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Detection of bacterial and viral organisms from the conjunctiva of cats with conjunctivitis and upper respiratory tract disease

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A variety of pathogens are involved in conjunctivitis in cats. In this study, the prevalence of feline herpesvirus (FHV), *Chlamydophila felis*, mycoplasmas, and aerobic bacteria on the conjunctival surface of cats with conjunctivitis and upper respiratory tract disease was investigated by polymerase chain reaction (PCR), immunofluorescent assay (IFA), and aerobic bacterial culture of ocular swabs. Forty-one cats were included of which 37 were found to be infected with an ocular organism. Single and multiple infections were present in 15 and 22 cats, respectively. FHV, mycoplasmas, and *C felis* were detected by PCR in 11 (27%), 20 (49%), and 23 (56%) cats, respectively. IFA detected 10 cats as positive for *C felis*. *Mycoplasma felis*, *Mycoplasma canadense*, *Mycoplasma cynos*, *Mycoplasma gateae*, *Mycoplasma lipophilum*, and *Mycoplasma hyopharyngis* were identified by genetic sequencing. The most common aerobic bacteria cultured included *Staphylococcus* species, *Streptococcus* species and *Micrococcus* species. The prevalence of mycoplasmas in cats with conjunctivitis was higher than previously reported, and four of the *Mycoplasma* species have not been described in cats so far.

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Different causes of conjunctivitis are recognised (eg, infection, neoplasia, trauma, foreign body, immune-mediated diseases). Of these, infectious agents are the most common causes in cats. Feline herpesvirus (FHV) and *Chlamydophila felis* are proposed to be the major primary pathogens.[1] Primary bacterial (other than *C felis*) conjunctivitis is considered to be rare.[2] However, in recent studies, *Mycoplasma* species were commonly isolated in cats with upper respiratory tract disease (URTD) and conjunctivitis.[3,4] Clinical signs of conjunctivitis vary in severity and include blepharospasm, conjunctival hyperaemia, chemosis, and serous to purulent ocular discharge of one or both eyes. In some cats, clinical signs persist or recur after initial improvement.

C felis has been reported as responsible for up to 60% of conjunctivitis cases in cats.[4,5] The immunofluorescent assay (IFA) has been used the identification of *C felis*, but today, polymerase chain reaction (PCR) is used as an alternative diagnostic tool.[3,5,6]

Mycoplasma species have also been isolated from the conjunctiva of cats with and without clinical signs.[7,8] In early studies, *Mycoplasma felis* was found with a prevalence of up to 25% among cats with conjunctivitis.[8,9] A recent study showed that the prevalence of *M felis* in cats with conjunctivitis was higher than in clinically healthy cats.[3] *Mycoplasma gateae* and *Mycoplasma arginini* were identified on the conjunctival surface in healthy cats.[7,10,11] For the detection of *Mycoplasma* species, bacterial culture has been the method of choice in the past, and species differentiation was performed by growth inhibition.[12] These methods, however, are not very sensitive. PCR is now also used to detect and differentiate *Mycoplasma* species. It has been shown that PCR is more sensitive, and results are obtained in a shorter period of time.[13,14]

The aims of this study were to determine the prevalence of FHV, *C felis*, *Mycoplasma* species, and aerobic bacteria on the conjunctiva of cats with conjunctivitis and URTD, to determine the sensitivity of IFA compared to PCR for detection of *C felis*, and to differentiate *Mycoplasma* species detected by means of PCR and

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Basic Local Alignment Search Tool (BLAST) program sequencing.

Material and methods

Cats

Cats with signs of feline URTD and bilateral conjunctivitis were included into the study. Conjunctivitis was characterised by conjunctival hyperaemia, chemosis, and serous to purulent ocular discharge. There were no restrictions regarding the duration of clinical signs, age, gender and breed. Exclusion criteria were unilateral conjunctivitis (as this more commonly associated with a primary ocular disease than with a primary infection), a history of any local or systemic treatment within 6 weeks prior to presentation or a history of other underlying ocular diseases.

