

## Mycoplasmas associated with canine infectious respiratory disease

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Canine infectious respiratory disease (CIRD) is a complex infection that occurs worldwide predominantly in kennelled dogs, and several bacterial and viral micro-organisms have been associated with outbreaks of CIRD. However, few studies have comprehensively examined the species of mycoplasma present in healthy dogs and those with CIRD. As part of an extensive study investigating the micro-organisms involved in CIRD, the species of mycoplasma present throughout the respiratory tract of dogs with and without CIRD were determined. Mycoplasmas were cultured from tonsillar, tracheal and bronchial lavage samples, and identified to the species level by PCR and sequencing. *Mycoplasma cynos* was demonstrated on the ciliated tracheal epithelium by *in situ* hybridization and was the only mollicute found to be associated with CIRD, but only in the lower respiratory tract. Isolation of *M. cynos* was correlated with an increased severity of CIRD, younger age and a longer time in the kennel.

## INTRODUCTION

Mycoplasmas are bacteria that lack a cell wall, but are enclosed by a lipid bilayer membrane. They colonize the mucous membranes of the respiratory and genital tracts as well as red blood cells, and are found in many animals and humans. To date, 15 species of mycoplasma have been isolated from or detected in dogs: *Acholeplasma laidlawii*, *Mycoplasma arginini*, *Mycoplasma bovigenitalium*, *Mycoplasma canis*, *Mycoplasma cynos*, *Mycoplasma felis*, *Mycoplasma feliminutum*, *Mycoplasma gateae*, *Mycoplasma haemocanis*, *Mycoplasma edwardii*, *Mycoplasma molare*, *Mycoplasma maculosum*, *Mycoplasma opalescens*, *Mycoplasma spumans* and *Ureaplasma canigenitalium*. In addition, other canine mycoplasmas have been isolated that do not cross-react with antisera to any of the known species, have distinct 16S rRNA genes, and have not yet been fully characterized (*Mycoplasma* sp. strains HRC 689 and VJC 358; Barile *et al.*, 1970; Kirchner *et al.*, 1990; Chalker & Brownlie, 2004).

In dogs, mycoplasmas are thought to be part of the normal bacterial flora in the upper respiratory tract (Rosendal, 1982), but there are conflicting reports about the presence of mycoplasmas in the lower respiratory tract of healthy dogs. Randolph *et al.* (1993) found that the lungs of up to 27% of healthy dogs were colonized, whereas other authors

have failed to detect mycoplasmas in the lower respiratory tract of healthy dogs (Rosendal, 1982). The role of individual *Mycoplasma* sp. in respiratory infections of dogs is not well understood, but they are thought to colonize the lungs during pneumonia (Rosendal, 1982). Of the species listed above, *M. bovigenitalium*, *M. canis*, *M. cynos*, *M. edwardii*, *M. feliminutum*, *M. gateae* and *M. spumans* have been isolated from dogs with respiratory disease (Armstrong *et al.*, 1972; Rosendal, 1978). Pneumonia in dogs has been reproduced by experimental endobronchial inoculation with an isolate of *M. cynos* that had been isolated from a dog with pneumonia, and also by exposure of non-infected dogs to dogs infected with *M. cynos* (Rosendal, 1972; Rosendal & Vinther, 1977). In addition, several cases have been described in which mycoplasmas have been isolated in pure culture from dogs with respiratory disease, although typing to the species level has not been performed (Randolph *et al.*, 1993; Chandler & Lappin, 2002).

Canine infectious respiratory disease (CIRD, kennel cough) is a complex disease involving a variety of pathogens that, when present concurrently, may act synergistically to enhance the severity of the disease (Appel & Binn, 1987). The infectious agents traditionally associated with CIRD are canine adenovirus, canine parainfluenza virus and the bacterium *Bordetella bronchiseptica*. Recently, however, *Streptococcus equi* subspecies *zooepidemicus* (Chalker *et al.*, 2003a) and canine respiratory coronavirus (CRCoV; Erles *et al.*, 2003) have both been found to be associated with CIRD in dogs. Outbreaks of CIRD are more common and severe in dogs that are housed communally in training

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Abbreviations: BL, bronchial lavage; CIRD, canine infectious respiratory disease; CRCoV, canine respiratory coronavirus; ISH, *in situ* hybridization.

institutions and rehoming centres. The majority of studies examining CIRDC have not included mycoplasmas as potential causative agents, and the few that have determined whether mycoplasmas were present did not identify the individual species, limiting their value. Techniques currently used to identify canine mycoplasmas include growth inhibition and immunofluorescence tests which require specific antisera, and are therefore limited to specialized laboratories. The identification of species is further complicated by the fact that the colonies of several canine mycoplasmas are morphologically identical, and infections with a mixture of species are common. Because of this, several colonies from each case must be identified to the species level to increase confidence in the presence or absence of individual species. All these factors have contributed to the lack of testing for mycoplasmas, and the extent to which mycoplasmas are involved in respiratory infections in dogs is unknown.

