

PAPER

Potential role of *Alternaria* and *Cladosporium* species in canine lymphoplasmacytic rhinitis

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OBJECTIVES: To evaluate the possible role of *Alternaria* and *Cladosporium* species in the pathogenesis of canine lymphoplasmacytic rhinitis by comparing the amount of specific fungal DNA in nasal mucosal biopsies between dogs without nasal neoplasia and those with lymphoplasmacytic rhinitis or nasal neoplasia.

METHODS: Quantitative real-time polymerase chain reaction (qPCR) assays detecting DNA from *Alternaria* and *Cladosporium* fungi were applied to nasal mucosal biopsies collected from dogs with lymphoplasmacytic rhinitis (n=8), dogs with nasal neoplasia (n=10) and control animals (n=10). A copy number for each sample was calculated using a standard curve of known copy number and differences amongst groups were assessed using Kruskal–Wallis tests.

RESULTS: No significant difference was found between the groups. Low levels of *Alternaria* DNA (10–100 copies/PCR) were detected in one sample; very low levels of DNA (<10 copies/qPCR) were detected in 6 samples, and 21 samples were negative. Low levels of *Cladosporium* DNA were detected in 2 samples; very low levels of DNA in 18; and 8 were negative.

CLINICAL SIGNIFICANCE: Results of this study reveal that *Alternaria* and *Cladosporium* species are part of the canine nasal flora, and that these fungi are probably not involved in the pathogenesis of lymphoplasmacytic rhinitis.

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INTRODUCTION

Idiopathic lymphoplasmacytic rhinitis (LPR) is a common cause of nasal discharge in dogs, characterised by lymphoplasmacytic to mixed inflammation of the nasal mucosa, for which no cause can be found (Windsor *et al.* 2004). The pathogenesis of this disease remains unknown. An increased expression of mRNA encoding interleukin (IL)-5 in the nasal mucosa of LPR dogs has been described (Peeters *et al.* 2007). A recent study has demonstrated dysregulation of genes encoding Toll- and Nod-like receptors in the nasal mucosa of affected dogs (Mercier *et al.* 2012). As a result, an aberrant immune response to inhaled

organisms or allergens may play a role in the pathophysiology of the disease.

Two studies have investigated the potential role of fungi in canine LPR. The first, using a universal fungal target gene quantitative real-time polymerase chain reaction (qPCR) (Windsor *et al.* 2006), demonstrated elevated levels of fungal DNA in nasal tissue from LPR dogs compared with controls, suggesting the involvement of fungi in the pathogenesis of the disease. The second study (Peeters *et al.* 2008), using qPCR assays detecting DNA from all *Penicillium* and *Aspergillus* species, did not find any difference between dogs with LPR and control animals. Nevertheless, other fungal species that could not be detected by the second study may still be involved in the pathogenesis of LPR.

It is generally agreed that human chronic rhinosinusitis (CRS) is an inflammatory rather than an infectious disease. Nevertheless, it has been postulated that microbes found in the nasal cavity (e.g. fungi, bacteria, viruses or their associated products) may play a role in initiating or perpetuating mucosal inflammation (Dosa *et al.* 2002, Gosepath *et al.* 2004, Shin *et al.* 2004). Dosa *et al.* (2002) cultured fungi from 83% of CRS patients and from 44% of healthy controls. Gosepath *et al.* (2004) showed the presence of *Alternaria* DNA in 100% of CRS patients but not in any healthy controls. Shin *et al.* (2004) exposed peripheral blood mononuclear cells (PBMCs) to *Alternaria* in vitro, and reported increased IL-5 and IL-13 production in 89% of CRS patients, but not in controls. *Cladosporium* is another fungus commonly isolated from CRS patients, as well as healthy controls (Ponikau *et al.* 1999, Buzina *et al.* 2003, Kim *et al.* 2005, Murr *et al.* 2006, Ragab *et al.* 2006, Shin *et al.* 2007). PBMCs from CRS patients stimulated specifically with *Cladosporium* antigens have also shown increased IL-5 production (Shin *et al.* 2004).

