racing Greyhounds in Florida, USA, and caused severe respiratory disease (Crawford et al., 2005).

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To date, the virus is endemic in many states of the USA (Payungporn et al., 2008; Barrell et al., 2010; Hayward et al., 2010; Holt et al., 2010; Seiler et al., 2010; Serra et al., 2011), but so far there are no endemic areas outside the USA (Kruth et al., 2008; Knesl et al., 2009; De Benedictis et al., 2010; Piccirillo et al., 2010).

To the authors' knowledge there are no published studies investigating the prevalence of CIRD-related pathogens in dogs in Germany. Furthermore, the prevalence of these infectious agents in healthy dogs has not previously been reported. Therefore, the aim of our study was to investigate the prevalence of CPIV, CAV-2, CIV, CRCoV, CHV-1, CDV and *B. bronchiseptica* in dogs with acute respiratory disease, and to compare the data to findings in healthy dogs.

Materials and methods

Study protocol

The study was performed as a prospective investigation from July 2011 to July 2012. The study protocol was approved by the Federal Institution for Animal Welfare (approval number 55.2-1-54-2532.3-33-12).

Animals

Nasal and pharyngeal swabs were collected from 151 dogs at the Clinic of Small Animal Medicine, Ludwig Maximilian University of Munich ($n = 90$), a private practice in Stockstadt/Main ($n = 3$), an animal shelter in Beckstetten/Jengen ($n = 29$) and the research kennels at the Department of Nutrition and Dietetics, Ludwig Maximilian University of Munich ($n = 29$). Of the 61 dogs with clinical signs CIRD, 16 (26.2%) lived in an animal shelter at the time of sampling and 45 (73.8%) were privately owned. All healthy dogs originated from an animal shelter (13/90; 14.4%), a research kennel (29/90; 32.2%), or private households (48/90; 53.3%).

Sixty-one dogs with CIRD and 90 healthy dogs were included in the study. Inclusion criteria for dogs diagnosed with CIRD were history of acute onset of respiratory signs (<14 days), including nasal discharge, sneezing, cough and dyspnoea. Dogs were excluded if an underlying cardiac condition, neoplasia, pleural effusion, or functional or anatomic abnormalities of the respiratory tract were diagnosed, based on the results of history, physical examination, and, if necessary, radiographic and/or echocardiographic examination. Healthy dogs were eligible for inclusion in the study if the physical examination was unremarkable, if they had not been vaccinated within 4 weeks before the sampling date and if they did not have a history of respiratory signs within the last 8 months.

Sampling procedure

Two nasal and two pharyngeal swabs (sterile cotton swabs, Sarstedt AG) were collected from healthy dogs and from dogs with CIRD. For nasal sampling, the swab was placed about 3 cm into one nostril; pharyngeal samples were collected by gently rolling the swab on the soft palate. One pair of nasal and pharyngeal swabs was submitted to Vet Med Labor GmbH, Division of IDEXX Laboratories in Ludwigsburg, Germany, for molecular detection of viral pathogens; the other pair of swabs was stored at –20 °C until real-time PCR analysis for *B. bronchiseptica* was performed at the PCR laboratory of the Clinic of Small Animal Medicine of the University of Munich. For virus detection, a combined analysis of the nasal and pharyngeal swab specimens from each dog was performed; for detection of *B. bronchiseptica*, nasal and pharyngeal swabs were analysed separately. It was not possible to take pharyngeal samples from three dogs, while nasal samples could not be taken from two dogs.