The principal aim of this study was to develop molecular-based diagnostic tests for the most common canine mycoplasmas in order to determine which species of mycoplasmas are present in healthy dogs and those with CIRDC.

## METHODS

**Study populations.** Two dog populations were included in the study. The primary population, (A), comprised 210 animals that formed part of a larger study to examine the micro-organisms present in outbreaks of CIRDC in which the involvement of *B. bronchiseptica*, *S. equi* subsp. *zooepidemicus* and CRCoV has been documented (Chalker *et al.*, 2003a, b; Erles *et al.*, 2003). Population (A) comprised animals from a well-established rehoming kennel (~600 dogs) with a history of endemic CIRDC. Samples were taken at regular intervals from 1999 to 2002. Dogs were euthanased for various welfare reasons, ranging from intractable behavioural problems to signs of severe respiratory disease, at a mean time period of 16 days after entry into the kennel. All dogs were graded for the severity of clinical respiratory signs prior to euthanasia in the following categories: (1) no respiratory signs,  $n=72$ ; (2) mild cough,  $n=36$ ; (3) cough and nasal discharge,  $n=77$ ; (4) cough and nasal discharge with depression and/or inappetence,  $n=9$ ; (5) cough and nasal discharge with depression and/or inappetence and clinical signs of lower respiratory tract disease,  $n=16$ . On day 1 of entry into the rehoming kennel, all dogs were routinely vaccinated against canine distemper virus, canine adenovirus type 2, canine parainfluenza virus, canine parvovirus and leptospirosis (Kavak DA<sub>2</sub>PIP69 and Kavak L, Fort Dodge Animal Health). Dogs were not vaccinated against *B. bronchiseptica*, and their vaccination history prior to entry into the rehoming centre was unknown. The estimated age and time in the kennel (in days) prior to euthanasia of each dog were also recorded. Dogs were housed in groups of three or fewer per kennel, but shared runs and air space with other dogs.

The additional dog population, (B), included in the study comprised 153 dogs visiting a training centre. Dogs entered the centre periodically for a few months of training, prior to being housed in family homes. All training dogs were less than 2 years of age, in excellent condition, and had complete clinical histories. Dogs were housed in groups of two or fewer per kennel, but shared runs and air space with other dogs. All dogs were regularly vaccinated against all the agents listed for population (A). Unlike population (A), in which CIRDC was endemic, outbreaks of CIRDC occurred only sporadically in the training

kennel and were of reduced severity. Dogs in the training kennel were monitored over a 12 month period from 2001 to 2002, and clinical respiratory disease graded as above. Tonsillar swabs were taken from a proportion of dogs in the kennel each month, and from dogs during an outbreak of CIRDC. A total of 153 dogs were swabbed in the following clinical categories: (1)  $n=110$ , (2)  $n=27$ , (3)  $n=16$ , (4)  $n=0$ , (5)  $n=0$ .

**Samples.** Dogs from population (A) were necropsied within 4 h of euthanasia. Bronchial lavage (BL) and tracheal samples were taken, sampled immediately and then stored at  $-70^{\circ}\text{C}$ . The method of BL sampling has been described previously (Chalker *et al.*, 2003a), but in brief ensures that the sample is not contaminated by micro-organisms higher up the respiratory tract. Tracheal tissue samples were stored in Hanks' balanced salt solution at  $-70^{\circ}\text{C}$ , and BL samples were stored in Hanks' balanced salt solution with the addition of 15% mycoplasma-free, heat-inactivated horse serum. Tonsillar swabs were not stored after use. Tissue samples were fixed in 10% formol saline and embedded in paraffin, and stored at ambient temperature until use.