Data and sample collection

All cats were examined clinically by the same examiner, and the degree of conjunctivitis was scored on a scale from 0 (no clinical signs) to 4 (very severe clinical signs; Table 1).[4] Prior to sampling, ocular discharge was removed using moist swabs. In total, three swabs were taken from the ventral conjunctival fornix of the right eye of each cat using slight pressure when rolling the swab over the surface. Swabs for bacterial culture were taken first, followed by the swabs for PCR and lastly by the swabs for IFA.

Bacterial culture

A dry sterile cotton swab (single plastic swab Amies Agar Gel 108C, Copan Italia SPA, Brescia, Italy) with transport medium was used. After sampling, the swabs were returned into their covers and stored for a maximum of 2 h at room temperature until culture was prepared. Culturing was performed aerobically under standard conditions using blood agar and selective media (Colistin/Nalidixic acid agar (CNA agar), Gassner agar, and Rambach agar). For further differentiation, microscopic examination, and biochemical reactions were performed using standard bacteriological test (eg, catalase reaction or cytochrome oxidase test) as well as commercially available kits (BioMérieux, Nürtingen, Germany; Oxoid, Wesel, Germany). Antimicrobial susceptibility was tested using the agar disc diffusion method according to the CLSI (Clinical and Laboratory Standards Institute) standard documents M31-A2 and M31-S1.[15,16]

Table 1. Scoring system used to describe the degree of conjunctivitis.

0	No clinical signs
1	Mild conjunctival hyperaemia
2	Moderate conjunctival hyperaemia and mild chemosis
3	Moderate to severe conjunctival hyperaemia and moderate chemosis
4	Severe conjunctival hyperaemia and severe chemosis

Multiplex real-time PCR for FHV and C felis

A sterile plain dry cotton swab (plain dry swab in peel pouches 155C, Copan Italia SPA, Brescia, Italy) was used for the PCR. The swabs were returned into their covers immediately after sampling, frozen and stored at −70°C until deoxyribonucleic acid (DNA) was extracted. The method used to isolate DNA for the detection of FHV, *C felis*, and *Mycoplasma* species has been described previously.[4] The three-way multiplex real-time PCR assay to detect FHV, *C felis*, and feline 28S ribosomal DNA (rDNA) was set up as follows: 12.5 ml 2× Hotstartaq (Qiagen, Crawley, UK), 1.5 ml of 50 mM magnesium chloride, 100 nM FHV primers and probe (Table 2), 100 nM *C felis* primers and probe (Table 2), 100 nM feline 28S primers and probe (Table 2), 5 ml genomic DNA, and water to bring the volume to 25 ml. Thermal cycling was performed in an iCycler iQ (Bio-Rad Laboratories, Hempstead, UK) for one cycle of 95°C for 15 min, followed by 45 cycles of 95°C for 10 s and 60°C for 60 s. Fluorescence was detected at 530, 620, and 680 nm at each annealing step (60°C). Data were analysed using the iCycler software version 3 as described previously.[17]

Nested PCR and BLAST sequencing for Mycoplasma species

The PCR assay to detect *Mycoplasma* species was performed using previously described primers (Table 2) for both the flank and the nested reactions.[18] The 50 µl reactions (both flank and nested) were carried out with 1 µl of sample DNA, and a final concentration of the following: 1 µM of each primer (Table 2), 800 µM deoxyribonucleotide (dNTP) mix, 1.5 mM $MgCl_2$, 1× PCR Buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris−HCl (pH 8.8 at 25°C), 0.01% Tween-20), and 3.75 U Taq polymerase (Biolase DNA Polymerase with 10× PCR Buffer; Bioline, Boston, MA, USA). The flank and nested reactions were carried out similarly, using a previously described protocol.[19] Known positive (ATCC 23391) and negative (PCR H_2O substituted for template) controls were run with the samples on each PCR assay. PCR products were analysed for the appropriately sized amplicons on a 2% agarose gel stained with ethidium bromide. DNA from positive conjunctival samples was purified using a commercially available kit (Qiagen Gel Purification Kit, Valencia, USA) and submitted for sequencing at a commercial laboratory (Macromolecular Research Core Laboratory, Colorado State University, Fort Collins, USA). The resulting sequences were then analysed by comparison to sequences in the GenBank using the BLAST program

Table 2. Primer and probe sequences used in the PCR assays.

