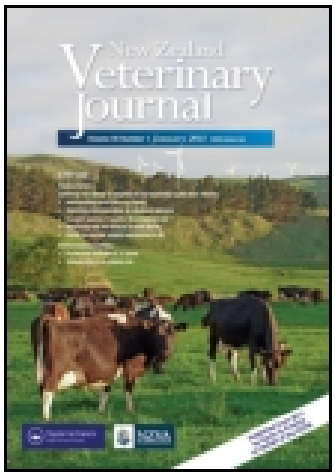

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Short Communication

The seroprevalence of canine respiratory coronavirus and canine influenza virus in dogs in New Zealand

O Knesl[*§], FJ Allan[†] and S Shields[‡]

Abstract

AIM: To determine whether canine respiratory coronavirus (CRCoV) and canine influenza virus (CIV) are present in dogs in New Zealand.

METHODS: Serum samples from 251 dogs of varying age, breed and clinical histories were tested for the presence of antibodies to CRCoV and CIV, using indirect fluorescent antibody (IFA) analysis. The population sampled represented a wide geographic area but principally encompassed the central and lower North Island of New Zealand.

RESULTS: Seventy-three of the 251 samples (29%) were seropositive for CRCoV. Dogs <2 years old were less likely to be seropositive for CRCoV than older dogs. None was seropositive for CIV.

CONCLUSIONS: This study revealed the presence of antibodies to CRCoV in dogs in New Zealand. Young dogs are less likely to be seropositive than older dogs, probably due to increased opportunity for exposure to CRCoV over time. Serum antibodies to CIV were not detected in any of the dogs sampled, suggesting that this virus is unlikely to be present in dogs in New Zealand.

CLINICAL RELEVENCE: Canine respiratory coronavirus is present in New Zealand. Although the role of this virus in canine infectious tracheobronchitis has not been fully elucidated, evidence suggests that it may have a causal role in this disease. Veterinarians should consider CRCoV as a differential diagnosis in cases of respiratory disease in dogs in New Zealand. While CIV appears not to be currently present in New Zealand, veterinarians should consider infection with this virus as a differential diagnosis in dogs presenting with respiratory signs.

KEY WORDS: ***Canine respiratory coronavirus, CRCoV, canine influenza virus, CIV, New Zealand, serology, indirect fluorescent antibody, IFA***

Introduction

Coronaviruses have been associated with respiratory disease in a number of species, including dogs and humans (Erles *et al*. 2003; Ellis *et al*. 2005; Priestnall *et al*. 2006). One study identified a novel canine respiratory coronavirus (CRCoV) in the respiratory tract of dogs in a large re-homing kennel in the United Kingdom (UK) where canine infectious tracheobronchitis was endemic and not controlled by the use of vaccines currently available against respiratory pathogens (Erles *et al*. 2003). This finding suggested a role for CRCoV and possibly other unidentified pathogens in the aetiology of canine infectious tracheobronchitis (Priestnall *et al*. 2006). CRCoV has been implicated as an important and prevalent infectious agent in many cases of respiratory disease in dogs in humane shelters in the UK (Erles *et al*. 2003, 2004; Erles and Bronwnlie 2005; Priestnall *et al*. 2006). A retrospective study in Canada provided further serological evidence that CRCoV may be causally associated with airway disease in dogs (Ellis *et al*. 2005), and studies in Japan and southern Italy suggested that CRCoV was prevalent amongst dogs there (Kaneshima *et al*. 2006; Yachi and Mochizuki 2006; Priestnall *et al*. 2007). CRCoV, a Group 2 coronavirus, is quite distinct from the canine enteric coronavirus (CCoV), a Group 1 coronavirus associated with gastrointestinal disease in puppies. Due to the dissimilarity in the major immunogenic viral spike protein between CRCoV and CCoV, neither infection with CCoV nor the use of vaccines currently marketed against CCoV is expected to illicit a neutralising or protective response to CRCoV infection (Erles and Brownlie 2008).

