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Detection of feline *Mycoplasma* species in cats with feline asthma and chronic bronchitis

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Abstract

Little is known about the aetiology of inflammatory lower airway disease in cats. The aim of this study was to investigate the role of *Mycoplasma* species in cats with feline asthma (FA) and chronic bronchitis (CB). The study population consisted of 17 cats with FA/CB, and 14 sick cats without clinical and historical signs of respiratory disease, which were euthanased for various other reasons. Nasal swabs, nasal lavage and bronchoalveolar lavage fluid (BALF) samples were taken from patients from both groups. *Mycoplasma* species culture with modified Hayflick agar and *Mycoplasma* polymerase chain reaction (PCR) were performed on all samples followed by sequencing of all *Mycoplasma* species-positive samples for differentiation of subspecies. PCR testing detected significantly more *Mycoplasma* species-positive BALF samples than *Mycoplasma* culture ($P = 0.021$). When cats with oropharyngeal contamination were excluded from comparison, the numbers of *Mycoplasma* species-positive BALF samples in the group with FA/CB (6/17) and the control group (4/9) were not significantly different ($P = 0.6924$). While all nasal samples of the cats with FA/CB were negative for *Mycoplasma* organisms, five samples in the control group ($P = 0.041$) were positive on PCR. Sequencing revealed *Mycoplasma felis* in all PCR-positive samples. *Mycoplasma* species can be detected in the lower airways of cats with FA/CB, as well as in the BALF of sick cats without respiratory signs. Further studies are warranted to investigate the possibility that *Mycoplasma* species represent commensals of the lower respiratory tract of cats.

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Introduction

Feline inflammatory lower airway disease is a frequently encountered disease complex in cats. Although a common clinical problem, the condition has been poorly defined.[1] Based upon the type of inflammatory response, two main disease forms have been described in cats: chronic bronchitis (CB) and feline asthma (FA).[2] While CB describes a neutrophilic inflammation of the lower airways accompanied by oedema and hypertrophy of the respiratory mucosa and excessive mucus production, FA represents a T cell-induced hypersensitivity reaction characterised by eosinophilic airway inflammation and bronchoconstriction.[1] Similar to the main triggers known for human asthma, various possible aetiologies have also been discussed for feline inflammatory airway disease, including drugs, environmental substances and toxins, exercise, stress, allergens and infections.[3]

Mycoplasma species are small bacteria lacking a peptidoglycan cell wall.[4] Belonging to the normal commensal flora of the upper airways in cats,[5,6] the organisms have also been detected in cases with conjunctivitis, upper respiratory infection[7–9] and lower respiratory tract infection in this species[10–13]; however, they also have been detected in cats with inflammatory bronchial disease.[14,15]

In humans, infections with *Mycoplasma pneumoniae* have recently been linked to asthma.[16] *M pneumoniae* is known to represent an important aetiological pathogen for childhood pneumonia.[17] Infections with the organism can precede the onset of asthma, exacerbate asthmatic

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signs and cause difficulties in asthma management.[16] Furthermore, *M pneumoniae* infection has been significantly associated with acute exacerbation of asthma.[18] Antibiotic treatment resulted in improvement of lung function in asthmatic patients who were polymerase chain reaction (PCR)-positive for *M pneumoniae* in their lower airways.[19] Several pathophysiological mechanisms have been discussed for how *Mycoplasma* species infections might trigger chronic bronchial disease. In the early phase of infection, *Mycoplasma* organisms interact with respiratory epithelial cells, leading to multifactorial alterations. *Mycoplasma pneumoniae* infection in children with wheezing episodes has been seen to induce an increase in the production of interleukin-5, a cytokine promoting eosinophilic airway inflammation and the development of airway hyperresponsiveness.[20]

The aim of this study was to investigate the role of *Mycoplasma* species in cats with FA and CB, and to compare the data with results from cats without clinical or historical signs of respiratory tract disease.

Materials and methods

Study design and animals

The prospective study was conducted between May 2009 and April 2011 with informed owner consent obtained for all patients. Seventeen cats diagnosed with FA/CB, and 14 sick cats without clinical and historical signs of respiratory tract disease were included in the study. All 31 cats were patients presented to the Clinic of Small Animal Medicine of the Ludwig Maximilian University of Munich, Germany.