Real-time PCR for detection of viruses

PCR for viral RNA and DNA of CPIV, CAV-2, CIV, CRCoV, CHV-1, and CDV was performed at Vet Med Labor GmbH, Division of IDEXX Laboratories, Ludwigsburg, Germany. Isolation and purification of total nucleic acid were conducted using the QIAamp DNA Blood BioRobot MDx Kit on an automated platform (BioRobot Universal, Qiagen GmbH). Real-time PCR at IDEXX Vet Med Labor was performed using the LightCycler 480 system (Roche) with proprietary forward and reverse primers and hydrolysis probes. Target genes for viral pathogen detection were as follows: (1) canine parainfluenza virus: haemagglutinin-neuraminidase gene; (2) canine adenovirus type 2: hexon gene; (3) canine influenza virus: haemagglutinin gene; (4) canine respiratory coronavirus: haemagglutinin-esterase gene; (5) canine herpesvirus: DNA polymerase gene; and (6) canine distemper virus: phosphoprotein (P) gene. Molecular diagnostics were carried out with six quality controls, including PCR positive controls of known quantity, PCR negative controls, negative extraction controls, an internal positive control spiked into the lysis solution to monitor the nucleic acid extraction efficiency and the presence or absence of inhibitory substances, RNA quality control and an environmental contamination monitoring control.

Real-time PCR for detection of Bordetella bronchiseptica

DNA isolation was performed with the QIAamp DNA Micro Kit (Qiagen GmbH) and eluted in 30 µL elution buffer (Buffer AE). PCR analysis was performed using a Applied Biosystems 7500 Real Time PCR System (Life Technologies GmbH) with 40 cycles, consisting of a denaturation step for 15 s at 95 °C and a combined annealing and amplification step at 60 °C for 60 s. The analysis used SYBR Green PCR Master Mix (QIAGEN GmbH) mixed with water, 5 pmol forward primer (5′-CCCCCGCACATTTCCGAACTTC-3′), 5 pmol reverse primer (5′-AGGCTCCCA AGAGAGAAAGGCTT-3′) and 3 µL of template in a total volume of 30 µL on a MicroAmp 96-well reaction plate (Life Technologies GmbH). A melting curve analysis was performed to confirm amplification of a single PCR product and the correct size was verified on a 2% agarose gel.

Statistical analysis

Data were evaluated using PASW Statistics 18 (SPSS). Groups were compared with Fisher's exact tests and χ^2 tests. Age comparisons between sick and healthy dogs were performed using *t* tests. The level of significance was set at $P < 0.05$. Prevalences of the pathogens were calculated with a confidence interval (CI) of 95%.

Results

Study population

Samples were collected from 61 dogs with CIRD and 90 clinically healthy dogs.

Age was not documented for three sick dogs and one healthy dog. The median age of dogs with CIRD was 3.5 years (3 months–16 years) and the median age of healthy dogs was 3.3 years (3 months–13 years; $P = 0.156$). In the sick dog group, 63.9% were male or castrated male; 36.1% were female or spayed female. The healthy dogs consisted of 40.0% male or castrated male and 60.0% female or spayed female dogs. There was a significantly higher proportion of male intact dogs in the CIRD group than in the healthy group ($P = 0.001$). Mixed breed was the most commonly recorded breed (39.3% of dogs with CIRD; 32.2% of healthy dogs). The clinical signs documented in dogs in the CIRD group included cough (86.7%), nasal discharge (62.2%), sneezing (26.7%) and dyspnoea (6.7%).

Information about vaccination status was available for 98.4% of dogs with CIRD and 96.7% of healthy dogs. A vaccine containing CPIV had been administered SC in 43.3% of dogs with CIRD and 54.0% of healthy dogs ($P = 0.241$). While none of the dogs with CIRD had been vaccinated against *B. bronchiseptica*, one healthy dog had received an intranasal vaccine with a live attenuated *B. bronchiseptica* strain ($P = 1.000$).

Detection of respiratory pathogens

Infections with CIRD pathogens were detected in 52/61 (85.2%) of dogs with CIRD and 47/90 (52.2%) healthy dogs ($P < 0.001$). While CPIV and CRCoV were the only viral pathogens detected in dogs with CIRD, CPIV and CAV-2 were also isolated from healthy dogs (Table 1). CIV, CHV-1 and CDV were not detected in any of the dogs.

When groups were compared, CPIV and CRCoV were significantly more frequently isolated from dogs with respiratory disease than from healthy dogs ($P < 0.001$ and $P = 0.002$, respectively). *B. bronchiseptica* was detected in dogs with CIRD and in healthy dogs, but a higher proportion of dogs with CIRD were positive for *B. bronchiseptica* than healthy dogs ($P < 0.001$).