	Primer or probe sequence (5′ → 3′)
C felis for	GAACTGCAAGCAACACCACTG
C felis rev	CCATTCGGCATCTTGAAGATG
C felis Taqman	FAM CGCTGCCGACAGATCAAATTTTGCC BHQ1
28S rDNA for	AGCAGGAGGTGTTGGAAGAG
28S rDNA rev	AGGGAGAGCCTAAATCAAAGG
28S rDNA Taqman	Texas Red TGGCTTGTGGCAGCCAAGTGT BHQ2
FHV for	GGACAGCATAAAAGCGATTG
FHV rev	AACGTGAACAACGACGCAG
FHV Taqman	CY5 AATTCCAGCCCGGAGCCTCAAT BHQ3
Myc for 1	ACACCATGGGAGCTGGTCAT
Myc rev 1	CTTC(AT)TCGACTT(CT)CAGACCAAGGCAT
Myc for 2	GTTCTTTGAAAACTGAAT
Myc rev 2	GCATCCACCA(AT)A(AT)ACTCT

BHQ = black hole quencher, CY5 = cyanine 5, FAM = carboxyfluorescein, for = forward, Myc = Mycoplasma, rev = reverse.

on the National Institutes of Health (NCBI) website (http://www.ncbi.nlm.nih.gov).

IFA

For IFA to detect *C felis*, a sterile plain dry cotton swab was used to collect epithelial cells, which were transferred onto a slide immediately after sampling, fixed with acetone, and evaluated for the approximate number of cells under the microscope. Sampling was repeated on the same day (within a maximum of 2 h) if slides contained less than 20 cells. The IFA (Imagen Chlamydia, Dako Diagnostics, Ely, Cambridgeshire, UK) was performed according to the manufacturer's guidelines. The test uses a group specific monoclonal antibody to detect *Chlamydophila psittaci* which is conjugated to fluoresceinisothiocyanate (FITC). The antibody containing solution was applied onto the slides and incubated for 15 min. The test was considered positive if intracytoplasmic bodies with a typical size and FITC-specific green fluorescence were detected by fluorescence microscopy.[5]

Statistics

Data concerning conjunctivitis score were normally distributed. Therefore, a Student *t* test was used to detect significant differences between groups of cats with either a single infection or multiple infections. To detect differences in the severity of conjunctivitis between groups of cats infected with a single pathogen, one-way analysis of variance (ANOVA) was performed. A *P*-value of ≤ 0.05 was considered statistically significant. Numbers for true positives (TP), false positives (FP), true negatives (TN), and false negatives (FN) were calculated; the following formulas were used to asses the sensitivity = $[TP/(TP + FN) \times 100]$ and the specificity = $[TN/(TN + FP) \times 100]$. For statistical analysis, the commercially available software package SPSS 13.0.0 (SPSS Inc, Chicago, USA) was used.

Results

Cats

Forty-one cats were included into the study. They originated from private households (nine cats; 22%), animal shelters (20 cats; 49%), and farms (12 cats; 29%). Farm cats lived as free roaming cats. All cats were domestic shorthairs ranging in age between 3 months and 16 years. The duration of clinical signs and the vaccination status could not be evaluated as information was lacking for the majority of cats.

Clinical examination

The mean score (±standard deviation) for conjunctivitis in all 41 cats was 1.7 ± 0.9 (range 1–4) (Table 3). The severity of conjunctivitis was mild (grade 1) in 21 cats, moderate (grade 2) in 10 cats, severe (grade 3) in nine cats, and very severe (grade 4) in one cat. Mean scores for conjunctivitis in cats infected with only FHV, *C felis*, or *Mycoplasma* species were 1.5 ± 0.5, 1.6 ± 0.8, or 1.8 ± 0.8, respectively ($P = 0.560$). Mean conjunctival disease score was significantly lower ($P = 0.047$) in cats infected with single pathogens (1.6 ± 0.7) than in cats infected with multiple pathogens (2.2 ± 1.0).

