MEDDOCS Open Access Publisher

ISSN: 2640-1223

Journal of Veterinary Medicine and Animal Sciences

Open Access | Research Article

Pan-Fungal Polymerase Chain Reaction and Fungal Precipitins Assays in West Highland White Terriers with Canine Idiopathic Pulmonary Fibrosis

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Received: Nov 16, 2022

Accepted: Dec 02, 2022

Published Online: Dec 05, 2022

Journal: Journal of Veterinary Medicine and Animal Sciences

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Keywords: Dogs; Lung; Fungi; Internal transcribed spacer; Electrosyneresis.

Abbreviations: BALF: Broncho-Alveolar Lavage Fluid; CIPF: Canine Idiopathic Pulmonary Fibrosis; HP: Hypersensitivity Pneumonitis; IPF: Idiopathic Pulmonary Fibrosis; ITS-2: Internal Transcriber Spacer-2; NSIP: Non-specific interstitial pneumonia; PCR: Polymerase Chain Reaction; UIP: Usual Interstitial Pneumonia; WHWT: West Highland White Terriers

Abstract

Objective: Canine Idiopathic Pulmonary Fibrosis (CIPF) is a progressive parenchymal lung disease of unknown origin mainly occurring in old West Highland White Terriers (WHWTs). The objective of this study was to investigate a potential association between fungal infection or sensitization and CIPF.

Methods: A conventional pan-fungal Polymerase Chain Reaction (PCR) assay targeting the conserved rDNA gene internal transcribed spacer-2 region was performed using DNA extracted from lung tissue samples from WHWTs affected with CIPF (n=26) and age-matched controls (n=14). Additionally, serum samples from 8 WHWTs affected with CIPF and 8 age-matched unaffected WHWTs were tested for precipitins against 10 species of environmental fungus using electrosyneresis on cellulose acetate.

Results: Fungal DNA was amplified in 8 (57%) controls and 15 (58%) WHWTs with CIPF (P=0.973). Sequences of good quality were obtained for 5 samples and matched with ≥ 97% homology with *Cladosporidium* spp. and *Alternaria* spp. in 2 distinct WHWTs with CIPF, and with *Aspergillus fumigatus*, unspecified fungi, and eukaryotic DNA from a plant (*Popolus* spp.) in 3 distinct controls. Results of the serological assay revealed the presence of ≥ 2 arcs of precipitins in 35 (44%) reactions in the CIPF group compared with 20 (25%) reactions in control group (P=0.013).

Conclusion: These results suggest that an association between CIPF and active fungal infection is unlikely as fungal DNA was equally amplified from lung of CIPF-affected WHWTs and controls. However, the higher proportion of serum positive precipitin reactions in CIPF may suggest a lung sensitization to inhaled fungal allergens and warrants further investigation.

Cite this article: Roels E, Barrera C, Millon L, Rajamäki MM, Talbot JJ, et al. Pan-Fungal Polymerase Chain Reaction and Fungal Precipitins Assays in West Highland White Terriers with Canine Idiopathic Pulmonary Fibrosis. J Vet Med Animal Sci. 2022; 5(2): 1120.

