Prevalence of canine infectious respiratory pathogens in asymptomatic dogs presented at US animal shelters

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Objectives: To determine the prevalence of nine canine infectious respiratory disease (CIRD) pathogens in asymptomatic dogs presented at animal shelters across the United States.

Methods: Ocular and oronasal swabs from asymptomatic dogs (*n* = 503) were tested using qPCR assay for *Bordetella bronchiseptica*, canine adenovirus type 2 (CAV-2), canine distemper virus (CDV), canine herpesvirus type 1 (CHV), canine influenza virus (CIV), canine parainfluenza virus (CPIV), canine respiratory coronavirus (CRCoV), *Mycoplasma cynos* and *Streptococcus equi* subsp *zooepidemicus*.

Results: A total of 240 (47·7%) asymptomatic dogs were PCR-positive for at least one CIRD pathogen. Prevalence of two-, three-, four-, and five-pathogen cases was 12·7, 3·8, 1·8, and 0·4%, respectively. *Mycoplasma cynos* (29·2%), *B. bronchiseptica* (19·5%), CAV-2 (12·5%), CDV (7·4%) and CPIV (3·2%) were the most commonly detected pathogens.

Clinical Significance: The prevalence of traditional and newly emerging pathogens associated with CIRD is poorly defined in clinically healthy dogs. This study determined that a high percentage of asymptomatic shelter dogs harbor CIRD pathogens, including the newly emerging pathogen *M. cynos* and the historically prevalent pathogen *B. bronchiseptica*.

INTRODUCTION

Canine infectious respiratory disease (CIRD) continues to challenge clinicians with its widespread prevalence and multi-agent aetiology, including several newly emerging pathogens, and occasional but persistent occurrence in appropriately vaccinated dogs. The commonly accepted CIRD clinical scenario, often referred to as "canine cough" or "kennel cough", involves primary infection with canine adenovirus type 2 (CAV-2), canine parainfluenza virus (CPIV) or *Bordetella bronchiseptica*, typically resulting in a relatively mild, self-limiting syndrome in uncomplicated cases. When co-infections are involved, CIRD can progress to a more severe and protracted disease (Erles *et al.* 2004, Ettinger & Kantrowitz 2005, Ellis *et al.* 2005, Buonavoglia & Martella 2007). Although multi-factorial infection often occurs in CIRD, *Bordetella bronchiseptica* by itself has been found to cause clinical respiratory disease, (McCandlish *et al.* 1978, Ellis *et al.* 2001), and is generally considered to be the single most prevalent and

important CIRD pathogen despite its usual role as a commensal agent (Keil & Fenwick 2000, Ford 2009).

In addition to these traditional CIRD pathogens, other infectious respiratory agents have been identified, either as newly emerging pathogens or as demonstrated contributors in cases of clinical disease. These include canine respiratory coronavirus (CRCoV) [Erles *et al.* 2004, Ellis *et al.* 2005, Buonavoglia & Martella 2007; Decaro *et al.* 2008, Erles & Brownlie 2008, Knesl *et al.* 2009], canine influenza virus (CIV) [Crawford *et al.* 2005, Buonavoglia & Martella 2007, Barrell *et al.* 2010, Harder & Vahlenkamp 2010, Holt *et al.* 2010], canine herpesvirus type 1 (CHV-1) [Erles *et al.* 2004, Buonavoglia & Martella 2007], and the bacterial pathogens *Mycoplasma cynos* and *Streptococcus equi* subspecies *zooepidemicus* (Chalker *et al.* 2003, Chalker *et al.* 2004, Chvala *et al.* 2007, Pasavento *et al.* 2008, Mannering *et al.* 2009, Priestnall & Erles 2011, Priestnall *et al.* 2011). More recently, a novel canine bocavirus (Sun *et al.* 2009, Kapoor *et al.* 2012, Pratelli & Moschidou 2012, Li *et al.* 2013) and a previously unrecognised

pneumovirus (Renshaw *et al.* 2010, Renshaw *et al.* 2011) have been isolated from dogs with clinical respiratory disease as well as from asymptomatic dogs, often in cases of co-infection with other respiratory viruses. The relative importance of these newer agents and their role in CIRD is not well understood (Renshaw *et al.* 2010, Pratelli & Moschidou 2012, Li *et al.* 2013), nor are they included in current commercial PCR pathogen assays. Given the evolving epidemiological landscape associated with CIRD, a current epidemiologic survey of CIRD pathogens and their relative prevalence in the US dog population would be useful information for clinicians and animal health scientists.

