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The association of *Streptococcus equi* subsp. *zooepidemicus* with canine infectious respiratory disease

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Abstract

Canine infectious respiratory disease (CIRD) is a multi-factorial infection that affects many kennelled dogs despite the wide use of vaccination. Current vaccines aim to protect against viral agents and a single bacterial agent, *Bordetella bronchiseptica*. We sought to examine the role of streptococcal species in CIRD. The isolation and identification of streptococci in the lower respiratory tract of clinically healthy dogs and those with CIRD were used to correlate the presence of specific streptococcal species with respiratory disease. In this study we report that the presence of *S. equi* subsp. *zooepidemicus* is associated with increasing severity of disease in a population of kennelled dogs with endemic CIRD.

Keywords: Canine infectious respiratory disease; β-Haemolytic; *Streptococcus canis*; *Streptococcus equi* subsp.; *zooepidemicus*

1. Introduction

Canine infectious respiratory disease (CIRD) is an infection that affects dogs of all ages and commonly occurs when large numbers of dogs are housed together in close confinement. The disease has high morbidity with the dry hacking cough characteristic of laryngitis in the early stages, nasal and/or ocular discharges, and variable anorexia and depression, which can progress to tracheobronchitis, pneumonia and even death in more severe cases. The disease has historically been regarded as a complex infection in which combined or sequential challenge with both viral (canine parainfluenzavirus; CPIV, canine adenovirus; CAV-2)

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and bacterial agents produces a synergistic enhancement of the clinical signs (Appel and Binn, 1987). The most common bacterial agent detected during the disease is *Bordetella bronchiseptica* (McCandlish et al., 1978), but other bacterial species such as *Pasteurella* spp., *Mycoplasma* spp. and β-haemolytic streptococci (βhS) have all been associated with disease (McCandlish et al., 1978; Rosendal, 1978; Thrusfield et al., 1991).

Many studies involving bacterial isolation from the upper (oral and nasal cavity) and lower respiratory tract (trachea and lungs) of both diseased and healthy dogs mention the presence of βhS (Smith, 1967; McCandlish et al., 1978; McKiernan et al., 1982; Azetaka and Konishi, 1988). However, despite the variety of species of βhS found in the upper respiratory tract of dogs, only a few investigations have focused upon the species of βhS involved in lower airway disease (Garnett et al., 1982; Angus et al., 1997). Although species of βhS in the canine respiratory tract were noted by Biberstein et al. (1980) this study neglected to distinguish between carriage in the upper and lower respiratory tract. Furthermore, even though isolation was from veterinary hospital patients the reason for referral and therefore any link to specific clinical conditions was omitted. The most common βhS in dogs, *S. canis*, a Lancefield Group G *Streptococcus*, is a normal commensal of the genital and respiratory mucosa as well as skin (Timoney, 1987; Quinn et al., 1999). *Streptococcus canis* has previously been isolated from the tonsils of 60–73% of healthy dogs (Smith, 1967; Sadatsune and Moreno, 1975; Biberstein and Hirsh, 1999). *Streptococcus canis* causes a variety of sporadic and opportunistic infections in dogs, including pneumonia, septicemia, abscesses, otitis, mastitis, pyometra, proctitis, toxic shock syndrome and necrotising fasciitis (Biberstein and Hirsh, 1999; Quinn et al., 1999).

In addition to *S. canis* βhS of other Lancefield Groups, such as A, C and E, have also been isolated from dogs (Biberstein et al., 1980). *Streptococcus equi* subsp. *zooepidemicus*, Lancefield Group C, is found as a commensal of the upper respiratory tract mucosa of mammals (Timoney et al., 1988; Quinn et al., 1999). It is associated with several disease syndromes including lower airway disease, foal pneumonia and cervicitis in horses (Chanter, 1997; Biberstein and Hirsh, 1999), pneumonia in llamas (Biberstein and Hirsh, 1999), septicaemia and arthritis in pigs (Timoney, 1987), mastitis in cows and goats (Timoney et al., 1988), septicaemia in poultry, pericarditis and pneumonia in lambs (Timoney, 1987), lymphadenitis in guinea pigs (Quinn et al., 1999) and glomerulonephritis in humans (Balter et al., 2000). In dogs *S. equi* subsp. *zooepidemicus* has been associated with wound infections, septicaemia (Quinn et al., 1999) and acute necrotising haemorrhagic pneumonia (Garnett et al., 1982). In this study we sought to establish which species of βhS are present in the respiratory tract of both healthy dogs and those with CIRD.