The aim of this study was to further investigate the potential role of fungi in the pathogenesis of canine LPR. qPCR assays were used to quantify *Alternaria* and *Cladosporium* fungal DNA in nasal mucosal biopsies from LPR dogs. This amount was compared with the amount found in nasal mucosal biopsies from dogs without LPR, but with another type of chronic nasal disease (dogs with nasal neoplasia) and from dogs without nasal disease.

MATERIALS AND METHODS

Animals

Dogs with LPR (n=8) had compatible chronic (3 months for 2 dogs; more than 6 months for the others) clinical signs and rhinoscopic findings (unilateral or bilateral, mild to moderate turbinate lysis with mucosal congestion and oedema, mucoid to mucopurulent secretions, and failure to demonstrate foreign body, neoplasia or fungal plaques in the nasal cavities), were seronegative for *Aspergillus fumigatus* antibody (agar gel double diffusion and enzyme-linked immunosorbent assay tests were both performed in each case) and had appropriate histopathological changes (lymphoplasmacytic inflammation of the lamina propria) in biopsy samples of the nasal mucosa. Initial diagnosis of LPR was also confirmed by the absence of progression of the nasal disease during a minimum follow-up period of 24 months. Dogs ranged in age from 1 to 13 years (median 9 years) and there were three females (one neutered) and five males (two castrated). Breeds represented included one each of Dalmatian, Yorkshire terrier, Cairn terrier, Jack Russell terrier, German shepherd dog, Staffordshire bull terrier, Dachshund and Pyrenean Mountain dog.

A control population of dogs without LPR, but with another chronic nasal condition was included in the study in order to evaluate the potential presence of *Alternaria* and *Cladosporium* DNA in nasal tissue from dogs suffering from another type of nasal disease. Therefore, 10 dogs with neoplasia (NEO) of the nasal cavity were included. In each dog, the diagnosis was made based upon visualisation of a mass during rhinoscopy and histopathological examination of nasal biopsy samples. These dogs ranged

in age from 7 to 13 years (median 10 years). There were five females (three neutered) and five males (three castrated). Breeds represented included four crossbreeds, two Golden retrievers and one each of Beauceron, Poodle, Fox terrier and Dobermann.

Ten dogs without nasal disease (client-owned dogs, euthanased for medical reasons, and with no history or signs of upper respiratory disease, immune-mediated disease or systemic immune suppression) [controls dogs (CTL)] were also included in this study. Owner consent was obtained for each dog. These dogs ranged in age from 3 to 15 years (median 9 years) and there were five females (three neutered) and five males (four castrated). Breeds represented included two Rottweilers, two crossbreeds and one each of Rheinland, Maltese bichon frise, Leonberger, Belgian shepherd, French sheepdog and Bernese Mountain dog.

Tissue samples

The nasal cavities were explored with a rigid endoscope (Cytoscope K Storz SL 308; Karl-Storz-Endoscopy Belgium SA) and, when possible, the frontal sinuses were investigated with a flexible bronchoscope (Fujinon BRO-YP2; Onys, SA). The frontal sinuses could not be explored in dogs without major turbinate lysis (i.e. all the NEO dogs, and all except two LPR dogs). All rhinoscopies and sinuscopies were performed by two investigators (CC and FB).

Perendoscopic biopsy samples were taken where the mucosa appeared to be the most inflamed in LPR dogs, from suspected neoplastic tissue in NEO dogs, or directly after euthanasia from the posterior third of the right nasal cavity in the CTL dogs.

Samples were placed in a 2.0-mL cryotube (CM.LAB), snap frozen in liquid nitrogen and stored at -80°C. Additional samples were placed in 10% neutral buffered formalin for histopathological examination by a board-certified veterinary histopathologist (MJD), without knowledge of their origin.

Fungal DNA quantification

DNA isolation

Freeze-dried ampoules of *Aspergillus fumigatus* (IMI number: 121660), *Alternaria alternata* (147408), *Cladosporium cladosporioides* (154278), *Cladosporium herbarum* (170353) and *Cladosporium sphaerospermum* (115790) were obtained from CABI Bioscience. Genomic DNA (gDNA) was isolated from these samples using the Macherey–Nagel NucleoSpin Blood Isolation Kit (ABgene) following reconstitution of the freeze-dried samples with nuclease-free water, as previously described (Peeters *et al.* 2008). Briefly, fungal suspensions were incubated with lysis buffer, proteinase K and a 5-mm stainless steel ball in a 2-mL Safe-Lock microcentrifuge tube (Eppendorf) at 56°C overnight in a shaking incubator (Vortemp 56EVC; Labnet) set at 10,000 × g, before completion of the extraction protocol as per the manufacturer's instructions. The DNA was eluted with 100 µL of elution buffer (from the extraction kit).