A comprehensive study of the CIRD flora of clinically normal, asymptomatic dogs has not been conducted in a large and diverse canine population. The purpose of our study was to prospectively determine the recent prevalence of nine recognised CIRD pathogens in asymptomatic dogs presented at animal shelters across the US.

MATERIALS AND METHODS

Sources of diagnostic data

Eleven municipal or private, non-profit animal shelters from geographically distinct regions in the United States participated in the study. Shelters were located in states in the West (three in California and one each in Arizona and Colorado), Midwest (one in Indiana), and East (two in Florida and one each in Pennsylvania, Virginia, and South Carolina). Diagnostic samples were obtained from clinically normal dogs presented at the shelters from December 2011 to May 2012, prior to their introduction to the resident shelter population. A total of 503 dogs were tested, 50 dogs from each of nine shelters, 35 dogs from a 10th shelter, and 18 dogs from an 11th shelter. Vaccination status of the study population was unknown. All dogs included in the study were asymptomatic and clinically healthy when admitted to the shelter. Dogs were excluded if they had any overt sign of disease, including inappetence, dyspnoea or other respiratory signs, signs of wasting, keratitis, oral ulceration, conjunctivitis or a rectal temperature above 38.6 C (101.5 F). Dogs were also excluded if they were below 1 year of age or did not have full adult dentition, were transferred from another shelter or were known or suspected to have been recently vaccinated. There were no restrictions for gender, breed or neutering status. Diagnostic samples were obtained before the dogs were introduced into the general shelter population or were vaccinated or treated therapeutically at the shelter.

Diagnostic sampling and testing

Diagnostic samples consisted of three swabs obtained from each asymptomatic non-sedated dog: a dry conjunctival swab, a deep nasal swab, and a deep pharyngeal swab. Swabs were combined in a sterile blood collection tube and submitted by overnight delivery to the same diagnostic laboratory. Samples were tested by PCR assay with a canine respiratory panel (RealPCR test code 2524, Idexx Laboratories, Inc.) for the nine CIRD pathogens included in the study: *B. bronchiseptica*, CAV-2, CDV, CHV, CIV H3N8, CPIV, CRCoV, *Mycoplasma cynos* and *S. equi* subsp *zooepidemicus*.

The IDEXX real-time PCR is a commercially available panel that targets nine respiratory pathogens which contribute to canine respiratory disease (test code 2524 RealPCR™ CRD panel). All assays were designed and validated according to industry standards (Applied Biosystems, User Bulletin #3) [Windsor *et al.*, 2006, Cunha *et al.* 2013]. Target genes for each application were: *B. bronchiseptica*: haemagglutinin fusion protein gene (*FhaB*), GenBank accession number AF140678; CPIV: haemagglutinin-neuraminidase, EF543647; CHV-1: DNA polymerase, CHU63459; CIV H3N8: haemagglutinin gene, CDQ124157; CRCoV: haemagglutinin esterase, AY423274; H1N1: haemagglutinin gene, GQ229373, based on the CDC protocol; *M. cynos*: ITS-1, AF412978; *S. equi* subsp. *zooepidemicus*: 3-phosphoshikimate 1-carboxyvinyltransferase, FM204884.