Infections with two or more pathogens were detected in 23/61 (37.7%) of dogs with CIRD and 2/90 (2.2%) healthy dogs (Table 2). Mixed infections with CPIV and *B. bronchiseptica*, and CRCoV and *B. bronchiseptica* were more common in dogs with CIRD than in healthy dogs ($P < 0.001$ and $P = 0.014$, respectively). Of the 23 dogs

Table 1
Detection of respiratory viruses and *Bordetella bronchiseptica* in dogs with canine infectious respiratory disease (CIRD) and in healthy dogs by PCR.

Pathogen	Dogs with CIRD $n = 61$ (%)	Healthy dogs $n = 90$ (%)	*P*
CPIV	23 (37.7) 95% CI, 26.6–50.3	7 (7.8) 95% CI: 3.6–15.4	<0.001
CAV-2	0	1 (1.1) 95% CI, <0.1–6.6	0.409
CIV	0	0	–
CRCoV	6 (9.8) 95% CI: 4.3–20.2	0	0.002
CHV-1	0	0	–
CDV	0	0	–
B. bronchiseptica	48 (78.7) 95% CI, 66.7–87.2	41 (45.6) 95% CI, 35.7–55.8	<0.001

CPIV, canine parainfluenza virus; CAV-2, canine adenovirus type 2; CIV, canine influenza virus; CRCoV, canine respiratory coronavirus; CHV-1, canine herpes virus-1; CDV, canine distemper virus; 95% CI, 95% confidence interval.

with CIRD that tested positive for CPIV, 14 (60.9%) had a history of vaccination against CPIV.

When analysis was performed comparing the prevalence of positive PCR test results for each pathogen among dogs living in private households and dogs from an animal shelter or research kennel, significantly more dogs from private households were PCR positive for *B. bronchiseptica* ($P < 0.001$; Table 3).

Discussion

CPIV, CRCoV and *B. bronchiseptica* were the only pathogens detected in dogs with acute respiratory signs in the present study and all three pathogens were significantly associated with respiratory disease when compared to healthy controls. CPIV was isolated from 37.7% of dogs with CIRD and was the most common viral pathogen detected. In contrast to the high prevalence of CPIV in our population of German dogs, a Japanese investigation reported positive CPIV results in 5/68 (7.4%) nasal swabs from dogs with acute respiratory disease (Mochizuki et al., 2008). Differences in geographical distribution or vaccination protocols might explain this.

In the present study, 43.3% of all dogs with CIRD and 60.9% of CPIV positive dogs with CIRD had a history of parenteral vaccination against CPIV. Since the time between vaccination and the onset of clinical CIRD was not documented, we could not determine if dogs developed clinical CPIV due to an insufficient immune response following vaccination or if some dogs tested positive for a vaccination strain of CPIV. It has been suggested that dogs vaccinated with a modified live CPIV vaccine could shed the virus for some time after vaccination (Ellis and Krakowka, 2012); the PCR used in the present study was unable to differentiate between vaccine and field strains of the virus. In dogs experimentally infected with CPIV, only mild clinical signs were observed in some animals and others remained subclinical (Appel and Binn, 1987). In our study, infection with CPIV was detected in seven healthy dogs. It has been postulated that most naturally acquired clinical infections with CPIV are co-infections with several pathogens (Appel and Percy, 1970). This was supported by the results of the present study, since only 4/23 (17.4%) dogs in the CIRD group had CPIV infection as a single agent, and 2/7 (28.6%) healthy CPIV positive dogs had a co-infection with *B. bronchiseptica* or CAV-2. It is possible that CPIV could impair the local host defence mechanisms in the canine respiratory tract, facilitating colonisation and infection with other respiratory pathogens, such as *B. bronchiseptica*, or vice versa (Appel and Binn, 1987).