Introduction

Canine idiopathic pulmonary fibrosis (CIPF) is a progressive fibrotic lung disease mostly reported in aged dogs of the West Highland white terrier (WHWT) breed [1-3]. The etiology of CIPF is currently unknown and the pathogenesis is poorly understood [1-3]. A genetic basis is suspected based on the breed predisposition and the recent identification of two genetic variants associated with CIPF [4]. Not all dogs of the WHWT breed develop CIPF at an advanced age, suggesting that triggering events may be involved in development of the disease. Interestingly, computed tomographic and histopathological findings of CIPF share characteristics of both human Usual Interstitial Pneumonia (UIP) and Non-Specific Interstitial Pneumonia (NSIP) patterns [5-8]. In humans, a UIP pattern is typical for Idiopathic Pulmonary Fibrosis (IPF) while NSIP pattern is commonly observed in hypersensitivity pneumonitis (HP) [9]. Hypersensitivity pneumonitis is an interstitial lung disease which results from sensitization to inhaled antigens such as fungal particles and can cause irreversible lung fibrosis in chronic stages [10-12]. Fungal species most frequently associated with the onset of HP include *Penicillium* spp. and *Aspergillus* spp. [13]. Besides HP, other human respiratory disorders can be triggered by fungal infections, particularly *Aspergillus* spp. [14,15]. Within the spectrum of pulmonary aspergillosis, which ranges from single aspergilloma to invasive aspergillosis, chronic fibrosing pulmonary aspergillosis has been recognised [14,15]. In addition to compatible imaging findings, diagnosis of pulmonary aspergillosis generally required serological or microbiological evidence implicating *Aspergillus* spp. infection [14]. This could be achieved by measuring serum *Aspergillus* specific immunoglobulins G or precipitins in blood, or by performing *Aspergillus* culture or polymerase chain reaction (PCR) on respiratory samples [14]. Given that no etiologies have been identified for CIPF, the aims of this study were to screen lung specimens from WHWTs affected with CIPF for fungal DNA and to assess for serologic evidence of environmental fungal exposure.

Methods

Case Selection

Lung tissue samples collected from 26 WHWTs affected with CIPF (15 males and 11 females aged from 9.1 to 16.3 years, median 13.6 years), and 14 control dogs (5 males and 9 females of various breeds including 4 WHWTs, 4 Jack Russell terriers, 1 Mixed breed, 1 Beagle, 1 Bulldog, 1 Newfoundland, 1 Maltese, and 1 American Staffordshire aged from 3.7 to 15.0 years, median 9.7 years) were included in the study for fungal DNA testing. Lung tissues used as controls were obtained from dogs of various breeds given that collecting lung specimens from healthy WHWTs was difficult to achieve in a clinical setting. Control dogs were euthanized for reasons unrelated to the study. These dogs had no history or clinical signs of lower respiratory disease at the time of euthanasia and no abnormalities were identified on necropsy and histopathological examination of their lungs. For all dogs, lung biopsies were aseptically obtained within 30 min after euthanasia, snap-frozen in liquid nitrogen and stored at -80 °C until further processing. Dogs included in this part of the study were recruited at the University of Liège (11 control dogs and 3 CIPF-affected WHWTs), the University of Helsinki (3 controls and 21 CIPF-affected WHWTs), and by other partners engaged in the European CIPF project (2 CIPF-affected WHWTs) [1].

Serum samples from 8 WHWTs affected with CIPF (7 males and 1 female; median age 13.0 years, range 10.7 – 15.0) and 8 unaffected WHWTs (4 males and 4 females; 9.7 years, 5.8 – 15.0) were selected for serological precipitin testing. Samples from dogs used for the precipitin assay were recruited at the University of Liège as environmental fungal exposure may vary across countries. Breed-matched controls were chosen to get rid of a possible confounding breed-effect on the serological results. Among CIPF-affected WHWTs included in the serological study, only one had lung tissue tested for fungal DNA. Blood samples were obtained in plain tubes. Thirty minutes after blood collection, tubes were centrifuged at 4 °C for 15 min at 1300 x g. Serum was harvested and aliquoted into 1.5 mL plastic cryotubes, and samples were stored at -80 °C until analysis.

The CIPF diagnosis was achieved according to a previously published approach and was confirmed by lung histopathology in all dogs [1]. Health status of the control WHWTs was assessed by thorough history, physical examination, hematology, serum biochemistry, echocardiography and thoracic high resolution computed tomography, which did not reveal significant abnormalities. The study was approved by the Committee of Experimental Animals of Western Finland (approval numbers: ESLH-2008_05403, date of approval: 27 June 2008; ESA-VI/7383/04.10.07/2013, date of approval: 13 November 2013) and by the ethical committee of the University of Liège, Belgium (approval number: 1435, date of approval: 14 March 2013). All samples were obtained with informed owner consent.