Real-time PCR tests were validated analytically and clinically according to industry standard protocols (Applied Biosystems) in order to obtain fully standardised real-time PCR tests. For the analytical validation, each assay was evaluated for six validation criteria including amplification efficiency, linearity, reproducibility intra-run, reproducibility inter-run, 2 square value, and signal-to-noise ratio of the fluorescent signal. Clinical samples were selected based on a reference method for each test, and a correlation study performed. For those tests without a reference method, PCR positive samples were confirmed by resequencing using outside primers. Diagnostic sensitivity and specificity based on comparison to reference testing methods were in the high 90s% or sometimes approached 100% (CDV, *S. zooepidemicus*)

RESULTS

The number of target CIRD pathogens per dog detected by PCR assay is shown in Table 1. Each participating shelter encountered clinically normal dogs that were PCR positive for viral and bac-

Table 1. Number of different canine infectious respiratory disease (CIRD) pathogens detected in asymptomatic dogs by geographic region and total study population

Parameter	Regional and overall results			
	East[a]	Midwest[b]	West[c]	Total
Number of shelters	5	1	5	11
Number of dogs tested	253	45	205	503
% dogs CIRD positive (*n*)	45·5 (115)	55·6 (25)	48·8 (100)	47·7 (240)
% positive for one pathogen (*n*)	28·5 (72)	13 (29·9)	29·8 (61)	29·0 (146)
% positive for two pathogens (*n*)	13·8 (35)	4·4 (2)	13·2 (27)	12·7 (64)
% positive for three pathogens (*n*)	2·8 (7)	1·1 (5)	3·4 (7)	3·8 (19)
% positive for four pathogens (*n*)	0·4 (1)	1·1 (5)	1·4 (3)	3·8 (19)
% positive for five pathogens (*n*)	0 (0)	0 (0)	1·0 (2)	0·4 (2)
% positive for 1–5 pathogens (*n*)	45·5 (115)	55·5 (25)	49·8 (100)	47·7 (240)

[a]East, Florida, Pennsylvania, South Carolina, Virginia
[b]Midwest, Indiana
[c]West, Arizona, California, Colorado

Table 2. Prevalence of individual canine infectious respiratory disease (CIRD) pathogens detected in asymptomatic dogs in single and multi-pathogen cases, in the total study population, and in CIRD-pathogen positive cases

Pathogen	% (*n*) CIRD pathogens detected per dog and PCR-positive dogs per pathogen					Overall CIRD pathogen prevalence		
	1 (*n* = 146)	2 (*n* = 64)	3 (*n* = 19)	4 (*n* = 9)	5 (*n* = 2)	No. cases detected	% of total dogs tested (*n* = 503)	% of CIRD pathogen positive dogs (*n* = 240)
B. bronchiseptica	23·3 (34)	59·4 (38)	78·9 (15)	100 (9)	100 (2)	98	19·5	40·8
CAV-2	11·0 (16)	35·9 (23)	73·7 (14)	88·9 (8)	100 (2)	63	12·5	26·3
CDV	11·0 (16)	7·8 (5)	36·8 (7)	77·8 (7)	100 (2)	37	7·4	15·4
CHV-1	0 (0)	3·1 (2)	10·5 (2)	0 (0)	0 (0)	4	0·8	1·7
CIV	0·7 (1)	1·6 (1)	0 (0)	0 (0)	0 (0)	2	0·4	0·8
CPIV	0·7 (1)	4·7 (3)	2·1 (4)	66·7 (6)	100 (2)	16	3·2	6·7
CRCoV	2·1 (3)	4·7 (3)	10·5 (2)	11·1 (1)	0 (0)	9	1·8	3·8
M. cynos	51·4 (75)	82·8 (53)	31·6 (6)	55·6 (5)	100 (2)	147	29·2	61·3
S. zooepidemicus	0 (0)	0 (0)	5·3 (1)	0 (0)	0 (0)	9	1·8	3·8

CAV-2 canine adenovirus type 2, CDV canine distemper virus, CHV canine herpesvirus type 1, CIV canine influenza virus, CPIV canine parainfluenza virus, CRCoV canine respiratory coronavirus

terial CIRD pathogens. In the overall study population of 503 asymptomatic, afebrile dogs, 240 (47·7%) were PCR positive for at least one of the nine CIRD pathogens tested. Prevalence of single-pathogen cases was similar across all geographic regions, 28·5% in the East, 29·8% in the East, 29·9% in the Midwest and 29·0% overall. In all regions, overall prevalence of two-pathogen cases was 12·7% of the total population. Individual dogs were PCR positive for as many as five of the CIRD target pathogens, but the overall prevalence declined abruptly for three- (3·8%), four- (1·8%) and five-pathogen (0·4%) cases.