Canine influenza virus is a newly emerging and highly contagious respiratory tract pathogen of dogs caused by Influenza A subtype H3N8 (Crawford *et al*. 2005; Smith and Daly 2005; Newton *et al*. 2007). This virus was first isolated from the lungs of racing Greyhounds that died of haemorrhagic pneumonia during outbreaks of respiratory disease at racetracks in Florida, United States of America (USA), during 2003 and 2004 (Crawford *et al*. 2005, 2007). Seroepidemiological studies indicated efficient and widespread horizontal transmission of CIV between pet dogs in the USA. As a naïve population, almost all dogs, regardless of breed or age, appeared to be susceptible due to lack of immunity. About 80% of dogs infected with CIV had clinical disease while 20% had subclinical infections (Crawford *et al*. 2007). Thus far, two clinical syndromes have been described, *viz* a mild form involving the upper respiratory tract and a more severe form in the lower respiratory tract, complicated by secondary infections. It is possible that some dogs with a dry cough associated with upper respiratory tract infection may have been infected with CIV (Crawford *et al*. 2007).

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CCoV	Canine enteric coronavirus
CIV	Canine influenza virus
CRCoV	Canine respiratory coronavirus
EIV	Equine influenza virus(es)
HRT18G	Human rectal tumour
IFA	Indirect fluorescent antibody

Canine influenza virus was determined to be very closely related to contemporary equine influenza viruses (EIV) (Crawford *et al.* 2005, 2007). Those authors concluded that the CIV outbreaks first reported in Greyhounds in March 2003 represented an unprecedented interspecies transfer of a complete EIV to dogs and consequent emergence of a new influenza virus associated with acute respiratory disease. A limited but fatal outbreak of respiratory disease attributable to H3N8 EIV infection in Quarryhounds from Britain was retrospectively confirmed in the autumn of 2002 (Smith and Daly 2005), and a further retrospective diagnosis of EIV infection was made among Foxhounds in Britain (Newton *et al.* (2007). Equine influenza occurs widely throughout the world, and until the recent outbreak in Australia in 2007, Australia and New Zealand were the only two countries with significant populations of horses that had been free of the virus (Daly and Mumford 2001).

To date, it has not been established whether either CRCoV or CIV are present in New Zealand. In light of the recent outbreak of equine influenza in Australia and the frequent movement of horses and dogs between Australia and New Zealand, we set out to determine whether CIV is currently present in New Zealand. In every country studied to date, there is a high prevalence of serum antibodies to CRCoV in samples collected from dogs. It was therefore our hypothesis that we would also find dogs seropositive to this virus in New Zealand.

Materials and methods

Blood samples

Blood samples obtained from 251 dogs that had been submitted by veterinary practitioners to a private veterinary diagnostic laboratory (New Zealand Veterinary Pathology Ltd, Palmerston North, NZ) were used. The samples obtained were from a range of breeds of dogs of various ages and with varying clinical histories. The blood samples were submitted as part of diagnostic work-ups for a range of clinical and non-clinical conditions, and histories of varying detail accompanied each sample. The population sampled represented a wide geographic area encompassing the central and lower North Island of New Zealand. Serum was harvested and frozen at –20°C until used.