DNA Extraction, ITS-2 Polymerase Chain Reaction and Sequence Analysis

Total DNA was extracted from lung tissue samples using a commercially available kit (DNeasy Blood & Tissue kit, QIAGEN Inc.). To evaluate DNA quality in each sample, a conventional PCR targeting a canine housekeeping gene, the ribosomal protein L32 (RPL32) gene was performed. The primer sequences for RPL32 are described elsewhere [16]. The total reaction volume was 50 μL containing 5 μL of template DNA, 0.1μL 20uM forward primer, 0.1μL 20uM reverse primer, 18 μL MilliQ water, and 25 μL MyTaq™ HS Red Mix (Bioline). Thermal cycler conditions consisted of 1 min at 95 °C; 35 cycles of 95°C for 15 s, 60 °C for 15 s, and 72 °C for 10 s; and a final extension step at 72 °C for 5 min. Then, a conventional pan-fungal PCR assay targeting the conserved rDNA gene internal transcribed spacer-2 (ITS-2) region of fungi was performed twice on two separate days on extracted lung DNA according to a previously published PCR protocol [17]. Primer sequences for the ITS-2 region are described elsewhere [17]. DNA of *Aspergillus fumigatus* cultured from soil was included as a positive control. Molecular grade water was used as a negative control. PCR products were separated by electrophoresis (30 min at 80V) on a 1% agarose gel made with 1 X Tris boric acid ethylenediaminetetraacetic acid (TBE) buffer and SYBR Safe DNA gel stain (ThermoFisher Scientific Inc.). Bands were visualised with a UV transilluminator (GelDoc, Biorad). PCR products of expected size (330 bp) were excised from the gel, and the DNA was purified using a commercially available kit (QIAquick PCR Purification Kit, QIAGEN Inc.). Pooled DNA from both PCR sets were submitted for Sanger sequencing (Macrogen Inc., Korea). Sequences obtained were assessed qualitatively by visual inspection of the chromatogram, edited and joined in a consensus sequence using BioEdit v7.0.5 (Ibis Therapeutics, Carlsbad, CA). Consensus sequences were queried against the GenBank database using the Basic Local Alignment Search Tool (BLAST, NCBI). A fungal identification

was made when a sequence matched the reference with ≥ 97% homology.

Electrosyneresis on cellulose acetate

Included sera were tested in batched analysis for the presence of precipitins against 10 environmental fungi extracts using electrosyneresis on cellulose acetate. Antigen extracts chosen were *Lichtheimia corymbifera, Wallemia sebi, Eurotium amstelodami, Aspergillus versicolor, Penicillium chrysogenum, Cladosporidium sphaerospermum, Stachybotrys chartarum, Mucor racemosus, Alternaria alternata,* and *Aspergillus fumigatus*. Antigen extracts were produced and tested as previously described [18]. Electrosyneresis was carried out as follows. Cellulose acetate sheets (Sartorius, Göttingen, Germany) were placed in the electrophoresis vat filled with buffered Tris glycin solution at pH 8.8; 15 µL of each serum was placed on three spots on the anode side and a 15 µL line of fungal antigen was placed on the cathode side. A current (110V) was then applied for 75 min and the sheets were stained with Coomassie blue. Results were interpreted qualitatively by one single observer by counting the number of precipitin arcs formed (≥ two arcs being considered as positive).

Statistical methods

Statistical analyses were performed using a commercially available software (XLSTAT, Addinsfot Inc.). Continuous variables were reported as median and range (minimum and maximum), and categorical data as proportions. Proportions were compared between WHWTs affected with CIPF and controls using the Fisher exact test or Chi-squared test according to the tested sample size. To account for multiple proportions comparison, the Marascuilo procedure was used, and the significance level was adjusted as *P*-value<0.05/n; n being the number of paired comparisons performed.