**Mycoplasma isolation and growth conditions.** Mycoplasmas were routinely cultured at  $37^{\circ}\text{C}$  in 95% nitrogen and 5%  $\text{CO}_2$  on Ureaplasma medium (Mycoplasma Experience; Windsor *et al.*, 1975) and Mycoplasma medium (ME media, Mycoplasma Experience; Hannan *et al.*, 1997), and in 5%  $\text{CO}_2$  and 95% air at  $37^{\circ}\text{C}$  in Ureaplasma and Mycoplasma broth (Mycoplasma Experience). Tracheal samples and tonsillar swabs were spread onto agar and then added to liquid media, whereas 0.05 ml BL was plated onto solid medium and 0.1 ml added to broth. All broth cultures were incubated for at least 7 days, and agar cultures for at least 28 days. Cultures were examined daily for the first 7 days and weekly thereafter.

Mycoplasmas were isolated from the air of the kennel of population (A) by leaving a Mycoplasma solid medium agar plate open for 1 h on top of the kennel, prior to incubation as above. Mycoplasmas from positive samples were purified, and individual clones stored at  $-70^{\circ}\text{C}$  prior to identification to species level. Due to the morphological similarity of canine mycoplasma colonies, several colonies from each sample were taken for further analysis. All mycoplasma type strains were obtained from the National Collection of Type Cultures (NCTC), Colindale, London (*A. laidlawii* PG8 NCTC10116, *M. arginini* G230 NCTC10129, *M. cynos* H381 NCTC10142, *M. edwardii* NCTC10132, *M. felis* CO NCTC10160, *M. feliminutum* PG15 NCTC10159, *M. gateae* C5 NCTC10161, *M. maculosum* PG15 NCTC10168, *M. molare* H542 NCTC10144, *M. opalescens* MHS408 NCTC10149, *M. spumans* PG13 NCTC10169, *Mycoplasma* sp. strain VJC358 NCTC11743), except for *Mycoplasma* sp. strain HRC 689, which was obtained from the University of Florida, and *U. canigenitalium* AE39, which was kindly donated by Mycoplasma Experience.

**Mycoplasma identification.** Due to the high similarity of the 16S rRNA genes of canine *Mycoplasma* species (Chalker & Brownlie, 2004), PCR tests were developed for the species listed in Table 1 to species-specific regions identified in the 16S/23S rRNA intergenic spacer region. Primers and amplification parameters are also listed in Table 1. All PCRs commenced with an initial denaturation at  $95^{\circ}\text{C}$  for 5 min and were followed by the specific annealing step listed in Table 1 and then by final extension at  $72^{\circ}\text{C}$  for 5 min. PCR reactions (50  $\mu\text{l}$ ) included 5.0  $\mu\text{l}$   $10\times$  magnesium-free buffer (0.1 M Tris/HCl, 0.5 M KCl, pH 8.3), 1.5 mM  $\text{MgCl}_2$  (Promega), 0.5  $\mu\text{l}$  (0.5 Units) Taq DNA polymerase (Promega), 0.2 mM PCR nucleotide mix (Promega), 0.025  $\mu\text{g}$  forward primer (Myc1; 5'-CA-CCGCCGTCACACCA-3'), 0.025  $\mu\text{g}$  reverse primer (see Table 1), and  $\sim 1$   $\mu\text{g}$  mycoplasma DNA (isolate or positive control type-strain) or 1  $\mu\text{l}$  water (negative control). DNA was extracted from mid-exponential-phase cultures of mycoplasmas (5–20 ml) using the DNeasy tissue kit (QIAGEN), according to the manufacturer's instructions.

**Table 1.** Canine *Mycoplasma* species PCR primers and reaction conditions

Species	Primer sequence	Cycle conditions (× 30)	Product size (bp)
<i>M. arginini</i> *	GTTGTATGACCTATTGTTGTC	95 °C 1 min, 50 °C 30 s, 72 °C 1 min	312
<i>M. canis</i> †	CTGTCGGGGTTATCTCGAC	95 °C 1 min, 55 °C 30 s, 72 °C 1 min	247
<i>M. cynos</i> ‡	GATACATAAACACAACATTATAATATTG	95 °C 45 s, 55 °C 30 s, 72 °C 20 s	227
<i>M. edwardii</i> ‡	CTGTCGGGGTTATCATGCGGAC	95 °C 45 s, 55 °C 30 s, 72 °C 20 s	250
<i>M. felis</i>	GGACTATTATCAAAAGCACATAAC	95 °C 45 s, 51 °C 30 s, 72 °C 20 s	238
<i>M. gateae</i> *	GTTGTATGACCTATTGTTGTC	95 °C 1 min, 50 °C 30 s, 72 °C 1 min	312
<i>M. maculosum</i> †	CCTATGATTGTTACAGATG	95 °C 1 min, 50 °C 30 s, 72 °C 1 min	432
<i>M. molare</i> ‡	AGCCTATTGTTTTGATTTG	95 °C 1 min, 55 °C 30 s, 72 °C 1 min	397
<i>M. opalescens</i> *	TAAGCTTTGTAGACCATAA	95 °C 1 min, 50 °C 30 s, 72 °C 1 min	236
<i>M. spumans</i> *	GTTGTATGACCTATTGTTGTC	95 °C 1 min, 50 °C 30 s, 72 °C 1 min	312
<i>Mycoplasma</i> sp. HRC 689	CTTGCGACCTAACAAAGTCC	95 °C 45 s, 51 °C 30 s, 72 °C 20 s	227