IFA procedure for CRCoV and CIV

Indirect fluorescent antibody procedures were conducted in a laboratory located in the USA. Monolayers of canine kidney cells of USA origin and human rectal tumour (HRT18G; American Type Culture Collection Cell Line, Manassas VA, USA) planted in 96-well tissue-culture plates were cultured to 30–70% confluency in Dulbecco's modified eagle's medium (Sigma-Aldrich Fine Chemicals Biosciences, St Louis MO, USA) supplemented with fetal bovine serum (Sigma-Aldrich Fine Chemicals Biosciences). Monolayers were inoculated with 25 tissue-culture infectious doses/well of CRCoV or CIV virus isolates originating from the USA. Infected and non-infected monolayers were incubated in a humidified incubator at 37°C for 2–5 days, and then fixed using 80% acetone (in water). Each canine serum sample was diluted 1:40 in phosphate buffered saline supplemented with 1% bovine serum albumin (w/v) (Steris Corporation, Mentor OH, USA) and 0.09% sodium azide (Mallinkrodt Chemicals, Hazelwood MO, USA) followed by two-fold serial dilutions to 1:1,280. The serum dilutions at a final volume of 100 µl/well were reacted with the acetone-fixed, CRCoV-infected and non-infected HRT18G and CIV-infected and non-infected canine kidney cells. Plates were incubated at room temperature for 40–60 minutes, after which time the serum dilutions were discarded and the plates rinsed twice in water to remove unreacted canine IgG. Antibodies bound to CRCoV-infected HRT18G cells or CIV-infected canine kidney cells were detected using a fluorescein isothiocyanate-labelled secondary antibody (rabbit anti-dog IgG) (Sigma-Aldrich, Jerusalem, Israel) diluted 1:250 in phosphate buffered saline supplemented with 1% bovine serum albumin (w/v) and 0.09% sodium azide (w/v), and added at 50 µl/well. The conjugate was incubated at room temperature for 40–60 minutes, followed by washing the plates twice in water. Endpoint CRCoV and CIV IFA titres, observed using immunofluorescence microscopy inverted microscope (Olympus IX71/IX51; Olympus, Southend-on-Sea, Essex, UK) fitted with a xenon 75-W lamp and fluorescein isothiocyanate filters, were the inverse of the last dilution of serum exhibiting definite CRCoV or CIV fluorescence. In instances where no virus-specific fluorescence at the 1:40 dilution was observed, dogs were considered seronegative or non-exposed to the virus. Each 96-well plate included both positive and negative control antibody. Bovine coronavirus direct fluorescent antibody (American BioResearch Inc, Pullman WA, USA) and a non-commercial source of CIV-positive sera were used as positive controls for CRCoV and CIV, respectively. Serum from dogs known to be CRCoV and CIV antibody-negative by IFA assay was used as negative control antibody.

Statistical analysis

Dogs were grouped in the following age groups: 0–2 years, 3–6 years, 7–10 years, and 11–18 years. The overall prevalence of dogs seropositive for both CRCoV and CIV was determined within each age group. A χ^2 test was used to determine whether there were any effects of age on the prevalence of dogs seropositive for CRCoV. In addition, a χ^2 test was used to determine whether there was an effect of age group on the proportion of samples that were seropositive within each of the antibody titre levels of CRCoV (<1:40, 1:160, 1:320, 1:640, and >1:1,280).

The χ^2 test was used to determine whether there was any difference in the prevalence of dogs seropositive for CRCoV between those with and those without a history of clinical signs consistent with respiratory disease. Minitab v15 (Minitab Inc, State College PA, USA) was used for statistical calculations.

Results

Of 251 sera, 73 (29%) were found to be seropositive for CRCoV. None was seropositive for CIV. The prevalence of samples with antibodies to CRCoV differed between dogs when grouped according to age (p=0.045), due mainly to fewer than expected antibody-positive dogs within the 0–2-year-old age group (Figure 1). However, there was no effect of age group on the proportion of seropositive samples within each of the CRCoV antibody titre levels (p=0.109).