Results

ITS-2 Polymerase Chain Reaction

PCR analysis of the 26 samples from WHWTs affected with CIPF and 14 control dogs resulted in a band of the expected size (330 bp) in 23 of all 40 (57.5%) lung samples [15 WHWTs with CIPF (57.7%) and 8 controls (57.1%), (*P*=0.973)], while five of 40 (12.5%) were negative, and 12 of 40 (30%) yielded non-distinct smeared amplification products at the 330 bp site and were excluded from further analysis **(Figure 1)**. Eighteen of 23 (78.2%) positive samples demonstrated multiple PCR product bands on electrophoresis. Bands at the 330 bp site were present in 11 of 23 (47.8%) positive samples. Sequencing of positive samples yielded mostly poor-quality mixed chromatograms (18/23, 78.3%) and were not queried against the GenBank database. BLAST analysis was performed on five good-quality sequences. *Alternaria* spp. (100% nucleotide identity) and *Cladosporidium* spp. (99% nucleotide identity) were each identified in two distinct WHWTs affected with CIPF. *Aspergillus fumigatus* (99% identity) was found in a control Newfoundland, and an unspecified isolate matching with *Bjerkandera* spp., *Thanatephorus* spp., *and Rhizoctonia* spp. (99% nucleotide identity) was identified in a control WHWT. The last positive sample, from a control Jack Russell terrier, matched with eukaryotic DNA from a plant (*Populus* spp.) (99% nucleotide identity).

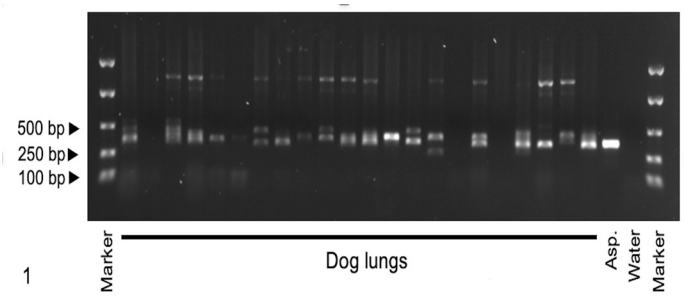

Figure 1: Analysis of lung samples collected from control dogs (lanes 2-15) and WHWTs affected with CIPF (lanes 16-23) with pan-fungal PCR ITS-2 using ITS3-ITS4 primers. Lanes 1 and 26: 2000-100 bp ladder (Marker); lane 24: positive control (soil *Aspergillus fumigatus*); lane 25: negative control (molecular grade water).

Electrosyneresis on cellulose acetate

Electrosyneresis was performed on 16 serum samples (8 control and 8 WHWTs with CIPF) against a panel of 10 environmental fungal antigens, leading to a total amount of 160 reactions from which 55 (34%) were positive (≥ two arcs of precipitins). Proportion of positive reactions (all fungal antigens confounded) was significantly higher in WHWTs affected with CIPF (35 positive results on total 80 reactions – 44%) in comparison with control WHWTs (20 positive results on total 80 reactions – 25%) (*P*=0.013). However, this comparison must be taken with extreme precaution as cross-reactivity between precipitin assays cannot be ruled out in this biological setting. Cross-reactivity amongst tested fungal antigens would imply dependency between samples and invalidate the statistical test. When fungal antigens were assessed separately using multiple comparison correction, no difference was noted between groups (Table 1). None of the dog tested had positive precipitin reaction against *Aspergillus fumigatus* **(Table 1)**. Only one WHWTs affected with CIPF was tested for both serum precipitins and lung ITS-2 PCR. This dog was positive for 8 out of 10 of the antigen fungal extracts tested and had an ITS-2 PCR positive for *Alternaria* spp.

Table 1: Details of positive precipitin reactions (≥ 2 arcs) in WHWTs affected with CIPF and controls.