2. Materials and methods

2.1. Study populations and sampling

The main study population ($n = 209$, bronchial alveolar lavage, BAL) comprised animals from a well-established re-homing kennel (∼600 dogs) with a history of endemic CIRD. On entry to the kennel all dogs were vaccinated with KAVAK DA_2 PiP69 (Fort Dodge) a live attenuated vaccine for distemper virus, CAV-2, CPIV and canine parvovirus

and KAVAK L against Leptospirosis. The presence of both canine coronavirus (CRCOV) and *B. bronchiseptica* has been demonstrated in dogs with CIRD in this centre (Chalker et al., 2003; Erles et al., 2003). Each week this kennel must sacrifice some dogs for welfare reasons and from these dogs 2–3 were selected arbitrarily for sampling. BAL samples were taken by the following method from a total of 209 individual dogs over a 2-year period from 1999 to 2001. Within 2 h of euthanasia the trachea was clamped just above the bifurcation to prevent any tracheal contamination of the lung during sampling. Using sterile catheter tubing 50 ml Hanks Balanced Salt solution was then placed into the left apical lung lobe. This lung lobe was then massaged manually for 30 s and the BAL withdrawn. At euthanasia dogs were also graded for the severity of clinical respiratory score into the following categories: (1) no respiratory signs, $n = 71$; (2) mild cough, $n = 37$; (3) cough and nasal discharge, $n = 76$; (4) cough and nasal discharge with depression and/or inappetence, $n = 9$; (5) suppurative bronchopneumonia, $n = 16$. After BAL sampling a section of lung tissue from the right distal lobe was taken for histological analysis. Formalin fixed (10% formalin saline) tissue blocks were embedded in paraffin, and standard haemtoxylin and eosin stained sections were viewed under a light microscope (40×, 100×, 400×). The presence or absence of intra-alveolar neutrophils was noted. The total number of days each dog spent in the kennel was recorded and time in the kennel was then calculated in weeks. The age and clinical condition on entry into the kennel of each animal was noted and a clinical condition composite score based on nutritional status, coat, demeanour, appetite and a general clinical examination (temperature, pulse rate, respiration rate) was graded as follows: good (1), poor (2), very poor (3). An additional dog population was included as a control group that comprised of household pet dogs with clinical respiratory symptoms referred to diagnostic bacteriology at the RVC over a 2-year period (1998–2000) ($n = 71$, BAL). Samples from the control group were collected using an endoscopically guided technique as described by Cocoran (1998). All samples in the study were kept at 4 °C until bacteriological testing, and testing was performed within 24 h of sampling excepting the calculation of CFU per millilitre that was performed on frozen BAL.

2.2. Bacterial isolation and identification

A 50 μl volume of BAL was plated in duplicate onto Columbia Blood Agar (Oxoid Ltd., Hampshire, UK) plates with 5% sterile sheep blood, and incubated both aerobically and anaerobically for 24 h at 37 °C. β-Haemolytic colonies were identified and then purified to single colonies. Gram-positive catalase-negative bacteria were identified as streptococci by colonial and cellular morphology, and then sero-grouped by latex bead slide agglutination (Oxoid Ltd., Hampshire, UK) into Lancefield Groups. Isolates were then identified to the species level by biochemical utilisation and enzymatic action using the API20STREP manual identification kit (bioMérieux UK Ltd., Basingstoke, UK). In order to detect mixed infections three colonies from the first 12 dogs in the study were tested by both latex bead slide agglutination and API20STREP. Serial dilutions of BAL in phosphate buffered saline (Sigma-Aldrich Co. Ltd., Dorset, UK) were plated in triplicate, incubated as described above and the CFU per millilitre BAL calculated. Growth of βhS was then graded as follows: none (0), <100 CFU per ml (1), 100–1000 CFU per ml (2), and >1000 CFU per ml (3).