Of the 251 samples tested, a history was submitted with the sample in 210 (84%) cases. Of those 210 samples, 63 (30%) dogs were seropositive to CRCoV. Of this subset, 2/9 (22%) dogs were seropositive and had a history of respiratory signs and 61/201

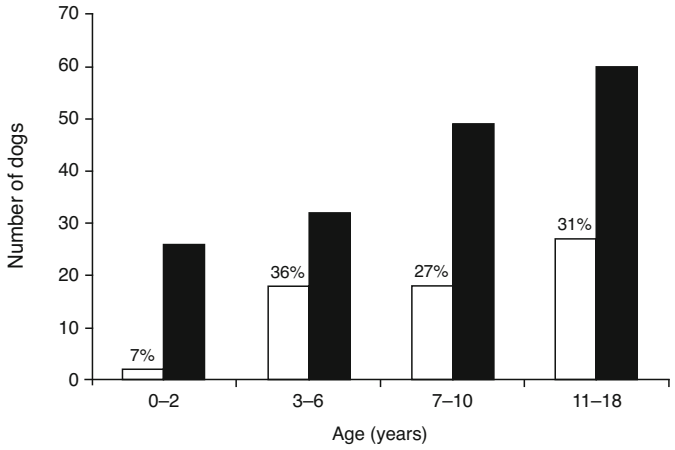

Figure 1. Distribution of serum samples with positive (□) or negative (■) antibody status for canine respiratory coronavirus (CRCoV) from 251 dogs in New Zealand differing in age. The prevalence of CRCoV within each age group is shown above the bars.

(30%) were seropositive but did not have a history of respiratory disease. There was no significant difference between the seroprevalence of these two groups (p=0.603).

Discussion

This is the first study that has demonstrated the presence of antibodies to CRCoV in samples collected from dogs in New Zealand. Furthermore, the study indicated a high prevalence (29%) of reactors to this virus in dogs in this country; this was in the range reported in dogs in other countries, *viz* 36% in the UK, 54.7% in the USA, 59.1% in Canada, and 30.4% in the Republic of Ireland (Priestnall *et al.* 2006; Erles and Brownlie 2008), 17.8% in Japan (Kaneshima *et al.* 2006), and 20–32.5% in Italy (Decaro *et al.* 2007; Priestnall *et al.* 2007).

Unlike CRCoV, CIV is either absent or of low prevalence in dogs in New Zealand. The CIV IFA serological assay used in this study is broadly reactive for antibodies to all antigens that are common across Influenza A subtypes and is not specific for CIV (Influenza A subtype H3N8). As such, we feel confident that dogs sampled had not encountered any Influenza A subtypes during the period of collection of the samples. Despite the apparent absence of CIV, veterinarians practising in New Zealand should be aware that the country has a naïve and highly susceptible population of dogs with respect to this virus. Whether the recent outbreak of EIV in Australia represents an additional risk for the development of CIV in New Zealand is unknown. The EIV outbreak in Australia resulted in some dogs becoming infected; seroconversion was seen in dogs in contact with infected horses, and while clinical signs were noted in those dogs, there was no evidence of horizontal transmission among the dogs (Dubovi and Njaa 2008). As such, those authors proposed that these cases were likely to be EIV in dogs rather than CIV infections. Australia was declared free of EIV on 30 June 2008, 6 months after the last case, and obtained international recognition of proof of freedom in December 2008 (Erles and Brownlie 2008). This would obviously reduce the risk of any potential spillover of EIV into dogs in Australia and thus New Zealand. Infection with CIV should, however, remain a differential diagnosis in any dog with respiratory signs.

Various studies have shown that infection with CRCoV can occur in dogs of all ages but dogs younger than 1 year of age are more likely than older dogs to be seronegative (Priestnall *et al.* 2006, 2007; Erles and Brownlie 2008). The seroprevalence of CRCoV has been found to increase in dogs older than 1 year of age, reaching a plateau between the ages of 2 and 8 years (Erles and Brownlie 2008). This pattern is thought to be a consequence of increasing likelihood of exposure to CRCoV, with increasing contact with infected dogs over time (Erles and Brownlie 2008), although the persistence of CRCoV antibody titres post-infection has not been determined. Our findings are consistent with this pattern as there was a lower prevalence of CRCoV-seropositive dogs in the 0–2-year-old age group than in the older age groups, and the prevalence within the older age groups was comparable with earlier reports in the literature (Figure 1).