Antigen extracts	Positive precipitin reaction		*P*-value
	WHWTs[a] with CIPF[b] (n=8)	Control WHWTs[a] (n=8)	
Lichtheimia corymbifera	6	1	0.041
Wallemia sebi	5	6	1.000
Eurotium amstelodami	2	0	0.467
Aspergillus versicolor	4	3	1.000
Penicillium chrysogenum	4	1	0.282
Cladosporidium sphaerospermum	4	5	1.000
Stachybotrys chartarum	3	1	0.569
Mucor racemosus	5	3	0.619
Alternaria alternata	2	0	0.467
Aspergillus fumigatus	0	0	n.a.

[a] WHWTs, West Highland white terriers. [b] CIPF, canine idiopathic pulmonary fibrosis

P-value<0.005 considered as statistically significant

Discussion

By using a pan-fungal PCR assay, we demonstrated the presence of fungal DNA in the same proportion of lung samples from WHWTs affected with CIPF and unaffected controls, suggesting that active fungal infection is unlikely associated with the disease. On the other hand, results of precipitins assays indicated an increased sero-prevalence of fungal exposure in the CIPF population compared with controls; hypothesizing no cross-reactivity between the fungal antigens tested. This may suggest a lung sensitization to recurrent inhaled fungal particles and requires further investigation. Lastly, neither control nor CIPF-affected WHWT displayed serological evidence of *Aspergillus fumigatus* sensitization, suggesting that *Aspergillus fumigatus* is unlikely to be implicated in CIPF pathology.

Human chronic fibrotic HP is one of the main differential diagnoses for IPF [19]. As similarities and differences exist between both diseases regarding clinical, radiological and histopathological features, other key elements should be considered in the diagnostic approach [9]. Most cases of fibrotic HP are environmental occupational diseases caused by repetitive exposure to various microbial species or chemicals [10]. Clinicians are therefore advised to search for offending antigen using precipitating antibodies or specific IgG measurement [12].

As cases of chronic HP have been associated with fungal sensitization in humans [13,20], we investigated the presence of immunologic sensitization to a panel of fungal antigens using precipitation reactions in CIPF and control WHWTs. Additionally, we assessed the presence of fungal DNA in lung tissue samples of CIPF and control dogs using culture-independent molecular technology. Lung samples were chosen over broncho-alveolar lavage fluid (BALF) samples in order to increase the likelihood of obtaining a positive reaction; BALF being suspected to contain a lower total DNA load than pulmonary tissue and carrying the risk for sampling contamination from the upper respiratory tract. The pan-fungal PCR assay used in this study has previously been shown useful for molecular identification of fungal pathogens invading animal tissue (including lung tissue) [17]. Positive amplification products of expected size were obtained in both CIPF and control dogs in similar proportions suggesting that an association between active fungal infection or heavy fungal lung colonization and CIPF is unlikely. Positive pan-fungal PCRs in both groups most likely reflect commensal fungal inhabitant of the lung; whereas environmental contamination could not be completely excluded but is unlikely as samples were collected aseptically. Good quality sequences for GenBank identification were obtained for only 5 out of 21 (24%) positive samples. Sequences identification revealed different fungi among individual dogs. In one sample, non-fungal eukaryotic DNA was identified due to the lack of specificity of the PCR employed, as previously observed [17]. Explanations for the high number of poor-quality sequences and for the presence of multiple and faint bands were already discussed earlier by Meason-Smith and collaborators (2017), determined to be most likely a consequence of the expected diversity of commensal fungi inhabiting the lung and of the low concentration of fungal DNA in non-infected lung tissue [17]. Non-specific host genomic amplification may also serve as an explanation for the multiple bands.