CRCoV was found in 9.8% of dogs with CIRD in the present study, indicating that the virus is circulating in the German dog population. To our knowledge, the prevalence of CRCoV and other respiratory viruses has not previously been investigated in the German dog population. Studies performed in Italy (Decaro et al., 2007), the UK (Erles et al., 2003), Japan (Yachi and Mochizuki, 2006) and Korea (An et al., 2010b) have detected CRCoV in respiratory samples from dogs with CIRD. Additionally, antibodies against CRCoV have been detected in dog populations worldwide, including Italy (Priestnall et al., 2007), New Zealand (Knesl et al., 2009), Korea (An et al., 2010b), and the USA, Canada, the UK and Ireland (Priestnall et al., 2006). CRCoV was only detected in dogs in the CIRD group in the present study and none of the healthy dogs were identified as carriers. However, these results must be interpreted in the light of the small sample size and the fact that CRCoV was not present as a single agent in any of the infected dogs. All six dogs that tested positive for CRCoV were co-infected with *B. bronchiseptica*; two of these dogs also tested positive for CPIV. This is similar to findings in Korea, in which CAV-1 and CAV-2, canine parvovirus 2a and CDV were detected as co-infections in dogs with CRCoV (An et al., 2010a). When Erles et al. (2003) first detected CRCoV in respiratory tissues of shelter dogs with acute respiratory disease in the UK, they suspected that the severity of clinical signs depended on additional factors, such as co-infections. In their study, almost all dogs that were antibody negative on entry to a shelter became CRCoV antibody positive within 3 weeks, but only 50% of them developed respiratory disease. This is in contrast to our findings, in which CRCoV was not detected in any healthy dogs.

In the present study, the respiratory pathogen with the highest prevalence was *B. bronchiseptica*, which was detected in 78.7% of dogs with CIRD. Of these, 52.1% were infected only with *B. bronchiseptica* strongly implying a primary pathogenic role for *B. bronchiseptica* in CIRD. It has been shown that *B. bronchiseptica* possesses the ability to cause respiratory disease after experimentally induced or naturally acquired infection, without concurrent viral or bacterial co-

Table 2
Detection of multiple pathogens in dogs with canine infectious respiratory disease (CIRD) and in healthy dogs by PCR.

Pathogen	Dogs with CIRD $n = 61$ (%)	Healthy dogs $n = 90$ (%)	*P*
Two or more pathogens	23 (37.7) 95% CI, 26.6–50.3	2 (2.2) 95% CI, 0.1–8.2	<0.001
CPIV and CAV-2	0	1 (1.1) 95% CI, < 0.1–6.6	0.409
CPIV and *Bordetella bronchiseptica*	17 (27.9) 95% CI, 18.1–40.2	1 (1.1) 95% CI, < 0.1–6.6	<0.001
CRCoV and *B. bronchiseptica*	4 (6.6) 95% CI, 2.1–16.1	0	0.014
CPIV, CRCoV and *B. bronchiseptica*	2 (3.3) 95% CI, 0.3–1.2	0	0.084

CPIV, canine parainfluenza virus; CAV-2, canine adenovirus type 2; CRCoV, canine respiratory coronavirus; 95% CI, 95% confidence interval.

Table 3
Detection of respiratory viruses and *Bordetella bronchiseptica* in dogs with canine infectious respiratory disease (CIRD) and in healthy dogs from private households and shelter/kennel environment.

Pathogen	Dogs from private households $n = 93$ (%)	Dogs from shelter/kennel $n = 58$ (%)	*P*
CPIV	17 (18.3) 95% CI, 11.7–27.3	13 (22.4) 95% CI, 13.6–34.7	0.537
CAV-2	0	1 (1.7) 95% CI, 0.3–9.1	0.384
CRCoV	6 (6.5) 95% CI, 3.0–13.4	0	0.083
B. bronchiseptica	65 (69.9) 95% CI, 59.9–78.3	24 (41.4) 95% CI, 29.6–54.2	<0.001

CPIV, canine parainfluenza virus; CAV-2, canine adenovirus type 2; CRCoV, canine respiratory coronavirus; 95% CI, 95% confidence interval.

infection (Thompson et al., 1976; Bemis et al., 1977; Keil and Fenwick, 1998).