FHV

FHV DNA was detected in 11/41 cats (27%). This was the sole infectious agent detected in five cats, whereas it was found in association with another agent in six cats. Co-infection with *C felis* was detected in one cat, whereas combined infection with FHV-1, C *felis* and *Mycoplasma* species was detected in five cats (Table 3).

Chlamydophila felis

C felis DNA was detected by PCR in 23/41 cats (56%; Table 3), but only 10/23 cats (43%) were positive when

Table 3. Distribution of pathogens among cats originating from private households, animal shelters and farms.

Origin	Private household (9 cats)	Animal shelter (20 cats)	Farm (12 cats)	Total (41 cats)
Conjunctivitis score	1.56 ± 0.73	1.60 ± 0.82	2.17 ± 1.03	1.76 ± 0.89
Detected pathogens				
No pathogen	1/9 (11%)	2/20 (10%)	1/12 (8%)	4/41 (10%)
Single pathogen	2/9 (22%)	13/20 (32%)	1/12 (8%)	15/41 (37%)
Multiple pathogens	6/9 (67%)	5 (25%)	10/12 (83%)	22/41 (53%)
Cats with respective pathogens				
FHV (PCR)	3/9 (33%)	5/20 (25%)	3/12 (25%)	11/41 (27%)
Chlamydophila felis (PCR)	5/9 (56%)	7/20 (35%)	11/12 (92%)	23/41 (56%)
Mycoplasma species (PCR)	5/9 (56%)	5/20 (25%)	10/12 (83%)	20/41 (49%)
Aerobic bacteria (culture)	6/9 (67%)	9/20 (45%)	1/12 (8%)	16/41 (39%)
Multiple infections				
FHV + *Chlamydophila felis*	–	1/20 (5%)	–	1/41 (2%)
Chlamydophila felis + *Mycoplasma* species	3/9 (33%)	–	8/12 (67%)	11/41 (27%)
3 Pathogens	1/9 (11%)	1/20 (5%)	2/12 (17%)	4/41 (10%)
Co-infections with primary pathogens and aerobic bacteria				
Single primary pathogen	2/6 (33%)	4/9 (44%)	–	6/41 (15%)
2 Primary pathogens	3/6 (50%)	1/9 (11%)	–	4/41 (10%)
3 Primary pathogens	1/6 (17%)	–	1/1 (100%)	2/41 (5%)

tested by IFA (Table 4). Eighteen samples could not be evaluated by IFA due to only small numbers of conjunctival cells present on the slide, even after re-sampling. The sensitivity and specificity of the IFA were 50.0% and 63.6%, respectively (Table 4).

Mycoplasma *species*

An amplicon (150, 200, 300, 400, or 500 bp) consistent with a *Mycoplasma* species was amplified from conjunctival swabs collected from 20/41 cats (49%; Table 3). In cats in which *Mycoplasma* species DNA was detected, co-infections with *C felis* were detected in 10 cats (50%), whereas infections with *Mycoplasma species*, *C felis*, and FHV were found in five cats (25%; Table 3). Twelve amplicons detected in nine cats were most homologous with *M felis*, *Mycoplasma canadense*, *Mycoplasma cynos*, *M gateae*, *Mycoplasma lipophilum*, or *Mycoplasma hyopharyngis* (Table 5). In 3/9 cats, two different *Mycoplasma* species were identified (Table 5). *M felis* was identified in 4/9 cats. In four cats, the amplified sequences showed homology with two *Mycoplasma* species between which BLAST was unable to differentiate.

Table 4. Comparison of Imagen Chlamydia IFA with PCR using following formulae to assess the sensitivity = [TP/(TP + FN) × 100] and the specificity = [TN/(TN + FP) × 100].