The same method was used for the extraction of gDNA from the nasal biopsies in the main experiment, except that biopsies were disrupted using a TissueLyser (Qiagen Ltd.) by shaking the tubes at 20 cycles/seconds for 2 minutes before the overnight incubation.

Primer and hydrolysis probe design

Primers and probes were designed using Primer 3 (Rozen & Skaletsky 2000) (www.genome.wi.mit.edu/cgi-bin/primer/)

Table 1. Primer and probe sequences used in the quantitative real-time PCR assays

Assay	Product length (base pairs)	Forward primer (5'-3')	Reverse primer (5'-3')	Probe sequence (5'-3')	Efficiency (%)
Alter	114	CTTTGCGTACTTCTTGTTC	GCCCAACACCAAGCAAAG	ACGCTCGAACAGGCATGCCC	99.3
Clado	90	AACTCTTGCCTAACTTTGCAGTC	ACAACGCTKAGGGGACRRAA	ACGCTCGAACAGGCATGCCC	96.6–98.6

primer3_www.cgi.) and M-Fold, as described previously (Peters *et al.* 2004). As high GenBank sequence similarities exist between fungal species of the same genus, it was not possible to design specific primers for *Alternaria* and *Cladosporium* species. Therefore, genus-specific primers and probes were designed, targeting the ITS1, ITS2 and 5.8S rDNA regions from *Alternaria* and *Cladosporium* (Table 1), using a consensus sequence derived from the 200 sequences obtained from GenBank by BLAST search.

Primers and probes were synthesised by Metabion (Martsried) and were reconstituted in EB buffer (10mM Tris–HCl pH 8.4; Qiagen Ltd.) before use.

Quantitative RT-PCR

Quantitative PCR was performed using GoTaq qPCR master mix (Promega). *Alternaria* gene amplification was performed using 200nM of each primer, 100nM of probe or SYBR Green I (1:100,000, Invitrogen), 4.5mM Magnesium and 5 µL of gDNA diluted as previously described (Peeters *et al.* 2007) in a final reaction volume of 25 µL. *Cladosporium* gene amplification was performed using 200nM of forward primer and 1200nM of reverse primer; concentration of the remaining reagents were the same. Sample incubations were performed in a Stratagene Mx3005P (Agilent Technologies) at 95°C for 15 minutes and then 45 cycles of 95°C for 15 seconds, 60°C for 15 seconds and 72°C for 30 seconds during which the fluorescence data were collected. The thermocycling protocol was extended when SYBR Green I was used by heating samples from 60 to 95°C in 0.5°C increments with a dwell time at each temperature of 11 seconds during which time the fluorescence data were collected in order to create a melt curve. Nuclease-free water passed through the DNA isolation was analysed in a similar manner to all other samples to control for sample contamination. A negative control of nuclease-free water and a positive control sample with a known Ct value were included with all sample runs to control for run-to-run Ct variation and all reactions were run in duplicate.

Fungal qPCR products from the most positive samples were sent for direct sequencing (GIGA research center) to confirm identity of the organism. The presence of amplifiable canine DNA was confirmed in all samples using an assay for canine β-actin, as described previously (Peters *et al.* 2007).

Reaction efficiency

Products from qPCR assays of each fungal species (*C. cladosporoides*, *C. herbarum* and *C. sphaerospermum* and *Alternaria alternata*) were separated by 2% gel electrophoresis to confirm that a single product band of the expected size was amplified by each reaction. The concentrations of the gel-purified qPCR products were quantified using the Qubit dsDNA BR Assay Kit (Invitrogen). A standard 10-fold dilution series from 10⁶ to 1 copy per PCR was

produced for each qPCR assay against each target fungal species with both SYBR Green I and the appropriate Taqman probes. In addition, each primer set was tested for cross-reactivity with gDNA of the non-target fungal genus (data not shown).