Inclusion criteria for cats with FA/CB were a history of clinical signs typical for this disease complex, including cough, tachypnoea, wheezing or episodes of dyspnoea, and a positive response to treatment with inhaled or systemic steroids. Cytology of bronchoalveolar lavage fluid (BALF) had to show increased numbers of eosinophils, mature neutrophils or a mixed eosinophilic/neutrophilic inflammatory pattern. Cats with a physiological cytology and with low numbers of physiological cells on BALF were included if clinical signs were typical for FA/CB, differentials had been excluded during diagnostic work-up and if they showed a long-term response to steroid treatment. Patients were excluded if they were diagnosed with heart failure, neoplasia, pleural effusion, pneumonia or other diseases of the respiratory tract except for FA/CB, or if they were infected with feline leukaemia virus (FeLV) or feline immunodeficiency virus (FIV). To confirm the diagnosis of FA/CB, to exclude other diseases and to obtain sample material for *Mycoplasma* species detection, several diagnostic tests were performed in all 17 cats: standardised physical examination, haematology, echocardiogram, thoracic radiographs in two views (right-lateral and ventrodorsal), Baermann faecal examination to rule out lungworm infection, and bronchoalveolar lavage (BAL) with cytological examination and bacterial culture of the BALF. A serum sample of all cats with FA/CB was tested for FIV antibodies and FeLV antigen (Snap Combo Plus Test; Idexx Laboratories).

Cats in the control group were euthanased for other reasons than respiratory or cardiac problems. They were included if they did not have a history of respiratory disease within the last 6 months and if physical examination of the respiratory tract was unremarkable. Reasons for euthanasia in the control group were lymphoma (n = 4), seizures (n = 2), pancytopenia (n = 2), neoplasia of the pancreas (n = 1), chronic renal disease (n = 1), peritonitis (n = 1), hyperthyroidism (n = 1), non-regenerative anaemia (n = 1) and diabetic ketoacidosis (n = 1).

Sample collection

Faecal samples of all cats of the FA/CB group were collected for three consecutive days for Baermann faecal examinaton and analysed according to a standardised protocol.[21]

To obtain BALF, cats of the FA/CB group were premedicated with atropine (Atropinsulfat, 0.5 mg/ml IV; B Braun) and midazolam (Midazolam-ratiopharm, 0.2 mg/kg IV; Ratiopharm) followed by propofol anaesthesia (Narcofol, 6–8 mg/kg IV; CP-Pharma Handelsgesellschaft). Cats were placed in a sternal position, intubated with a sterile autoclaved endotracheal tube and pre-oxygenated for 3–5 mins. A sterile catheter (CH 4.5, 1.0 × 1.5; B Braun Vet Care) was inserted into the endotracheal tube and gently forwarded into the lower airways until resistance was noticed. The lavage was performed by applying 3 ml of sterile sodium chloride solution (Isotone Kochsalz-Lösung, 0.9%; B Braun Vet Care) through the catheter followed by recovery of the fluid with a sterile collection container (Lukens Schleimprobenbehälter; Olympus) that interconnected the catheter and the mechanical suction apparatus. The blind lavage procedure was repeated 1–2 times in one area until at least 1 ml of BALF was recovered. In all 14 cats in the control group, BAL was performed within 5 mins of euthanasia. The procedure was carried out as described above, except for the fact that BALF was recovered by manual suction with a syringe.

Diagnostic tests performed on BALF samples of all 31 cats included cytology, bacterial culture, *Mycoplasma* species culture and PCR for *Mycoplasma* species detection. In all PCR-positive samples, sequencing was conducted to determine the *Mycoplasma* species involved.

Sterile nasal swabs (sterile swab with Amies transport medium; Sarstedt) were taken from 12 cats with FA/CB and from 12 control cats after the BAL. Nasal lavage was performed in 13 cats with FA/CB and in 14 cats in the control group after the BAL with 3 ml of sterile sodium

chloride solution (Isotone Kochsalz-Lösung, 0.9%; B Braun Vet Care). Nasal swabs were submitted for *Mycoplasma* species culture, while nasal flush samples were used for DNA extraction and *Mycoplasma* PCR. Sequencing was performed in all PCR-positive samples to determine the *Mycoplasma* species.

