Short communication

Serological evidence of *Mycoplasma cynos* infection in canine infectious respiratory disease

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Abstract

A high proportion of dogs suffer from respiratory disease when they are placed in kennels for vacation or re-homing. The role of *Mycoplasma cynos* as an initiating agent in canine infectious respiratory disease was investigated by examining the serological response of dogs to this organism at the time of entry into a large re-homing kennel. Forty-two paired serum samples from dogs (21-day interval) were examined for antibody to *M. cynos* using Western blotting. The development of antibody in the serum was related to clinical disease recorded over the same period. Sixty seven per cent of the dogs showed a two-fold or greater rise in antibody to *M. cynos* during the first 3 weeks in the kennel. Reactivity with a 45 kDa antigen was dominant. Of those showing a positive serological reaction, 80% had recorded clinical respiratory disease while 20% remained healthy. The findings of this study show that an antibody response to *M. cynos* is common in dogs entering the re-homing kennel and is positively related to the development of clinical respiratory disease.
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1. Introduction

Canine infectious respiratory disease (CIRD) is a clinical syndrome frequently encountered when dogs are held in large numbers, over prolonged periods in relatively high density (Appel and Binn, 1987). Dogs show a dry cough, anorexia, vomiting and depression. This usually lasts for a few days to some weeks and in more severe cases, tracheobronchitis, pneumonia and deaths occur.

The disease has been regarded as a complex infection. Challenge with viral and bacterial agents produces a synergistic effect leading to the clinical signs (Kontor et al., 1981; Wagener et al., 1984). The most common bacterial agent detected during this disease is *Bordetella bronchiseptica* (Bemis et al., 1977a; McCandlish et al., 1978; Thrusfield, 1992). However, in many cases of CIRD *B. bronchiseptica* is not present in the respiratory tract (Ueland, 1990) and their involvement is not supported by serological data (Chalker et al., 2003a,b).

Mycoplasmas are recognised agents of respiratory disease in a wide range of animal species including

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man. Generally, the disease is mild but has been shown to predispose the animal to more severe secondary infection and to exacerbate concurrent infections (Thacker et al., 1999). Mycoplasmas have been implicated as potential agents of canine respiratory disease (Greig, 1954; Rosendal, 1978). *Mycoplasma cynos* was isolated from two kennelled dogs with pneumonia (Rosendal, 1972) and was shown to induce pneumonia with severe inflammation of bronchi and respiratory tract tissue (Rosendal, 1978).

Until recently there has been limited information regarding the aetiological role of mycoplasmas in natural canine disease (Rosendal, 1972, 1978, 1982; Randolph et al., 1993). However, an association between *M. cynos* isolated from the lower respiratory tract and the severity of canine infectious respiratory disease (CIRD) in kennelled dogs has now been demonstrated (Chalker et al., 2004). Only *M. cynos* was significantly associated with respiratory disease.

In this paper we report the finding of serological evidence of exposure to *M. cynos* in dogs, during the first 21 days following entry into a large re-homing kennel, in which a high proportion of the animals develop mild or moderate respiratory disease.

2. Materials and methods

2.1. Animals

Dogs in the study were gifted or received as strays at a large re-homing kennel over a 3-year period. Samples of clotted blood were obtained from dogs on entry. Serum was separated and stored at $-70\,^{\circ}C$. Paired samples were obtained 21 days later from those animals that had not yet been re-homed. The general health of the animals, including evidence of respiratory disease, was recorded on entry, at days 7 and 21 after entry. Episodes of clinical respiratory disease were recorded according to a scale of 0–5 in which 0 was healthy and 5 was severe respiratory disease. Healthy animals were those which showed no evidence of disease throughout the 21-day period. Negative control samples were obtained from healthy Beagles in a colony with no history of infectious respiratory disease.

2.2. Organisms and culture conditions

M. cynos NCTC 10142 and *Mycoplasma felis* NCTC 10160 were obtained from the National Collection of Type Cultures, London, UK. The organisms were cultured on solid medium from Mycoplasma Experience Ltd, Reigate, UK and cultured for antigen preparation in the liquid medium of Kobisch and Friis (1996).