\*†‡These assays can be combined as multiplex reactions.

All PCRs were validated by testing for amplification with the following range of canine mycoplasmas and other bacterial species: *M. arginini*, *M. canis*, *M. cynos*, *M. edwardii*, *M. feliminutum*, *M. felis*, *M. gateae*, *M. molare*, *M. maculosum*, *M. opalescens*, *M. spumans*, *Mycoplasma* sp. strain HRC689, *U. canigenitalium*, *Escherichia coli*, *B. bronchiseptica* and *S. equi* subsp. *zooepidemicus*. A PCR for *M. spumans*, *M. gateae* and *M. arginini* was performed as a multiplex assay followed by restriction endonuclease digestion with *SspI* to specifically identify *M. spumans* by the generation of a 173 bp fragment. The 12 mycoplasma isolates that were provisionally identified as either *M. arginini* or *M. gateae*, together with those samples from which products were not amplified by any of the above PCRs, were speciated by sequencing a partial sequence of the 16S rRNA gene or the intergenic spacer region using methods described previously (Chalker & Brownlie, 2004), and by comparison of the resulting sequence data to known species. As only one species of *Ureaplasma* has been isolated from dogs, *Ureaplasma* isolates were identified by the production of urease and by their distinct colonial morphology on *Ureaplasma* solid medium.

***Mycoplasma cynos* riboprobe construction and *in situ* hybridization (ISH).** The last 145 bp of the 16S rRNA genes, the entire 16S/23S rRNA spacer region, and the first 36 bp of the 23S rRNA gene of *M. cynos* were amplified as a single amplicon using the PCR reaction described by Chalker & Brownlie (2004). The 450 bp fragment (sequence AF443606) was ligated into pGemT-EASY, which was used to transform *E. coli* JM109. Transformants were incubated on selective medium: Luria broth with the addition of 100 µg ampicillin ml<sup>-1</sup> (Sigma), 50 µg X-Gal ml<sup>-1</sup> (Sigma) and 0.01 M IPTG (Sigma). Positive transformants were identified by PCR, and the insert was confirmed as *M. cynos* DNA by sequencing. The plasmid containing the insert was then purified and digested with *NofI*. A digoxigenin-labelled riboprobe based on this plasmid was then synthesized with a Riboprobe Combination System (Roche), according to the manufacturer's instructions. Probe specificity was determined by dot-blot analysis, using a range of bacterial RNA (*M. arginini*, *M. canis*, *M. cynos*, *M. edwardii*, *M. gateae*, *M. felis*, *M. molare*, *M. maculosum*, *M. opalescens*, *M. spumans*, *A. laidlawii*, *B. bronchiseptica*, *Clostridium perfringens*, *E. coli* and *S. equi* subsp. *zooepidemicus*) that had been isolated as follows. Bacterial cells were lysed in 1 ml Tri Reagent (Sigma) for 5 min at room temperature, mixed with 0.2 ml chloroform, incubated for 10 min at room temperature, and centrifuged at 10 000 g at 4 °C for 15 min. The upper aqueous phase was added to 0.5 ml 2-propanol, incubated for 10 min at room temperature, and centrifuged at 10 000 g at 4 °C for 10 min. The resulting RNA pellet was washed with 1 ml 75 % ethanol and centrifuged at 10 000 g at 4 °C for 5 min. Finally, the

RNA pellet was air-dried, resuspended in 50 µl diethylpyrocarbonate-treated water, and stored at -70 °C prior to use. The probe hybridized to *M. cynos* RNA and weakly to *M. felis* RNA and *A. laidlawii* RNA.