BALF cytology

All cytology samples were evaluated by the same person (IZ). Cytocentrifuge-concentrated smears (5 mins at 95 g) were prepared immediately after collection and stained with modified Wright's stain after air drying. Cytological evaluation was performed with a light optical microscope (Olympus BX 51; Olympus Deutschland) following a standardised protocol. The slide with the best quality was chosen and 500 inflammatory cells per slide were counted (5 × 100 cells on different areas of the slide). The predominating cell type was documented based upon reference values previously established for interpretation of feline BALF cytology.[22,23] An eosinophilic inflammation was diagnosed if BALF cytology revealed >20% of eosinophils; a diagnosis of CB was established if >7% non-degenerative neutrophils were present. Samples with >20% eosinophils and >7% neutrophils were classified as mixed eosinophilic/neutrophilic inflammation. Samples with macrophages as the predominating cell type, <20% eosinophils and <7% neutrophils were classified as physiological. A standardised search for microorganisms was performed (100 fields with 100-fold magnification). *Simonsiella* species organisms and squamous cells on cytology were interpreted as oropharyngeal contamination. Samples classified as oropharyngeal contamination were samples displaying *Simonsiella* species organisms and squamous cells with an otherwise physiological cell population. Samples were classified as low cellularity if they contained only a few physiological cells, but were too dilute to perform a standardised cell count.

Bacterial cultures

Bacterial and *Mycoplasma* species cultures were performed at the Institute for Infectious Diseases and Zoonoses of the Ludwig Maximilian University of Munich, Germany. For bacterial culture, 50 µl BALF was applied to an agar plate. Test systems used for the detection of aerobic and anaerobic bacteria included Gassner and Rambach agar, sheep blood agar with colistin and nalidixin acid, and Bordet–Gengou agar for selective culture of *Bordetella bronchiseptica*. After inoculation, agar plates were incubated at 37°C for 48 h.

For *Mycoplasma* species culture, nasal swabs were first streaked onto a modified Hayflick agar and then rotated in a Hayflick bouillon in which the swab remained. BALF samples were applied to the Hayflick agar and to the bouillon. Fifty microlitres of sample fluid was applied to the agar plate and 50 µl was added to 3 ml of bouillon. Plates and bouillon were incubated under carbon dioxide nebulisation for 10 days, and then a gas pack (Anaerocult C for microbiology; Merck) humidified with 6 ml water was added before samples were incubated in an incubator at 37°C. Evaluation of plates and bouillon for *Mycoplasma* species colonies was performed on days 2, 4, 7 and 10 with a stereomicroscope.

PCR

DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen). The BALF samples were extracted using an adapted protocol for viscous samples. Briefly, the BALF sample (1–2 ml) was mixed with 30 µl acetylcystein solution (100 mg/ml) and vortexed to reduce viscosity. The sample was pelleted by centrifugation and re-suspended in 1 ml phosphate-buffered saline (PBS). The sample was centrifuged again and the cell pellet was re-suspended in 200 µl PBS. Nasal flush samples were also centrifuged and the cell pellet was re-suspended in 200 µl PBS. DNA extraction was performed according to the manufacturer's instructions. The DNA was eluted in 100 µl elution buffer and stored at −20°C. For the PCR, feline 28s recombinant DNA was amplified in each sample to confirm successful DNA extraction. The primer pair feline-28s-F (forward primer) and feline-28s-R (reverse primer) (Table 1) was mixed with Hot Star Taq Mastermix (Qiagen), and the DNA extracted from BALF and nasal flush sample. Amplification was done on an Eppendorf Mastercycler for 30 cycles with an annealing temperature of 60°C. For *Mycoplama* species detection, the primer pair Mycopl16sF (forward primer) and Mycopl16sR (reverse primer) (Table 2) was used, which is able to amplify, among others, *Mycoplasma arthridis, Mycoplasma canadense, Mycoplasma cynos, Mycoplasma felis, Mycoplasma gateae, Mycoplasma hyopharyngis* and *Mycoplasma lipophilum.* DNA samples were mixed with primers and the Hot Star Taq Mastermix, and PCR products were amplified for 30 cycles with an annealing temperature of 58°C, as described above.

Sequence analysis

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), according to the manufacturer's instructions. The cleaned samples were mixed with Mycopl16sF as the sequencing primer and sequenced by Eurofins MWG Operon. The sequence identities were determined using BLAST.