Prevalence data for individual CIRD pathogens (Table 2) show that *B. bronchiseptica* and *M. cynos* were most commonly detected. *B. bronchiseptica* was detected in 19·5% (98/503) of the dogs overall and in 40·8% (98/240) of those that were CIRD-pathogen positive. *M. cynos* was even more prevalent, occurring in 29·2% (147/503) of the overall population and 61·3% (147/240) of the dogs that were CIRD-pathogen positive. The next most commonly detected pathogens in the total study population were CAV-2 (12·5%), CDV (7·4%), and CPIV (3·2%). CRCoV (1·8%), CHV (0·8%), CIV (0·4%) and *S. zooepidemicus* (0·2%) were relatively uncommon in asymptomatic dogs.

As the number of CIRD pathogens detected per dog increased, *B. bronchiseptica*, *M. cynos*, CAV-2, CDV and CPIV were increasingly more prevalent in multi-pathogen cases. For example, *B. bronchiseptica* was present in 23·3% of single-pathogen cases, 59·4% of two-pathogen cases, 78·9% of three-pathogen cases and 100% of four- and five-pathogen cases. Similar trends were noted for the viral pathogens CAV-2, CDV and CPIV. *M. cynos* prevalence declined from 82·8% when two pathogens were involved to 31·6% when three pathogens were involved, but showed an increasing trend overall as the number of pathogens increased. *M. cynos* was present in 55·6 and 100% of four- and five-pathogen cases, respectively.

There was no noteworthy regional variation in the prevalence of individual CIRD pathogens, with two exceptions. CAV-2 was present in only one of the 205 dogs tested in the five western shelters, a 0·5% prevalence. In contrast, CAV-2 was identified in dogs from all five shelters in the East, with a 4·7% (12/253) prevalence in dogs tested, and in the Midwest shelter, which

had a 6·7% (3/45) CAV-2 prevalence. CDV prevalence was 2·2% (1/45) in the Midwest shelter and 1·2% (3/253) in the East shelters. However, CDV was detected in 12 dogs (5·9%) in the West region, 10 of which were from one shelter in California. Only one of the CDV-positive dogs had a quantitative PCR indicative of active infection (>1million CDV RNA particles per swab), confirming that the dog was incubating CDV despite being asymptomatic at presentation (Elia *et al.* 2006). A follow-up call to the shelter revealed that the dog died of clinical canine distemper 2 weeks after the swab was taken. No other dogs had quantitative PCR values indicative of active infection and no other follow-up calls were made to assess clinical disease progression. This study focused on the PCR test results of dogs at admission to shelters and did not include a plan to follow these animals over time. Animal shelters work hard to find a home for healthy dogs and cats and most of these animals would be lost to follow-up after adoptions.

When the nine target pathogens were analysed for prevalence in the 62 dogs in which two CIRD pathogens were detected, nine different two-way combinations were identified (Table 3) out of 36 possible dual combinations. *B. bronchiseptica* and *M. cynos* were each identified in four of the two-way combinations. When grouped together, these two pathogens were present in seven of the nine 2-way combinations. CDV and CAV-2 were identified in three of the two-way combinations and CPIV in two 2-way combinations. *B. bronchiseptica* and *M. cynos* were involved in 59·4 (38/64) and 82·8% (53/64) of the two-way cases, respectively. CAV-2 was involved in 35·9% (23/64) of the dual-pathogen cases. Breakdown by region revealed no noteworthy geographic variances in prevalence of individual pathogens involved in two-pathogen cases.