Statistical analysis

A copy number for each sample was calculated using a standard curve of known copy number and differences amongst the groups were assessed using the Kruskal–Wallis test (level of significance $P < 0.05$).

RESULTS

Histopathology

Control dog mucosa had normal histological structure. Mucosa from LPR dogs had relatively well-preserved architecture, focal oedema, and a mild to moderate infiltrate of lymphocytes and plasma cells, although neutrophils were occasionally predominant. Fungal hyphae were not observed. The diagnosis in NEO dogs included carcinoma in six (including five cases of adenocarcinoma and one of squamous cell carcinoma), sarcoma in three (one each of myxosarcoma, osteochondrosarcoma, haemangiosarcoma) and melanoma in one.

Quantitative PCR assay validation

All qPCR assays produced a single melt curve peak when run with SYBR Green I and single band products of appropriate size were obtained with agarose gel electrophoresis (data not shown). All reactions had efficiencies between 96.6 and 99.3% (Table 1) both with SYBR Green I and Taqman probe. The species-specific assays showed no cross-reactivity with the non-target species, and the three *Cladosporium* species were detected by the “Clado” assay, while *A. alternata* was detected by the “Alter” assay.

Fungal DNA quantification

Low levels of *Alternaria* DNA (10–100 copies/PCR) were detected in one sample (one CTL); very low levels of *Alternaria* DNA (<10 copies/qPCR) were detected in six samples (three CTL, one NEO and two LPR) while 21 samples (six CTL, nine NEO and six LPR) yielded a negative result (Fig. 1). Low levels of *Cladosporium* DNA (10–100 copies/qPCR) were detected in two samples (both CTL); very low levels of DNA (<10 copies/qPCR) were measured in 18 samples (five CTL, five NEO and eight LPR), and eight samples (three CTL, five NEO) yielded a negative result. No significant difference in the amount of fungal DNA was found between the groups.

Sequencing confirmed the fungal genus, but not the species, because of the very high sequence homology between the species.

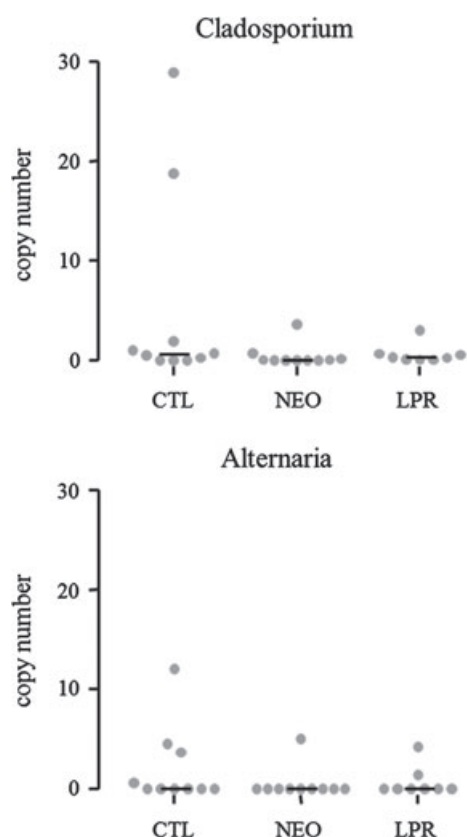


FIG. 1. Plots of the copy number of *Alternaria* and *Cladosporium* DNA in nasal biopsies assessed by quantitative PCR in control dogs (CTL, n=10), dogs with nasal tumours (NEO, n=10), and dogs with idiopathic lymphoplasmacytic rhinitis (LPR, n=8). The horizontal black line corresponds to the median value of the group

DISCUSSION

The results of this study have demonstrated that *Alternaria* and *Cladosporium* DNA can be detected in the nasal mucosa of dogs. Previously, fungi have been found to be part of the canine nasal flora (Windsor *et al.* 2006, Peeters *et al.* 2008); however, this is the first time that *Alternaria* and *Cladosporium* species have been shown to be part of the nasal flora of dogs with and without nasal disease. Similarly, in man, fungi were initially thought to be an abnormal constituent of the nasal flora; however, it has been shown that fungi are present in the respiratory tract (Braun *et al.* 2003b, Buzina *et al.* 2003, Catten *et al.* 2001, Dosa *et al.* 2002, Lackner *et al.* 2005, Murr *et al.* 2006, Ragab *et al.* 2006) of both healthy and diseased individuals.