Statistical evaluation

Statistical evaluation was performed with the software GraphPad Prism Version 5.00 (GraphPad Software). The Kolmogorov–Smirnov and D'Agostino tests were used to test for parametric distribution of numeric data. Data comparison of normally distributed data was performed

Table 1 Nucleotide sequences of feline 28s forward and feline 28s reverse primers

Primer	Sequence
Feline-28s-F	CGCTAATAGGGAATGTGAGCTAGG
Feline-28s-R	TGTCTGAACCTCCAGTTTCTCTGG

Table 2 Nucleotide sequences of MycopI16sF forward and MycopI16sR reverse primers

Primer	Sequence (5′–>3′)
MycopI16sF	GTCGATGAAGGACGTGATTA
MycopI16sR	GGCAGATTCAGACAGGGTTT

with a non-paired *t*-test. The Mann–Whitney test was used for data not normally distributed. Ratios were evaluated with Fisher's exact test. The level of significance was set at <0.05 for all comparisons.

Results

Study population

The median age of cats with FA/CB was 8.0 years (mean 6.4 years) and the median age of the control cats was 12.0 years (mean 13.2 years); this difference was not significant ($P = 0.662$). The cats with FA/CB included seven male castrated, one intact male and nine female spayed cats; the control group consisted of six male castrated and eight female spayed cats. Breed distribution in the FA/CB group included 15 domestic shorthair (DSH) cats, one British Shorthair and one Persian mix. The control group consisted of 11 DSH, one Devon Rex, one Maine Coon and one Persian mix. Clinical signs of the cats with FA/CB included cough (14/17), tachypnoea (2/17) and dyspnoea (1/17).

Cytological examination of BALF samples

While cats with FA/CB more often showed an eosinophilic and eosinophilic/neutrophilic inflammation, cats from the control group were more often diagnosed with oropharyngeal contamination of the BALF (Table 3).

Detection of Mycoplasma species

Significantly more *Mycoplasma* species-positive samples were detected by PCR in the nasal flush samples of control cats than those of the FA/CB group (Table 4). No significant difference could be detected when *Mycoplasma* species PCR-positive BALF samples were compared between both groups. There was also no significant difference between groups regarding *Mycoplasma* species PCR-positive BALF samples when cats with oropharyngeal contamination were excluded from comparison and *Mycoplasma* species-positive BALF samples of cats with FA/CB (6/17) and controls (4/9) were compared ($P = 0.6924$). Furthermore, there was no significant difference between the groups when all cats showing oropharyngeal contamination, samples with low cellularity and the two cats in the FA/CB group with a physiological BALF cytology were excluded from the comparison ($P = 1.000$). Culture results of *Mycoplasma* species-positive nasal and BALF samples were not significantly different between groups.

Comparison of PCR and culture

In nasal flush samples, there was no significant difference between number of PCR-positive (5/27) and culture-positive (2/24) samples of all cats ($P = 0.425$). In contrast, BALF samples were significantly more often PCR-positive (13/31) than culture-positive (4/31) ($P = 0.021$).

Sequencing of Mycoplasma species-positive samples

Sequencing was performed in all *Mycoplasma* species PCR-positive samples. In all 18 samples, *M felis* was identified as the only species.

Discussion

Detection of *Mycoplasma* species in the lower respiratory tract of cats has been linked to pneumonia in several studies.[10–13] As *Mycoplasma* organisms have not so far been found in the lower airways of healthy cats, it has been discussed whether they represent primary respiratory pathogens in the cat, rather than opportunistic invaders.[6,22] *Mycoplasma* species have also been isolated from cats with inflammatory bronchial disease and discussed as a potential trigger for FA or CB.[14,15,24] In this study, *Mycoplasma* species were detected in the BALF of cats with FA/CB, as well as in samples from cats without clinical respiratory abnormalities. However, samples in the control group were taken shortly after euthanasia and 5/14 obtained BALF samples showed oropharyngeal contamination on cytology, while in the FA/CB group none of the 17 samples was contaminated with oropharyngeal material. One explanation for this finding could be the fact that the cricopharyngeal muscles relax post mortem, which might facilitate draining of saliva into the lower airways, leading to oropharyngeal contamination with *Simonsiellea* species and squamous cells. However, when cats with oropharyngeal contamination were excluded from the statistical evaluation, the remaining *Mycoplasma* species-positive BALF samples of cats with FA/CB and controls were still not different. However, it could also be possible that post-mortem oropharyngeal contamination occurred in even more cats than only in the ones detected by light microscopy, and that numbers of squamous epithelial cells and *Simonsiella* organisms were too low for detection in some cats. Quantitative real-time PCR for the detection of *Mycoplasma* species could have been helpful to further investigate this possibility and to differentiate between