*In situ* hybridization (ISH) was performed on formalin-fixed, paraffin-embedded 4 µm sections of tracheal tissue on Superfrost Plus slides (BDH). Briefly, slides were incubated at 60 °C for 1 h, dewaxed in xylene, and rehydrated by treatment in 100 % ethanol, 70 % ethanol and finally water. Proteinase K treatment for 20 min at 37 °C was used to expose RNA, and enzyme activity was stopped by immersion in water and then 100 % ethanol, prior to air drying. Just prior to covering, 0.5 µl (50 pg) riboprobe or 0.5 µl control (hybridization buffer alone: 5 ml formamide, 1 ml 50 % dextran sulphate, 1 ml 20 × SSC) in 49.5 µl hybridization mix (12.5 µl TES: 0.05 M Tris, pH 8.0, 0.0005 M EDTA, 0.5 M NaCl, 2 µl 10 mg ml<sup>-1</sup> denatured salmon sperm DNA, 35 µl hybridization buffer) was applied to each slide. The slides were then heated at 80 °C for 10 min to denature the nucleic acid, and the riboprobe was hybridized at 68 °C for 2 h. Slides were then washed at 68 °C in 4 × SSC (pH 7.0) for 5 min, twice with 1 × SSC for 5 min, and then finally in 0.1 × SSC at room temperature for 10 min. Slides were then blocked at room temperature for 30 min and incubated with anti-digoxigenin alkaline phosphatase Fab fragments (1 : 500 in blocking solution) for 30 min. The colour reaction was developed with DIG substrate (Roche) in 1 ml DIG buffer 3, following the manufacturer's instructions, and stopped by rinsing in water. Slides were then counter-stained with picric acid in ethanol (1 : 5) for 1 min, immersed in water and then in acetone for 20 seconds. Slides were then dehydrated in xylene and ethanol prior to mounting with DPX resin. Tracheal sections were deemed positive for *M. cynos* by the observation of dark-staining clumps along the epithelial surface. ISH was performed on tracheal sections from 50 dogs in population (A).

**Statistical analysis.** A significance level of probability of a type 1 error ( $\alpha$ ) of 0.05 was assumed for all analyses. The association between the presence of mycoplasmas and CIRD and the association between the presence of *M. cynos* and CIRD, clinical respiratory score, age and time in the kennel were analysed using a two-tailed Fisher's exact test.

## RESULTS

### Isolation and identification of *Mycoplasma* species

The species and number of mycoplasmas isolated from populations (A) and (B) are shown in Tables 2 and 3. The

**Table 2.** Mycoplasmas from tracheal and bronchial lavage samples in population (A)

Values in the Healthy and CIRD columns show the number of isolates of each species related to the total number of dogs sampled; percentage values are given in parentheses. BL, bronchial lavage sample; T, tracheal sample. The *P* value was calculated using a two-tailed Fisher's exact test.

Species		Healthy (%)	CIRD (%)	<i>P</i>
<i>M. arginini</i>	T	2/70 (2.8)	2/136 (1.4)	0.601
	BL	3/69 (4.2)	3/135 (2.2)	0.415
<i>M. canis</i>	T	13/59 (18.1)	24/114 (17.4)	1.0
	BL	9/63 (12.5)	18/120 (13.0)	1.0
<i>M. cynos</i>	T	7/65 (9.7)	33/105 (23.9)	0.016
	BL	7/65 (9.7)	30/108 (21.7)	0.036
<i>M. edwardii</i>	T	14/58 (19.4)	14/124 (10.1)	0.855
	BL	3/69 (4.2)	7/131 (5.1)	1.0
<i>M. felis</i>	T	1/71 (1.4)	0/138 (0)	0.343
<i>M. gateae</i>	T	0/72 (0)	1/137 (0.7)	1.0
<i>M. maculosum</i>	T	6/66 (8.3)	8/130 (5.8)	0.563
	BL	1/71 (1.4)	6/132 (4.3)	0.426
<i>M. spumans</i>	T	11/61 (15.3)	20/118 (14.5)	1.0
	BL	4/68 (5.6)	15/123 (10.9)	0.31
<i>Mycoplasma</i> sp. HRC 689	T	2/70 (2.8)	3/135 (2.2)	1.0
	BL	1/71 (1.4)	2/136 (1.4)	1.0
<i>Mycoplasma</i> sp. VJC 358	T	0/72 (0)	1/137 (0.7)	1.0
	BL	0/72 (0)	1/137 (0.7)	1.0
Ureaplasma	T	4/67 (5.6)	17/119 (12.5)	0.161
	BL	9/62 (12.7)	20/116 (14.7)	0.834
Unidentified	T	2/70 (2.8)	4/134 (2.9)	1.0
	BL	2/70 (2.8)	4/134 (2.9)	1.0