2.3. Statistical analyses

A significance level or probability of a type I error (α) of 0.05 was assumed for all analyses. The presence of *S. equi* subsp. *zooepidemicus* with the age, clinical condition on entry to the kennel, weeks in the kennel, the presence of intra-alveolar neutrophils and clinical respiratory scores was analysed using Prism (version 3.0, GraphPad Software Inc., San Diego, USA) statistical analysis software χ^2 testing. The correlation of bacterial growth and respiratory score was determined by use of the combined mean scores for *S. equi* subsp. *zooepidemicus* growth for each respiratory score, analysed with Prism one way ANOVA (non-parametric) testing. The presence of *S. canis*, *S. equi* subsp. *zooepidemicus* and respiratory disease in the sampled kennelled dogs with time in weeks was also calculated.

3. Results

β-Haemolytic streptococci were isolated from both study populations, and isolation from the BAL of household pets was markedly different from the kennelled dogs (1.4% household, 23.9% kennel, χ^2 analysis $^{***}P = 0.000$). All βhS isolates were found to be *S. canis* or *S. equi* subsp. *zooepidemicus*. Mixed infections with differing Lancefield Groups or species were not found, furthermore all individual plates yielded colonies of uniform morphology. Both *S. canis* and *S. equi* subsp. *zooepidemicus* were isolated from the kennelled dogs, whereas only a single isolate of *S. equi* subsp. *zooepidemicus* and no *S. canis* were isolated from the household pets. *Streptococcus equi* subsp. *zooepidemicus* was found to be the predominant βhS species in the kennelled dogs (92.0%). The carriage of both *S. canis* and *S. equi* subsp. *zooepidemicus* was examined in the kennelled dogs within each grade of clinical respiratory score (Fig. 1). *S. canis* was present in dogs both with and without clinical scores, and isolation did not increase with disease severity. By contrast, healthy

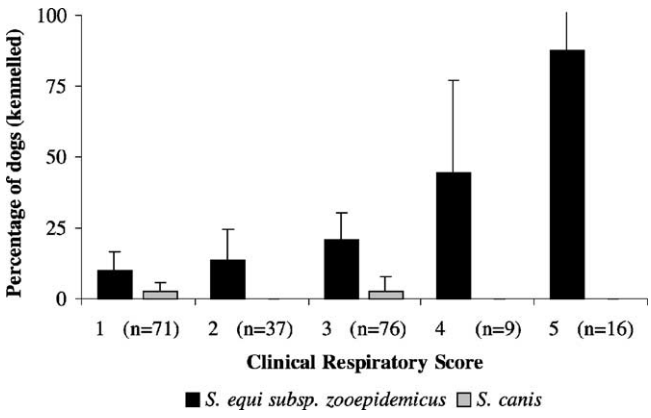

Fig. 1. Isolation of *S. canis* and *S. equi* subsp. *zooepidemicus* from 209 kennelled dogs with clinical respiratory score (*n*: total number of dogs in each group). Err bars represent confidence intervals (95%).

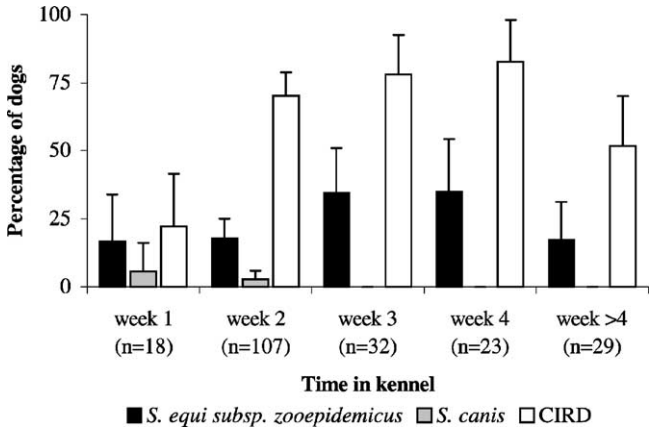

Fig. 2. Percentage of dogs with CIRD, *S. canis* or *S. equi* subsp. *zooepidemicus* with time in the kennel (*n*: total number of dogs in each group from a total of 209 dogs). Err bars represent confidence intervals (95%).