Table 3 Cytology results of bronchoalveolar lavage fluid samples of cats with feline asthma/chronic bronchitis and control cats

Cell type	Patients	Controls	*P*
Physiological	11.8% (2/17)	42.9% (6/14)	0.097
Neutrophilic inflammation	11.8% (2/17)	0% (0/14)	0.488
Eosinophilic inflammation	35.3% (6/17)	0% (0/14)	0.021
Eosinophilic/neutrophilic inflammation	35.3% (6/17)	0% (0/14)	0.021
Low cellularity	5.9% (1/17)	21.4% (3/14)	0.304
Oropharyngeal contamination	0% (0/17)	35.7% (5/14)	0.012

Table 4 Polymerase chain reaction (PCR) and culture results for *Mycoplasma* species detection in nasal flush/nasal swabs and bronchoalveolar lavage fluid (BALF) samples of cats with feline asthma/chronic bronchitis and control cats

	Patients	Controls	*P*
Nasal flush/swabs			
PCR *Mycoplasma* species-positive	0% (0/13)	37.5% (5/14)	0.041
Culture *Mycoplasma* species-positive	0% (0/12)	16.7% (2/12)	0.196
BALF			
PCR *Mycoplasma* species-positive	35.3% (6/17)	50.0% (7/14)	0.481
Culture *Mycoplasma* species-positive	11.8% (2/17)	7.1% (2/14)	1.000

cats with low and high numbers of *Mycoplasma* organisms.

In the group of cats with FA/CB, two cats with a physiological cell pattern and one cat with low numbers of cells on BALF cytology were also included; three cats with samples of low cellularity were included in the control group. The reason for the inclusion of these patients in the FA/CB group was that all three cats showed typical signs consistent with a diagnosis of FA/CB (chronic cough), other respiratory diseases could not be detected on diagnostic work-up and they responded well to steroid treatment. In addition, two of the three cats showed eosinophilia on blood work. To investigate the possibility that results might have been influenced by the inclusion of insufficiently classified BALF samples, comparison was repeated excluding the physiological cytology samples of the cats with FA/CB and all samples with low cellularity and oropharyngeal contamination, which still resulted in no difference between groups. However, the results have to be interpreted with caution, as the sample size for this comparison was small.

Mycoplasma species were detected by PCR in more than a third of nasal samples from control cats without respiratory signs. Similar to the findings of this study, *Mycoplasma* species have been previously cultured from the upper airways of cats with respiratory tract infection[7,8] and the upper respiratory tract of healthy cats,[5,6] indicating a role as a commensal organism in the upper airways of cats. In contrast to detection in the lower airways, *Mycoplasma* species were only detected in nasal samples of control cats, but not in samples of cats with FA/CB in this study. All control cats were assessed as respiratory healthy by history, clinical examination and BALF diagnostics; however, these animals suffered from other severe diseases and some had received immunosuppressive therapy prior to euthanasia, potentially influencing the growth of *Mycoplasma* species because of systemic immunosuppression. In contrast to that, most cats in the FA/CB group had not been pretreated with steroids or other immunosuppressive agents, potentially explaining the difference between both groups regarding the recovery of *Mycoplasma* organisms from the nasal cavity. Another factor influencing the detection rate of *Mycoplasma* species in this study could be the sample technique chosen for *Mycoplasma* detection. Johnson and Kass[25] demonstrated that in some cats with chronic rhinosinusitis, *Mycoplasma* species could only be detected in nasal biopsy samples and not in nasal flush samples. Therefore, the *Mycoplasma* prevalence in the FA/CB group and in the control group might have been underestimated with the sampling technique used in this study.