As well as considering the prevalence of dogs seropositive to CRCoV, we also wished to determine whether there was an effect of age group on the proportion of seropositive samples within different titre levels of CRCoV antibody. Low titres in older dogs may reflect antibodies acquired during past exposure rather than recent infection, and correspondingly high titres in older dogs may be suggestive of frequent re-exposure to the virus. However, no effect of age group on the magnitude of the titres was found.

Canine infectious tracheobronchitis, also known as kennel cough, is a multi-aetiological condition which can be caused by viruses and bacteria, including mycoplasmas (Erles *et al.* 2003; Ellis *et al.* 2005; Ford 2006; Priestnall *et al.* 2007; Zeugwetter *et al.* 2007). In a re-homing kennel, Erles *et al.* (2003) found that endemic canine infectious tracheobronchitis could not be controlled by routine vaccination against some of the known pathogens, namely canine parainfluenza virus, canine adenovirus type 2, and *Bordetella bronchiseptica*. Those authors found that dogs that had no antibodies to CRCoV on entry into the kennel had a significantly increased probability of developing respiratory disease. This finding suggests that CRCoV may be involved in the pathogenesis of canine infectious tracheobronchitis.

We were interested in determining whether there was an increased seroprevalence to CRCoV in dogs that had a history consistent with respiratory disease, but this was not seen. There are many potential reasons for this. Firstly, antibody persistence in dogs infected with CRCoV is unknown. Dogs seropositive to CRCoV but lacking a clinical history consistent with respiratory disease may reflect past exposure to CRCoV. Secondly, an apparent lack of association between seroprevalence for CRCoV and clinical disease could be explained by sampling acutely infected dogs before they have had a chance to seroconvert. Access to paired acute and convalescent serum samples was not available in this retrospective study; such samples would be required in order to further understand this association more clearly. Thirdly, the lack of association could have been due to the fact that dogs with a history of respiratory signs may have had non-respiratory disease, e.g. heart disease, or may have had primary respiratory disease of a non-infectious nature, e.g. neoplasia, or infectious respiratory disease due to another cause. The retrospective nature of this study made it impossible to separate out these possibilities. Additionally, it is important to consider the close relationship between CRCoV and bovine coronavirus (Erles *et al.* 2003). Dogs experimentally infected with bovine coronavirus did not suffer from significant disease (Kaneshima *et al.* 2007). As such, a fourth possibility to consider is that, given this was a serological study, it was not pos-

sible to differentiate between infection with CRCoV or bovine coronavirus. While infection with bovine coronavirus would be expected to be a rare event, the only way to rule it out would be to isolate the virus from infected dogs.

Controlled canine respiratory coronavirus challenge studies in dogs have not been reported to date, and although there appears to be an association between CRCoV infection and canine infectious tracheobronchitis, causality has yet to be definitively confirmed (Erles and Brownlie 2008). Despite this, infection with CRCoV should be considered a differential diagnosis in cases of apparent 'vaccine failure' in outbreaks of respiratory disease in fully vaccinated dogs (Ellis *et al*. 2005). It remains to be seen whether vaccination against CRCoV would confer additional protection against canine infectious tracheobronchitis. Available vaccines containing CCoV are unlikely to provide any protection from disease associated with CRCoV due to significant antigenic dissimilarity (Erles and Brownlie 2005; Kaneshima *et al*. 2006).

In conclusion, the serological findings described here indicate CRCoV is present in dogs in New Zealand. There is a growing body of evidence suggesting that a number of novel pathogens, including CRCoV, may well play a role in the complex and multifactorial nature of canine infectious tracheobronchitis. Serum antibodies to CIV were not detected in any of the samples collected from dogs, suggesting that this virus is unlikely to be present in dogs in New Zealand.

Declaration of interest

While the study was part funded by Pfizer Animal Health, the authors, two of whom (Knesl and Shields) are employees of Pfizer Animal Health, had full discretion in the study and the content of this paper.

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