dogs were less likely to have *S. equi* subsp. *zooepidemicus* in the lower respiratory tract than diseased animals (χ^2 analysis, $^{**}P = 0.004$) and the isolation of *S. equi* subsp. *zooepidemicus* increased dramatically with increasing clinical respiratory score, from 9.7% in dogs with no symptoms to 87.5% in those dogs with suppurative bronchopneumonia (χ^2 analysis, $^{***}P = 0.000$). Dogs with higher respiratory scores were also more likely to have a greater mean *S. equi* subsp. *zooepidemicus* bacterial growth score than clinically healthy dogs (one way ANOVA analysis $^{***}P = 0.000$. $R^2 = 0.194$, $F = 22.265$). The age and clinical condition of the animal on entry to the kennel had no affect on the isolation of *S. equi* subsp. *zooepidemicus* (χ^2 analysis, age $P = 0.341$, clinical condition on entry $P = 0.295$).

The percentage of dogs with CIRD in the kennel increased dramatically from 21.1% in week 1 to 70.1% in week 2, and CIRD did not decrease in the population until after the fourth week (Fig. 2). Although no significant difference was detected, the number of dogs with *S. equi* subsp. *zooepidemicus* in the lung increased by 20.6% with time in the kennel from 16.7% in week 1 to 34.4% in week 3 (Fig. 2), whereas no such trend was seen with *S. canis*.

Histological analysis revealed that dogs with *S. equi* subsp. *zooepidemicus* were more likely to have intra-alveolar neutrophils than those without *S. equi* subsp. *zooepidemicus* (χ^2 analysis, $^{**}P = 0.006$). In dogs with higher bacterial scores, acute suppurative or necrotizing pneumonia with moderate to marked macrophage aggregation was often noted, similar to the findings of Garnett et al. (1982) in dogs with *S. equi* subsp. *zooepidemicus* induced haemorrhagic streptococcal pneumonia (HSP). No bacterial cells were apparent on H and E stained sections.

4. Discussion

In this study we focused upon the species of βhS present in the lower respiratory tract of household and kennelled dogs, with and without respiratory disease. Although

S. canis is the predominant βhS of the respiratory tract in dogs (Biberstein et al., 1980) and was isolated from the lower respiratory tract of some kennelled dogs in this study, it was not associated with CIRD in the kennelled dogs. In contrast, an increased isolation of *S. equi* subsp. *zooepidemicus* was associated with increasing CIRD severity. Dogs with any respiratory symptoms were more likely to have *S. equi* subsp. *zooepidemicus* in the lower respiratory tract than more healthy animals in the kennel and *S. equi* subsp. *zooepidemicus* was found in a lower proportion of the household pets than the kennelled dogs.

Streptococcus equi subsp. *zooepidemicus* has previously been associated with HSP in dogs (Garnett et al., 1982). The HSP syndrome was a severe infection in a closed colony of beagles, in which sudden death ensued without prior clinical scores. Necropsy findings included abundant haemorrhagic exudates within the trachea and bronchial tree, with diffuse dark reddening of the lungs. In addition, there were ecchymotic haemorrhages of a range of other tissues. The disease was reproduced by intra-tracheal inoculation with *S. equi* subsp. *zooepidemicus* in one dog. Interestingly in this study, dogs with higher *S. equi* subsp. *zooepidemicus* growth scores were more likely to have intra-alveolar neutrophils and share histological features of the lungs described by Garnett et al. (1982) in HSP than those dogs with low growth scores.