The sampling technique might have had an influence on the interpretation of BALF cytology samples and the results of *Mycoplasma* species detection. Only one area of the lungs was sampled, and the sample procedure was performed blind on all cats, as none of the FA/CB cats showed focal bronchopulmonary changes. A recent study showed that the differential cell count of BALF can significantly differ between different lung segments in cats.[26] Therefore, the grade and type of inflammation might have been misinterpreted by sampling only one

segment, and inflammation might have been missed in some of the control cats with the sample technique used in this study.

M felis was detected as the only species in all *Mycoplasma*-positive samples in this study. This *Mycoplasma* species has been previously detected in cats with upper[7,8] and lower respiratory tract disease.[15] In this study, *M felis* was detected in cats with and without signs of respiratory tract disease, suggesting that this species might represent a non-pathogenic *Mycoplasma* species in the feline respiratory tract, potentially acting as a secondary pathogen if host defence mechanisms are impaired. Because *M felis* was the only *Mycoplasma* species detected in this study, no conclusion can be drawn regarding the pathogenicity of other species in feline respiratory disease.

In contrast to the findings in past studies,[6,22] this study revealed *Mycoplasma* species not only in the BALF of 35% of cats with FA/CB, but also in 50% of sick cats without clinical signs of respiratory disease by PCR. The most likely explanation of why previous investigations failed to detect *Mycoplasma* organisms in healthy cats is the use of PCR as a more sensitive detection method in this study compared with detection by culture in older studies. When diagnostic tests were compared in this study there was no significant difference between the number of PCR-positive and culture-positive nasal samples. This is in agreement with results of a study comparing PCR and the culture technique for *Mycoplasma* species detection in feline nasal flush and nasal biopsy samples, where concordance of results of both techniques was demonstrated in 19/20 samples.[27] In contrast, there were significantly more PCR-positive (13/31) than culture-positive (4/31) samples in BALF in this study. A similar finding was described in a recent study in which *Mycoplasma* species could not be cultured in any of 76 cats with lower airway disease, although 15.4% of patients were PCR-positive for the organism.[15] Based on these results, we recommend the use of PCR rather than culture for the detection of *Mycoplasma* species in lower airway samples of cats to increase sensitivity.

In this study, a non-quantitative PCR method was used for the detection of *Mycoplasma* species; however, quantitative analysis could be helpful in assessing the number of *Mycoplasma* organisms in feline respiratory samples. In future studies, the use of quantitative PCR might help to distinguish *Mycoplasma* colonisation from *Mycoplasma* infection in cats, and therefore facilitate interpretation of a positive *Mycoplasma* species result in a patient.

The major limitation of this study is that the control group consisted of cats presented with various diseases and not healthy animals, and samples were taken post mortem. Although control cats did not show any respiratory signs on history, had an unremarkable physical examination of the respiratory tract, and no signs of respiratory disease on BALF cytology and routine bacterial culture, in these systemically ill cats primary respiratory disease or secondary involvement of the respiratory tract could not be totally excluded by the diagnostic tests performed. It would have been helpful if thoracic imaging or post-mortem examination and histopathology had been performed in these cats; unfortunately, these diagnostics were not available for most cats. Furthermore, it is possible that systemically ill cats showed colonisation of the upper and lower respiratory tract with *Mycoplasma* organisms owing to impairment of systemic and local defence mechanisms, potentially leading to a higher number of *Mycoplasma*-positive cats compared to a healthy population. In addition, some cats in the control population were treated with immunosuppressive agents prior to euthanasia, potentially facilitating the growth of *Mycoplasma* species in the airways. Therefore, results obtained from this population of sick cats probably do not represent the *Mycoplasma* status in healthy cats, which were not chosen as a control group because of ethical considerations. In addition, information about antibiotic treatment prior to sampling was not available for most cats, and antibiotic therapy was not elected as an exclusion criterion for the study population; however, antimicrobial therapy could have influenced the results and decreased the detection rates of *Mycoplasma* species by culture and PCR in both groups.

Conclusions

We were able to show that *Mycoplasma* species can be detected in the lower airways of cats with FA/CB, as well as in the BALF of sick cats without clinical and historical signs of respiratory disease. Further studies are warranted to investigate the possibility that *Mycoplasma* species are commensals of the lower respiratory tract of cats, but there is insufficient evidence from this study to make this conclusion.

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