2.3. Preparation of antigen

Antigen was prepared by centrifugation of 30 ml of culture at 15,000 rpm for 15 min, 4 °C. The pellet was then washed twice in HEPES saline buffer (10 mM *N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES), 150 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, pH 7.3) and resuspended to 300 μl in HEPES saline containing 1 μl protease inhibitor cocktail (P8465, Sigma) and stored at $-70\,^{\circ}C$ until use. For electrophoresis, antigen was mixed with one-half volume of sample buffer (180 mM Tris·Cl, 30% (v/v) glycerol, 6% (w/v) SDS, 15% (v/v) β-mercaptoethanol, pH 8.3), heated to 100 °C for 5 min and 45 μl was loaded into each well.

2.4. SDS polyacrylamide gel electrophoresis and immunoblotting

SDS-PAGE was conducted according to standard procedures (Rycroft et al., 1991). Separated antigen was electro-transferred overnight onto reinforced nitrocellulose membrane (Biorad). Non-specific staining of transferred antigen was carried out with 0.2% (w/v) Ponceau S in 1% (w/v) trichloroacetic acid solution. Strips were cut from the blot and blocked for 10 min with 5% skim milk powder in TNT (150 mM NaCl, 20 mM Tris–HCl pH 8.0, 0.05% Tween 20). Canine serum was applied to each antigen strip at a dilution of 1:500 for 4 h and anti-canine IgG conjugated to Horse radish peroxidase (Serotec) was used at a dilution of 1:2000 for one hour. Final development was as previously described (Rycroft et al., 1991). Analysis of blots was carried out using the Image Master Elite system (Pharmacia Biotech). Band intensity was quantified using the "peak height" comparison between the blot of day 1 sample and day 21 sample.

2.5. Statistical analysis

The Fishers exact test was used to determine significance of seroconversion in relation to disease state. The non-parametric, Mann–Whitney rank sum test was used to compare increase in peak height values in the two groups: healthy and diseased using a 95% confidence limit.

3. Results

Forty two paired canine serum samples were examined for antibody to the type strain of *M. cynos* using Western blotting. The paired samples were taken on the day of entry into the kennel and 21 days later. An immunodominant antigen of 45 kDa was clearly prominent in the blots (Fig. 1). Many of the dogs showed reactivity to this antigen in both the entry and 21-day sera while others showed no reactivity from either serum. Fifteen of the dogs (36%) showed a two-fold or greater increase in antibody to a 45 kDa antigen and in some cases this was a marked increase.

Of the 42 dogs, 26 (62%) became clinically sick with respiratory symptoms during the 21-day period after admission, while 16 (38%) remained healthy. Of the 26 diseased dogs, 46% had become seropositive to *M. cynos* while 54% had not seroconverted. Of the 16 clinically healthy dogs, only 3 (19%) had serocon-
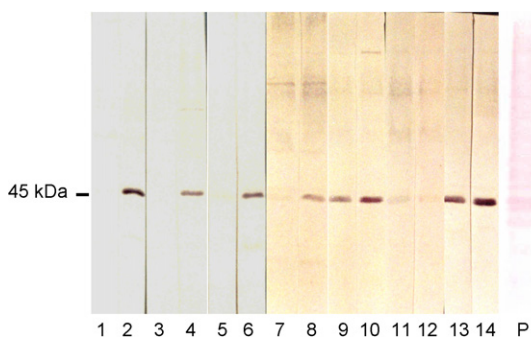

Fig. 1. Immunoblot of whole, solubilised *Mycoplasma cynos* antigen showing reactivity with representative paired samples of canine serum. Odd numbered lanes are probed with day 1 sera; even numbered lanes are probed with day 21 sera. 1 and 2, dog 1736; 3 and 4, dog 5366; 5 and 6, dog 6705; 7 and 8, dog 3471; 9 and 10, dog 1741; 11 and 12, dog 3825; 13 and 14, dog 2089. P, total *M. cynos* protein profile stained with Ponceau S.

Table 1
Serological response in paired canine sera in relation to clinical status

	Diseased	Healthy	Total
Seroconverted (>two-fold increase)	12 (29%)	3 (7%)	15
No response (<two-fold increase)	14 (33%)	13 (31%)	27
Total	26	16	42

verted compared to 13 (81%) which had not (Table 1; $p = 0.07$). As a comparison, the same sera were analysed against *M. felis* antigen by immunoblotting. A larger number of *M. felis* antigens were recognised by the canine serum samples. However, there was no discernable difference between the days 1 and 21 serum samples with respect to the number or intensity of the antigens detected.