CIRD has historically been considered a complex disease, involving both bacterial and viral agents. Indeed, several agents have been described in this kennelled population of dogs, including CRCOV (Erles et al., 2003), *B. bronchiseptica* (Chalker et al., 2003) and *S. equi* subsp. *zooepidemicus* (this study). Although the pathogenic potential of CRCOV has not yet been clarified, data by Erles et al. (2003) shows that CRCOV predominates in those dogs with mild respiratory disease (score 2) and similarly Chalker et al. (2003) found that *B. bronchiseptica* predominates in those dogs with moderate disease (score 3). *Streptococcus equi* subsp. *zooepidemicus* is associated more commonly with only the more severe cases of CIRD (scores 4–5) indicating it may act as a secondary invader. Indeed, βhS species have previously been described as secondary invaders in the CIRD 'complex' (McCandlish et al., 1978). However, it is still not known if *S. equi* subsp. *zooepidemicus* plays a primary role in respiratory disease in these animals or merely invades the respiratory tract following damage by other pathogens. Epidemiological evidence suggests that in the horse *S. equi* subsp. *zooepidemicus* may be a primary pathogen in respiratory disease (Wood et al., 1993; Chanter, 1997) but it is generally considered to be an opportunistic pathogen (Walker and Timoney, 1998; Anzai et al., 2000). Even if *S. equi* subsp. *zooepidemicus* is not a primary cause of CIRD in these dogs, the high isolation rate from dogs with suppurative bronchopneumonia (87.5%) supports the hypothesis that *S. equi* subsp. *zooepidemicus* is responsible for the more severe clinical signs seen in this kennel. The low isolation from household pets (1.4%) with respiratory disease indicates this agent is probably not a common respiratory infection and could be a problem particular to this kennel. Although any previous kennelling was not taken into consideration it is likely that some of the household pet dogs in this study have been kennelled at one time. The role played by *S. equi* subsp. *zooepidemicus* in other cases of CIRD in kennelled dogs has not been ascertained.

The isolation of *S. equi* subsp. *zooepidemicus* from these dogs increases with time in the kennel, indicating the lungs of these dogs are becoming infected with this bacterium. Such infection could be occurring from either sub-clinical infections of the upper respiratory tract or from a single pathogenic strain. A PCR typing system for the gene of the variable M-like

SzP protein enables the separation of the 15 known sero-types of *S. equi* subsp. *zooepidemicus* into five distinct groups, HV1-5 (Walker and Timoney, 1998). Analyses with this typing system by Anzai et al. (2000) found that single clonal variants of *S. equi* subsp. *zooepidemicus* are found in the pneumonic equine lung whereas several types are found in the tonsils of healthy horses. It would be of interest to sub-type the *S. equi* subsp. *zooepidemicus* isolates involved in this outbreak of CIRD to determine whether a single clonal variant is present in the diseased population, and also to examine the relationship, if any, that canine *S. equi* subsp. *zooepidemicus* isolates have to those causing respiratory disease in horses and other animals. *Streptococcus equi* subsp. *zooepidemicus* associated pneumonia occurs in horses of all ages and acute haemorrhagic pneumonia in older horses that have been stressed by transportation (Anzai et al., 2000). In this outbreak of CIRD younger dogs and those in poor clinical condition on entry to the kennel were equally susceptible to infection with *S. equi* subsp. *zooepidemicus* as the older dogs and those that were healthy on entry.

In this kennel antibiotic therapy is given for a range of infections, and treatment is not routinely given to dogs with CIRD except in cases of severe bronchopneumonia. It is possible that treatment could have influenced the bacterial spectrum noted in this study. However the examination of natural outbreaks of respiratory disease can provide valuable information that cannot be obtained by other means.

CIRD is known to be a multi-factorial disease involving several agents including CAV-2, CPIV, *B. bronchiseptica* and *Mycoplasma* spp. In this kennel in which large numbers of dogs from a variety of locations are brought together and housed, several pathogens are present and the severity of the disease may reflect this. Future studies may determine whether other pathogens such as *Mycoplasma* spp. are also present in these dogs.

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