Similar to the results for two-pathogen cases, *B. bronchiseptica* and *M. cynos* were disproportionately represented in dogs in which three CIRD pathogens were detected. Out of 84 possible three-way combinations involving the nine CIRD target pathogens, 10 different three-pathogen combinations were identified in dogs that were CIRD-pathogen positive. Seven of the three-way combinations involved *B. bronchiseptica* and six involved *M. cynos*. Only one 3-way combination did not involve

Table 3. Canine infectious respiratory disease pathogens detected in asymptomatic dogs in which two pathogens were detected

Two-pathogen combinations detected [a, b]	Regional and total % (*n*) of two-pathogen combinations			
	East[c]	Midwest[d]	West[e]	Total
B. bronchiseptica + CAV-2	6·0 (2)	0 (0)	11·1 (3)	8·1 (5)
B. bronchiseptica + CDV	0 (0)	0 (0)	3·7 (1)	1·6 (1)
B. bronchiseptica + CPIV	0 (0)	0 (0)	3·7 (1)	1·6 (1)
B. bronchiseptica + *M. cynos*	37·1 (13)	50·0 (1)	63·0 (17)	50·0 (31)
M. cynos + CAV-2	37·1 (13)	50·0 (1)	7·4 (2)	25·8 (16)
M. cynos + CDV	0 (0)	0 (0)	7·4 (2)	3·2 (2)
M. cynos + CIV	2·9 (1)	0 (0)	0 (0)	0 (0)
M. cynos + CPIV	2·9 (1)	0 (0)	3·7 (1)	3·2 (2)
M. cynos + CRCoV	2·9 (1)	0 (0)	0 (0)	0 (0)
CDV + CRCoV	5·7 (2)	0 (0)	0 (0)	3·2 (2)
CAV-2 + CHV	5·7 (2)	0 (0)	0 (0)	3·2 (2)
All two-pathogen combinations	54·7 (35)	3·1 (2)	42·2 (27)	100·0 (64)

[a] *S. zooepidemicus* was not detected in dogs where two CIRD pathogens were involved
[b] CAV-2 canine adenovirus type 2, CDV canine distemper virus, CHV canine herpesvirus type 1, CIV canine influenza virus, CPIV canine parainfluenza virus, CRCoV canine respiratory coronavirus
[c] East, Florida, Pennsylvania, South Carolina, Virginia
[d] Midwest, Indiana
[e] West, Arizona, California, Colorado

B. bronchiseptica or *M. cynos*, a case where CDV, CAV-2 and *S. zooepidemicus* were detected. Eight of the three-way combinations involved CAV-2, four involved CDV, two involved CPIV and one involved CRCoV. Prevalence of three-way combination cases was 3·8% (19/503). Cases involving four or five CIRD pathogens were uncommon in asymptomatic dogs. Five different combinations of four or five CIRD pathogens were identified. All five involved *B. bronchiseptica* and four involved *M. cynos*. The combined prevalence of four- and five-way combination cases was 2·2% (11/503).

DISCUSSION

Nearly half of the asymptomatic dogs presented at participating shelters in each region were PCR-positive for at least one CIRD pathogen (Table 1). Although the fact that asymptomatic dogs can harbour respiratory pathogens is well known, the numbers of asymptomatic of dogs that were PCR-positive for CIRD pathogens may be surprising to some clinicians. Of the dogs that were PCR-positive, single- or dual-pathogen cases comprised the great majority, a combined prevalence of 87·5% (210/240). Cases involving three pathogens or more were relatively rare, involving only 30 of 240 PCR-positive dogs and 6·0% (30/503) of the entire study population.