In this study, no difference in the presence and the amount of fungi was detected between LPR dogs, NEO dogs and dogs without nasal disease.

Ponikau *et al.* (1999) found that fungi could be cultured from nasal mucus in almost 100% of patients with CRS; which is similar to that found in most healthy humans (Catten *et al.* 2001, Braun *et al.* 2003a, Kim *et al.* 2005, Murr *et al.* 2006, Ragab *et al.* 2006), calling into question the role of fungi as a significant aetiological factor in human CRS. As fungi are ubiquitous

in nature and are present equally in the nose and sinuses of both CRS patients and healthy controls, it was hypothesised that it is not the presence or absence of fungi that is relevant for disease development, but rather the fungal species or fungal load. However, there appears to be no difference in the range of fungal genera grown (Buzina *et al.* 2003, Kim *et al.* 2005, Murr *et al.* 2006) or quantity of fungal DNA (Catten *et al.* 2001, Scheuller *et al.* 2004, Murr *et al.* 2006) between patients with CRS and uninfected subjects.

Moreover, Orlandi *et al.* (2009) recently examined the relationship between CRS and fungi in a more heterogeneous group of CRS patients (variable disease severity and residing areas). Contrary to Shin *et al.* (2004), they found a significant production of IL-5 by PBMCs in CRS patients following *Alternaria* exposure, but also in control patients. In addition, *Alternaria*-induced levels of IL-13 did not differ between CRS patients and controls. However, the methods used by Orlandi *et al.* (2009) (patient selection criteria, fungal extracts used and different test settings) differed significantly from those of Shin *et al.* (2004). Consequently, additional work must be undertaken in order to understand whether fungi have a role in the pathogenesis of CRS and, if so, in which types of patients.

Studies investigating the effect of antifungal therapy (amphotericin B) in the treatment of CRS also call into question the role of fungi in the pathophysiology of human CRS. Despite initial evidence of benefit of amphotericin B in two uncontrolled trials (Ponikau *et al.* 2002, Ricchetti *et al.* 2002), a subsequent uncontrolled prospective trial (Helbling *et al.* 2006) and four double-blinded placebo-controlled studies using the same drug administered by nasal lavage (Ponikau *et al.* 2005; Ebbens *et al.* 2006, Liang *et al.* 2008) or intranasal sprays (Weschta *et al.* 2004, Helbling *et al.* 2006) in CRS patients with and without nasal polypsis either failed to show benefit (Weschta *et al.* 2004, Ebbens *et al.* 2006, Helbling *et al.* 2006, Liang *et al.* 2008) or showed, at best, only modest irrelevant radiographic benefit without symptomatic improvement. Despite these results, some argue that failure of antifungal medications does not necessarily disprove that fungi may be the major aetiological factor in CRS, as failure may be explained by ineffective dosage or delivery (Shirazi *et al.* 2007). In LPR dogs, anecdotal evidence suggests beneficial improvement using orally administered itraconazole (Kuehn 2009).

Some authors have suggested that fungi may have a disease-modifying rather than a disease-causing role, for instance by inducing changes in mucosal immunity of CRS patients (Claeys *et al.* 2003, Dong *et al.* 2005, Lane *et al.* 2006). In dogs with LPR, disorders in adaptive and innate immunity were recently described (Peeters *et al.* 2007, Mercier *et al.* 2012); however, the involvement of fungi in the pathogenesis of the disease is not proven.

There are some limitations to this study. First, it was impossible to design specific qPCR assays for each individual fungal species; therefore, it is possible that a significant involvement of a particular fungal species may have been missed. Secondly, as computed tomography was not performed to help definitively diagnose LPR, in theory a foreign body, subclinical neoplasia or

sinonasal aspergillosis could potentially have been misdiagnosed. Nevertheless, as all LPR dogs had negative aspergillosis serology and as a minimum follow-up of 24 months failed to reveal any worsening of clinical signs, these differential diagnoses are very unlikely. Low case numbers are another limitation of this study; as a consequence, either the study is underpowered to make the observed difference significant, or *Alternaria* and *Cladosporium* are not implicated in the pathogenesis of canine LPR.

Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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