Results of IFA	Number of specimens	Number of results obtained by PCR	
		Positive	Negative
Positive	10	6	4
Negative	13	6	7
Total	23	12	11

Culture results

Positive cultures of conjunctival swabs were obtained in 16/41 cases (39%). One bacterial species was cultures in 11 samples, whereas two or three bacterial species were identified in three and two samples, respectively (Table 4). Aerobic bacteria without additional organisms were cultured in four cats; co-infections with either one or two and more bacterial organisms were identified in six cats each (Table 4). *Streptococcus* species (5/15), *Staphylococcus* species (4/15), and *Micrococcaceae* (4/15), were present most often. In two swabs each, *Corynebacterium* species, *Pseudomonas* species, *Enterobacteriaceae*, and unclassified Gram-negative rods were found (Table 4).

Discussion

In this study, the prevalence of FHV, *C felis*, *Mycoplasma* species, and aerobic bacteria in cats with conjunctivitis and URTD was evaluated. The number of cats positive for FHV (27%) is within the range which

Table 5. *Mycoplasma* species differentiation based on BLAST sequencing of 12 amplicons isolated from nine cats (M = Mycoplasma, rRNA = ribosomal ribonucleic acid).

Patient number	Amplicon size	Accession number	Mycoplasma strain	% Homology
7	200	AY741674.1	*M felis* strain ATCC 23391	94–96
				93
16	400	AY800341	*M canadense*	97
		AF443609	*M gateae*	
23	200	AY729935	*M lipophilum*	94
		AY816345	*M hyopharyngis*	90
26	200	AY274256	*M felis* strain MF6 16S rRNA	100
32	200	AY741674.1	*M felis* strain ATCC 23391	96
	400			97
34	150	AY800341	*M canadense*	99
		AY973562	*M gateae*	
37	200	AY816345	*M hyopharyngis*	95
	500			
38	200	AY741674.1	*M felis* strain ATCC 23391	96
	400			92
39	300	AF538682	*M cynos*	94

has been reported to be between 14% and 54%.[3,20–26] *C felis* has been found in 0–59% of cats with conjunctivitis and URTD.[3,21,22,24,26–28] The *C felis* prevalence of 56% in this study is at the upper end of the range of previous published studies in which cats with conjunctivitis only were included. The hygiene status has been identified as one of the URTD major risk factors in catteries, animal shelters, and private households. It could be noted that a higher prevalence of FHV and *C felis* was associated with a less than excellent hygiene status.[29] As 78% of the cats originated from a multi-cat household (animal shelter or farm), the hygiene status could have influenced the prevalence in the present study. The survival time in the environment is relatively short for FHV and the elementary bodies of *C felis*. Close contact between cats could have maintained a high infectious rate.

IFA is a common method to identify *Chlamydiae*.[6,30] The commercially available test performed in this study is routinely used to detect the human species *Chlamydia trachomatis*, *C psittaci*, and *Chlamydophila pneumoniae*. It was also used to assess *Chlamydia/Chlamydophila* species prevalence in turkeys and cats.[5,31] However, in this study, test results could only be obtained in 23/41 cats. Too low cell numbers were collected in all other cats resulting in 18 samples with inadequate quality. Reasons such as small sampling site, low shedding of infected cells, and trapping of cells on the swab have to be considered. However, the same test was used in another study in which the same sampling method was used without these problems.[5] One study suggested taking two swabs, the first to clean the sampling site and the second to obtain the sample; using this method, a higher number of positive results were present on the second sample.[32] In the present study, the area surrounding the eyes was cleaned with a moist swab before sampling, and even re-sampling did not yield in better results. Another reason for the discrepant results of IFA and PCR may be a low number of elementary bodies (EB). One experimental study demonstrated that a good visualisation of EB is achieved in samples containing 10^5–10^7 EB/ml, whereas in less concentrated samples (10^1–10^4 EB/ml), fluorescence was more difficult to detect.[32] Duration of clinical signs seem to influence the shedding of EB as the detection of EB becomes more difficult in chronic cases.[6] Although the duration of clinical signs was not known, a possible chronic presence of clinical signs could have influenced the shedding of the organisms in cats of the present study. In these cases, the more sensitive PCR is more effective in the detection of a small number of EB.[17] Thus, PCR was shown to be more useful as a diagnostic tool to detect *C felis* than IFA.