prevalence of CIRD was significantly associated with the isolation of *M. cynos* from the trachea and lung, increasing from 9.7% to 21.7% (lung,  $P=0.036$ ) and from 9.7% to 23.9% (trachea,  $P=0.016$ ) in dogs with CIRD. No other mycoplasmas were significantly associated with CIRD in population (A). No particular mycoplasma species was associated with CIRD in population (B); however, unidentified mycoplasmas were isolated from a greater number of tonsillar swabs in population (B) than from the samples from lower in the respiratory tract in population (A).

### *M. cynos*

In population (A), *M. cynos* was isolated more frequently from samples from both the trachea and lung in dogs with higher clinical scores, was highest in dogs with moderate disease (clinical score 3–4; Fig. 1) and was significantly more likely to be isolated from the lungs or trachea of dogs with a clinical score of 3 than those with a score of 1 (lung,  $P=0.011$ ; trachea,  $P=0.001$ ). *M. cynos* was less frequently isolated from older dogs (Fig. 2) and was significantly more likely to be isolated from the trachea of dogs less than 1 year old ( $P=0.045$ ). The frequency of isolation of

**Table 3.** Mycoplasmas from tonsillar swab samples in population (B)

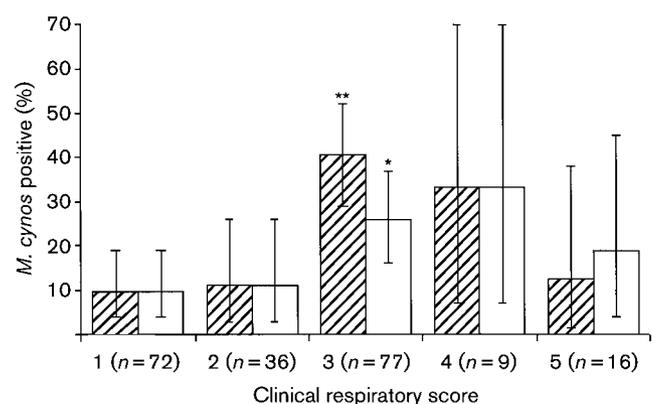
Values in the Healthy and CIRD columns show the number of isolates of each species related to the total number of dogs sampled; percentage values are given in parentheses. The *P* value was calculated using a two-tailed Fisher's exact test.

Species	Healthy (%)	CIRD (%)	<i>P</i>
<i>M. canis</i>	24/86 (21.8)	13/30 (30.2)	0.298
<i>M. cynos</i>	1/109 (0.9)	0/43 (0)	1.0
<i>M. edwardii</i>	47/63 (42.7)	18/25 (41.9)	1.0
<i>M. felis</i>	1/109 (0.9)	1/42 (2.3)	0.484
<i>M. maculosum</i>	5/105 (4.5)	4/39 (9.3)	0.269
<i>M. molare</i>	2/108 (1.8)	0/43 (0)	1.0
<i>M. spumans</i>	15/95 (13.6)	4/39 (9.3)	0.591
Ureaplasma	23/87 (20.9)	10/33 (23.3)	0.145
Unidentified	17/93 (15.5)	14/29 (32.6)	0.025

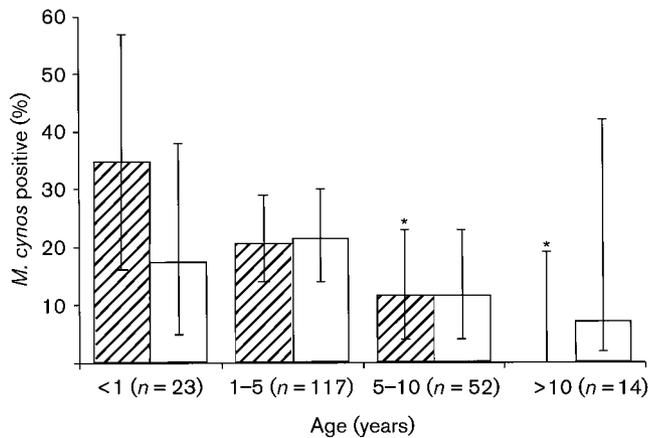
*M. cynos* in dogs was correlated with time spent in the kennel (Fig. 3). *M. cynos* was more likely to be isolated from the trachea of dogs remaining in the kennel than from the trachea of those dogs housed in the kennel for less than 1 week ( $P=0.028$ ). *M. cynos* was the only species of mycoplasma to be isolated from the air of the kennel.