A recent retrospective study reported a prevalence of *B. bronchiseptica* of 20/99 (20.2%) in bronchoalveolar lavage fluid (BALF) from German dogs with respiratory signs (Steinfeld et al., 2012). There are several possible explanations for the relatively low prevalence in that study compared to ours. First, Steinfeld et al. (2012) included dogs with acute and chronic respiratory signs, and therefore some dogs might have had non-infectious respiratory diseases. Secondly, bacterial culture results were reported, but this method is less sensitive than PCR (Reizenstein et al., 1993). Additionally, in our study, specimens were collected from the upper respiratory tract, an area colonised by different resident bacterial species to those expected from BALF specimens in dogs with respiratory disease; the prevalence of *B. bronchiseptica* might have been different in our study if samples from the lower airways had been collected for analysis.

In the present study, information regarding antimicrobial treatment was not available for most dogs in the CIRD group, but it is possible that some of the dogs had been treated prior to presentation, leading to negative test results for *B. bronchiseptica* in dogs that were originally positive. Thus, we might have underestimated the true prevalence of this bacterium in dogs with CIRD. Although significantly more dogs with respiratory signs tested positive for *B. bronchiseptica*, almost half of healthy dogs (45.6%) were also identified as carriers. Since vaccination within the last 4 weeks was an exclusion criterion for the study and only one healthy dog had received an intranasal vaccination against *B. bronchiseptica*, the positive test results cannot be explained by recent vaccination in our study population. In a British study, *B. bronchiseptica* was cultured from BALF specimens in 62.8% of dogs with clinical signs of respiratory disease and from 34.6% of healthy dogs in the same shelter (Chalker et al., 2003). Thus, healthy dogs can be carriers of *B. bronchiseptica* and, therefore, might represent a potential source of infection for susceptible dogs.

Only one clinically healthy dog tested positive for CAV-2, and CIV, CHV-1 and CDV were not detected from the study population. Based on these results, these pathogens do not seem to play a significant role in CIRD in the German dog population; however, our results must be interpreted carefully because of the relatively small sample size. In addition, the selection of dogs for inclusion in the study might have influenced these data. Since only 26.2% of dogs with CIRD and 46.6% of healthy dogs were from a shelter or kennel environment, and the rest lived in private households, it is possible that infectious agents that can be endemic in multi-dog facilities might have been underdiagnosed in this investigation. When privately owned dogs and shelter/kennel dogs were compared, there were more *B. bronchiseptica* positive dogs living in private households and all CRCoV positive dogs were privately owned. This indicates that CIRD is not only a problem when large numbers of dogs are housed together, as the expression 'kennel cough' implies, but privately owned dogs are susceptible to infection and can act as carriers.

A limitation of our study was that geographical differences in the prevalence of pathogens could not be assessed, since most dogs originated from Southern Germany. It would be interesting to test a larger sample of shelter dogs for respiratory pathogens in future studies and to include dogs from other regions of Germany. Another limitation of our study was the limited sampling period. Since most of the viral pathogens involved in CIRD can only be detected for 7–10 days after infection and peak shedding usually occurs during the incubation period (Buonavoglia and Martella, 2007), specimens from dogs tested outside this window could have yielded false negative PCR results and thus led to underestimation of pathogen prevalence. In nine dogs from the CIRD group, no pathogens were detected, perhaps because specimens were collected outside the viral shedding period, or perhaps these dogs were infected with viral or bacterial pathogens not included in the diagnostic panel used.

Conclusions

This study demonstrated that CPIV and *B. bronchiseptica* represent the most common infectious agents in dogs with CIRD in Southern Germany. However, clinically healthy dogs can also be subclinical carriers of these two pathogens, and therefore could act as a source of infection for susceptible dogs. While CPIV and CRCoV commonly occur as part of mixed infections with multiple pathogens, the role of *B. bronchiseptica* as a primary respiratory pathogen was confirmed.

Conflict of interest statement

Vet Med Labor GmbH Division of IDEXX Laboratories, performed the real-time PCR for detection of viruses in this study. Vet Med Labor GmbH played no role in the study design, in the collection and interpretation of data, or in the decision to submit the manuscript for publication. Dr Balzer is employed at Vet Med Labor GmbH, Division of IDEXX Laboratories. He provided information on viral testing for the Materials and methods section of the manuscript. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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