The predominance of single- and dual-pathogen cases draws attention to which individual CIRD agents were most often involved (Table 2). In the overall study population, *B. bronchiseptica* (19·5%) and *M. cynos* (29·2%) were more prevalent, respectively, than CAV-2 (12·5%), the next most frequently

diagnosed pathogen. CDV was the next most commonly detected (7·4%). In single-pathogen cases, either *B. bronciseptica* and or *M. cynos* together were involved nearly 75% of the time (Table 2). Broadly speaking, these results indicated that in healthy dogs, two CIRD pathogens (*B. bronchiseptica* and *M. cynos*) were commonplace, two other pathogens (CAV-2 and CDV) were present in a lower but clinically relevant minority, and five pathogens (CHV, CIV, CPIV, and CRCoV, and *S. zooepidemicus*) were rare.

The prevalence data suggest that CIRD pathogens do not associate randomly. Only 9 of the 36 possible two-way pathogen combinations and 10 of the 84 possible three-way combinations occurred in the study population. These multi-pathogen cases predominantly involved *B. bronchiseptica* and *M. cynos*, suggesting that the presence of these agents may facilitate co-infection and increase the likelihood of clinical CIRD, which typically involves interaction between bacterial and viral pathogens (Ettinger & Kantrowitz 2005). For example, studies have shown that *B. bronchiseptica* colonisation in dogs is far from innocuous, but is the initial event which quickly leads to ciliostasis, infection and impairment of mucociliary defences (Anderton *et al.* 2004).

B. bronchiseptica and CPIV have long been considered key agents in the aetiology of clinical CIRD (Erles *et al.* 2004, Ettinger & Kantrowitz 2005, Buonavoglia & Martella 2007). This prominent role in dogs with clinical CIRD was not affirmed by the low prevalence of CPIV in asymptomatic dogs in our study (Table 2). Our results indicate that *B. bronchiseptica* and *M. cynos* are found in the respiratory tract of asymptomatic dogs (Table 2). Other studies confirm that *B. bronchiseptica* and *M. cynos* are more prominent in severe CIRD cases and when co-infections are involved. Isolation of *M. cynos* in dogs with CIRD is correlated with increased disease severity and lower respiratory tract involvement, younger age, longer length of time in a shelter (Chalker *et al.* 2004, Chvala *et al.* 2007) and is found as a co-infection with other CIRD pathogens (Chvala *et al.* 2007). *B. bronchiseptica*-associated CIRD is associated with younger age, long duration of recovery, comingling of mixed canine populations, lower-respiratory involvement and coinfection with CAV-2 or CPIV (Decaro *et al.* 2004, Ettinger & Kantorwitz 2005, Radhakrishnan *et al.* 2007, Mochizuki *et al.* 2008).

There was little evidence for regional variations in CIRD prevalence. The exceptions occurred in shelters in the West region, where there was a virtual absence of CAV-2 and a relatively high prevalence of CDV. The latter was the result of 10 positive CDV cases at one shelter, which resulted in a disproportionately high prevalence of this pathogen in the western region. The clinically normal, afebrile, CDV-positive dog that later developed clinical disease at a California shelter should remind shelter staff to conduct syndromic surveillance for any signs of developing illness, isolate animals displaying signs consistent with contagious disease to reduce exposure to the rest of the population and vaccinate on intake, including CDV vaccine. A severe CDV outbreak with *B. bronchiseptica* and CAV-2 involvement in kenneled dogs reported by European investigators further underscores the continued prevalence of CDV in the canine population and

high mortality risk that exists when CDV is involved in CIRD (Decaro *et al.* 2004).

It is important to note that the PCR results were based on non-invasive samples taken from non-sedated clinically healthy dogs but, because positive results were obtained in nearly half the tested dogs, these pathogens must be regarded as commonplace. However it is important to consider that positive results may represent convalescence, subclinical infection, colonisation or carriage of these infectious agents. Although colonisation with *B. bronchiseptica* or *M. cynos* may be asymptomatic, potentiating factors such as environmental stress, impaired immune function, nutritional deficits or co-mingling may trigger primary or secondary infection progressing to clinical disease in healthy dogs harbouring these pathogens. This study highlights the importance of vaccinating animals as they are brought into shelter facilities and best practices that minimise exposure, like reducing length of stay and providing housing that minimises stress and high traffic interactions.

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Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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