### Localization of *M. cynos* in the respiratory tract

Of the 50 dogs analysed for *M. cynos* by ISH, six (12%) had visible patches of staining indicative of mycoplasma infection along the ciliated epithelium (Fig. 4). *M. cynos* was isolated from five of the six dogs (83.3%) that had tracheal colonization detectable by ISH but was only isolated from



**Fig. 1.** Comparison of *M. cynos* isolation with clinical score. The number of dogs in each group (*n*) is displayed, and 95% confidence intervals are shown as error bars. An asterisk indicates a significant difference in *M. cynos* isolation from the lung or trachea compared to dogs with a clinical score of 1. No other group differed significantly from dogs with a clinical score of 1. Hatched bars, tracheal samples; white bars, lung samples.

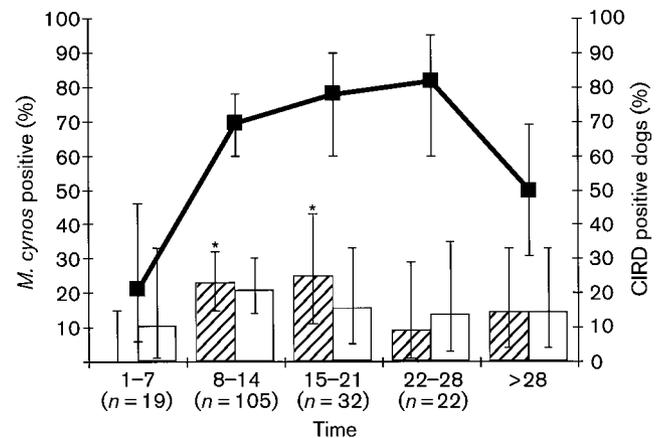


**Fig. 2.** Relationship between *M. cynos* isolation and age. The number of dogs in each group (*n*) is displayed, and 95% confidence intervals are shown as error bars. An asterisk indicates a significant difference in *M. cynos* isolation from the lung or trachea compared to dogs less than 1 year old. No other age group differed significantly from dogs less than 1 year old. Hatched bars, tracheal samples; white bars, lung samples.

seven of the 44 dogs (15.9%) that had no detectable tracheal colonization by ISH. Thus isolation of *M. cynos* was significantly more frequent in dogs with positive ISH sections ( $P=0.002$ ), and dogs from which *M. cynos* was isolated were more likely to be positive by ISH than dogs from which *M. cynos* was not isolated ( $P=0.0001$ ).

## DISCUSSION

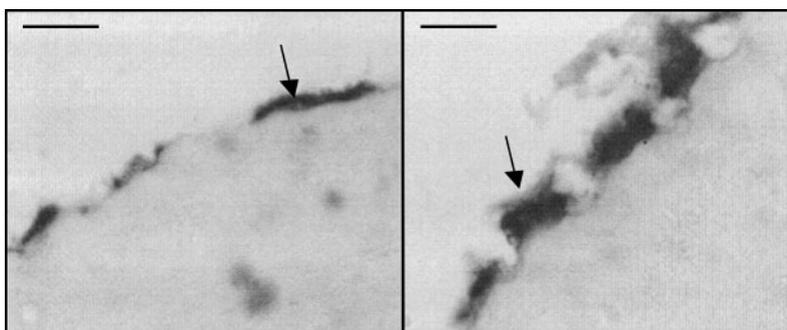
We found that a diverse range of *Mycoplasma* species can be isolated from healthy and diseased dogs, and that a variety of species can be isolated from throughout the respiratory tract. However, only *M. cynos* was significantly associated with respiratory disease, and only in one kennel. *M. cynos* was originally isolated from an outbreak of enzootic pneumonia in kennelled dogs in which two healthy dogs were exposed to an infected litter (Rosendal, 1972). In our study, *M. cynos* was isolated from the lungs and trachea of healthy and diseased dogs, and isolation from either site was significantly associated with CIRDC. *M. cynos* was most



**Fig. 3.** Relationship between *M. cynos* isolation and CIRDC and time in kennel. The number of dogs in each group (*n*) is displayed and 95% confidence intervals are shown as error bars. An asterisk indicates a significant difference in *M. cynos* isolation from the lung or trachea from dogs that had been in the kennel for less than 7 days. No other group differed significantly from dogs that had been in the kennel for more than 7 days. ■, CIRDC; hatched bars, tracheal samples; white bars, lung samples.