Precipitins against fungal antigens were tested to investigate serological evidence of fungal environmental exposure in both WHWTs affected with CIPF and controls. In human HP, precipitins testing takes part in the diagnostic approach of the disease as it allows to identify environmental triggers that may be responsible for the clinical signs [21]. Other more sensitive serological testing may also be employed such as Western blot or ELISA targeting specific agents, but they may be less specific than traditional precipitins assays and have not yet been developed for use on canine serum [22]. As dogs spend time breathing close to vegetal material as their instinctive behavior, they may be exposed repeatedly to fungal allergens that could lead to an exacerbated immune response in genetically predisposed individuals. Precipitin assay carried herein against a panel of environmental fungus revealed a higher proportion of positive reactions in CIPF population compared with breed and age-matched controls. This finding may suggest a greater exposure to fungal antigens in dogs affected with CIPF, which could reflect a greater lung sensitization to aerosolized fungal allergens in diseased patients. However, as explained above, this result should be taken with precaution as cross-reactivity between fungal antigens tested could have biased the results. Interestingly, even though not statistically significant, the proportion of dogs with evidence of *Lichtheimia corymbifera* exposure was higher in WHWTs affected with CIPF compared with controls. *Lichtheimia corymbifera* is a filamentous fungus commonly involved in farmer's lung HP able to trigger inflammatory mediation at the alveolar epithelial cell level based on *in-vitro* studies [23-25]. Accordingly, chronic exposure to this specific fungal antigen in WHWTs could contribute to repetitive alveolar injury favoring development of pulmonary fibrosis in the long-term. At the opposite, none of the dogs tested displayed immunologic sensitization to *Aspergillus fumigatus,* suggesting that this specific fungus is unlikely involved in CIPF.

Limitations of the present study are related to the inherent performances of the PCR and precipitins techniques employed. More advanced sequencing techniques, such as next generation sequencing (e.g. lung mycobiota study), are likely to have greater success in identification of multiple concurrent species of fungal DNA from lung tissue. Such studies would allow to compare the diversity and relative abundance of fungal organisms between CIPF and controls and identify possible alteration of the mycobiota composition in diseased patients. Precipitin testing allows qualitative determination of specific antibodies against a panel of antigens. The electrosyneresis method used in the present study is routinely run in human clinical setting in France (CHRU human University hospital Besançon) but was not specifically validated in dogs. However, as this test is based on simple qualitative detection of precipitating antigen-antibody complexes, a validation was not judged necessary. Using quantitative methods such as ELISA to measure fungal IgG antibodies titers would have permit a more sensitive comparison between CIPF and controls. Another limitation was the small population size used for the precipitin assay; however, given the low prevalence of CIPF, this patient series can be considered as relevant. Furthermore, serum samples employed for precipitin assay were collected from different dogs than the ones whose lungs served for fungal PCR assay (except from one single dog). Although no matched samples were available, we considered that the use of two datasets from different dogs was adequate since testing precipitins was intended to assess previous allergens exposure while fungal PCR on lung sample assessed present inhabitant of the lung.

Conclusions

The present study showed no difference between WHWTs affected with CIPF and controls for the presence of fungal DNA

in lung samples suggesting that an association between active fungal infection and CIPF is unlikely. However, a higher seroprevalence for environmental fungal antigens was found in the CIPF population and requires further considerations. Serological testing for *Lichtheimia corymbifera* should be considered in a larger population of dogs by using a more sensitive method, e.g. ELISA or Western blot, before any conclusion can be made. *Aspergillus fumigatus* unlikely play a role in CIPF as there was no serological evidence of exposure.

Conflicts of interest statement

The authors have no conflict of interest to report.

Acknowledgements

We gratefully thank Allerton Fergus from Willows Veterinary Centre and Referral Service, West Midlands, UK and Bomassi Eric from Veterinary Hospital Centre Coderliers, Meaux, France for providing lung samples material used in this study. We also kindly thank Mahdis Aghazadeh from Sydney School of Veterinary Science, Faculty of Science, and Marie Bashir Institute, University of Sydney, Australia for her technical assistance in PCR procedures. We thank Audrey Laboissière from Faculty of Medecine, UMR6249 Chrono-Environnement, University of Bourgogne_franche_Comté for her technical assistance in electrosyneresis procedures.

Funding

This work was supported by a grant from the 'Fonds de la Recherche Scientifique (FNRS)' (Grant number: FC96092).

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