The clinical status of the animals was related to the increase in antibody levels. Comparison of the numerical values of the increase in band peak intensity for the two groups (healthy versus diseased) by the Mann–Whitney test showed the difference in the two groups to be statistically significant ($p < 0.05$).

4. Discussion

Dogs entering kennels where there is a high density of other animals are frequently susceptible to respiratory disease. This has been termed "kennel cough", canine infectious tracheobronchitis or CIRD, and has been associated with a number of infectious agents including canine parainfluenza virus (CPIV), *B. bronchiseptica*, canine distemper virus, canine herpesvirus and canine adenovirus Type 2 (Thrusfield, 1992). However, instances of disease have been documented in which none of these agents or any other recognised pathogen can be found and it is likely that other, unrecognised agents may initiate disease in these cases. Recently, canine respiratory coronavirus (CRCoV) has been implicated in CIRD (Erles et al., 2003; Priestnall et al., 2006). Evidence that infection with *M. cynos* may play a role in CIRD was recently reported (Chalker et al., 2004). To support this finding we sought serological evidence of exposure to *M. cynos* in the early stages of disease.

While some dogs in this study showed antibody as evidence of past exposure to *M. cynos* or a related organism, others were free of this antibody on entry to the home. Of the 15 dogs that underwent a seroconversion, 12 suffered respiratory symptoms while only 3 remained healthy. Proportionally this was 46% of the dogs with respiratory disease compared to 19% that remained apparently healthy. This difference was statistically significant when the values of the antibody increase from the two groups were compared. This finding indicates that the antibody response to *M. cynos* occurring following entry into the re-homing kennel is more likely among dogs that show clinical disease. Nevertheless, overt disease may not be necessary for an initiating agent to allow colonisation by secondary pathogens. In other mycoplasma diseases it is well recognised that while the initial mycoplasma infection is mild, secondary bacterial infection may become much more likely following the initial infection (Ciprian et al., 1988, 1994; Amass et al., 1994).

Of the 27 dogs which did not show increasing antibody, almost equal numbers showed signs of disease or remained disease-free. Clearly, not all of these animals recognised or became infected with *M. cynos*. This is consistent with our previous findings that *M. cynos* could be isolated from trachea and bronchial lavage in a proportion of both healthy and diseased dogs (Chalker et al., 2004). It is also consistent with the finding that a longer time spent in the kennel was positively correlated with increased infection with *M. cynos*.

In each of the three cases of animals recorded as healthy, but which underwent seroconversion, antibody to the *M. cynos* antigen was present at day 1, indicating the possibility of past exposure to this agent in the life of the animal. This is consistent with the possibility that pre-existing antibody to *M. cynos* may confer some protection against CIRD.

We cannot rule out the possibility that the dominance of the single protein band in the antibody response of the dogs is due to infection of the animals with an antigenically related organism, other than *M. cynos*. Although reactivity with a greater number of bands was seen with *M. felis*, this was not seen to increase at the time of entry to the home when respiratory disease occurred. It is also consistent with the culture of *M. cynos* from the respiratory tract of post-mortem cases in our previous work (Chalker et al., 2004).

The disease history and vaccination history of the animals sampled was unknown. All animals are vaccinated against CPIV on entry to the home. However, approximately 80% of animals go on to suffer some degree of respiratory disease during the first weeks after entry to the kennel. The agent or agents responsible for this disease remain unclear since different organisms may be identified from different cases. In some cases *B. bronchiseptica* was present (Chalker et al., 2003a) while in others β-haemolytic streptococci were found (Chalker et al., 2003b). In other cases, no obvious bacterial cause could be detected. Mycoplasmas have been known for some time to modulate host susceptibility to secondary infections (Kaklamanis and Pavlatos, 1972; Thacker et al., 1999). In view of this, the presence of other initiating agents could be suspected and mycoplasma agents could be involved.

In conclusion, the serological data presented here support the previous suggestion, based on isolation and pathology data (Chalker et al., 2004), that in a proportion of dogs, *M. cynos* is involved in the early stages of canine infectious respiratory disease and may act as an initiating agent of CIRD.

Acknowledgement

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