Mycoplasma species are found more often in cats with conjunctivitis compared to clinically healthy cats.[3] Prevalence has been reported to be 16% and 25%.[3,8] As discussed for *C felis*, close contact between cats in the same household may have lead to a higher prevalence in the present study. This is the first study evaluating the prevalence of *Mycoplasma* species among cats with conjunctivitis in Germany. Thus, different geographic distribution of these pathogens in the cat population may play a role as well.

In previous studies, species differentiation found *M felis* in the majority of cats with conjunctivitis.[8,9] *M gateae* and *M arginini* were found in conjunctival swabs of mainly healthy cats and only to a lesser extent cats with conjunctivitis.[7,10] Four of the *Mycoplasma* species detected in this study have not been described to occur in cats so far and have not been mentioned as pathogens of the conjunctiva in other

species. *M canadense* was isolated from mastitis in dairy cows, from granulomatous vulvitis in heifers, from an aborted foetus, and from semen of bulls.[33–37] No lesions in the respiratory tract of cows were found after experimental infection.[38] *M hyopharyngis* was detected on the surface of the mucus membranes of the respiratory tract of asymptomatic pigs.[39,40] *M cynos* was found in the lower respiratory tract of dogs in correlation with degenerative changes in the bronchial epithelium.[41,42] Although these species were isolated from the respiratory tract of cattle, pigs, and dogs, respectively, the presence in cats may be incidental, and additional research (eg, experimental infection studies) is necessary to evaluate their role and pathogenicity in cats.

BLAST finds regions of local similarity between nucleotide and protein sequences and calculates the statistical significance of matches.[43] An exact determination of the present *Mycoplasma* species was not possible in three cases of this study. Close phylogenetic relationships based on 16S rRNA analysis had been described to exist between *M gateae* and *M canadense* (*Mycoplasma hominis* cluster) and *M lipophilum* and *M hyopharyngis* (*M lipophilum* cluster).[13,44–47] Although a variety of search tools are available in BLAST, similarity of genome sequences between mycoplasmas limits the exact prediction of the species.

In a study evaluating the bacterial flora of the conjunctiva in cats with concurrent inflammation, 34% of 38 cats yielded a positive culture which is comparable to the present study.[6] The percentage of positive cultures from healthy cats has been reported to be 33%, 58%, and 67%.[48–50] There seem to be surprisingly low percentages as more positive cultures would be expected due to the inflammatory process and the following secondary invasion. It has been suggested that natural local host defence mechanisms are responsible for the low prevalence of bacterial infections.[48]

The bacterial spectrum present in this study is similar to studies evaluating nasal or oropharyngeal swabs taken from cats with URTD.[51–53] Their presence on the conjunctiva may be explained by distribution due to grooming of the cats. Although aerobic bacteria are thought to be secondary invaders, in four cats, bacterial culture was positive for *Streptococcus canis*, *Staphylococcus aureus*, *Pseudomonas* species or *Klebsiella ozeanae* in the absence of primary pathogens. Although these bacteria are part of the normal bacterial flora, they may have a certain potential to cause respiratory tract problems, but their influence on the pathogenesis of conjunctivitis is unknown.[54–56] As there were another four cats, in which no organisms were identified, false negative PCR results for primary pathogens may also be an explanation.

There was no significant difference in the severity of clinical signs between cats with different primary pathogens (single infections); however, cats with multiple infections had significantly more severe signs. The damage to conjunctival cells due to replication of several pathogens may be a suitable explanation. In addition, the status of infection (acute or chronic), the pathogenicity of the strains, and the viral and bacterial load have to be considered as well.

In this study, *C felis* and *Mycoplasma* species were found frequently in cats with conjunctivitis. BLAST sequencing identified four *Mycoplasma* species which were not reported to occur in cats before. The pathogenetic importance of these species is unknown, and further studies are needed to evaluate the pathogenicity of *Mycoplasma* species in cats with conjunctivitis.

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