common in dogs with moderate signs of disease and was less common both in milder and in more severe cases. Infection with *M. cynos* may have been preceded or superseded by infection with another micro-organism. In fact, CRCoV was found to predominate in dogs with mild clinical disease in this kennel, *B. bronchiseptica* in dogs with moderate disease and *S. equi* subsp. *zooeidemicus* in dogs with severe clinical disease (Erles *et al.*, 2003; Chalker *et al.*, 2003a, b). A previous study found that a greater number of bacteria were isolated from dogs with more severe clinical disease (Chalker *et al.*, 2003b), and this may have made isolation of *M. cynos* more difficult due to the increased number of bacterial colonies also growing on the medium. In such cases, the use of *Mycoplasma* species-specific PCRs directly on clinical samples or filtering samples through a 0.2  $\mu\text{m}$  filter prior to plating may improve the detection of *M. cynos*.

The association between isolation of *M. cynos* and the



**Fig. 4.** Demonstration of *M. cynos* by *in situ* hybridization. A tracheal section was stained by ISH with a *M. cynos* probe. Arrows indicate *M. cynos* ISH-probe staining on the tracheal ciliated epithelial border. Bars, 1  $\mu\text{m}$ , 0.25  $\mu\text{m}$ .

duration of stay in the kennel indicates that dogs were being infected with this bacterium during the first two weeks in the kennel. However, a decline in the isolation of *M. cynos* was seen in dogs kept longer than 21 days, which may reflect an active immune response to this bacterium. This concurs with the results of a previous study, in which antibodies to *M. cynos* were detected 4–5 weeks after infection, corresponding with the elimination of the organism from the lower respiratory tract (Rosendal, 1978). A later investigation noted a greater susceptibility to mycoplasma infection in younger dogs (Randolph *et al.*, 1993), and our study found a lower prevalence of *M. cynos* infection in older dogs.

Experimental endobronchial inoculation of dogs with *M. cynos* has been shown to produce localized pneumonia with destruction and loss of the bronchial epithelial cilia and alveolar infiltration with neutrophils and macrophages (Rosendal & Vinther, 1977), and in our study *M. cynos* was detected on the tracheal ciliated epithelium by ISH. It is known that *M. cynos* can persist in the lung for up to 3 weeks following infection (Rosendal & Vinther, 1977), and that it can also be isolated from conjunctiva (Rosendal, 1973), tonsils, and even kennel aerosols. The capacity of *M. cynos* to persist in the environment is unknown, but other *Mycoplasma* species can survive for weeks to months outside the host, and the environment could therefore be a source of infection (Nagatomo *et al.*, 2001). Indeed, *M. cynos* was isolated from kennel aerosols in this study.

It was not possible to identify several mycoplasmas that were isolated in our study because PCR amplification was not achieved with the primers used. This may be due either to the presence of potentially novel *Mycoplasma* species with sequences insufficiently similar to the primers or to inhibition of the PCR.

In conclusion, *M. cynos* is associated with CIRDC, and younger dogs are more likely to be infected with *M. cynos* than older dogs. Canine infectious respiratory disease is a complex syndrome, and in the main study population in this investigation *B. bronchiseptica*, CRCoV, *S. equi* subsp. *zooepidemicus* and *M. cynos* were all associated with CIRDC (Chalker *et al.*, 2003a, b; Erles *et al.*, 2003). Further work is required to understand the interaction between these organisms during infection and to investigate whether other pathogens may be involved. Rosendal (1982) stated that natural cases of pneumonia with *M. cynos* as the sole agent had not been described, and that this bacterium could be a contributory factor. This suggestion agrees with our findings, as *M. cynos* was more strongly associated with moderate signs of respiratory disease than with milder disease or severe bronchopneumonia. *M. cynos* was isolated more frequently from the lower respiratory tract than the upper respiratory tract, and bronchial or tracheal lavage samples may be of more use than upper respiratory tract swabs for the detection of *M. cynos*. The study of canine mycoplasmas has perhaps been neglected due to the difficulties in isolation associated with mixed infections

and the difficulty of identification to species level. This is the first study to comprehensively investigate the presence of individual *Mycoplasma* species in dogs with respiratory disease. The overall importance and distribution of *M. cynos*, the mechanisms of pathogenicity and nature of the immune response to this pathogen are currently unknown.

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